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The thermodynamics of protein stability

Cold destabilization as a general phenomenon

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A theoretical analysis of the temperature/stability profiles of proteins shows that, where a two-state model represents the denaturation, and where the free energy of denaturation $\Delta G(T)$ shows a strong temperature dependence, then the protein becomes subject to both high- and low-temperature destabilization. In the simplest case $\Delta G(T)$ is parabolic, therefore the high temperature T_H, where $\Delta(G(T_H)=0$, is complemented by a low temperature T_L, where $\Delta G(T_L)=0$. It is generally stated that the partial molal heat capacity change ΔC accompanying the heat denaturation is positive and independent of the temperature. This implies that heating the protein through T_L results in a negative ΔC which seems physically unsatisfactory. The constant ΔC model is explored and a physically more realistic model is advanced which allows for a temperature-dependent ΔC which changes sign at some temperature within the range of stability of the native protein; $\Delta G(T)$ then has the form of a skewed parabola. Experimental heat capacity data for native lysozyme and for a flexible polymer lend support to this model. The molecular basis of cold inactivation of proteins is discussed in the light of the thermodynamic analysis.

1. Introduction

The origin of the marginal stabilities of functional (native) proteins is still very much a matter of speculation [1]. Comment has often been made that the stability relative to an inactive (denatured) state seldom exceeds 50 kJ mol^{-1}, equivalent to three or four hydrogen bonds. It follows that the stabilizing interactions must be almost balanced by factors which destabilize the native, folded state. Various attempts have been made to draw up 'balance sheets' of the various types of interactions which might contribute to the stability of small proteins [2], the two most important being the configurational free energy of the macromolecule and hydrophobic interactions. Other contributions include hydrogen bonding within the peptide chain and with the solvent, polyelectrolyte and van der Waals interactions and configurational constraints on the solvent. Few, if any, of these contributions can be unambiguously determined by experiment, nor can they be reliably calculated. This is particularly true for hydrophobic effects the quantitative estimation of which is fraught with uncertainties [3].

Computer simulation methods are being increasingly applied to studies of protein structure, dynamics and stability. The known (crystal) structure usually forms the starting point for such computations. Hydration contributions to the stability of the macromolecular configuration cannot be accommodated in any realistic manner, nor can hydrophobic effects. It must be borne in mind that even for the 'simple' water dimer, upwards of

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20 different potential functions have been proposed [4] since Rahman and Stillinger [5] reported the first liquid water simulations in 1971. Many of these functions are still in use, thus confirming the somewhat rudimentary state of the art.

Since native proteins tend to be stable only within limited ranges of environmental conditions, most experimental approaches utilize perturbations of the native state by changes in temperature, pressure, pH, salt concentration or the solvent medium. In recent years alterations in the primary sequence by point mutations have gained in popularity as a probe of stability and its perturbation [6].

The destabilization of native proteins by high temperatures is a favourite tool for studies of both the thermodynamic and kinetic aspects of protein unfolding and refolding. Calorimetry yields the most direct thermodynamic information; such techniques have been used to good effect by Privalov [7] and his associates. Probably for reasons of experimental convenience, most protein stability studies are performed by spectroscopic or optical activity techniques. The molecular interpretation of such data is most often made by assigning the spectral features to particular species in the context of a chemical model. Derived thermodynamic quantities, such as ΔH must then be shown to be consistent with the same quantities determined by calorimetry [7].

2. Thermodynamics of thermal denaturation

The thermal unfolding and inactivation of several small globular proteins has been studied in detail and reviewed on several occasions (see, for instance, refs. 6 and 7). Essentially, the overall process can often be represented by a two-state equilibrium of the type $N \rightleftharpoons D$, where N is the fully native and active protein (usually an enzyme), and D an inactive, denatured state. The molecular details of the N-state in solution can be specified quite reliably, because the time-averaged atomic coordinates of the peptide chain and of a considerable number of the associated water molecules are believed to resemble those in the protein crystal.

Useful descriptions of D-states are lacking. In the limit, an unfolded protein might resemble a random-flight coil, so that its dimensions in a theta-solvent could be calculated from its degree of polymerization. However, it is known that different denaturing treatments produce polypeptides with different degrees of unfolding [8]. Investigations of thermally unfolded states are also complicated by the rapid and irreversible aggregation of the chains, leading to flocculation and precipitation. In the language of polymer statistics, such aggregation is equated with the condition that at the denaturation temperature (T_H) water is a poor solvent for the chemical residues of which the polymer is composed, with a Flory-Huggins interaction parameter $\chi > 0.5$. Under favourable circumstances, reversible unfolding can be experimentally separated from subsequent irreversible processes [9].

Detailed studies of the reversible denaturation of chymotrypsinogen and ribonuclease enabled Brandts [10] and Brandts and Hunt [11] to demonstrate that the two-state N-D model was applicable and that the free energy of denaturation exhibits an unusual temperature dependence. For any given pH, $\Delta G(T)$ can be fitted by a simple power series

$$\Delta G(T) = A + BT + CT^2 + \dots \tag{1}$$

where $\Delta G(T) = G^D(T) - G^N(T)$ is referred to as the free energy of denaturation; this is shown in fig. 1a. (Strictly speaking, $\Delta\mu$, the change in the protein chemical potential is the quantity of significance.) At this point it should be noted that, since the ionization product of water K_w is itself a function of temperature, such $\Delta G(T)$ profiles as have been reported, ostensibly at constant pH, do in fact incorporate the effects of the change in pK_w with temperature.

For chymotrypsinogen Brandts [7] reported an 'almost parabolic' relationship, and this has since been confirmed for several other small proteins. Actually, at any given pH, ΔG can only be measured over a limited temperature range, because of the cooperative nature of the N-D transition. However, Brandts observed that at any given temperature, ΔH is apparently independent of pH, and this enabled him to construct, from measure-

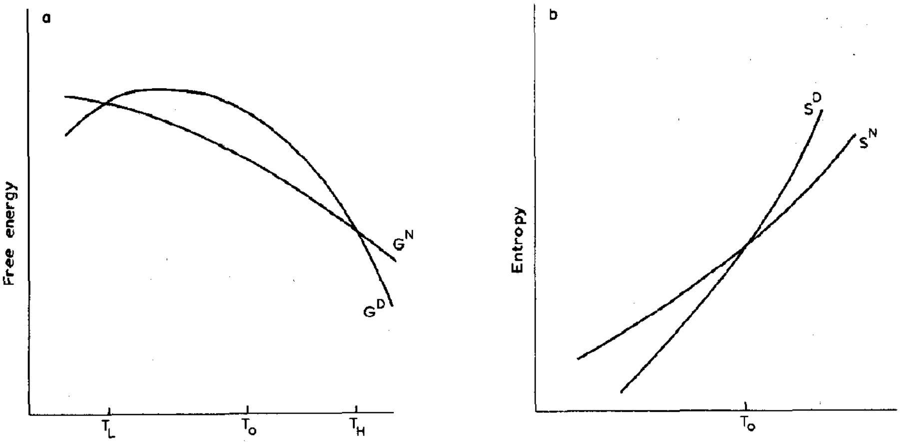

Fig. 1. (a) Free energy and (b) entropy profiles for a two-state equilibrium system which can give rise to N-D transitions at T_L and T_H, respectively.

ments at different pH values, composite $\Delta G(T)$ curves over the temperature range 273–353 K.

To analyse the curves in fig. 1a, we use the identity

$$f(x)=f(x_0)+(x-x_0)f'(x_0)+\int_{x_0}^{x}\mathrm{d}y\int_{x_0}^{y}f''(z)\,\mathrm{d}z \tag{2}$$

where the primes indicate derivatives. We have, for either N or D, and in the neighbourhood of some reference temperature T_0, to be specified later,

$$G(T)=G(T_0)-(T-T_0)S(T_0)-\int_{T_0}^{T}\mathrm{d}t\int_{T_0}^{t}C(u)\,\mathrm{d}u/u \tag{3}$$

where $C=C_p$ is the partial molal heat capacity. It is convenient to introduce the following reduced temperature θ:

$$\theta\equiv(T-T_0)/T_0 \tag{4}$$

We write $G[\theta]=G(T)$, etc., and also introduce the approximation

$$C(T)=C(T_0)+(T-T_0)C'(T_0)=C[0]+T_0\theta C'[0] \tag{5}$$

to make the discussion simpler, although it would be easy to keep a general form. With these changes eq. 3 becomes

$$G[\theta]=G[0]-\theta T_0S[0]-T_0s(\theta)C[0]-(1/6)T_0^2\theta^3C'[0] \tag{6}$$

where we define

$$s(\theta)\equiv(1+\theta)\ln(1+\theta)-\theta \tag{7}$$

which has the shape of a skewed parabola in the range $-1<\theta<1$, as shown in fig. 2.

We now form the N – D difference

$$\Delta G[\theta]=\Delta G[0]-\Delta C[0]s(\theta)T_0-(1/6)T_0^2\theta^3\Delta C'[0] \tag{8}$$

where we have chosen T_0 so that $\Delta S[0]=0$. That this choice is possible and convenient can be seen

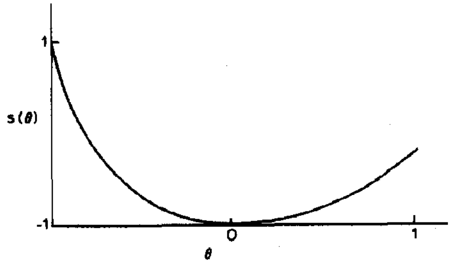

Fig. 2. The function $s(\theta)$, as defined by eq. 7.

from fig. 1a where T_0 may be identified as the temperature at which the two curves have the same slope; the corresponding entropy profiles are shown schematically in fig. 1b. Also, the data that lead us to construct fig. 1a have $\theta_L = (T_L - T_0)/T_0$ and $\theta_H = (T_H - T_0)/T_0$ in the range from $-0.2 < \theta < 0.2$; this is the range of θ with which we shall be concerned here.

From eq. 8 and fig. 2 we see that in the neighbourhood of $\theta = 0$ the free energy of denaturation in a skewed parabolic function of θ, the reduced temperature. The inclusion of a modest $\theta^3 \Delta C'$ contribution only affects the skew of the parabola. Thus, with or without the ΔC term, the two-state model thermodynamics predicts that $\Delta G(\theta)$ has the form of a distorted parabola which is consistent with the various results of Brandts [10,11] and Privalov [7]. Depending on $\Delta G[0]$ and the details of $\Delta C(T)$, this parabola may have zero at two temperatures corresponding, respectively, to the temperature T_H for heat denaturation and T_L for cold denaturation of the native protein.

3. Cold denaturation

A significant consequence of Brandts' experimental studies was the prediction that a relationship of the type shown in eq. 1 demanded that, in addition to the much-investigated thermal transition, there should exist a low-temperature transition at some temperature T_L, also given by eq. 1. Although the phenomenon of cold inactivation of enzymes is well known, until recently no thermodynamic information was available about low-temperature-induced ND transitions. The main reason is that for all proteins that have been studied in detail, the predicted T_L values lie well below the equilibrium freezing point of water, which makes T_L inaccessible to experimental determination in a homogeneous system. For obvious reasons, freezing must be avoided, because freeze denaturation is due not so much to low temperature as to the large increase in concentration of the protein and all other water-soluble species (mainly salts) during freezing [12].

Unlike heat denaturation, which is probably of little physiological or ecological relevance, the cold inactivation of proteins is implicated in cold tolerance and cold resistance of overwintering species. The process, as it applies to insect cold acclimation, has been studied by Storey et al. [13]. Jaenicke [14] has discussed the different forms which the $\Delta G(T)$ parabola can take, in connection with enzyme function under extremes of temperature, while Franks [12] has emphasized the physiological and ecological significance of reversible cold inactivation.

By employing the water-in-oil droplet emulsion technique to inhibit the nucleation of ice in the undercooled solution [15], Franks and Hatley [16] were able to monitor the stability of chymotrypsinogen at low temperatures and to identify T_L at 240 K. Subsequently, Hatley and Franks [17] extended these studies to lactate dehydrogenase in cryosolvents (aqueous methanol). More recently, Privalov et al. [18] have reported the cold unfolding of metmyoglobin, where the onset of the transition can, under suitable buffer conditions, be as high as 273 K. On the other hand, Fink and Painter [19] were unable to detect a cold denaturation of ribonuclease A in 70% aqueous methanol, where, on the basis of the parabolic $\Delta G(T)$ relationship, they expected it to lie near 228 K. Instead, they report that even at 203 K substantial enzyme activity could still be observed. However, an analysis of the ribonuclease stability profile [20], coupled with the respective effects of methanol on T_L and T_H of lactate dehydrogenase [21] suggests that 228 K would at best correspond to the onset of the N-D transition but not its completion. Regarding the reported enzyme activity at 203 K, presumably high enzyme concentrations would be required to achieve any detectable activity at such a low temperature. It is known that high protein concentrations enhance the stability against denaturation. The possibility must exist, therefore, that Fink and Painter stabilized ribonuclease against cold inactivation by the need to use high protein concentrations.

On the common assumption that ΔC for an order-disorder transition is always positive, then ΔH^*, which is positive for heat denaturation, is expected to decrease with decreasing temperature, pass through zero and then become negative, which is indeed observed experimentally. For chymotrypsinogen ΔH^* changes from 502 kJ mol^{-1} at 313 K to zero at 283 K and to -284 kJ mol^{-1} at 240 K [16]. The molecular origin of an exothermic order-disorder transition (negative latent heat of fusion) poses interesting problems.

4. Analysis of the $\Delta G(T)$ profile

In this section we explore the relation of the $\Delta G(T)$ function to an important aspect of the cold denaturation process. First we consider the case in which $\Delta C'(T) = 0$, as is usually assumed, and then the case $\Delta C'(T) = \Delta C'$, a constant value. Finally, we consider the case of $\Delta C'(T)$.

A. ΔC is independent of temperature:

$$\Delta C(T) = \Delta C(T_0) \text{ or } \Delta C[\theta] = \Delta C[0] \tag{9}$$

According to Privalov [7,18] this condition holds for N-D transitions of proteins. It is then possible to have $\Delta G(T_H) = 0$ as well as $\Delta G(T_L) = 0$. In view of fig. 1a, this is only possible if $\Delta C(T_0) < 0$. For a simple phase transition this condition seems to be anomalous, because it implies that $\Delta C(T_L) < 0$. However, it is probably not forbidden.

If $\Delta G(\theta) = 0$, then, according to eq. 8:

$$s(\theta) = (\Delta G[0])/(\Delta C[0]) \tag{10}$$

When this equation has two solutions, they are at θ_H and θ_L, such that

$$s(\theta_H) = s(\theta_L) \tag{11}$$

Thus, if θ_H is known, θ_L is the other root of this equation which may be solved, for example, by the use of fig. 2.

For chymotrypsinogen at pH 3, $T_H/T_L = 1.37$, so that the true parabolic relationship would not be expected to hold. However, since T_H/T_L decreases with decreasing pH, the parabolic approximation is probably warranted at low pH [16]. It is remarkable that such a curvilinear $\Delta G(T)$ relationship is obtained even when ΔC is independent of the temperature. It also implies that heating the system through T_L decreases the heat capacity, which seems unlikely, although not impossible. It seems more reasonable to lift the restriction that $\Delta C(T) = \Delta C(T_0)$.

Now we employ the full eq. 8. At a zero of $G[\theta]$ it reduces to

$$\Delta G[0] = \Delta C[0] s(\theta) T_0 - (1/6) T_0^2 \theta^3 \Delta C'[0]$$

If θ_H is known, we obtain θ_L by an iterative solution of

$$s(\theta_L) = s(\theta_H) - (T_0 \Delta C[0])^{-1} (1/6) T_0^2 \Delta C'[0] \times \left[\theta_H^3 - \theta_L^3\right]$$

Then

$$\Delta C[\theta_L] = \Delta C[0] + T_0 \theta_L \Delta C'[0] \tag{12}$$

with a corresponding expression for $\Delta C[\theta_H]$. Bearing in mind that according to eq. 8 the folding (renaturation) process is considered both at T_L and T_H, then eq. 12 shows that heating through T_L (renaturation) increases the partial heat capacity, just as does heating through T_H (denaturation).

Considering once again the two-state denaturation model, then for any given degree of denaturation x,

$$G = xG_D + (1-x)G_N$$

from which the heat capacity is obtained by differentiating twice:

$$C/T = \left[xC_D + (1-x)C_N\right]/T - \Delta G(T)(\mathrm{d}^2x/\mathrm{d}T^2) + \Delta S(T)(\mathrm{d}x/\mathrm{d}T) \tag{13}$$

Neglecting the relaxation terms in eq. 13, we write

$$C_B[\theta] = xC_D[\theta] + (1-x)C_N[\theta]$$

where the subscript B refers to 'baseline' values where $x = 0$ or 1, for the fully native and the fully denatured protein, respectively. Now when $x = 0$, then $\theta = 0$, but when $x = 1$, θ can take the values of $+1$ or -1. Thus,

$$C_B[1] > C_B[0] > C_B[-1]$$

and

$$C_D[1] > C_N[0] > C_D[-1]$$

Assigning a linear temperature dependence to C_D, we can write

$$C_D[0] + k_{DH} > C_N[0] > C_D[0] + k_{DL}$$

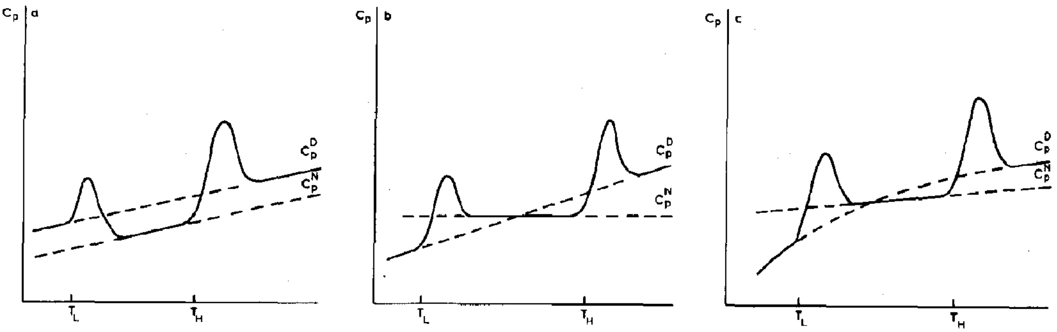

Fig. 3. Schematic DSC power-time curves for protein folding at T_L and unfolding at T_H: (a) according to the assumption of a constant ΔC (see eq. 9); (b) according to the model proposed in eq. 8, with a constant $\Delta C'$; (c) a general representation, based on the general form of eq. 8 with $\Delta C'(T)$. The model in panel c would require solution by iteration.

where k_{DH} and k_{DL} are, respectively, positive and negative increments which satisfy the inequality. Also

$$\Delta C[0] + k_{DH} > 0 > \Delta C[0] + k_{DL}$$

The thermodynamic consequences of the two-state denaturation model are displayed in the form of DSC power-time curves in fig. 3. Thus, heating the solution from some temperature $T < T_L$ gives rise to a renaturation endotherm at T_L with a corresponding enthalpy ΔH_L^*. This has been observed (although only partially) for metmyoglobin [17]. On further heating, the protein exhibits the well-known heat denaturation endotherm (ΔH_H^*) at T_H. Fig. 3a shows the temperature profiles of C_N and C_D, based on the assumption of a constant ΔC. Fig. 3b describes the simplest case of eq. 8, i.e., a constant $\Delta C'(T)$ and fig. 3c represents the general form of eq. 8.

5. Partial heat capacities of proteins and model polymers

The above analysis suggests that the common assumption that $d(\Delta C)/dT = 0$ is an oversimplification and that a linear relationship which has been claimed to exist between ΔH_H^* and temperature [1,7] cannot be taken for granted. Although over the limited stability range of the N-state it may be a permissible approximation, the extrapolation of ΔH^* far beyond the range of stability of the native state is unwarranted. Thus, in any integration of equations of the type described by eq. 3, it should not be assumed that $C^N(T) = C^D(T)$ and that ΔC is a constant, independent of temperature.

Unfortunately, there are as yet no experimental data against which the above models can be rigorously tested. Becktel and Schellman [6] have discussed the effects on $\Delta G(T)$ of a linear variation of ΔC with temperature in the neighbourhood of T_H. The stability profile would then be given by

$$\Delta G(T) = \Delta G_0(T) - b/2(T_H - T)^2$$

where $\Delta G_0(T)$ refers to the condition of constant ΔC and b describes the linear increment per degree in ΔC. They conclude that for a hypothetical protein, with $T_H = 323$ K at which temperature $\Delta H_H^* = 382$ kJ mol^{-1} and with a constant ΔC increment of 8.4 kJ mol^{-1} K^{-1}, a 10% increase in ΔC over the temperature range 298–348 K is unlikely, because cold denaturation should then occur at $T > 273$ K which, according to the authors, is not usually found at neutral pH and in the absence of perturbants. On the other hand, their analysis of experimental errors leads them to suggest that any change in $\Delta C(T)$ less than this (< 17 J K^{-2}) could probably not be detected. They do not consider the case of $\Delta G(T_L) = 0$ or the possibility that ΔC becomes negative at low temperatures (see section 4).

The measurement of partial heat capacities of proteins over a range of temperature is a matter of some difficulty, mainly because of their high molecular weights. It requires scanning calorimeters of high sensitivity to determine small heat capacity differences between a buffer solution and a dilute protein solution. For this reason most experimental protein investigations are limited to measurements of $\Delta H_{\mathrm{H}}^{*}$. On a few occasions ΔC values have been reported, but Pfeil and Privalov [22] have carried out the only comprehensive study of the partial heat capacities of a native protein (lysozyme) in the N-state as function of pH and temperature. They were able to construct the G-pH-T surfaces of the two states and hence to evaluate ΔG as a function of temperature and pH. They quote $C^{\mathrm{N}} = 19.136$ kJ mol^{-1} K^{-1} at 298 K with a temperature coefficient of 0.120 kJ mol^{-1} K^{-2}, independent of pH. ΔC is given as 6.56 kJ mol^{-1} K^{-1}, independent of pH and temperature, so that at 298 K, $C^{\mathrm{D}} = 25.696$ kJ mol^{-1} K^{-1}, with the same temperature coefficient as C^{N}. Their results correspond to the condition imposed by eq. 9 which yields a regular parabolic $\Delta G(T)$ profile, as suggested by their results. The actual $\Delta G(\mathrm{pH}, T) = 0$ contour is of complex appearance but shows distinct indications of a low-temperature region where, under appropriate conditions of pH, the N-state is unstable, although the region is experimentally inaccessible because T_{L} would lie well below the homogeneous ice nucleation temperature (233 K) for all pH values.

No independent published $C^{\mathrm{D}}(T)$ data exist against which the postulate of constant ΔC or the full eq. 8, can be tested, especially at or below T_{L}. However, very recent measurements, although covering only a limited temperature range, suggest that $C^{\mathrm{D}}(T)$ is indeed of a curvilinear form, whereas $C^{\mathrm{N}}(T)$ is linear to within the experimental uncertainty (P.L. Privalov, personal communication). Thus, at some low temperature the extrapolated curves would be expected to intersect.

Denatured globular proteins are believed to be substantially flexible macromolecules, with a considerable freedom of rotation about the dihedral angles [7]. This also appears to be the case for cold-denatured myoglobin [18]. To investigate the $C(T)$ behaviour of a flexible macromolecule over a wide temperature range, without the complexity of an N-D transition, we chose polyvinyl pyrrolidone (PVP) as a suitable candidate. Although the molecular architecture of the vinyl backbone differs from that of the polypeptide chain, the dimensions of both PVP and denatured globular proteins in aqueous solution can be described quite adequately by the laws of polymer statistics [23]. We therefore suggest that PVP is a suitable model and that $C^{\mathrm{PVP}}(T) \approx C^{\mathrm{D}}(T)$.

Recent partial molal heat capacity data for undercooled aqueous solutions of PVP of average degree of polymerization 390 over the temperature range 235–275 K demonstrate that, although $\mathrm{d}C/\mathrm{d}T$ is indeed positive, $\mathrm{d}^2C/\mathrm{d}T^2$ is large and negative [24]. Thus, when a 10% PVP solution is cooled, C changes sign at 253 K and reaches -600 J basemol^{-1} K^{-1} at 235 K, corresponding to -234 kJ mol^{-1} K^{-1}! At 273 K, $\mathrm{d}C/\mathrm{d}T$ is only of the order of 0.8 kJ mol^{-1} K^{-1} and is sensibly independent of concentration. The limiting partial heat capacity of PVP at 298 K is estimated as 58.5 kJ mol^{-1} K^{-1}, compared to 25 kJ mol^{-1} K^{-1} for denatured lysozyme. Since lysozyme has a degree of polymerization of 112, the average C^{D} per mol residue would be 223 J K^{-1}, compared to 150 J K^{-1} for PVP. These values are reasonably consistent with the average number of internal degrees of freedom of amino acid and vinyl pyrrolidone residues, respectively, assuming a similar average contribution from residue hydration interactions.

Still on the assumption that the flexible PVP molecule is a reasonable model for an unfolded peptide chain, these results demonstrate that ΔC must change sign at some temperature within the range $T_{\mathrm{L}} < T < T_{\mathrm{H}}$. Hypothetical DSC power-time curves for the native and denatured protein states and the postulated N-D transitions according to the two models are shown in fig. 3. Heating the solution through T_{H} (i.e., supplying kinetic energy to the system) is accompanied by the absorption of heat and an increase in the partial heat capacity, as would be expected for any order-disorder transformation. According to Privalov [7], the model in fig. 3a would adequately account for the experimental data, but his measurements are limited to the temperature range in the region of T_{H}. We believe that over a more extended temperature range, particularly in the neighbourhood of T_{L}, fig. 3b provides a more satisfactory account of $\Delta C(T)$, because any heat-induced transition, even of a disorder-order type, at T_L would be expected to exhibit a positive ΔC. If the PVP data can serve as a guide, then it is clear that fig. 3b is still a somewhat crude approximation: in the first place C^N should be allowed to increase with temperature, but also C^D probably decreases with decreasing temperature in a strongly nonlinear manner. A possible representation of the $C(T)$ behaviour is shown in fig. 3c; this could also explain the observation that ΔC appears to be independent of temperature in the region of T_H. Even so, the simple model in fig. 3b can account in a qualitative manner for both N-D transitions which we believe to be a general feature of globular proteins.

6. The molecular basis of cold denaturation

The phenomena of cold unfolding and cold dissociation are not confined to proteins. Similar effects are observed for micellar solutions of surfactants, where critical micelle concentrations exhibit minima at given temperatures, e.g., 299 K for SDS and for dodecyl pyridinium chloride [25]. The denaturation behaviour of proteins is also analogous to lower and upper critical demixing phenomena (closed solubility loops) frequently encountered in aqueous polymer solutions, e.g., polyethylene glycols. A common feature of the molecules (and ions), including PVP, which exhibit both cold and heat destabilization in aqueous solution is the delicate balance between hydrophobic and polar hydration interactions [24].

Friedman and Krishnan [26] have discussed the $C(T)$ curves of tetraalkylammonium halides, which resemble that of PVP, in terms of the relative contributions of hydrogen bonding and hydrophobic hydration. The negative C values indicate that at low temperatures water-solute hydrogen bonding provides the major contribution, and water is a 'good' solvent in the Flory-Huggins sense. These interactions favour maximum exposure of polar groups to the solvent and promote cold unfolding. With increasing temperature, hydrogen bonding becomes progressively weaker, in favour of intra- and intermolecular hydrophobic interactions which, at physiological temperatures, provide the major contributions to C and confer stability on the folded state. At still higher temperatures the input of kinetic energy will destroy the delicate balance between the hydrophobic interactions (stabilizing) and the configurational entropy (destabilizing) on which the N-state depends.

The above reasoning suggests that the low- and high-temperature-induced D-states might differ in the sense that at T_L the protein folding is destroyed but secondary (hydrogen-bonded) domains are maintained intact. Such an interpretation is supported by the remarkable degree of reversibility of cold denaturation, even at high protein concentrations [16,17]. On the other hand, the CD and NMR spectra reported for cold- and heat-denatured metmyoglobin [18] exhibit remarkable similarities but differ from those for the N-state, lending support to the existence of a common D-state at $T < T_L$ and $T > T_H$. A reliable structural description of cold-inactivated proteins will be feasible only after more detailed studies of the N-D transition at low temperatures have been performed. There can, however, no longer be any doubt about the existence of the phenomenon or its physiological and ecological importance.

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