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THERMODYNAMIC BASES OF THE STABILITY OF PROTEIN STRUCTURE

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One can judge the stability of any structure by studying its disruption; thus the stability of a protein can be determined by studying its denaturation. Since a protein molecule is a macroscopic system, the disruption of its structure should be regarded as a change of the macroscopic state of the system.

The native protein structure can be disrupted by changing different intensive variables specifying the external conditions, such as temperature, pressure, pH, and concentration of denaturants. However, the information on the energetic basis of a protein structure can be obtained only by using temperature as a variable, because temperature and energy, or enthalpy, of a system are the conjugate intensive and extensive variables determining the state of a macroscopic system. The functional dependence between these two basic thermodynamic parameters includes all thermodynamic information on the macroscopic states of a system in the considered temperature range and permits us to analyze in detail the process of disruption of the native protein structure and the reverse process of formation of this structure.

Experimentally, the temperature dependence of enthalpy can be determined only by direct measurements of the thermal energy absorbed upon heating of the studied material, i.e. only by calorimetric measurements of the excess heat capacity of this material, $\langle \Delta C_p(T) \rangle$, in the considered temperature range:

$$\langle \Delta H(T) \rangle = \int_0^T \langle \Delta C_p(T) \rangle \, dT \qquad (1)$$

Since in the case of protein one is interested in the intramolecular disruption process, it is necessary to measure the heat capacity of the protein in a solution dilute enough that the effect of the interaction between proteins is negligible. But in such a solution the heat effect that should be measured, the thermal energy absorbed by the protein molecule upon heating, is

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also small, especially against the background of intensive heat absorption by the solvent, which dominates in dilute solutions. Therefore, studies of protein thermodynamics required the development of a special supersensitive calorimetric technique, which is now known as scanning microcalorimetry (reviewed in ref. 1).

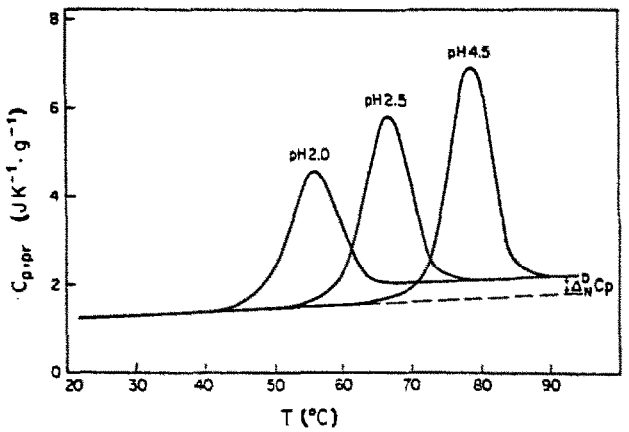

Fig. 1. Temperature dependence of the partial heat capacity of lysozyme in solutions with various pH values (ref. 2).

The native structure is stable up to a certain critical temperature, which depends on the environmental conditions (e.g. pH, ionic strength, presence of denaturants), and then disrupts with intense heat absorption (Fig. 1). Detailed thermodynamic analysis of the heat absorption profile shows that two macroscopic states predominate in the denaturation process, native and denatured. The population of all the intermediate states does not usually exceed 5%, which shows that they are highly unstable. Thus, a small globular protein represents a single extremely cooperative macroscopic system (ref. 2).

Since a single-domain small globular protein has only two discrete macroscopic states, native (N) and denatured (D), one can describe this macroscopic system by two surfaces in the phase space corresponding to extensive thermodynamic functions of these states. The transition between these states is determined by the differences of enthalpy,

$$\Delta_N^D H(T,pH,a_i) = H^D(T,pH,a_i) - H^N(T,pH,a_i), \quad (2)$$

entropy,

$$\Delta_N^D S(T,pH,a_i) = S^D(T,pH,a_i) - S^N(T,pH,a_i), \quad (3)$$

and Gibbs energy,

$$\Delta_N^D G(T,pH,a_i) = G^D(T,pH,a_i) - G^N(T,pH,a_i)$$

$$= \Delta_N^D H(T,pH,a_i) - T\Delta_N^D S(T,pH,a_i) \quad (4)$$

The midpoint of the transition is determined by the condition

$$\Delta_N^D G(T,pH,a_i) = \Delta_N^D H(T,pH,a_i) - T\Delta_N^D S(T,pH,a_i) = 0 \quad (5)$$

If the temperature is used as a variable, then the enthalpy of transition between the native and denatured states can be determined experimentally from the area of the heat absorption peak (Fig. 1). As follows from equation 7, the entropy of transition is

$$\Delta_N^D S(T_G) = [\Delta_N^D H(T_G)]/T_G \quad (6)$$

where T_G is the temperature at which concentrations of the native and denatured proteins are equal and $\Delta_N^D G=0$.

One of the most specific features of protein denaturation is a significant heat capacity increment that always accompanies this process (Fig. 1). As

$$\Delta C_p = (\partial \Delta H)/(\partial T), \quad (7)$$

$$\Delta C_p/T = (\partial \Delta S)/(\partial T), \quad (8)$$

it follows that the enthalpy and entropy difference of the native and denatured states should strongly depend on temperature:

$$\Delta_N^D H(T) = \Delta_N^D H(T) + \int_{T_G}^{T} \Delta_N^D C_p dT, \quad (9)$$

$$\Delta_N^D S(T) = \Delta_N^D S(T) + \int_{T_G}^{T} [(\Delta_N^D C_p)/T] dT \quad (10)$$

The dependence of the denaturation enthalpy on temperature can be observed directly by studying, at different temperatures, protein denaturation caused by varying pH or denaturant concentration. The enthalpy is always a universal function of temperature if its value is properly corrected for the heat effect of ligand binding (ref. 3) (Fig. 2). It follows that the denatured states of protein obtained by heating, pH variation, or the activity of denaturants are similar from the thermodynamic point of view. In all cases the heat capacity increments of denaturation are indistinguishable.

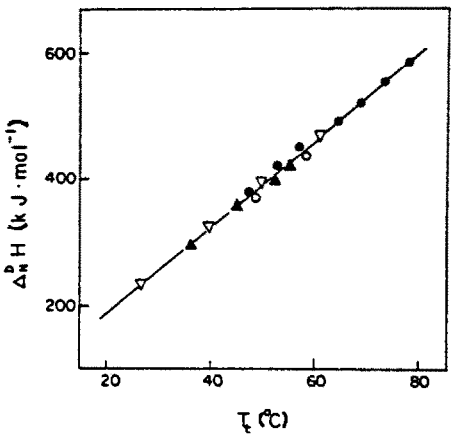

Fig. 2. Enthalpies of lysozyme denaturation obtained by various methods and under different conditions plotted against the temperature of denaturation. *Circles* indicate solutions without guanidinium chloride (GuHCl): *open*, denaturation by temperature at fixed pH; *filled*, denaturation by pH at fixed temperatures. *Triangles* indicate solutions with GuHCl: *filled*, denaturation by temperature at fixed concentration of GuHCl; *open*, denaturation by GuHCl at fixed temperatures (ref. 3).

It appeared in earlier experiments that the heat capacity increment of denaturation does not depend on temperature (see Fig. 1), and this was confirmed by the observed linear dependence of the denaturation enthalpy on temperature (Fig. 2). However, recent studies carried out over a broad temperature range (ref. 4) showed that the heat capacity of the native protein and that of the denatured protein do not change in parallel as temperature increases (Fig. 3). While the heat capacity of the native state is likely to be a linear function of temperature (in any case in the range from zero to 80°C, in which the native state can be

practically studied), the heat capacity of the denatured state is a nonlinear function, which asymptotically approaches some constant level at high temperature. Linear extrapolation of the heat capacity of the native state above 80°C indicates that $\Delta^D_N C_p$ is likely to decrease to zero at about 140°.

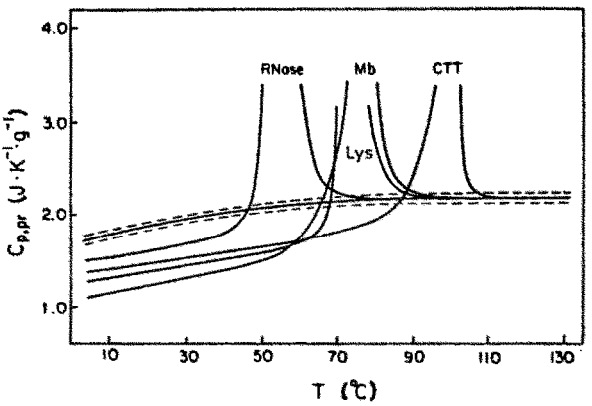

Fig. 3. Temperature dependence of the partial specific heat capacity of pancreatic ribonuclease (RNase), hen egg white lysozyme (Lys), sperm whale myoglobin (Mb), and catalase from *Thermus thermophilus* (CTT). The flattened curves represent RNase and Lys with disrupted disulfide cross-links and apomyoglobin whose polypeptide chains have a random coil conformation without noticeable residual structure (ref. 4).

The main consequence of the decrease in the heat capacity increment of denaturation with temperature increase is that the enthalpy and entropy of protein denaturation are increasing functions of temperature that asymptotically approach definite levels at about 140°C (Fig. 4). These levels are likely to be universal for the specific values of the enthalpy and entropy of all compact globular proteins (refs. 4, 5). If one neglected the temperature dependence of $\Delta^D_N C_p$, then the specific enthalpy and entropy of all globular proteins would come to the same values, but at about 110°C. However, in this case one could hardly suggest any physical meaning for these universal values, as the enthalpy and entropy functions increase continuously above 110°C. Since in reality an infinite increase of these functions is improbable, the assumption that $\Delta^D_N C_p$ decreases with temperature increase is more justified than the assumption that it is independent of temperature.

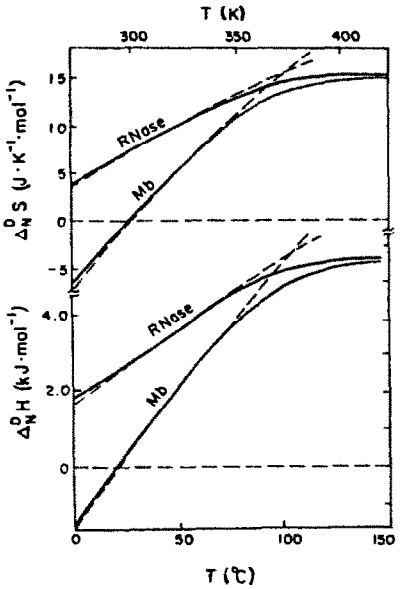

Fig. 4. Temperature dependence of the $\Delta_N^D H$ and $\Delta_N^D S$ functions of RNase and Mb when $\Delta_N^D C_p$ is assumed to be decreasing (solid line) or constant (*dotted-and-dashed line*) as the temperature increases (ref. 5).

Another peculiarity that follows from equations 9 and 10 is that both the $\Delta_N^D H$ and $\Delta_N^D S$ functions decrease upon temperature decrease and at some temperature become zero and then change their sign. Temperature T_S, at which $\Delta_N^D S=0$, must always exceed temperature T_H, at which $\Delta_N^D H=0$. Indeed, for simplicity let us assume in the first approximation that $\Delta_N^D C_p$, which is not very sensitive to the temperature in the vicinity of T_S and T_H, does not depend on temperature. In this case from equations 9 and 10 we have:

$$\Delta_N^D H(T) = \Delta_N^D H(T_G) - (T-T_G)\Delta_N^D C_p, \qquad (11)$$

$$\Delta_N^D S(T) = [\Delta_N^D H(T_G)]/T_G + \Delta_N^D C_p \ln T/T_G. \qquad (12)$$

Thus for $\Delta_N^D G(T)$,

$$\Delta_N^D G(T) = \Delta_N^D H(T) - T\Delta_N^D S(T)$$

$$= [(T_G-T)/T_G]\Delta_N^D H(T_G) + \int_{T_G}^{T} \Delta_N^D C_p(T)dT - T\int_{T_G}^{T} \Delta_N^D C_p(T)d(\ln T)$$

$$\cong [(T_G-T)/T_G]\Delta_N^D H(T_G) + (T-T_G)\Delta_N^D C_p - T\Delta_N^D C \ln (T_G/T). \qquad (13)$$

Then

$$T_H \cong T_G - [\Delta_N^D H(T_G)]/\Delta_N^D C_p, \qquad (14)$$

$$T_S \cong T_G/([\Delta_N^D H(T_G)]/(\Delta_N^D C_p T_G) + 1) = T_G^2/(2T_G-T_H), \qquad (15)$$

and for the difference of these values

$$T_S - T_H = [[\Delta_N^D H(T_G)]/(\Delta_N^D C_p T_G)]^2 T_G, \qquad (16)$$

which is always positive (ref. 6).

The temperature shift of the enthalpy and entropy functions is very important for stabilization of the native protein structure.

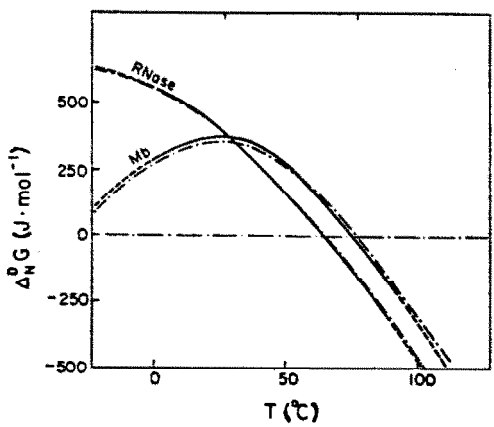

Fig. 5. Temperature dependence of the $\Delta_N^D G$ function of RNase and Mb when $\Delta_N^D C_p$ is assumed to be decreasing (*solid line*) or constant (*dotted-and-dashed line*) as the temperature increases (ref. 5).

Stability of protein is usually expressed in the Gibbs energy values, since $\Delta_N^D G$ is the work required for disruption of the native protein structure. As can be seen from Fig. 5, the extremum of $\Delta_N^D G$ (equation 13) is not very sensitive to the $\Delta_N^D C_p$ dependence on temperature. The maximum $\Delta_N^D G$ is reached when

$$(\partial\Delta_N^D G)/(\partial T) = -\Delta_N^D S = 0, \qquad (17)$$

i.e. at temperature T_S. Thus, the native state of protein is most stable at the temperature where the entropy difference of the native and denatured states is zero, and it is stabilized only by the enthalpy difference of these states (ref. 3).

At temperatures above and below T_S, $\Delta_N^D G$ should decrease and, correspondingly, should decrease the stability of the native state. One can expect, therefore, that zero stability of the native state is reached at two difference temperatures, at high temperature T_G, at which the heat denaturation of protein is observed, and a low temperature T_G',

$$T_G' = T_G^2/([2\Delta_N^D H(T_G)/\Delta_N^D C_p] + T_G) \cong T_G^2/(3T_G - 2T_H), \qquad (18)$$

at which cold should also cause the breakdown of the native structure. The distinguishing feature of these two processes is that while heat denaturation proceeds with heat absorption and thus with increase of the enthalpy and entropy, cold denaturation proceeds with a release of heat, i.e. with enthalpy and entropy decrease, since below T_S and T_H both these functions change their sign.

Cold denaturation of proteins, which follows from the thermodynamic formalism, appears paradoxal, since one can hardly expect a priori that the breakdown of the ordered native structure could result in an entropy decrease, i.e. in an increase in the order of the system. Therefore experimental demonstration of cold denaturation phenomena was regarded as a crucial test for the correctness of the protein thermodynamic theory presented above. However, direct observation of the proposed phenomena was a complicated experimental task, since the predicted values of T_G' for all the studied proteins were far below the freezing point of aqueous solutions.

The direct demonstration of protein denaturation with heat release upon cooling was recently achieved by supercooling aqueous solutions of protein below 0°C and studying them by various experimental techniques, including scanning microcalorimetry. It has been shown for a number of globular proteins under different solvent conditions [myoglobin (ref. 6),

apomyoglobin (ref. 7) and staphylococcal nuclease (ref. 8)] that upon cooling, the compact protein structure unfolds, releasing heat (Fig. 6). This process is highly reversible; if the cooled protein solution is heated, the protein folds back, absorbing the heat. All the studied cases (Fig. 7) are satisfactorily described by the thermodynamic equations presented above, which confirms that the denaturation of a single-domain protein can be regarded in good approximation as a transition between the two macroscopic states, which differ significantly in their heat capacity.

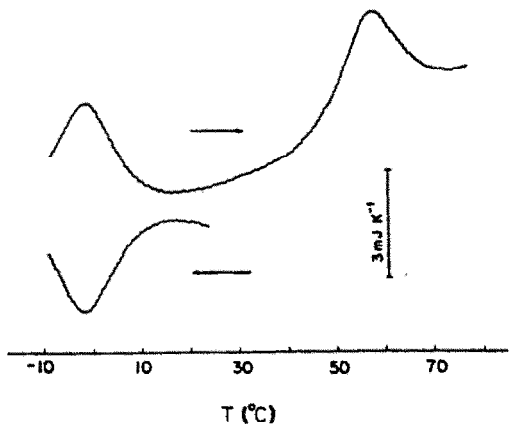

Fig. 6. The heat effect observed upon cooling and subsequent heating of apomyoglobin solution (ref. 7).

One of the most general characteristics of protein denaturation is the heat capacity increment, which is the same upon heat, cold, acid, or guanidinium chloride (GuHCl) denaturation and is specific for a given protein. As for the enthalpy and entropy of denaturation, they can be positive, negative, and even zero, depending on temperature. However, it is most surprising that at high temperatures the specific values of these functions are similar for all globular proteins studied. What do the asymptotic values of the denaturation enthalpy and entropy mean and why are they apparently universal for very different proteins? Why should the denaturation enthalpy and entropy depend so much on temperature and consequently have negative values at low temperature?

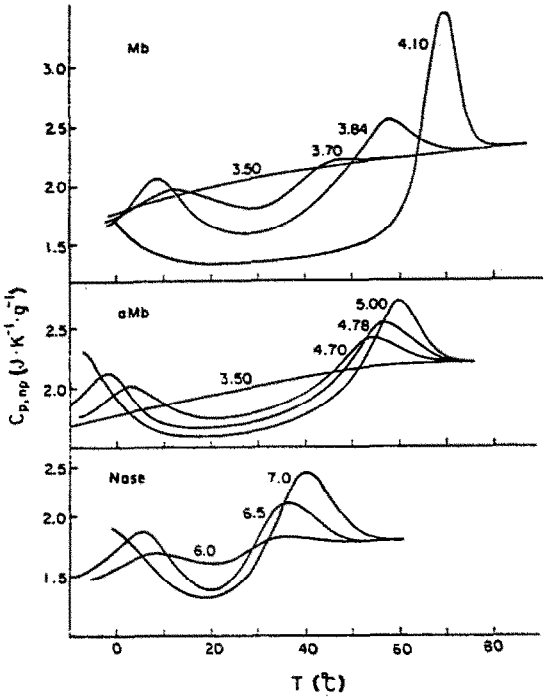

Fig. 7. The temperature dependence of the partial heat capacities of metmyoglobin (Mb) (ref. 6), apomyoglobin (aMb) (ref. 7), and staphylococcal nuclease (Nase) (ref. 8) in solutions with different pH values.

Since entropy is a measure of disorder in a system, the negative entropy of denaturation might only mean that at low temperature the native state is less ordered than the denatured one, notwithstanding the much higher order in the arrangement of the polypeptide chain in the native protein. This paradox can be resolved if one takes into account that the partial entropy of a protein molecule in solution is determined not only by its conformation, but also by the state of the solvent in the vicinity of the protein.

Dissolution of a nonpolar molecule in water leads to a decrease in the entropy of the system owing to ordering of the water molecules, while a decrease in this order with temperature gives the solution excess heat capacity. It is assumed that unfolding of the compact protein structure with the exposure of internal nonpolar groups to water should also lead to the ordering of water molecules; the extent of this ordering should

decrease with increasing temperature. An extra energy expenditure upon the gradual "melting" of water ordered by the exposure of nonpolar groups of the unfolded protein is indeed the only reasonable explanation for most of the denaturation heat capacity increments observed upon a temperature increase (ref. 6).

On the other hand, if the observed heat capacity increment of denaturation is caused mainly by melting water ordered by exposed nonpolar groups of protein, one would expect that the influence of these groups on the surrounding water should vanish at some temperature T_0. I propose that this should be the temperature at which the specific enthalpies and entropies of denaturation of various proteins become equal, which is about 140°C according to recent calorimetric studies. This suggestion was confirmed by the finding that at about the same temperature the entropy of transfer of various nonpolar substances from the liquid phase to water becomes zero, i.e. these substances no longer affect the order of the water (ref. 6).

If one neglects the dependence of the denaturation heat capacity increment on temperature, then T_0 decreases to about 110°C (Fig. 4). This modification does not notably affect the $\Delta_N^D G$ function (Fig. 5). Therefore, considering protein stability, in the first approximation one can neglect the temperature dependence of $\Delta_N^D C_p$ and present the $\Delta_N^D H$ and $\Delta_N^D S$ functions in the following way:

$$\Delta_N^D H(T) = \Delta_N^D H(T_0) - (T_0-T)\Delta_N^D C_p, \qquad (19)$$

$$\Delta_N^D S(T) = \Delta_N^D S(T_0) - [\ln (T_0/T)]\Delta_N^D C_p$$

$$\approx \Delta_N^D S(T_0) - [(T_0-T)/T]\Delta_N^D C_p - \tfrac{1}{2}[(T_0-T)/T]^2\,\Delta_N^D C_p \qquad (20)$$

Here $\Delta_N^D H(T_0)$ and $\Delta_N^D S(T_0)$ are temperature-independent parts of the enthalpy and entropy of protein denaturation that do not include the effects of water ordering by the protein's nonpolar groups. Then, for the Gibbs energy of stabilization of the native protein structure we have:

$$\Delta_N^D G(T) = \Delta_N^D H(T) - T\Delta_N^D S(T)$$

$$\cong \Delta_N^D H(T_0) - T_N^D S(T_0) - \tfrac{1}{2}\Delta C_p[(T_0-T)^2/T]. \qquad (21)$$

In this expression, only the first term is positive, i.e. the enthalpy of protein unfolding in the absence of water solvation effects from the protein's non-polar groups. This term represents the temperature-independent contribution of van der Waals and hydrogen bonds in the stabilization of the protein's compact structure. The second term, which is negative and increases with temperature, represents the disordering action of dissipative forces. The third term merely expresses the contribution of water solvation by nonpolar groups in the stabilization of the protein's native structure. The most remarkable feature of this term is that it is negative and that its value decreases to zero as the temperature increases to T_0 (Fig. 8). Therefore at all temperatures below T_0, water solvation by protein nonpolar groups leads to a decrease in the stability of the native compact state; stability is maintained only by the enthalpic interactions, i.e. by van der Waals and hydrogen bonding.

This conclusion disagrees with the widespread opinion that water solvation by nonpolar groups is responsible for the hydrophobicity of these groups and for the stability of the compact state of a protein molecule. Hydrophobic interactions are usually taken to include, in fact, not only the effect of the hydration of nonpolar groups, but also the van der Waals interaction between these groups, which is far from negligible, contrary to what was previously supposed (for details see ref. 6). It is important that these two effects are of opposite sign and of a different range; the van der Waals interaction is short-range, while the hydration effect is long-range. Therefore, the hydrophobic interaction should be attractive at short distances and repulsive at long distances (exceeding the size of a water molecule). This might be one of the reasons for the extreme cooperativity of a tightly packed native domain.

It is remarkable that for all proteins studied the value of the $\Delta_N^D G$ function determining the stability of the native state does not exceed 50 $kJ \cdot mol^{-1}$ (ref. 3). Since the cooperative domain usually includes about 100 amino acid residues, it appears

that the contribution of each of the residues in stabilization of the native structure does not exceed 500 J per mole of residue.

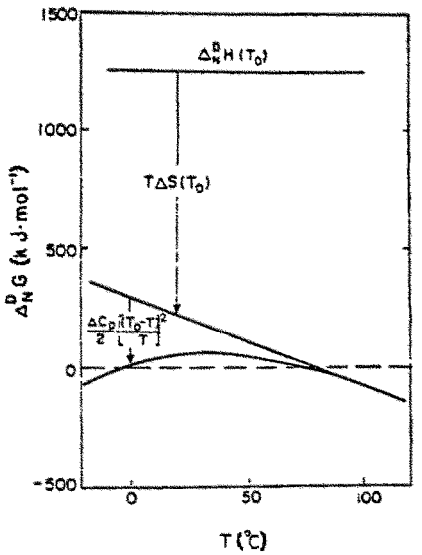

Fig. 8. Contributions of the dissipative forces $T\Delta S(T_0)$ and the water solvation effect $(\Delta C_p/2)[T_0-T)/T]^2$ to the stabilization of the native state of globular protein (ref. 6).

This value is five times less than that of the energy of thermal motion at room temperature. It follows that protein has an ordered native structure only because protein is a cooperative system whose components can change their state only cooperatively. In other words, the stability of such a system is determined by an integral contribution of all the components of the system. The stability of a cooperative domain exceeds by almost 20 times the energy of thermal motion; this stability is quite sufficient to ensure the existence of the ordered structure of the domain. It is this requirement for stability that seems to determine the lower limit of the size of the cooperative unit: The must include at least 50 amino acid residues to be stable enough at physiological temperature.

Thus the secret of the stability of the native structure of protein is not in the magnitude of intramolecular interactions, which are always too weak to withstand individually the

dissipative action of thermal motion, but in the effective cooperation of these interactions.

At present we know little about the mechanism of cooperation of the intramolecular interactions in proteins. Extreme cooperativity when all the elements of the system are integrated into a single unit seems to be achieved only in molecules with tight and unique packing of groups. In other words, extreme cooperativity is a peculiarity of the aperiodic structure. It looks as if only such a structure can provide the complex interlacing of all the short- and long-range interactions between groups in the polypeptide chain that is necessary for their cooperation.

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