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The extra-stability of thermophilic globular proteins: a thermodynamic approach☆

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Abstract

In this paper a general thermodynamic analysis of the Gibbs energy change associated with the two-state denaturation process of small globular proteins is presented. The proposed "parabolic approximation" to the Gibbs energy change, apart from an analytical relationship for calculating the hot and cold denaturation temperatures, emphasizes the limiting thermodynamic mechanisms that a globular protein can exploit to increase its thermostability. These mechanisms are critically discussed, by stressing the fact that, actually, they are mutually dependent, due to the strong temperature-dependence of denaturation enthalpy and entropy changes.

Keywords: Denaturation enthalpy; Gibbs energy change; Globular proteins; Protein denaturation; Protein extra-stability; Protein tertiary structure; Thermal stability; Two-state denaturation

1. Introduction

The quantitative understanding and rationalization of the relationship between a specific folding pattern and the thermodynamic stability of protein tertiary structure are strictly bonded to the classic problem of protein folding, since Anfinsen demonstrated the applicability of "thermodynamic hypothesis" to this biologically fundamental process [1]. The native, fully active structure of a globular protein, being due to a subtle balance between stabilizing destabilizing interactions, results in only marginal stability: the Gibbs energy of stabilization, for proteins from mesophilic organisms,

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corresponds to 20–60 kJ mol^{-1} at room temperature [2]. However thermophilic bacteria are able to live in extreme temperature conditions and their proteins are still fully active at temperatures above 70–80°C [3]. The strong correlation between structure and function requires a very effective interlocking between rigidity and flexibility. The description of this fine tuning mechanism is a hard challenge as it involves a very great number of weak noncovalent interactions.

It is intriguing and fascinating to try to understand how Nature has optimized the balance between stabilizing and destabilizing interactions in order to permit life even in these extreme conditions. The investigations devoted to the elucidation of factors responsible of this extrastability have acquired great interest from a technological point of view. Really they can give a theoretical rationalization and a guide for the modern methods of genetic engineering applied to create mutant proteins that, apart from a fully biological activity, possess a greater thermal stability [4, 5].

In this paper, starting from the well estabilshed experimental findings that the profile of Gibbs energy change associated with a two-state denaturation process as a function of temperature is represented by a skewed parabola [6], a simple and direct thermodynamic analysis is developed to emphasize how the different main parameters determine the stability range of the tertiary structure of globular proteins. Even if the two-state denaturation process is not a rule for proteins, an analysis based on this model can be used in an heuristic manner to shed light on more complex processes. This approach enables identification of the limiting thermodynamic conditions that can guarantee an extra-stability of native conformation. Finally, through the analysis of experimentally characterized cases, the limitation of a strict separation and independence of the limiting mechanisms is critically discussed.

2. Thermodynamic analysis

The thermodynamic stability of a globular protein that unfolds in a reversible manner according to a two-state transition $N \Leftrightarrow D$ is quantitatively measured by the denaturation Gibbs energy function $\Delta_d G^\circ(T) = G_D(T) - G_N(T)$, where G_D and G_N are the Gibbs energies of denatured and native states, respectively. This function represents the work necessary to destroy the native structure. From experimental investigations with both spectroscopic and calorimetric methods [7] it has always been found that the profile of $\Delta_d G^\circ$ function is highly characteristic. Indeed the Gibbs energy change assumes the form of a skewed parabola with a point of maximum and two temperatures where $\Delta_d G^\circ = 0$, usually called cold and hot midpoint denaturation temperatures [8–12]. In Fig. 1 is reported the plot of $\Delta_d G^\circ$ versus T, obtained for bovine pancreatic ribonuclease A at pH 5.0, 0.1 M acetate buffer [13]. Schellman and Becktel [14] called such plots "protein stability curves".

The maximum of the thermodynamic stability of native conformation with respect to the denatured one occurs at $T = T_{max}$. In the case of globular proteins from mesophilic organisms this temperature is always lower than the physiological temperature (i.e. where these macromolecules carry out their biological functions), and usually lower or close to room temperature. This means that under physiological conditions the

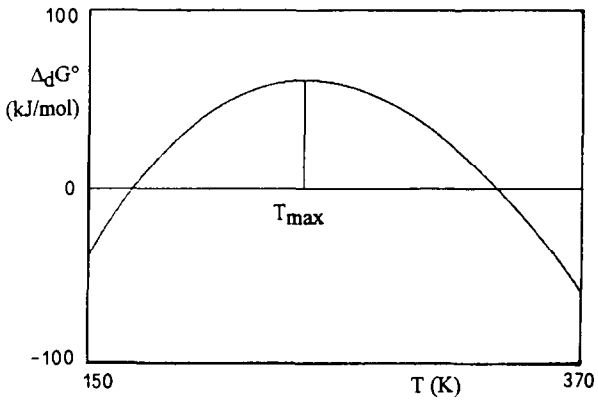

Fig. 1. Stability curve, $\Delta_d G°$ vs T, of bovine pancreatic ribonuclease A at pH 5.0, 0.1 M acetate buffer, determined from DSC measurements. The experimental themrodynamic parameters are: $T_{hd} = 61.3°C$, $\Delta_d H°(T_{hd}) = 456$ kJ mol^{-1}, $\Delta_d C_p^\circ = 6.0$ kJ (K mol)$^{-1}$.

proteins are usually less rigid or more flexible than at T_{max}. The physiological temperature is intermediate between T_{max} and the temperature of incipient equilibrium denaturation or very near to the latter.

The stability of globular proteins is strongly dependent on temperature, pressure, pH, and the presence of denaturing or stabilizing agents. The present analysis takes into account only the temperature-dependence of $\Delta_d G°$. It may be significant to expand in Taylor power series the function $\Delta_d G°(T)$ around its maximum, T_{max}, to better characterize the condition of maximum thermodynamic stability of the native structure. Elementary thermodynamics dictates that T_{max} coincides with T_s, the temperature where the denaturation entropy change is zero and in the following we will use always T_s instead of T_{max}. The general expression for the Gibbs energy of denaturation results:

$$\Delta_d G°(T) = \Delta_d G°(T_s) + [\partial \Delta_d G°/\partial T]_{T_s}(T - T_s) + 1/2[\partial^2 \Delta_d G°/\partial T^2]_{T_s}(T - T_s)^2 + 1/3![\partial^3 \Delta_d G°/\partial T^3]_{T_s}(T - T_s)^3 + \ldots\ldots \quad (1)$$

Clearly because T_s corresponds to the point where $\Delta_d S° = 0$:

$$\Delta_d G°(T_s) = \Delta_d H°(T_s) \quad (2)$$

Then the point of maximum thermodynamic stability of the native structure is entirely due to an enthalpic contribution. This term may be evaluated with conformational energy calculations [15, 16], keeping in mind that it is an oversimplification to assume that the denatured state is a random coil [17], as confirmed by recent reports [18–20].

Other terms of power expansion are readily calculated assuming, as usual, that the denaturation heat capacity change $\Delta_d C_p^\circ$, is temperature-independent. Although, Privalov et al. have shown, by performing DSC measurements in the range $-5°C$–$120°C$, that $\Delta_d C_p^\circ$ is temperature-dependent [21], its temperature-dependence does not significantly affect the Gibbs energy function [14]. So with $\Delta_d C_p^\circ$ = constant, the

expression for $\Delta_d G^\circ(T)$ becomes:

$$\Delta_d G^\circ(T) = \Delta_d H^\circ(T_s) - 1/2(\Delta_d C_p^\circ/T_s)(T - T_s)^2 + 1/6(\Delta_d C_p^\circ/T_s^2)(T - T_s)^3 + \ldots \quad (3)$$

The accuracy of this relationship is verified for a number of simulated and experimental two-state transitions: the profile of $\Delta_d G^\circ(T)$ calculated with Eq. (3) practically matches that obtained from the exact thermodynamic equation:

$$\Delta_d G^\circ(T) = \Delta_d H^\circ(T_{hd})(1 - T/T_{hd}) + \Delta_d C_p^\circ[T - T_{hd} - T \ln(T/T_{hd})] \quad (4)$$

that can be drawn by determining the experimental values of T_{hd}, $\Delta_d H^\circ(T_{hd})$ and $\Delta_d C_p^\circ$ by means of DSC or spectroscopic measurements, where T_{hd} stands for the hot denaturation temperature (for a detailed thermodynamic treatment of a two-state transition and some tests to ascertain its validity, see Refs. [14] and [22]).

However, as can be seen from Fig. 2, the quadratic expression also gives a good approximation of the thermodynamic $\Delta_d G^\circ(T)$ function. On the basis of this finding, in the following we will make use, for the denaturation Gibbs energy function of the expression:

$$\Delta_d G^\circ(T) \approx \Delta_d H^\circ(T_s) - 1/2(\Delta_d C_p^\circ/T_s)(T - T_s)^2 \quad (5)$$

This will be called the "parabolic approximation" of the denaturation Gibbs energy change. From this equation it is possible to derive the two denaturation temperatures, cold and hot, by imposing the condition $\Delta_d G^\circ(T_{c,hd}) = 0$, where $T_{c,hd}$ represents the values of the two roots of the quadratic equation. With simple algebraic calculations the following analytical expression is obtained:

$$T_{c,hd} = T_s \pm [2\Delta_d H^\circ(T_s)\, T_s/\Delta_d C_p^\circ]^{1/2} \quad (6)$$

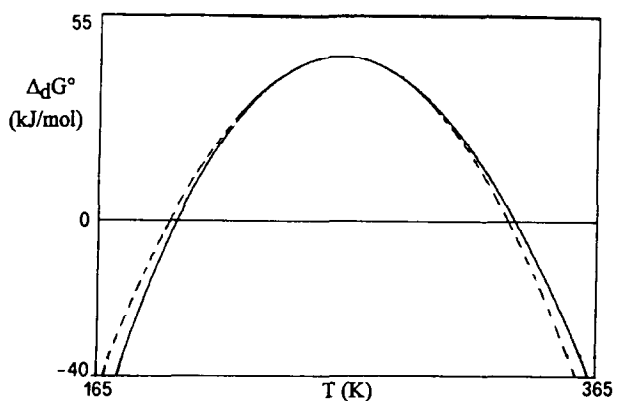

Fig. 2. Comparison between the stability curve, $\Delta_d G^\circ$ vs T, obtained from the thermodynamic relationship, Eq. (4), continuous line, and that calculated using Eq. (5), broken line. The values of thermodynamic parameters are: $T_{hd} = 333$ K, $\Delta_d H^\circ(T_{hd}) = 400$ kJ mol^{-1}, $\Delta_d C_p^\circ = 5.0$ kJ (K mol)$^{-1}$; $T_s = 262$ K and $\Delta_d H^\circ(T_s) = 44.4$ kJ mol^{-1}.

This relationship enables the establishment of the thermodynamic conditions required to enlarge the temperature range where the native fully active structure is stable. It must be noted that, with the adopted parabolic approximation, the range is symmetrical with respect to $T_{max} = T_s$, and its enlargement causes a raising of T_{hd} and a lowering of T_{cd}.

The limiting thermodynamic mechanisms for extra-stability of native structure with respect to the temperature are:

a) an increase of the value of $\Delta_d H^\circ(T_s)$, that means the extra-stability is due to purely energetic factors;

b) a decrease of the vlaue of $\Delta_d C_p^\circ$, that means the extra-stability can be ascribed to entropic factors, because a decrease of $\Delta_d C_p^\circ$ may be correlated with a decrease of $\Delta_d S^\circ(T_{hd})$. Indeed, considering the MacLaurin power series of $\Delta_d S^\circ$ and retaining only the first two terms, gives:

$$\Delta_d S^\circ(T_{hd}) \approx [\Delta_d C_p^\circ / T_s](T_{hd} - T_s) \tag{7}$$

This relationship, although approximate, supports the idea of linking a decrease of denaturation heat capacity change to a decrease of denaturation entropy change at $T = T_{hd}$. It is possible to give an interpretation at molecular level of this fact. It is firmly estabilshed that the large and positive values of $\Delta_d C_p^\circ$ are due to the exposure to water contact of apolar groups previously buried in the protein "core" [23,24]. A lower value of $\Delta_d C_p^\circ$ for a protein relative to that of another homologous reference protein must be associated with a lower degree of exposure to water of apolar side-chains in the denatured state. That is, the denatured state is less disordered and characterized by a reduced number of conformational degrees of freedom. This, as consequence, corresponds to a lower value of $\Delta_d S^\circ(T_{hd})$ because the denatured state possesses some degree of structural order.

These limiting cases, derived from the analysis of Eq. (6), are shown in Fig. 3 and 4, which illustrate the two mechanisms that enable enlargement of the range of native

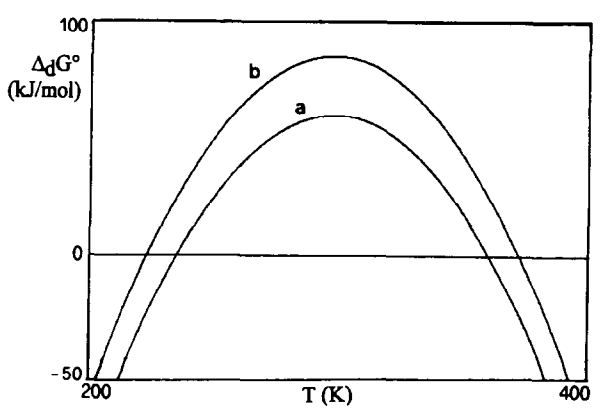

Fig. 3. Comparison between two stability curves calculated with Eq. (5), changing only the value of $\Delta_d H^\circ(T_s)$. The thermodynamic parameters are: $T_s = 298$ K; $\Delta_d H^\circ(T_s) = 80$ kJ mol^{-1} for curve a, and $\Delta_d H^\circ(T_s) = 60$ kJ mol^{-1} for curve b; $\Delta_d C_p^\circ = 9.0$ kJ (K mol)$^{-1}$.

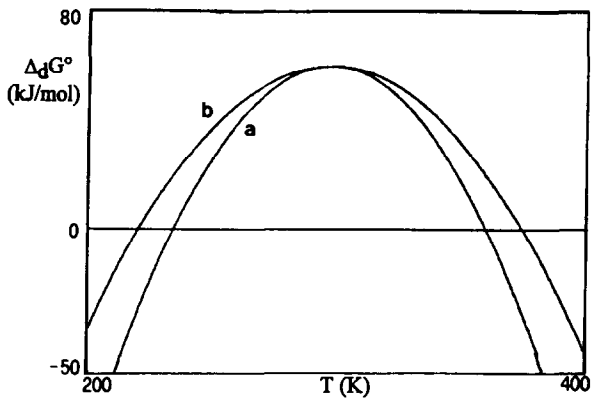

Fig. 4. Comparison between two stability curves calculated with Eq. (5), changing only the value of $\Delta_d C_p^\circ$. The thermodynamic parameters are: $T_s = 298$ K; $\Delta_d H^\circ(T_s) = 60$ kJ mol^{-1}; $\Delta_d C_p^\circ = 9.0$ kJ (K mol)$^{-1}$ for curve a, and $\Delta_d C_p^\circ = 6.0$ kJ (K mol)$^{-1}$ for curve b.

structure stability. In Fig. 3 the difference between curves a and b is only due to a different value of $\Delta_d H^\circ(T_s)$, so the whole stability curve of thermostable protein is translated upward relative to that of a mesophilic one. In Fig. 4 the difference between curves a and b is only due to a different value of $\Delta_d C_p^\circ$, and the stability curve of thermostable protein is broader and flatter than that of the mesophilic counterpart.

The value of T_s is also of paramount importance for the thermostability of globular proteins, as emphasized by Eq. (6) also. Indeed, an increase of T_s causes, by keeping the other parameters constant, the hot denaturation to occur at higher temperatures, even if the cold denaturation happens, in contrast with the preceding cases, at higher temperatures also. The profiles of $\Delta_d G^\circ(T)$ calculated for two different values of T_s, leaving fixed the other parameters, are reported in Fig. 5; the whole stability curve is practically translated along the temperature axis. Obviously, greater thermal stability can also be obtained by changing, in a concerted manner, the values of $\Delta_d H^\circ(T_s)$, $\Delta_d C_p^\circ$ and T_s (i.e. by exploiting at the same time all the limiting mechanisms pointed out).

3. Discussion

The proposed thermodynamic analysis is of general validity and suggests how to raise the hot denaturation temperature with the purpose of thermal stabilization of modified enzymes for biotechnological applications. Following Eq. (6) the thermostability of globular proteins can be increased:

a) by making $\Delta_d H^\circ(T_s)$ greater; that can be realized by stabilizing the native state with a higher number of intramolecular hydrogen bonds and salt bridges, as pointed out some years ago by Perutz and Raidt [25, 26]. Furthermore an increase in the number of aromatic–aromatic interactions can provide a significant stabilization of native structure, as suggested by Burley and Petsko [27]. These ideas are confirmed by

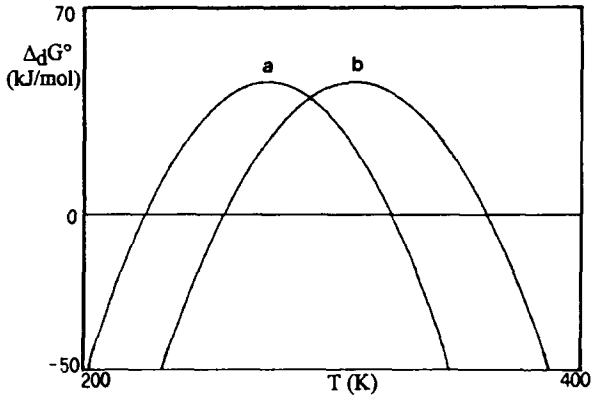

Fig. 5. Comparison between two stability curves calculated with Eq. (5), changing ony the value of T_s. The thermodynamic parameters are: $T_s = 275$ K for curve a, and $T_s = 310$ K for curve b; $\Delta_d H°(T_s) = 45$ kJ mol^{-1}; $\Delta_d C°_p = 9.0$ kJ (K mol)$^{-1}$.

a comparison of X-ray structures of RNAase H from *Escherichia coli* and *Thermus thermophilus* [28], of subtilisin BPN′ and thermitase [29] (i.e. there are 14 salt bridges and 13 aromatic–aromatic interactions in thermitase compared with three salt bridges and three aromatic–aromatic interactions in subtilisin BPN′). The importance of aromatic interactions is also stressed by the results obtained on mutant forms of T4 lysozyme [30], and phage λ repressor [31].

b) by making $\Delta_d S°(T_{hd})$ lower; that can be realized allowing lower conformational freedom of the polypeptide chain in the denatured state, for instance with the insertion of covalent cross-links which create strong hindrance to the complete unfolding and exposure of the tertiary structure to water. Indeed Scheraga et al. [32], and Rupley et al. [33] found a strong increase of denaturation temperature for RNAase A and hen lysozyme after the insertion of covalent cross-links in the polypeptide chains. Furthermore Scheraga et al. showed that the stabilization is mainly due to entropic factors [32]. This mechanism is operative also in the case of point mutations such as Gly → Xaa or Xaa → Pro, assuming that the contribution of a given residue to the conformational entropy is proportional to its available area in a Ramachandran conformational map [34].

c) by raising the value of T_s, the temperature where the entropy of the native state equals that of denatured state; what this means in structural terms is uncertain because a quantitative correlation between entropy changes and structure does not exist. Indeed, apart from the disordering of secondary and tertiary structures, a large contribution to the denaturation entropy comes from the reorganization of a very great number of water molecules around previously buried apolar side-chains [35]. For this reason it is hard to translate structural information in the prediction of thermodynamic stability.

However, the strong temperature-dependence of thermodynamic functions makes ambiguous the use of Eq. (6) for quantitative comparisons between thermophilic

proteins and mesophilic counterparts. Actually, the proposed limiting mechanisms to make a globular protein thermostable are not independent of each other, because the thermodynamic functions that describe the denaturation process are strongly temperature-dependent, as $\Delta_d C_p^\circ$ is large and positive. For example if, passing from mesophilic to thermophilic protein, the hot denaturation temperature increases, it is necessary to recalculate, with experimentally determined values of $\Delta_d H^\circ(T_{hd})$, $\Delta_d S^\circ(T_{hd})$, and $\Delta_d C_p^\circ$, the tempearature T_s. Surely T_s varies and this causes also a variation of $\Delta_d H^\circ(T_s)$, in going from mesophilic to thermophilic protein. Furthermore, if there is only a difference of $\Delta_d C_p^\circ$ between two homologous proteins, but they have the same values of T_{hd} and $\Delta_d H^\circ(T_{hd})$, this would result in different temperature-dependence of the functions $\Delta_d H^\circ$ and $\Delta_d S^\circ$ for the two proteins, and as a consequence different values of T_s and $\Delta_d H^\circ(T_s)$. This situation is emphasized in Fig. 6. From these remarks it results that the variations of thermodynamic parameters characterizing the denaturation process are strongly correlated and interdependent, owing to the temperature-dependence. This interdependence stresses that the comparison between mesophilic and thermophilic proteins must be carefully treated with deep and detailed investigations. Clearly, all that has been said on the limiting mechanisms for gaining thermostability remains true and valid, with the fundamental warning that they are not, actually, separable and independent. Thus it is likely that small but right variations in the structural and thermodynamic parameters can guarantee the strong adaptability to very different external conditions shown by the tertiary structures of polypeptide chains.

Our analysis gives theoretical support to the experimental findings of Nojima et al. [36, 37]. These researchers, by performing spectroscopic measurements as a function of temperature in the presence of GuHCl, have compared the thermodynamic stability of two globular proteins extracted from a thermophilic microorganism, cytochrome C and phosphoglycerate kinase (PGK), with the homologs from mesophilic organisms.

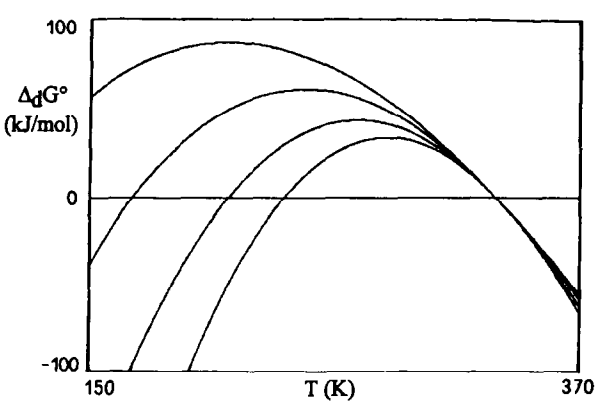

Fig. 6. Stability curves obtained from the exact thermodynamic relation, Eq. (4), for a two-state transition, keeping fixed $\Delta_d H^\circ(T_{hd})$ and T_{hd}, and changing only the values of $\Delta_d C_p^\circ$. On reducing $\Delta_d C_p^\circ$, the values of T_s and $\Delta_d H^\circ(T_s)$ change dramatically.

They, on the basis of experimental results and reasonable hypotheses, reached qualitative conclusions similar to our thermodynamic rationalizations. Indeed, the Japanese authors found that cytochrome C from Thermus thermophilus differs from cytochrome C from mesophilic organisms in the value of $\Delta_d G^\circ$ at 25 °C and in the temperature of maximum stability, and they stated that this protein exploits mechanisms a) and c) to gain its thermostability. Instead PGK from Thermus thermophilus exploits principally mechanism b), with a certain contribution of mechanism a), (i.e. the heat capacity change of thermophilic protein is 45% lower than the value of the mesophilic homolog).

The formula derived for $T_{c,hd}$ can be compared to that valid for a fusion process. Indeed for a fusion process it is:

$$\Delta_f G(T_f) = 0 \Rightarrow T_f = \Delta_f H / \Delta_f S \tag{8}$$

The fusion temperature can be raised either by making $\Delta_f H$ greater, or by making $\Delta_f S$ smaller. For instance, in the fusion process of synthetic polymers an increase of the macromolecule conformational rigidity in the melted state, by inserting monomers with a reduced number of degrees of freedom in order to decrease the entropy change associated to the process, enables a significant increase in the fusion temperature. In this respect, comparison between the fusion temperatures of rigid aromatic and flexible aliphatic polyesters is very impressive [38]. But a great difference does exist with regard to the denaturation process of globular proteins. For a fusion process the temperature-dependence of thermodynamic functions is small. Instead for globular proteins $\Delta_d H^\circ$ and $\Delta_d S^\circ$ are so strongly temperature-dependent that $\Delta_d G^\circ$ presents a maximum and two transition temperatures. It is very unsual that the same physical variable, namely the temperature, causes an order - disorder transition twice in the same system.

Finally, we have thought to analyze with this thermodynamic approach some experiments of point mutations selected to insert or delete disulfide bridges in tertiary structure. The insertion of a disulfide bridge should cause a decrease of $\Delta_d S^\circ$ because it reduces the conformational freedom of the polypeptide chain in the denatured state and principally falls in mechanism b suggested by our analysis to increase thermal stability. Some authors have also derived theoretical relationships to determine the entropy loss due to the insertion of a cross-link in a polymer chain [39, 40]. A detailed analysis of experimental results on these mutant proteins can give profitable clarification of adaptation mechanisms at high temperatures.

Matthews et al. have prepared mutant forms of T_4 lysozyme with one, two and three disulfide bridges inserted [41]. The hot denaturation temperature increases by 23.5 °C at pH 2.0 and 20.0 °C at pH 5.0 passing from wild-type protein, without disulfide bridges, to the mutant form with three disulfide bridges (i.e. at pH 5.0 the mutant form has $T_{hd} = 86$ °C, a very high temperature). The insertion of disulfide bonds strongly increased the thermal stability, and the authors concluded that the cause was exclusively entropic, in agreement with theoretical predictions. But a correct comparison of thermodynamic stability between wild-type and mutant protein could not be performed because the value of $\Delta_d C_p^\circ$ for the mutant form was not deter mined [41]. In general, most studies on point mutations suffer from incomplete thermodynamic analysis. The determination of T_{hd} and $\Delta_d H^\circ(T_{hd})$ is insufficient for

calculating $\Delta_d G^\circ$ as a function of temperature and for establishing which combination of the proposed mechanisms is operative. To overcome this problem it would be necessary to use differential scanning calorimetry. A single DSC measurement enables direct determination of the parameters T_{hd}, $\Delta_d H^\circ(T_{hd})$, and $\Delta_d C_p^\circ$, and the performance of a complete thermodynamic analysis if the hot denaturation is a two-state transition.

Hinz and Moses performed detailed DSC measurements on bovine pancreatic trypsin inhibitor, BPTI [42], that possesses three disulfide bridges, and a modified form, lacking the 14–38 disulfide bond and with the Cys-14 and Cys-38 carboxymethylated, RCOM-BPTI [43]. For wild-type BPTI at pH 5.0, 1 mM glycine buffer and 100 mM NaCl, the thermodynamic parameters were: $T_{hd} = 102\,^\circ C$, $\Delta_d H^\circ(T_{hd}) = 292\ kJ\ mol^{-1}$ and $\Delta_d C_p^\circ = 1.65\ kJ\ K\ mol^{-1}$. This protein is very stable and can be assimilated to a protein from thermophilic bacteria. For RCOM-BPTI at pH 5.0, 1 mM potassium phosphate buffer and 100 mM NaCl, the thermodynamic parameters were: $T_{hd} = 80\,^\circ C$, $\Delta_d H^\circ(T_{hd}) = 220\ kJ\ mol^{-1}$ and $\Delta_d C_p^\circ = 1.55\ kJ\ (K\ mol)^{-1}$. The destabilization caused by disulfide bond deletion is principally due to enthalpic factors, contrary to theoretical arguments. The mechanism a) seems to be operative, but it must also be noted that the very small value of $\Delta_d C_p^\circ$ for both the proteins strongly broadens their "stability curves". Moreover, recently, DSC measurements have been performed on modified forms of hen and human lysozyme lacking a disulfide bond [44, 45]. The results of these measurements are contradictory. Cooper et al. [44] found that the strong destabilization of hen lysozyme can be attributed totally to an increase in the entropy difference between native and denatured states whereas Kuroki et al. [45] found that the destabilization of human lysozyme is caused by enthalpic factors. These contrasting findings point out that the effects of dislufide bridges, as the other interactions that determine protein structure and function, are subtle, multifaceted, and difficult to extract from the whole [46].

The whole developed analysis is valid only for globular proteins whose denaturation process is well represented by a two-state transition $N \Leftrightarrow D$. It is well known that many thermostable proteins are oligomeric, constituted by the association of single polypeptide chains by means of noncovalent interactions [47]. It is clear that in these cases the possible mechanisms for increasing the thermal stability can be different, because the system shows greater complexity [48, 49]. Indeed, Klump et al. [49] have directly measured by DSC the enthalpy change associated to the "activation process" and the enthalpy change associated with the denaturation of glutamate dehydrogenase, an hexameric enzyme from the marine hyperthermophile Pyrococcus furiosus, that optimally grows at 100 °C. The DSC peaks are centered at 57 °C for activation and 113 °C for denaturation and both are representative of multi-step processes.

In conclusion, efforts must be made to better characterize the thermodynamics of denaturation processes of thermophilic proteins and elucidate the correlation with the mesophilic homologous counterparts in order to learn from Nature how to engineer more stable and yet fully active proteins. This exciting task requires wide and detailed investigations in this interesting field of biophysical chemistry.

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