Do Globular Proteins Require Some Structural Peculiarity To Best Function at High Temperatures?

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Abstract: Our knowledge of protein thermodynamics is limited to proteins from mesophilic sources. We propose a model showing how proteins from thermophilic organisms may be best adapted to function at temperatures that usually determine the unfolding of mesophilic proteins. We find that the ratio between unfolding enthalpy and entropy evaluated at the respective convergence temperatures is almost constant among mesophilic globular proteins. While this result is an expected one for proteins that were shown to obey unfolding enthalpy−entropy convergence, it is less plain for those proteins whose residual enthalpy and entropy at the respective convergence temperatures are quite far from the convergence values. This ratio can be considered a melting temperature that reflects the crystal-like protein packing. It seems to be a universal property of globular proteins, irrespective of their different origins. On this basis we suggest that the residual unfolding enthalpy and entropy, which were shown to be associated with hydrogen bond and van der Waals interactions, might play a major role in the thermal stabilization of proteins from organisms living under extreme conditions.

There is a large body of thermodynamic data accumulated on mesophilic proteins. On the contrary, it is not sufficiently clear what energetic mechanisms underlie the thermal resistance of proteins from thermophilic organisms, which are best adapted to function at temperatures close to 100 °C. The stabilization of these proteins seems to result from the appropriate combination of the weak interactions typical of "normal" proteins. On the basis of a model that dissects the unfolding free energy into melting and hydration contributions, we analyze how the temperature dependence of the free energy profile is affected by these individual terms. This seems to provide for an intriguing mechanism driving the "anomalous" behavior of thermophilic proteins.

Thermodynamics of Protein Melting

A number of findings have accredited the view that globular proteins can be considered as "crystal molecules", owing to the solid-like packing of their interiors.[1−6] Besides packing density, intrinsic compressibility may be the property of choice for evidencing this feature. Recent studies show convincingly that the isothermal compressibility coefficient of the protein interior is similar to that of solid organic polymers.[7] Further support of this view comes from some thermodynamic findings. It has been recently shown that the convergence unfolding entropy typical of globular proteins ($\Delta S^* \cong 18$ J·mol^{-1}·K^{-1})[8] resembles the fusion entropy of small organic crystals.[9,10] Accordingly, protein unfolding is conveniently schematized by a two-step pathway:[11,12] (i) melting of the crystal-like core into a liquid-like state; (ii) hydration of nonpolar moieties following the disorganization of the liquid-like state. On this basis it is evident that the melting temperature characterizing the thermal unfolding of proteins in water does not represent a "pure" melting temperature, because it is to some extent affected by the hydration of moieties that are excluded from water contact in the native state. Another point is that the fusion entropy must have an enthalpic counterpart. It seems to us that this aspect has not been fairly accounted for to date.

The enthalpy−entropy dissection proposed by some authors[8,9] suggests that this role could be played by ΔH^*, the convergence unfolding enthalpy,[8] which was attributed to forces typical of melting processes, i.e., hydrogen bonding and dispersion interactions. Recently, we have shown[13] that the enthalpy−entropy convergence phenomenon can be described by just one equation, suggesting that the temperature-independent parts of the unfolding free energy (ΔH^* and ΔS^*) are closely related:

$$\Delta H^*/\Delta S^* = (T_h^* - T_h)/\ln(T_s^*/T_s) \qquad (1)$$

Here, T_h^* and T_s^* are the isoenthalpic and isoentropic convergence temperatures, at which ΔH^* and ΔS^* are evaluated, respectively.[8] T_h and T_s were defined as the temperatures at which the unfolding enthalpy and entropy go to 0, respectively.[14] The interpretation of ΔH^* as a melting enthalpy is also dictated by the observation that the isoenthalpic temperature of protein unfolding (T_h^*) can be predicted using liquid amides as a model.[15,16]

The dissolution of liquid amides in water provides a straightforward explanation of the supposed anomaly of proteins, i.e., that the temperature dependence of the unfolding enthalpy shows substantial differences as compared to the enthalpy associated with the dissolution of liquid hydrocarbons in water.[8,17] It is also clarified that the desolvation of polar

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surfaces, sequestered with nonpolar moieties within the protein interior, contributes favorably to unfolding, thus opposing the role played by hydrophobic interactions. These contributions exactly counterbalance each other at the T_h* of protein unfolding, where the total solvation enthalpy of internal residues is 0. Thus, it is likely that forces in which the residual enthalpy (ΔH*) originates are to be properly associated with packing of residues within the protein structure.[15,16] As a consequence, van der Waals interactions, for which a predominant role in the hydrophobic behavior has been recently claimed,[8,18] are restored to their original significance, without the need for a new view of the hydrophobic effect. The prediction of T_h* through the thermodynamics of liquid amide dissolution clarifies what this temperature actually represents and strengthens the view that the hydration of the buried surface upon unfolding can be modeled by liquid organic molecules, according to the classical approach.[17,19] The analysis offered here is different from that recently proposed, which thoroughly investigates the folding energetics through the transfer thermodynamics of gaseous compounds.[20,21] However, it has the advantage of resorting to experimental evidence, which leads us back to the classical view of hydrophobicity, without adding more complication to untangling the folding energetics. As a matter of fact, the observation that the hydration enthalpy of gaseous compounds vanishes at a temperature close to the T_h* of protein unfolding[22] has given rise to misconceptions about the role played by hydrophobicity in protein organization, also suggesting that unfolding and dissolution of organic crystals in water closely resemble gas transfer into water.[22] This appears to be an entirely coincidental occurrence.[16] Another point, which has been recently discussed by Muller,[23] is that the dissection of the unfolding heat capacity change ($\Delta C_p°$) into polar and nonpolar contributions[20] implies that the polar solvation enthalpy also vanishes at the same temperature as the nonpolar enthalpy. This is not required by the amide model, where nonpolar moieties are the unique contributors to $\Delta C_p°$ and polar groups contribute a nearly constant value to the total solvation enthalpy.

Here, we extend the use of eq 1 by evaluating T_h and T_s for a large set of globular proteins, including those originally analyzed.[8,13] These temperatures can be calculated by thermodynamic quantities available in the literature (for references, see Table 1). The values reported in Table 1 allow the calculation of the ratio ΔH*/ΔS*, with T_h* = 377 K and T_s* = 385 K.[13] This ratio, whose dimensions are those of a temperature, is obviously constant for proteins showing the convergence phenomenon,[8] because all the parameters in eq 1 are constant. However, there is no expected reason why this "temperature" should be constant for the set of proteins examined, most of which depart from the convergence behavior. Nevertheless, Table 1 shows that ΔH*/ΔS* is almost the same among globular proteins, with an average value of 324.7 ± 8.0 K. This means that T_h* and T_s* are peculiar of protein unfolding and that ΔH* and ΔS* are not independent from each other. Together they constitute a residual unfolding free energy ($\Delta G^* = \Delta H^* - T\Delta S^*$) which vanishes at a temperature shared by all globular proteins, irrespective of the individual values of ΔH* and ΔS*.

Table 1. Thermodynamic Properties of Globular Proteins

protein	T_h (K)	T_s (K)	$\Delta C_p°$ (J·mol^{-1}·K^{-1})	$\Delta H^*/\Delta S^*$ (K)
ribonuclease A[10]	243.5	255.4	43.5	325.3
lysozyme, chicken[10]	258.9	267.8	51.7	325.3
Tendamistat[48]	260.1	271.0	39.2	332.9
protein G, domain B1[40]	261.2	273.6	46.4	339.0
plasminogen, fr K4[10]	262.8	270.5	51.7	323.5
ribonuclease T1[49]	263.1	268.2	66.4	315.1
parvalbumin, carp[10]	268.4	280.4	46.0	342.6
protein G, domain B2[40]	270.4	279.5	51.8	332.9
ser retinol bind. prot[50]	271.3	279.6	58.2	330.4
α-amylase[51]	273.3	278.7	76.2	320.9
β-trypsin[10]	275.0	280.8	57.7	323.2
myoglobin, opossum[52]	275.8	283.8	36.6	331.8
α-chymotrypsin[10]	276.2	280.1	57.7	316.9
lysozyme T4[53]	277.2	279.8	65.6	312.7
arabinose bind. prot[51]	279.1	282.3	43.8	315.5
myoglobin, rat[52]	280.7	288.4	40.5	333.3
myoglobin, armadillo[52]	282.5	289.0	39.2	329.5
papain[10]	282.5	290.2	60.1	334.3
Staph. nuclease[10]	284.1	288.0	61.3	320.0
myoglobin, horse[52]	284.5	291.0	51.0	330.5
carb. anhydrase[10]	285.4	289.8	63.3	322.5
diphtheria tox, fr A[54]	285.8	287.3	75.9	311.6
diphtheria tox, fr B[54]	286.7	289.6	52.6	317.1
myoglobin, raccoon[52]	286.9	293.0	44.4	330.0
cytochrome *c*, horse[10]	288.3	294.0	67.3	328.9
cytochrome *c*, tuna[51]	292.1	296.8	73.1	326.3
myoglobin, carp[52]	292.7	296.0	52.1	320.7
lactalbumin[55]	294.3	297.8	54.4	322.0
pepsinogen[10]	294.7	299.1	73.3	326.0
myoglobin, s. whale[10]	297.5	301.2	74.5	323.9
subtilisin BPN′[56]	313.1	313.7	73.0	312.0
subtilisin BPN′, var[56]	318.9	319.8	59.3	313.1
average ± SD				324.7 ± 8.0

ΔG* is devoid of any hydration contribution, thus representing the energetics associated with van der Waals and hydrogen bond interactions. Hence we suggest that this temperature reflects the "pure" melting of the solid-like protein core. It is nearly constant, notwithstanding that the internal architecture of individual globular proteins can be quite different.

The species independence of the ratio ΔH*/ΔS* could be questioned if one considers that the values of ΔH* and ΔS* are calculated assuming the constancy of $\Delta C_p°$. Actually, in the range 0−130 °C, the unfolding heat capacity increment is not temperature independent.[24,25] This fact could also affect the values of T_h and T_s of individual proteins. However, in the limited temperature range from 20 to 80 °C, $\Delta C_p°$ can be assumed to be temperature independent[10] because it was found that its change is lower than the experimental uncertainty.[14] In any case, the amount of the change becomes appreciable only above ~80 °C.[25] The majority of T_h and T_s values fall within the range from 0 to 50 °C, which allows us to conclude that these temperatures cannot be very different from figures reported in Table 1. It was also shown that the temperature dependence of $\Delta C_p°$ does not affect appreciably the position and the shape of the unfolding free energy curve along the temperature axis.[10] This again drives us to the same conclusion. As a consequence, consideration of the temperature dependence of $\Delta C_p°$ should not produce significant alterations of the ratio ΔH*/ΔS*.

Thermodynamics and Evolution of Globular Proteins

We wish to focus on a further peculiarity arising from the data in Table 1. At a glance, the difference between T_s and T_h

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vanishes at a temperature near 320 K. It must be noted that the maximum unfolding free energy occurs at T_s, where its value is given by $\Delta C_p^\circ(T_s - T_h)$.[14] Here, ΔC_p° represents the unfolding heat capacity change, which arises from the water exposure of surface buried within the protein interior.[8-19] Thus the maximum stability of the folded state is 0 when $T_s = T_h$, i.e., the existence of the folded state is hindered. On this basis globular proteins from mesophilic sources appear to have evolved so that their maximum stability temperature does not exceed 320 K.

This aspect deserves some comment, as long as we are concerned with evolutionary arguments. It was hypothesized that evolution has selected globular proteins with an unfolding free energy small enough to make their degradation feasible as well as to allow a high degree of flexibility (essential for function) and to avoid their getting trapped into incorrectly folded structures.[26] However, the unfolding free energy cannot be so small as to allow a high susceptivity to mutations which would lead to the protein unfolding at room temperature.[26] These arguments were raised by Becktel and Schellman[14] and have been sustained recently by Muller,[23] with a particular emphasis on the narrow range spanned by protein melting temperatures. However, our knowledge of protein thermodynamics is quite limited, since it is mostly restricted to proteins from mesophilic sources. The problem of how proteins from thermophilic organisms evolved to best function at temperatures near or above the boiling point of water[27-32] is still unresolved and seems to escape these considerations.

Is it necessary to invoke a somewhat peculiar stabilization mechanism for proteins working at high temperatures? Many efforts have been concerned with this issue, but the general conclusion is that properties of thermophilic proteins result from a variety of stabilizing effects due to an appropriate combination of the weak interactions commonly involved in protein stabilization.[33,34] The necessity of an increased "hydrophobicity" of the protein core has often been invoked,[35-37] because classically the burial of the nonpolar surface is believed to drive protein stability. However, from the analysis of a large set of globular proteins, the increase of the melting temperature absurdly showed a good correlation with the reduction of buried surface.[38] In our opinion, the particular set of amino acids at protein disposal does not allow stabilization at very high temperatures through the exclusive increase of protein "hydrophobicity". It is unlikely that the unfolding heat capacity change, which reflects hydrophobic behavior, can fall outside ~40-80 J·mol^{-1}·K^{-1}.[39] This can be also appreciated from the third column of Table 1. As a consequence, hydrophobicity cannot drive protein stabilization at temperatures beyond about 90 °C,

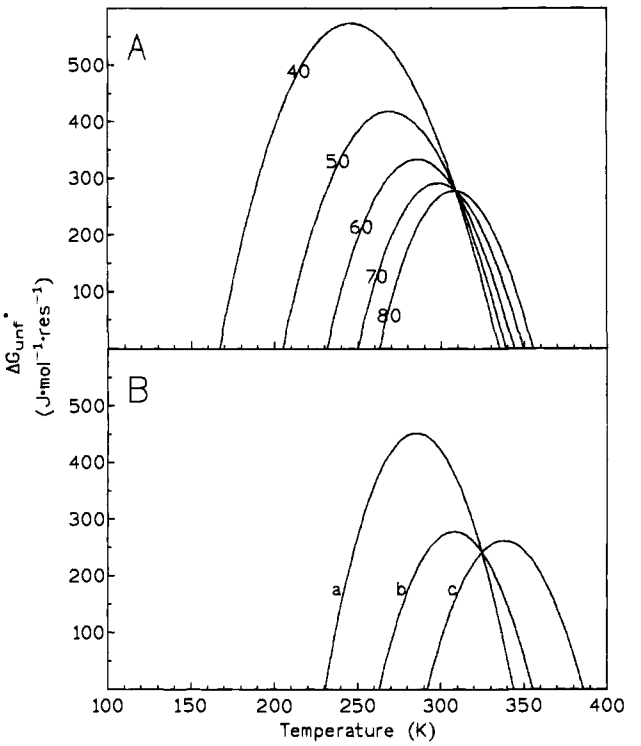

Figure 1. (A) Effect of ΔC_p° on the unfolding free energy of model proteins obeying the convergence behavior. The curves are drawn according to the equation $\Delta G_{unf}^\circ = \Delta H^* - T\Delta S^* + \Delta C_p^\circ[T - T_h^* - T\ln(T/T_s^*)]$, with $\Delta H^* = 5800$ J·mol^{-1}, $\Delta S^* = 17.9$ J·mol^{-1}·K^{-1}, $T_h^* = 377$ K, and $T_s^* = 385$ K. Each curve is identified by the respective ΔC_p° value. (B) Effect of ΔG^* on the unfolding free energy of model proteins. The curves are drawn as before, but with $\Delta C_p^\circ = 80$ J·mol^{-1}·K^{-1} and different pairs of values for ΔH^* and ΔS^*. ΔH^* values are (a) 7800, (b) 5800, and (c) 3400 J·mol^{-1}. ΔS^* was evaluated according to the ratio $\Delta H^*/\Delta S^* = 324.7$ K. Experimental values of ΔH^* fall between 3386 (subtilisin BPN′ variant) and 7826 (α-amylase) J·mol^{-1}. This is easily verified by the relationship $\Delta H^* = \Delta C_p^\circ(T_h^* - T_h)$ using the data reported in Table 1.

at least for proteins following the enthalpy-entropy convergence behavior. This is shown in Figure 1A, where we consider model proteins with different ΔC_p°. As can be seen, the increase of ΔC_p° determines a progressive shift of the unfolding free energy curve toward higher temperatures with a concomitant decrease of the stability maximum. The highest accessible value of the thermal unfolding temperature (T_m) is about 360 K ($\Delta C_p^\circ = 80$ J·mol^{-1}·K^{-1}).

At this point we have to explain how this physical limit can be overcome. We have already pointed out that globular proteins share a nearly constant melting temperature of their core. Nevertheless, ΔH^* and ΔS^* can span a range of values, with the consequence that ΔG^* does not contribute equally to the stability of individual proteins. This is shown in Figure 1B using different pairs of values for ΔH^* and ΔS^*, with $\Delta H^*/\Delta S^* = 324.7$ K (this is the average value calculated from the set of proteins given in Table 1) and a fixed ΔC_p°. As can be seen, decreasing values of both ΔH^* and ΔS^* shift the unfolding free energy curve toward higher temperatures to a larger extent than that achievable by increasing ΔC_p° alone. We suggest that this mechanism, implying reduction of van der Waals and hydrogen bond interactions, could be adopted by thermophilic proteins to best function at temperatures that usually determine the unfolding of mesophilic proteins. Actually, it should be sufficient to reduce only ΔS^*, because it is the only opposer against folding (together with the desolvation of the peptide moiety following surface burial). The condition that ΔH^*

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becomes smaller is dictated by the constancy of the ratio $\Delta H^*/\Delta S^*$. A close inspection of Figure 1 also shows that a high T_m is not synonymous with larger maximum stability. We can appreciate that curves with the highest T_m have a smaller maximum free energy. In this regard, it has been shown that globular proteins of limited stability do have high thermal unfolding points, irrespective of a folded state stabilized by forces remarkably typical of other globular proteins.[40]

Concluding Remarks

Homologous proteins from mesophilic sources do not seem to be adequate to check the hypothesized stabilization mechanism. For example, the seven myoglobins reported in Table 1 appear to have been engineered by evolution so that they retain the ability to best function below the boiling point of water. Mutations within this set of proteins produce changes in ΔH^* and ΔS^* which are not sufficient to shift notably the unfolding free energy curve toward higher temperatures. The situation is even worse when we attempt the analysis of randomly generated mutants of lysozyme T4.[41] The largest change of T_m amounts to less than 2 °C, which is clearly insufficient to check our hypothesis. On this basis, we are forced to conclude that a satisfactory thermodynamic explanation could be achieved exclusively when a comparison between homologous proteins from mesophilic and thermophilic sources is assessed. Unfortunately, there are insufficient thermodynamic studies on this task to date. We believe that additional efforts must be directed toward this end.

Nevertheless, some authors have argued that a basic way to make a protein thermostable (i.e., with a higher T_m) is to flatten the temperature profile of the unfolding free energy.[42,43] The same suggestion comes from more recent studies,[30,34] which report that the unfolding free energy curve for thermophilic proteins seems to be flattened rather than shifted, without substantial changes of the maximum free energy. This reduced temperature dependence implies a diminution of ΔC_p°, since $\delta\Delta G^\circ/\delta T = -\Delta S^\circ$ and $\delta\Delta S^\circ/\delta \ln T = \Delta C_p^\circ$ at constant pressure. As shown in Figure 1A, a reduced T_m should be expected on this basis, as a consequence of shifting the curve to lower temperatures, as well as a larger maximum free energy. Thus, it seems evident that the temperature profile flattening without any modification other than an increased T_m must necessarily involve those contributions to the unfolding free energy which do not directly depend on the hydration of buried surface, i.e., ΔH^* and ΔS^*. This is clarified in Figure 2, showing that the effect linked to a reduced ΔC_p° can be counterbalanced only if ΔH^* and ΔS^* become smaller, whenever we want to flatten the free energy profile and leave unchanged the maximum free energy.

The scheme outlined here allows straightforward analysis of distinct aspects of protein unfolding, suggesting a simple way to distinguish between solid-like and liquid-like features. The involvement of hydrogen bonding and van der Waals interactions (represented by ΔG^*, the residual free energy) in the stabilization of the folded state at high temperatures comes as a necessary consequence of the impossibility of burying

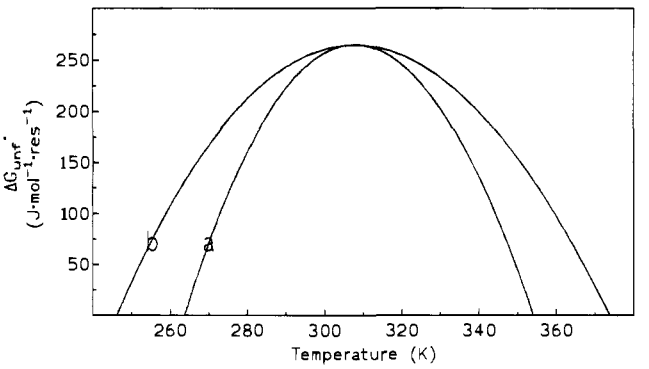

Figure 2. Flattening the unfolding free energy profile. Curve a is drawn as before, with $\Delta C_p^\circ = 80$ J·mol^{-1}·K^{-1}, $\Delta H^* = 5800$ J·mol^{-1}, and $\Delta S^* = 17.9$ J·mol^{-1}·K^{-1}, which give $T_h = 304.5$ K and $T_s = 307.8$ K. Curve b is drawn using $T_h = 301.2$ K, $T_s = 307.8$ K, and $\Delta C_p^\circ = 40$ J·mol^{-1}·K^{-1}, according to the equation $\Delta G_{unf}^\circ = \Delta C_p^\circ[T - T_h - T \ln(T/T_s)]$, under the condition that the maximum free energy $[\Delta G_{max}^\circ = \Delta C_p^\circ(T_s - T_h)]$ is unchanged. This gives $\Delta H^* = \Delta C_p^\circ(T_h^* - T_h) = 3032$ J·mol^{-1} and $\Delta S^* = \Delta C_p^\circ(\ln T_s^* - \ln T_s) = 9.0$ J·mol^{-1}·K^{-1}.

nonpolar surface at will. The limited set of amino acids available and geometrical constraints associated with the spherical shape of globular proteins allow the burial of a nearly constant fraction of that surface (~58%),[42] which means a ΔC_p° as large as 80 J·mol^{-1}·K^{-1} per residue at the most.[39] This implies that the highest T_m achievable through the burial of nonpolar surface is about 360 K (see Figure 1A). Neither can we forget that polar and nonpolar surfaces are sequestered together in the course of folding, from which follows the necessity of paying an enthalpic penalty to desolvate polar moieties. Thus, it seems very likely that the stabilization mechanism we propose for thermophilic proteins could be effective, even if in concomitance with other effects.

We recognize that our arguments reside primarily on the unfolding enthalpy dissection,[8,9] which singles out dispersion forces and hydrogen bonding as the sources of the residual unfolding enthalpy. This seems to be supported by the recent amide model,[15,16] although it has been questioned in other papers.[22,23] In this regard, some authors[45,46] found that most of the residual unfolding enthalpy was likely to arise from hydrogen bonding within the protein interior, as originally supposed.[8-10] In spite of its simplicity, our model predicts that the adaptation of proteins from organisms living in extreme conditions to best function at temperatures near or above the boiling point of water does not require introduction of any structural peculiarity. The same conclusion has been attained recently by a similar approach,[47] which nevertheless appears to split up the unfolding free energy in a way quite different from that offered here. This

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involves considerable disparity in the balance of forces responsible for protein organization. We believe that our approach has the merit of reconciling protein energetics with the classical model of hydrophobic solvation.[17,19] Of course, it is subject to further improvement, mostly concerning the correlation between enthalpy and entropy changes associated with the solid-like packing of the protein structure. On this track we could probe deeper into the problem of protein (thermo)stability as well as into the biology of extremophilic organisms.

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