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Isoenthalpic and isoentropic temperatures and the thermodynamics of protein denaturation

(hydrophobicity/protein stability)

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ABSTRACT **The standard enthalpy or entropy change upon transfer of a small nonpolar molecule from a nonaqueous phase into water at a given temperature is generally different for different solute species. However, if the heat capacity change is independent of temperature, there exists a temperature at which the enthalpy or the entropy change becomes the same for all solute species within a given class. Similarly, the enthalpy or the entropy change of protein denaturation, when extrapolated to high temperature assuming a temperature-independent heat capacity change, shows a temperature at which its value becomes the same for many different globular proteins on a per weight basis. It is shown that the existence of these temperatures can be explained from a common formalism based on a linear relationship between the thermodynamic quantity and a temperature-independent molecular property that characterizes the solute or the protein. For the small nonpolar molecule transfer processes, this property is the surface area or the number of groups that are brought in contact with water. For protein denaturation, it is suggested that this property measures the polar/nonpolar mix of the internal interaction within the protein interior. Under a certain set of assumptions, this model leads to the conclusion that the nonpolar and the polar groups of the protein contribute roughly equally to the stability of the folded state of the molecule and that the solvent-accessible surface area of the denatured form of a protein is no more than about two-thirds that of the fully extended form.**

1. Introduction

A wealth of highly accurate thermodynamic data is now available from modern calorimetry on protein denaturation and on transfer processes of small nonpolar molecules (1). Privalov (2) found that the protein data show a curious feature. The specific enthalpy changes upon denaturation at a given temperature are significantly different for different proteins. However, if these values are extrapolated to ≈110°C assuming that the heat capacity changes are independent of temperature, then they converge to a common value for most of the globular proteins studied (2). The entropy changes per unit weight similarly converge to a common value, again at ≈110°C (2). Similar features are detectable in the small molecule data also. It is well known (3–6) that the enthalpy changes upon dissolution in water of liquid nonpolar compounds are nearly zero at room temperature. Since the heat capacity change for this process is large and different for different solute species, the enthalpy change varies rapidly with temperature at different rates for different solute species. Thus room temperature is the only temperature at which the enthalpy changes are nearly the same, zero in this case, for all solute species. The temperature at which the entropy change for the same process becomes zero is also found to be common for a number of different hydrocarbon solute species if the heat capacity change is assumed to be independent of temperature (6). Intriguingly, this temperature is ≈110°C, the same as Privalov's convergence temperature for the protein denaturation (2).

If the standard entropy change, ΔS, for a process attains a common value at a temperature T_s^* for a number of different solute species transferred or for a number of different protein molecules denatured and if the heat capacity change, ΔC, for the process is assumed to be independent of temperature, one has

$$\Delta S = \Delta C(\ln T - \ln T_s^*) + \Delta S^*, \quad [1]$$

where T is the temperature and ΔS^* is the value of ΔS at T_s^* and common to all species considered. Therefore, if ΔS is plotted against ΔC for a number of different solute species at one temperature, the plot will show a straight line, with the slope given by $\ln(T/T_s^*)$ and the y intercept by ΔS^*. Murphy *et al.* (7) made such plots, which we shall refer to as the MPG entropy plot, for the protein denaturation, for the transfer of nonpolar molecules from gas to water ($g \rightarrow w$ process) and from the pure liquid phase to water ($l \rightarrow w$ process), and for the transfer of several cyclic dipeptides with hydrophobic side chains from the pure solid to water ($s \rightarrow w$ process). They found that each of these processes gives a good straight line. Furthermore, although the y intercept was unique for each of these processes, the slopes were all the same. This indicates that T_s^* not only exists but is also the same for all of these processes, including the protein denaturation. The common value of T_s^* Murphy *et al.* (7) report is 112°C. A similar relation for the enthalpy is

$$\Delta H = \Delta C(T - T_h^*) + \Delta H^*, \quad [2]$$

where T_h^* and ΔH^* have the same value for all solute species considered for a given process. Murphy *et al.* (7) found that the analogous plot for enthalpy, the MPG enthalpy plot, also gives a straight line for the protein denaturation. The slope of this line indicated that T_h^* was equal to T_s^* in accordance with Privalov's observation (2). This is different from T_h^* for the small molecule $l \rightarrow w$ transfer process, which is near room temperature (6).

T_h^* and T_s^* are the temperatures at which the changes in enthalpy and entropy, respectively, become the same for a class of molecular species undergoing the same process. In analogy to the isosbestic point in absorption spectroscopy, we shall refer to these as the isoenthalpic and isoentropic temperatures, respectively. One must carefully note that these are not necessarily the same as the temperatures at which the enthalpy or the entropy change becomes zero. These latter will be denoted as T_h and T_s, respectively, without the superscript *. T_h (or T_s) equals T_h^* (or T_s^*) only when ΔH^* (or ΔS^*) is zero, which happens to be nearly the case for the $l \rightarrow w$ process but not for other processes.

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Why do the isoentropic and isoenthalpic temperatures occur? Since the correlations are a type of linear relationship, can they be related to the linear relation between the thermodynamic quantity and the surface area of the molecule such as that found for the free energy (8, 9) and for the heat capacity (10)? A simple mathematical procedure shows that such linear relation indeed produces these temperatures and connects the two parameters of the MPG plots—the slope and the y intercept—to the coefficients of the linear relation. The proof is based on the property of a function that is linear in two variables, say, T and X. The rate of change with X of this kind of function is itself linear with respect to T so that it must pass through zero at some T. At this T, the value of the original function will be independent of X.

The plan for the rest of this paper is as follows. Section 2 describes the mathematical proof. Section 3 deals with the thermodynamics of small molecule transfer processes. For these processes, the variable X that produces the isoenthalpic and isoentropic temperatures is clearly a size measure, such as the surface area or the number of groups, of the solute molecule. The relations derived in section 2 can be used to understand relations among, and in some cases to evaluate the absolute value of, the observed parameters of the MPG plots. The protein denaturation is taken up in section 4. The variable X cannot be a size measure in this case. A model free energy function for the denaturation process is proposed based on the similarities and differences of the MPG plots compared with the small molecule transfer processes, and the consequences of this model are then explored.

2. Linear Relation Produces Isoenthalpic or Isoentropic Temperature

Suppose that the enthalpy change ΔH_i of a process involving the molecular species i is a linear function of some physical property, X_i, of the molecule,

$$\Delta H_i = a_h X_i + b_h, \quad [3]$$

where the coefficients a_h and b_h are functions of temperature but "species-independent" (i.e., common to all molecular species within a given class). The property X_i would typically be a size measure of the molecule such as the number of carbon atoms, the number of hydrogen atoms (5), or the surface area of the molecule, but it can be any other property of the molecule as long as it is independent of temperature. Since the heat capacity is obtained by the temperature derivative of enthalpy, it too is a linear function of X_i with species-independent coefficients,

$$\Delta C_i = a_c X_i + b_c, \quad [4]$$

where a_c and b_c are the temperature derivatives of a_h and b_h, respectively. We now assume that the heat capacity change is independent of temperature. This is of course not true in many cases. The relation that we shall derive is, therefore, generally an extrapolation behavior; it is what one expects if the thermodynamic behavior observed over a small range of temperature were extrapolated assuming that the heat capacity change is independent of temperature. Then ΔH_i, a_h, and b_h are all linear with respect to temperature, and their rates of change with temperature are given by ΔC_i, a_c, and b_c, respectively. Assuming that a_c is nonzero, one can therefore write

$$a_h = a_c(T - T_h^*), \quad [5]$$

where T_h^* is a constant. b_h can then be written as

$$b_h = b_c(T - T_h^*) + b_h(T_h^*), \quad [6]$$

where $b_h(T_h^*)$ is the value of b_h at T_h^*. Inserting these expressions into Eq. 3, and using Eq. 4, one obtains

$$\Delta H_i = \Delta C_i(T - T_h^*) + b_h(T_h^*), \quad [7]$$

which is the same as Eq. 2 in a slightly different notation. The function b_h is the value of ΔH in the limit of zero X. It becomes independent of temperature when b_c is zero (i.e., when ΔC is proportional to X). T_h^* is the temperature at which a_h becomes zero and ΔH becomes independent of X.

The entropy correlation can be obtained by an exactly analogous procedure using ln T for T. Thus, suppose that the entropy change for a species i is given by

$$\Delta S_i = a_s X_i + b_s, \quad [8]$$

with species-independent coefficients a_s and b_s. Then ΔC_i, being the derivative of ΔS_i with respect to ln T, satisfies Eq. 4 and, if ΔC_i is independent of temperature and a_c is nonzero, one readily obtains,

$$\Delta S_i = \Delta C_i(\ln T - \ln T_s^*) + b_s(T_s^*), \quad [9]$$

where T_s^* is a simple constant defined by the equation

$$a_s = a_c(\ln T - \ln T_s^*). \quad [10]$$

A notable feature of these relations is that they do not contain X explicitly. Thus, if there is any temperature-independent property to which the enthalpy (or the entropy) change is related linearly with species-independent coefficients, a linear MPG plot will be observed. Obviously, neither of the two parameters of the plot is affected by the scale change of, or the units used to measure, this property. Murphy and Gill (11) obtained similar results for the $s \rightarrow w$ process using a group additivity approach. Their procedure corresponds to using the number of groups in a molecule for X.

If the free energy change is linearly related to X with species-independent coefficients, i.e.,

$$\Delta G_i = a_g X_i + b_g, \quad [11]$$

then both the enthalpy and the entropy changes will be linearly related to X_i. T_h^* and T_s^* then both exist and are related to each other by

$$a_g = a_h - Ta_s = a_c[(T - T_h^*) - T \ln(T/T_s^*)]. \quad [12]$$

As with other equations, this equation generally holds at all temperatures only as an extrapolation assuming that the heat capacity change is independent of temperature. The expression within the square bracket of this equation is well known if T_h^* and T_s^* are replaced by T_h and T_s, respectively (6, 7, 12). Examination of this expression shows (*i*) that a_g/a_c is a slowly increasing function of temperature below T_s^* and reaches its maximum value at T_s^*, (*ii*) that, if $T_h^* = T_s^*$, a_g/a_c is negative at all temperatures except at T_s^* where it is zero, (*iii*) that a_g/a_c is never positive at any temperature unless $T_h^* < T_s^*$, and (*iv*) that a_g/a_c at any one temperature increases as T_h^* decreases from T_s^*.

3. The Small Molecule Transfer Processes

For the transfer processes of small nonpolar molecules into water, the isoenthalpic and isoentropic temperatures undoubtedly arise from the well-known linear relationship between the free energy and the surface area (8, 9, 13–15) or the number of hydrogen atoms (5, 16) of the solute molecule.

For the $g \rightarrow w$ process, Ooi *et al.* (17) assumed that the changes in the various thermodynamic quantities are propor-

tional to the surface area of the solute molecule transferred and determined the proportionality constants by the least squares fit over many experimental data. Using the reported values of a_g, a_h, and a_c for the hydrocarbons, T_s^* and T_h^* for the $g \rightarrow w$ process are calculated to be 132°C and 94°C, respectively. The T_s^* value is significantly different from 112°C from the MPG plot and indicates that the absolute value of a_s/a_c of Ooi *et al.* (17) is slightly too large; this is probably caused by the neglect of the b_s term implied by the assumption of proportionality (see below). When the a_c value is not available or is inaccurate, the T_h^* value can be computed from T_s^* using only the ratio of a_h and a_s. Dec and Gill (16) gave the values of a_h and a_s, but not a_c, per number of hydrogen atoms in the molecule. Use of these values gives 90°C for T_h^*. This value of T_h^* and that obtained from the data of Ooi *et al.* (17) are close to the common temperature at which the lines of enthalpy vs. temperature plot cross each other for the dissolution of gaseous benzene derivatives in water (1).

The $l \rightarrow w$ or $s \rightarrow w$ process may be considered in two steps; the vaporization or sublimation followed by the $g \rightarrow w$ process. The fact that the T_s^* values are the same for all three processes is an expected result if a_s and a_c for the $g \rightarrow w$ process are much larger than those for processes that do not involve the aqueous phase. On the other hand, the T_h^* values are expected to vary since the enthalpy changes for the vaporization and sublimation will vary with the size of the molecule. According to Eq. **12**, the deviation of T_h^* from the common value of 112°C for T_s^* reflects the magnitude of a_g. Thus, the fact that the T_h^* value for the $l \rightarrow w$ process is lower than that for the $g \rightarrow w$ process implies that the size-dependence of the free energy change is larger for the $l \rightarrow w$ process than for the $g \rightarrow w$ process. In addition, the interaction energy between the solute and solvent depends not only on the contact area with the solvent but also on other factors such as the polarizability of the solute molecule. One therefore generally expects the enthalpy correlation to be poorer than the entropy correlation even for one process type. The T_h values for the $l \rightarrow w$ process indeed show a segregation between the aromatics and the alkanes (6).

Gill *et al.* (10) found an analytical formula for the heat capacity change for the $g \rightarrow w$ process that agrees well with experimental data. According to this formula, the heat capacity change is *proportional* to the accessible surface area of the solute molecule; i.e., b_c is zero for this process and, therefore, b_h and b_s are independent of temperature. Since the heat capacity changes for the nonaqueous transfer processes are small (18), b_h and b_s are expected to be nearly independent of temperature for all transfer processes to water.

For the $l \rightarrow w$ process, ΔH^* and ΔS^* are both zero (6). This means that $T_h^* = T_h$ and $T_s^* = T_s$ and that b_h, b_s, and b_g are zero and the enthalpy, entropy, and free energy changes, as well as the heat capacity change, are all proportional to X. Eqs. **5**, **10**, and **12** can then be written in terms of the total quantities as well as by the rate of change of these quantities with X. For example, Eq. **10** becomes $\ln(T_s/T) = -a_s/a_c = -\Delta S_i/\Delta C_i$, a relation first recognized by Sturtevant (19). Apparently, this is true only for the liquid-to-liquid phase transfer process; for other processes, at least ΔS^* is nonzero, which means that the entropy change is not proportional to X, as mentioned in connection with the data of Ooi *et al.* (17) for the $g \rightarrow w$ process.

The b_s value for the $g \rightarrow w$ process can be estimated theoretically, if one assumes that the linear relation between ΔS and X extends down to the infinitesimally small solute size. The entropy change at this limit is just that of transferring a mathematical point. The free energy change upon transferring a mathematical point from the gas to a liquid phase is rigorously given by $-RT \ln(1 - \xi) + RT \ln(RT/p^\circ v)$, where R is the gas constant, ξ is the volume packing density of the liquid, p° is the constant of 1 atm (1 atm = 101.3 kPa), and v is the molar volume of the liquid. The first term is the work of inserting a fixed point in the liquid (20). The second term represents the difference in the translational free energy arising from the different volume available to the solute molecule, a mathematical point in this case, in the gas and the liquid phases (21, 22). The entropy change is given by the temperature derivative of this expression. Assuming that ξ is 0.363 for water at room temperature (23), the value of b_s calculated from this formula is −71.0 J per degree per mol at 25°C and −69.5 J per degree per mol at 112°C. These values compare favorably with the y intercept of the $g \rightarrow w$ line in figure 1 of Murphy *et al.* (7).

4. Protein Denaturation

Because of the large size of a globular protein molecule, the protein denaturation is like a phase transfer process wherein groups interior to the protein are brought out in contact with water. Since many of these groups are nonpolar, the thermodynamics of protein denaturation is expected to be related to that of the transfer processes of small nonpolar molecules. In fact, since Kauzmann (3), the hydrophobic effect has been considered to be one of the main forces that drive the protein folding process (24). The accessible surface area of an amino acid side chain buried upon folding is linearly related to the free energy of transfer of the group from water to a nonaqueous medium (25). The fact that the T_s^* value of protein denaturation is the same as that for the nonpolar molecule transfer processes further indicates the prominence of the hydrophobic effect in the thermodynamics of protein denaturation.

It seems, therefore, initially reasonable to suppose that the underlying molecular characteristic X that produces the linear MPG plots is a quantity that measures the amount of exposure of the nonpolar groups to water when a protein molecule unfolds. It turns out, however, that use of such a quantity for X creates great confusion. Because T_h^* and T_s^* are closely similar for the protein denaturation process, a_g/a_c is negative at room temperature, according to Eq. **12**. Since a_c is positive in small molecule transfer processes, one must conclude, as did Privalov and Gill (1) and Murphy *et al.* (7), that the hydrophobic effect, which discourages contact of nonpolar molecules with water, now destabilizes the protein. This conclusion is, however, contrary to the weight of evidence steadily accumulated over 30 years since Kauzmann (3). [Dill (26) objected to the use of the term "hydrophobicity" in Murphy *et al.* (7), and Privalov *et al.* (27) now favor the term "hydration." However, the fundamental problem implied by the fact that T_h^* and T_s^* are the same for the protein denaturation cannot be resolved by semantics alone.]

One way out of this dilemma is to suppose that the non-non-polar (i.e., polar) interaction is also important for protein stability and that protein molecules that are stabilized by a large hydrophobic effect tend to have fewer or weaker polar interactions. Since the polar interaction will be mainly enthalpic, its effect will be to shift T_h^* without altering T_s^*. The molecular characteristic X could then be a quantity that measures the nonpolar fraction of the total interactions, rather than the magnitude of the nonpolar interaction alone. Unlike the latter, the nonpolar fraction need not scale with the size of the molecule. This is a desired feature since the linear MPG plots for the protein denaturation process are observed only when specific, rather than molar, changes in enthalpy or entropy are used (2). Thus, the property X for the protein denaturation is not a property that scales with the size of the molecule in any case.

In order to test this supposition and quantify the nonpolar fraction, one needs a single physical property to measure the extent of both the polar and the nonpolar interactions on a common basis. In this report, we use the accessible surface area buried upon folding for this purpose, since it is the best known measure for assessing the magnitude of the nonpolar effect. The energy of a hydrogen bond, which will be the main contributor to the polar interaction, is a sensitive function of its geometry and not simply proportional to the buried surface area of the atoms forming the bond. Nevertheless, when a number of proteins are compared on a per weight basis, the average hydrogen bond energy of a protein may still be proportional to the polar surface area buried in it. Use of the number of hydrogen bonds and of the hydrophobic contacts will probably yield similar results.

Thus, suppose that the free energy change upon denaturation, ΔG, is given by

$$\Delta G/M = (f_n g_n + f_p g_p)\Delta a - Ts_{conf}, \quad [13]$$

where M is the molecular weight of the protein; Δa is the change in the total accessible surface area (often referred to as surface area or simply as area), both polar and nonpolar, per unit weight of the protein upon unfolding; f_n and f_p are the nonpolar and polar fractions of Δa, respectively; g_n and g_p are the free energy change upon exposure of unit area of nonpolar and polar character, respectively; and s_{conf} is the conformational entropy change per unit weight. g_n and g_p represent some average strength of the polar and nonpolar interactions, respectively, and are assumed to be species-independent. g_p, in particular, includes the difference between intraprotein and protein–water hydrogen bond energies, averaged over all proteins on a per polar surface area basis. Δa is also assumed to be species-independent. If one models the denatured protein as a fully extended chain (see below, however), the statistical relation between areas and the molecular weights of proteins (28) can be used to calculate the value of Δa. It varies from 0.98 to 1.20 Å^2/Da for the proteins used by Murphy *et al.* (7), the molecular weights of which vary from 11,500 to >8 times as large. s_{conf} is assumed to be independent of species and temperature. Notice that the form of Eq. **13** is similar to that proposed by Brandts (29) to interpret the denaturation thermodynamics of chymotrypsinogen, although he used the number of residues exposed p rather than the area exposed Δa as the primary variable for measuring the degree of unfolding.

This equation can be written in a form that is linear in f_n,

$$\Delta G/M = [(g_n - g_p)f_n + g_p]\Delta a - Ts_{conf}. \quad [14]$$

The variable f_n is then assumed to be our X. The surface area exposed upon unfolding is the same as that buried upon folding. f_n is, therefore, clearly related to the number of nonpolar contacts in the interior of the native protein, a property that was found to correlate with the heat capacity change upon denaturation (2). If the denatured protein is again modeled as a fully extended chain, the f_n values for ribonuclease, lysozyme, and myoglobin calculated from the data of Lee and Richards (30) are 0.44, 0.49, and 0.53, respectively. The experimental denaturation heat capacity changes follow the same order.

This model then predicts linear MPG plots for both enthalpy and entropy. The temperatures T_h^* and T_s^* are those at which $h_n - h_p$ and $s_n - s_p$ become zero, respectively; i.e., the polar and nonpolar parts become equal. Since $T_h^* = T_s^*$ experimentally, g_n also equals g_p at this common temperature. (The actual nonpolar and polar contributions to the free energy change upon denaturation are given by $f_n g_n$ and $f_p g_p$, which are not exactly the same even at T_s^*.) According to this model, the term that is labeled ΔG°_{hyd} in Murphy *et al.* (7) does not measure the hydrophobic or hydration part of the free energy change for protein denaturation. Rather, it basically measures $a_g = g_n - g_p$, the difference between the average strengths of nonpolar and polar interactions per area.

The consequences of this model can be explored further by making the following additional assumptions: (*i*) that the nonpolar part is the same, on a per area basis, as that given by the $l \rightarrow w$ small molecule transfer process, (*ii*) that the polar part makes only a temperature-independent enthalpic contribution, and (*iii*) that Δa is independent of temperature. Assumption (*i*) is essentially the same as that of Baldwin (6). Assumption (*ii*) makes the analysis simple; the heat capacity change due to polar interactions is unlikely to be zero (11, 31), but the essential features of the model will not be altered as long as the heat capacity and the entropy changes are small compared with their nonpolar counterparts. Baldwin (6) found that, if the nonpolar part is subtracted from the total change for the denaturation, the remainder is nearly independent of temperature for the denaturation of lysozyme. Assumption (*iii*) is potentially serious and future studies will have to be directed on examining consequences of having Δa dependent on temperature. The remainder of this section makes all three assumptions.

The isoenthalpic and isoentropic temperatures are given by

$$T_h^* = T_{hn}^* + h_p/c_n \quad [15]$$

and

$$\ln(T_s^*/T) = -s_n/c_n, \quad [16]$$

where we have written T_{hn}^* for T_h^* of small molecule $l \rightarrow w$ process, $h_x = -T^2\partial(g_x/T)/\partial T$, $s_n = -\partial g_n/T$, and $c_n = \partial h_n/\partial T$. Eq. **16** directly shows that T_s^* for the protein denaturation is the same as that for the small molecule $l \rightarrow w$ process. Eq. **15** shows that the difference between T_h^* and T_{hn}^* is indeed caused by the polar interaction. If $g_p = g_n(T_s^*)$, one has $h_p = g_p = g_n(T_s^*) = h_n(T_s^*)$ and T_h^* becomes equal to T_s^*. We have of course used the converse argument earlier that $g_p = g_n(T_s^*)$ because $T_h^* = T_s^*$. The coincidence of T_h^* and T_s^* for protein denaturation, therefore, appears in this model as a consequence of the fact that the free energy changes upon exposure of the polar and the nonpolar groups happen to be closely similar on a per area basis at T_s^*.

The value of g_n may be taken to be ≈25 cal per mol per Å^2 (1 cal = 4.184 J) at room temperature (8). By using Eq. **12** with 22°C for T_h^* for the small molecule $l \rightarrow w$ process (6), the value of g_n at T_s^* then becomes 29 cal per mol per Å^2. In the present model, this is also the value of g_p at all temperatures. The surface area of a main-chain peptide group is ≈45 Å^2 (30). If it is assumed that these groups are the sole contributor to the polar interactions, the above value of g_p corresponds to about 1.3 kcal/mol of peptide group. This is a reasonable value for one internal (uncharged) hydrogen bond per peptide group (32). Because the temperature dependence of g_n is small, it is nearly the same as g_p even at room temperature. If the polar and nonpolar parts become buried independently, this means that there will be no fractionation of polar and nonpolar surfaces upon protein folding. This expectation is consistent with the observation (28, 30) that polar/nonpolar composition of the surface area of the native protein is the same as that of the fully extended chain. This also gives a justification for assuming that f_n is independent of temperature, a required property of X.

The model gives definite interpretations of the y intercepts of the MPG plots. As suspected by Privalov (2), ΔS^* gives $s_{conf.}$ ΔH^* gives $h_p\Delta a$ and, since h_p is known, the value of Δa. By using the value given above for g_p and the experimental value of 13 cal/g for ΔH^* (2), Δa becomes 0.45 Å^2/Da. If the formulas given by Miller *et al.* (28) are used, the areas of the

native and the fully extended forms are, respectively, 0.28 to 0.50 and 1.48 Å^2/Da for the set of proteins used by Murphy *et al.* (7). Thus the area change upon denaturation is about half of the difference between the fully extended and native forms. This roughly compares with 0.634 obtained by Brandts (29) for the parameter p in his free energy expression for chymotrypsinogen. The surface area increases roughly by a factor of two upon unfolding, and this is consistent with earlier estimates made from entirely different experimental data (33, 34). The area of the denatured protein is no more than about two-thirds that of the fully extended form.

At T_s^*, g_n and g_p are equal. Since f_n is nearly 0.5, actual contributions, $f_n g_n$ and $f_p g_p$, are also approximately equal. As the temperature is lowered from T_s^*, the nonpolar contribution to the free energy decreases slowly whereas the polar contribution remains constant. Thus, both the polar and the nonpolar effects favor the folded form but the latter becomes weaker at lower temperatures. At some low temperature, the reduction in the nonpolar contribution can become sufficient for the total free energy to change its sign, at which point the cold denaturation will occur. Since the temperature dependence of the nonpolar contribution is small, this will happen only if the sum of the polar and nonpolar contributions to the free energy is closely balanced by the conformational entropy change. This is indeed the case since the value of $T_s^* \Delta S^*$, which equals $T_s^* s_{conf}$, is 14.4 cal/g (7) and that of ΔH^*, which equals $[f_n g_n + f_p g_p]\Delta a$ at T_s^*, is 13 cal/g (2). That the former is slightly greater than the latter means that T_s^* is beyond the (high-temperature) denaturation temperature.

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