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Relation between the convergence temperatures T^*_h and T^*_s in protein unfolding

(liquid hydrocarbon model)

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ABSTRACT **A challenge in understanding the thermodynamics of protein unfolding is to explain the 1979 puzzle posed by Privalov. Why do values of the specific enthalpy and specific entropy of unfolding both converge to common values at approximately the same temperature ($T^*_h \cong T^*_s$) when extrapolated linearly versus temperature? In 1986, a liquid hydrocarbon model gave an explanation for convergence of the specific entropies at T^*_s: it happens because the contribution of the hydrophobic effect to the entropy of unfolding goes to zero at T^*_s. The reason for convergence of the specific enthalpies at T^*_h and for the equality $T^*_h \cong T^*_s$ has remained, however, a matter for speculation; recently, some explanations have been given that are based on models for polar interactions in protein folding. We show here that the relation $T^*_h \cong T^*_s$ can be derived straightforwardly without making any assumptions either about polar interactions or about splitting the hydrophobic interaction into two terms—one for the "hydrophobic hydration" and the other for the residual effect, as suggested recently. Thus, the liquid hydrocarbon model explains both halves of Privalov's puzzle. A similar conclusion has been reached independently by A. Doig and D. H. Williams (personal communication). It has been proposed recently that a correction should be made for the relative sizes of a hydrocarbon solute and water when computing the thermodynamic properties of the hydrophobic interaction from a solvent transfer experiment. This correction affects the temperature at which the entropy of transfer equals zero, and it is important to evaluate its effect on the convergence temperature T^*_s. We show that making the size correction does not change the conclusion, reached earlier, that the liquid hydrocarbon model explains the convergence of the specific entropies of protein unfolding.**

A liquid hydrocarbon model was shown in 1986 (1) to explain one-half of Privalov's puzzle (2): why do the specific entropies of protein unfolding (entropy per g of protein) converge to a common value for a set of globular proteins when extrapolated linearly to T^*_s = 112°C? Calorimetric and solubility data for the dissolution of liquid hydrocarbons in water show that the unitary entropy of the transfer process becomes zero at a common temperature (T_s = 112°C), which equals, within error, the convergence temperature for the specific entropies of protein unfolding (1). Thus, in unfolding, entropy convergence for the set of proteins studied occurs at the temperature where, in the study of model hydrocarbons, the hydrophobic interaction is purely enthalpic and occurs without any entropy change. The change in conformational entropy upon unfolding is believed to be the other major factor, in addition to the hydrophobic interaction, that contributes to the entropy change. Thus, entropy convergence at 112°C implies that the specific change in conformational entropy upon unfolding has similar values for different proteins in the set studied by Privalov.

This result indicated that the liquid hydrocarbon model could be used to give the temperature dependence of the hydrophobic interaction in protein folding. Each of the thermodynamic quantities $\Delta H°$, $\Delta S°$, and ΔCp for dissolution of a liquid hydrocarbon in water (where $\Delta H°$ and $\Delta S°$ are standard enthalpy and entropy changes and ΔCp is change in heat capacity) is proportional to the water-accessible surface area of the hydrocarbon (3–8). This proportionality, used together with the solvent transfer model of Kauzmann (9) and Tanford (10–12), allows the contribution of the hydrophobic interaction to $\Delta H°$, $\Delta S°$, and ΔCp for protein unfolding to be computed, if the change in nonpolar, water-accessible surface area upon unfolding is known. The solvent transfer model states that the hydrophobic interaction of, for example, a phenylalanine side chain with water can be modeled by the interaction of toluene with water, and that burial of the side chain through folding can be modeled by the transfer of toluene from water to a nonpolar solvent.

Relation Between "Hydrophobic Hydration" and ΔCp

In 1986 it appeared, however, that the liquid hydrocarbon model was unable to explain the second half of Privalov's puzzle: why do the specific enthalpies of unfolding also converge to a common value at $T^*_h = T^*_s$ for this set of proteins? This apparent failure of the liquid hydrocarbon model played an important role in later thinking about how to model the hydrophobic interaction in protein folding. In 1990, Murphy, Privalov, and Gill (13, 14) proposed that the properties of liquid hydrocarbons do not provide an appropriate model for protein folding. They argued instead that the equality $T^*_h = T^*_s$, observed in protein unfolding experiments, provides the correct starting point for analysis, and, based on the observation of a single convergence temperature $T^*_h = T^*_s$, they defined a quantity called the hydrophobic hydration (13–15). It can be regarded as the water-ordering effect of a dissolved hydrocarbon and it has, by definition, $\Delta H° = 0$, $\Delta S° = 0$, and therefore $\Delta G° = 0$, at $T^*_h = T^*_s$.

The properties of model hydrocarbons show, however, that ΔCp for the hydrophobic interaction is not zero at T_s (16). This point is illustrated in Table 1. Because values of ΔCp decrease at high temperatures (14), a curved extrapolation is needed to find T_s when taking account of this temperature dependence. Thus, the exact value of T_s is not certain, but it has been given as ≈413 K (14). At this temperature Table 1 shows that the values of ΔCp for benzene and toluene are about 0.6 times their values at 298 K (17), and the gas → liquid values of ΔCp for some noble gases at temperatures above 413 K are, in most cases, as large as 0.4 times the value at 298 K. Moreover, two models (7, 16) that correlate other properties of the hydrophobic interaction and that predict the temperature dependence of ΔCp both predict that such large values of ΔCp at high temperatures are reasonable. Thus, it

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Table 1. Measurements of ΔCp at high temperatures

Solute	T, K	$\Delta Cp(T)/\Delta Cp(298\ K)$	Ref.
Benzene	413	0.66†	17
Toluene	413	0.62†	17
Neon	520	0.42‡	18
Argon	520	0.41‡	18
Krypton	520	0.28‡	18
Methane	520	0.35‡	19
Argon	463	0.7‡	19

†Ratio of ΔCp at high temperature to ΔCp at 298 K; the transfer process is from liquid hydrocarbon to aqueous solution.
‡The transfer process is from the gas phase to aqueous solution.

seems clear that ΔCp is not zero at T_s. Most workers agree that the most direct measure of the water-ordering effect of a dissolved hydrocarbon is a large, positive value of ΔCp. Thus, there is a serious conceptual difficulty in defining a hydrophobic hydration that is characterized by $\Delta H°$, $\Delta S°$, and $\Delta G° = 0$ at T_s but for which ΔCp is not zero at T_s. In the following section, we show that the relation $T_h^* \cong T_s^*$ can be derived without splitting the hydrophobic interaction into two terms.

Relation Between T_h^* and T_s^*

In deriving a relation between T_h^* and T_s^*, we treat ΔCp for unfolding as independent of temperature. Thus, we are considering the values of T_h^* and T_s^* that are found by linear extrapolation from moderate temperatures (20°C–70°C). The assumption ΔCp = constant has been made in other recent treatments of this problem, both by Lee (20) and by Murphy and Gill (8). Unlike these treatments, we do not consider here the role of polar interactions in folding but we also do not assume that ΔCp results solely from the exposure of nonpolar surface to water, since recent evidence shows otherwise (8, 21, 22). We first make use of the well-known relation for unfolding

$$\Delta H°(T_m) = T_m \Delta S°(T_m), \quad [1]$$

where T_m is the temperature midpoint of the unfolding transition, and the Gibbs energy change $\Delta G°$ of unfolding is zero. Next, we consider the values of $\Delta H°$ and $\Delta S°$ at T_s^*, the entropy convergence temperature,

$$\Delta H°(T_s^*) = \Delta H°(T_m) + \Delta Cp(T_s^* - T_m), \quad [2]$$

$$\Delta S°(T_s^*) = \Delta S°(T_m) + \Delta Cp \ln \frac{T_s^*}{T_m}. \quad [3]$$

Because (T_s^*/T_m) is close to 1, we can expand its logarithm,

$$\ln \frac{T_s^*}{T_m} = \frac{T_s^* - T_m}{T_m} - \frac{(T_s^* - T_m)^2}{2T_m^2} + \ldots. \quad [4]$$

By equating the values of $\Delta H°(T_m)$ in Eq. 1 and 2, and then making use of Eqs. 3 and 4, we have the final result

$$\Delta H°(T_s^*) = T_m \Delta S°(T_s^*) + \Delta Cp \frac{(T_s^* - T_m)^2}{2T_m} + \ldots. \quad [5]$$

If T_m = 331 K (see below), then the second term on the right in Eq. 5 is 4.4 ΔCp, and thus Eq. 5 can be written

$$\Delta H°(T_s^* - 4.4) \cong T_m \Delta S°(T_s^*). \quad [6]$$

Eq. 5 and 6 state that different proteins should have the same value of $\Delta H°$ per g of protein at a temperature about 4°C below T_s^*, if their values of T_m (in degrees Kelvin) are close to 331 K and if their values of $\Delta S°$ at T_s^* are equal. The values of T_m will not, in general, be equal but calculations based on Eq. 6 show that modest differences in T_m have little effect. Proteins have unique values of T_m only in fixed solvent conditions: T_m depends strongly on pH in particular. On the other hand, $\Delta H°$ depends on temperature and is often independent of pH (2). The values of $\Delta S°$ and T_m should be taken at the same pH, when $\Delta H°$ is pH independent and T_m depends on pH. The values of T_m that correspond to Privalov's extrapolations of $\Delta H°$ and $\Delta S°$ versus temperature are given in his figure 10 (2) in the form of plots of $\Delta H°$ per mol versus T_m. When the average T_m for each protein is taken from the midpoint of its plot of $\Delta H°$ versus T_m, then the mean T_m for the entire set of proteins is 331 K (±9 SD). The SD in T_m is <3% of the mean. Consequently, the variation in T_m can be neglected in using Eqs. 5 and 6 for Privalov's set of proteins to a good approximation.

To check these equations against experimental data, we use Sturtevant's data for hen lysozyme (23) because they have been used earlier and $\Delta S°(T_s^*) = \Delta S°_{res}$ (see Eq. 11 below) is known (1): $\Delta S°_{res}$ = 2290 J·mol^{-1}·deg^{-1}. In Sturtevant's experimental conditions, hen lysozyme has a high T_m (349 K), and so we use Eq. 5 to compare predicted and observed values of $\Delta H°$ at T_s^*. The "observed" value is found by linear extrapolation of the measured values to be 811 J·mol^{-1}. The two terms on the right of Eq. 5 are computed to be 799 and 12, and thus the predicted value of $\Delta H°$ at T_s^* is 811 J·mol^{-1}, using ΔCp = 6530 J·mol^{-1}·deg^{-1} (23), and the predicted and observed values agree.

The overall conclusion is that values of $\Delta H°$ for unfolding of different proteins (of the kind considered in Privalov's data set) should converge approximately to a common value a few degrees below T_s^*. Because the liquid hydrocarbon model shows $T_s^* = T_s$ and explains why convergence of the unfolding entropies occurs at T_s^*, this model provides a straightforward explanation of both halves of Privalov's puzzle. No assumption is needed about the relative weights of polar and nonpolar interactions, or about splitting the hydrophobic interaction into two terms, one of which should be temperature independent.

Effects of Polar Interactions

Some other recent treatments of this problem (8, 20, 22) conclude that specific properties of polar interactions in folding explain $T_h^* \cong T_s^*$. Lee (20) presents an interesting analysis of the conditions needed for the existence of T_h^* and T_s^*. He shows that, for a given family of molecules, if ΔCp is independent of temperature and if some molecular property x exists such that $\Delta H°$, $\Delta S°$, and ΔCp are all linear functions of x, then convergence temperatures will be found both for $\Delta H°$ and $\Delta S°$. Lee proposed that, in protein unfolding, a plausible candidate for the molecular property x is the fraction of the change in water-accessible surface area that is nonpolar. He then used the observed relation $T_h^* \cong T_s^*$ to analyze the balance between polar and nonpolar interactions in folding.

Both Murphy and Gill (8) and Spolar *et al.* (22) use specific models for polar interactions in folding. Murphy and Gill state that, although the hydrophobic effect they measure in these experiments is different from the hydrophobic hydration defined earlier (15), it also differs by definition from the hydrophobic interaction that is modeled in solvent transfer experiments (see below). Murphy and Gill use calorimetric data for the dissolution in water of crystalline cyclic dipeptides, while Spolar *et al.* use data for the dissolution in water of liquid amides. Herzfeld (24) and Lee (20) have both pointed out inherent advantages of using liquid → liquid transfer data, rather than solid → liquid or gas → liquid data, in modeling interactions that occur in protein folding. Ben-Naim (25) and

Roseman (26) discuss problems that arise in modeling polar interactions. Ben-Naim argues that the enthalpy change for forming a peptide hydrogen bond should have quite different values in an isolated α-helix exposed to water as compared to a helix buried inside a protein away from water. Roseman shows that when polar and nonpolar groups are neighbors, large deviations can occur from simple additivity relations based on the behavior of only one kind of group. Recent work on the hydrophobic interaction in protein folding, and on the significance of $T_h^* \cong T_s^*$, has been reviewed by Creighton (27).

A different approach to the problem has been taken by Doig and Williams (28). They find that $\Delta H°$ and $\Delta S°$ of unfolding are correlated with the number of disulfide bonds per g of protein and that these correlations affect the interpretation of the relation $T_h^* \cong T_s^*$.

Solute and Solvent Size Correction to Data for the Hydrophobic Interaction

Based on their experimental work, DeYoung and Dill (29) proposed that a correction should be made in solvent transfer experiments for the relative sizes of solute and solvent, and their proposal has been supported by a theoretical analysis made by Sharp, Honig, and coworkers (30–32). When the correction is made, the coefficient relating the Gibbs energy of transfer to the change in accessible nonpolar surface area nearly doubles (29–32). Moreover, since the correction directly affects the entropy of transfer, it enters into the evaluation of T_s and the relation between T_s^* and T_h^*.

In the original development of the hydrocarbon model (1), the standard state of a dissolved hydrocarbon in water was so defined that the Gibbs energy and entropy changes for transfer from the pure liquid to the aqueous solution are given by

$$\Delta G° = -RT \ln X, \quad [7]$$

$$\Delta S° = \frac{\Delta H°}{T} + R \ln X, \quad [8]$$

where X is the solute mol fraction in the saturated solution. Recently, Sharp *et al.* (31) proposed that the standard state should be revised so as to give these quantities values that depend on r, the molar volume ratio $\bar{V}_{solute}/\bar{V}_{water}$, in a way that is similar to the Flory-Huggins theory—i.e.,[§]

$$\Delta G°' = -RT \ln X - RT \ln r - RT(1 - r), \quad [9]$$

$$\Delta S°' = \frac{\Delta H°}{T} + R \ln X + R[\ln r + (1 - r)]. \quad [10]$$

We are concerned here with the effect of the size correction on the convergence of the specific entropies of unfolding of different proteins.

The entropy change given by Eq. **8** appears in Eq. **10** as that portion of $\Delta S°'$ that remains when $r = 1$ and the size correction vanishes. We assume that r can be considered independent of temperature. Then it follows that $\Delta S°$ and $\Delta S°'$ have the same temperature dependence, given by $[\partial(\Delta S)/\partial(\ln T)]_p = \Delta Cp$. Values of $\Delta S°$ (Eq. **8**) become zero at a common temperature, T_s, for different liquid hydrocarbons (1, 14). Comparison of Eqs. **8** and **10** shows that, for a series of nonpolar solutes with different sizes that obey this relation, their values of $\Delta S°'$ will not become zero at any common temperature. As noted above, the size correction for any single solute is temperature independent.

[§]See *Note Added in Proof.*

Consider next the application of Eqs. **7** and **9** to protein unfolding. As before (1), we write

$$\Delta S°_{unf} = \Delta S°_{hyd} + \Delta S°_{res}, \quad [11]$$

$$\Delta G°_{hyd} = \Delta Cp_{hyd}\left[(T - T_h) + T \ln\left(\frac{T_s}{T}\right)\right], \quad [12]$$

where $\Delta S°_{hyd}$ and ΔCp_{hyd} result from the change in water-accessible nonpolar surface area caused by unfolding. We avoid the assumption made earlier (1) that $\Delta Cp_{unf} \cong \Delta Cp_{hyd}$, because polar groups have been shown to contribute significantly to ΔCp_{unf} (8, 21, 22). Because the $\Delta S°$ values for hydrocarbons of different sizes become zero at T_s, $\Delta S°_{hyd}$ in Eq. **11** should be zero at T_s, and then $\Delta S°_{unf} = \Delta S°_{res}$.

To examine the contribution of the size-correction term to $\Delta S°_{unf}$, it is necessary to determine how such a size correction should be made for the folded and unfolded forms of a protein. The problem has been discussed recently (32). Just as for hydrocarbon solutes, the size correction term for a protein should be temperature independent. Therefore, it can be absorbed into $\Delta S°_{res}$ in Eq. **11**. As noted above, $\Delta S°_{res}$ is determined chiefly by the large change in conformational entropy upon unfolding. Earlier (1), the conclusion was drawn that the specific values of $\Delta S°_{res}$ are similar for different proteins, because $\Delta S°_{hyd}$ vanishes at T_s (112°C) and the specific entropies of protein unfolding converge, approximately, at 112°C. This conclusion remains unchanged when the size correction is made but $\Delta S°_{res}$ now includes, in addition to the change in conformational entropy upon unfolding, the size correction term. Our conclusion is that this size correction makes no essential difference to the previous deduction (1) that T_s^* for protein unfolding equals T_s in the liquid hydrocarbon model.

A different size effect is predicted by scaled particle theory, which, in its original form (see ref. 33 and references therein), considers hard-sphere solutes and solvents and predicts the Gibbs free energy change for transferring the solute from its ideal-gas phase to a solution of hard spheres. The theory has been adapted by Pierotti (34) to treat hydrocarbon solutes in aqueous solutions. The small size of the H_2O molecule endows aqueous solutions with unusual properties, according to scaled particle theory, and Lucas (35) and Lee (36, 37) have proposed that these properties make a major contribution to the hydrophobicity of hydrocarbon solutes in aqueous solution. Molecular dynamics simulations of the formation of small and medium-sized cavities in water compare favorably with the predictions of scaled particle theory (38, 39); extension of this work to include larger cavities and a range of temperatures is needed.

Experimental Models of the Hydrophobic Effect

Recently, there has been discussion of what experimental systems should be used to model the hydrophobic effect in protein folding (8, 13–16, 22, 24, 27). This issue is related to the problem of whether the hydrophobic interaction itself, or some component of the hydrophobic interaction, is destabilizing for protein folding (13–16, 20, 27). The approach taken here follows from the work of Kauzmann (9) and Tanford (10–12) (see also ref. 24) in giving an operational definition of the hydrophobic interaction in protein folding based on the solvent transfer experiment. Interpretation of the transfer experiment is simplified by choosing a liquid hydrocarbon both as the solute undergoing transfer and as the nonpolar solvent from which transfer occurs. Defined in this way, the hydrophobic interaction stabilizes protein folding at all temperatures: otherwise, the liquid hydrocarbon would have to be highly soluble in water.

When used in interpreting the thermodynamics of protein unfolding, solvent transfer experiments give the proportionality coefficient (3, 4) between the water-accessible surface area (40) of a nonpolar solute and the change in Gibbs energy for transferring the solute from a nonpolar solvent environment to water. To make use of this proportionality (3, 4), the change in nonpolar surface area upon unfolding must be known. Nonpolar surface in an unfolded protein is not, in general, fully exposed to water (20, 28) and one of the unsolved problems in this field is how to determine the fraction that is exposed.

The solvent transfer experiment does not model all of the events that occur when a hydrophobic side chain previously buried in the interior of a protein becomes exposed to water. The interior of a protein is close packed, as in an organic crystal (41), and stronger van der Waals interactions may be made between the side chain and neighboring atoms inside the protein than between a hydrocarbon solute and molecules of a nonpolar solvent (42). Cavities may exist inside the hydrophobic core of a protein, and the presence of a cavity is an important factor contributing to the $\Delta G°$ of unfolding (43). A hydrophobic side chain is found in a unique rotamer configuration when the side chain is buried inside a protein, whereas free rotation about appropriate side-chain bonds occurs when the protein is unfolded. This effect also contributes significantly to the $\Delta G°$ of unfolding (32, 44). These different effects can be recognized and treated separately from the hydrophobic effect that is modeled by solvent transfer experiments.

Note Added in Proof. See the recent discussion of Flory-Huggins theory in interpreting partition coefficients (45).

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