Proc. Natl. Acad. Sci. USA
Vol. 83, pp. 8069–8072, November 1986
Biochemistry

Temperature dependence of the hydrophobic interaction in protein folding

(hydrocarbon model)

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Contributed by Robert L. Baldwin, July 21, 1986

ABSTRACT **Accurate calorimetric data for the thermodynamics of transfer of six liquid hydrocarbons to water have been combined with solubility data to provide a model for the temperature dependence of the hydrophobic interaction in protein folding. The model applies at temperatures for which the change in heat capacity (ΔCp) is constant. The extrapolated value of the temperature (T_s) at which the entropy of transfer ($\Delta S°$) reaches zero is strikingly similar (T_s = 112.8°C ± 2.2°C) for the six hydrocarbons. This finding provides an interpretation for the empirical relation discovered by Sturtevant: the ratio $\Delta S°/\Delta Cp$ measured at 25°C is constant for the transfer of nonpolar substances from nonaqueous media to water. Constancy of this ratio is equivalent to T_s = constant. When applied to protein folding, the hydrocarbon model gives estimates of the contributions of the hydrophobic interaction to the entropy and enthalpy changes on unfolding and, by difference, estimates of the residual contributions from other sources. The major share of the large enthalpy change observed on unfolding at high temperatures comes from the hydrophobic interaction. The hydrophobic interaction changes from being entropy-driven at 22°C to being enthalpy-driven at 113°C. Finally, the hydrocarbon model predicts that plots of the specific entropy change on unfolding versus temperature should nearly intersect close to 113°C, as observed by Privalov.**

The thermodynamic properties of the unfolding reactions of globular proteins are now known accurately as a function of temperature through calorimetric studies. Many of these data have been obtained by Privalov and co-workers, and they are summarized and analyzed by him (1). The unfolding reactions of different proteins display certain common properties. The enthalpy of unfolding depends on the temperature at which unfolding occurs, which can be varied by adjusting pH or guanidine hydrochloride concentration. The unfolding enthalpy is small at room temperature but increases rapidly with temperature, becoming large at high temperatures. ΔCp, the difference in heat capacity between the native and unfolded forms, is independent of temperature in the range studied (up to 80°C). Plots of the specific enthalpy of unfolding (enthalpy per g) versus temperature intersect at a common high temperature for several globular proteins [at 110°C, see figure 24 of Privalov's review (1)]. The slope of the plot, which is ΔCp per g of protein, is linearly related to the fraction of hydrophobic residues. These proteins also show an approximate intersection point near 110°C when the specific entropy of unfolding is plotted against temperature (see figure 26 in ref. 1).

Some of these properties are understood but others are obscure. The large and positive value of ΔCp is commonly attributed to the hydrophobic interaction, although other factors may contribute to ΔCp (2, 3). As early as 1935, Edsall (4) observed that the transfer of a nonpolar compound from an organic medium to H_2O is characterized by a large positive value of ΔCp. The nature of the large enthalpy change in unfolding at high temperatures is unknown. The reason for the intersection near 110°C in plots of the specific enthalpy and entropy of unfolding is also not known. Privalov (1) suggested that it must result from the properties of the hydrophobic interaction.

The purpose of this article is to show that data for the thermodynamics of solution of liquid hydrocarbons in H_2O provide a model for the temperature dependence of the hydrophobic interaction in protein folding. Accurate calorimetric data are available for the heat of solution in H_2O of seven liquid hydrocarbons in the range 15°C–35°C (5). The seven hydrocarbons show quite uniform behavior, and an equation of state has been given (6) that describes their thermodynamic properties as functions of a single variable, n_H, the number of H atoms per molecule. I show here that a hydrocarbon model based on these data provides explanations for some of the thermodynamic properties of protein unfolding reactions. In particular, the hydrocarbon model predicts that the major share of the large enthalpy change observed on protein unfolding at high temperatures comes from the hydrophobic interaction.

As defined here, "hydrophobic interaction" refers to the process in which a hydrophobic side chain of an unfolded protein is taken out of H_2O and is buried in the interior of a protein through folding. When this definition is used, the hydrophobic interaction can be modeled by solvent transfer experiments, as shown originally by Kauzmann (7), and developed by Tanford (8–11) and other workers (12, 13). The solvent transfer model is open to criticism, particularly because the interior of a protein differs in obvious respects from an organic liquid (14–17). Nevertheless, following an earlier analysis (18), Rose *et al.* (19) find a close correlation between the average extent of burial of an amino acid side chain and its hydrophobicity on the scale of Nozaki and Tanford (10). The liquid transfer process is used here as a semi-quantitative model for the burial of hydrophobic side chains during folding, and criticisms of the model are discussed later.

Transfer of a hydrocarbon from the pure liquid to H_2O can be divided into two steps: (*i*) transfer from the liquid hydrocarbon into the vapor phase and (*ii*) transfer from the vapor phase into H_2O. Data for the second step of the transfer process have been given for compounds that serve as models for amino acid side chains (20). When the overall process is analyzed theoretically (21–26), it is necessary to consider each step separately. Experimentally, the overall transfer process can be modeled by a single experiment, either by a liquid partition experiment or by a solubility experiment. The

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Abbreviations: T_s, temperature at which $\Delta S° = 0$; T_h, temperature at which $\Delta H° = 0$. ($\Delta S°$ and $\Delta H°$ refer to the standard-state entropy and enthalpy changes for transfer of a hydrocarbon from the liquid hydrocarbon to H_2O.)

equations used to analyze these experiments are presented by Tanford (11).

Properties of the Liquid Hydrocarbon Model

The standard Gibbs energy of transfer (per mol), $\Delta G°$, is related to the solubility X by

$$\Delta G° = -RT \ln X, \quad [1]$$

where X is the mol fraction of hydrocarbon dissolved in H_2O. The purpose of using the mol fraction scale is to avoid including in $\Delta G°$ a term involving the entropy of mixing (7, 11). It has been argued that a number density scale such as the molar scale should be used instead (27, 28). For consistency with earlier work in the literature, I use the mol fraction scale here. Numerical examples of the change in $\Delta G°$ caused by shifting to Ben-Naim's concentration scale have been given (26). The standard-state entropy of transfer, $\Delta S°$, is obtained from

$$\Delta S° = \Delta H°/T + R \ln X, \quad [2]$$

where $\Delta H°$ is the standard enthalpy of transfer. Data for $\Delta S°$, $\Delta H°$, and X at 25°C are given in Table 1 for the six liquid hydrocarbons considered. [Propylbenzene, which also was studied (5) is omitted here for lack of solubility data.]

Data for seven liquid hydrocarbons show $\Delta H°$ to be a linear function of temperature in the range studied, 15°C–35°C, with ΔCp constant (5):

$$\Delta H°(T_2) = \Delta H°(T_1) + \Delta Cp(T_2 - T_1). \quad [3]$$

When ΔCp is nonzero, $\Delta S°$ like $\Delta H°$ must be a function of ΔCp and temperature. If ΔCp is constant, then

$$\Delta S°(T_2) = \Delta S°(T_1) + \Delta Cp \ln(T_2/T_1). \quad [4]$$

For the transfer of a nonpolar substance to H_2O at 25°C, $\Delta S°$ is negative and ΔCp is positive; therefore, $|\Delta S°|$ decreases with temperature and approaches zero at a high temperature.

Sturtevant (2) used model compound data to analyze the changes in entropy and heat capacity that occur in protein unfolding reactions. For the transfer of a nonpolar substance to water, he found an empirical relation between $\Delta S°$ and ΔCp, measured at 25°C: $\Delta S°/\Delta Cp = -0.263 \pm 0.046$. His relation corresponds to finding a constant temperature at which $\Delta S°$ goes to zero. If T_2 is taken to be T_s, the temperature at which $\Delta S°$ is zero, then Eq. 4 becomes

$$-\Delta S°/\Delta Cp = \ln(T_s/T). \quad [5]$$

Thus, if T_s is constant, the ratio $\Delta S°/\Delta Cp$ is constant. The temperatures at which $\Delta S°$ and $\Delta H°$ go to zero are denoted here as T_s and T_h, following the notation used by Becktel and Schellman to describe the thermodynamics of folding of T4 lysozyme mutants (J. A. Schellman, personal communication). The values of $\Delta S°$, ΔCp, and T_s are given in Table 1. The six liquid hydrocarbons show closely similar values of T_s: 386.0 K or 112.8°C ± 2.4°C. From Sturtevant's relation (2), obtained from data for a wide range of nonpolar compounds, one finds T_s = 387.8 K or 114.6°C ± 18°C.

Eq. 5 can be tested with data taken at different temperatures. Table 2 provides such a test for benzene in H_2O, for which the necessary solubility data (30) and calorimetric data (5) are available in the range 15°C–35°C. Table 2 shows that the same value of T_s for benzene is found from data taken at different temperatures. This is not surprising, because ΔCp is known to be constant in this range and Eq. 5 assumes only that ΔCp is constant.

What is the meaning of a constant value of T_s found for different nonpolar substances? When $\Delta S°$ is zero, the solution shows ideal entropy of mixing by definition. T_s is the temperature at which an aqueous solution of a nonpolar substance becomes a regular solution, with $\Delta S° = 0$ but with $\Delta H°$ nonzero [cf. Shinoda and Fujihira (31)]. There are reasons for suspecting that ΔCp decreases at high temperatures (see below). In this case, T_s is a hypothetical temperature. (In any case, T_s is above the boiling point of H_2O.) If ΔCp decreases at high temperatures, then T_s is the temperature at which $\Delta S°$ would go to zero if the solution obeyed the same rules at high temperatures as in the range for which ΔCp is constant. Shinoda and Fujihira (31) and Shinoda (32) consider the behavior of liquid hydrocarbons dissolved in H_2O at high temperatures: they postulate that ΔCp should decrease at high temperatures and that $\Delta S°$ should reach zero in the range 120°C–160°C.

The seven liquid hydrocarbons show similar values of T_h, the temperature at which the solubility is a minimum and $\Delta H° = 0$ (5). The average value of T_h for the six hydrocarbons in Table 1 is 22.2°C ± 5.5°C. $\Delta H°$ at any temperature T in the range where ΔCp is constant can be calculated from the simple relation

$$\Delta H° = \Delta Cp(T - T_h). \quad [6]$$

Table 1. Thermodynamic properties of liquid hydrocarbons dissolved in water (25°C)

Substance*	Solubility,† mol fraction × 10^{-4}	$\Delta H°$,‡ kJ·mol^{-1}	ΔCp,‡ J·mol^{-1}·deg^{-1}	$\Delta S°$,§ J·mol^{-1}·deg^{-1}	T_h,¶ °C	T_s,‖ °C
Benzene	4.01	2.08	225	−58.06	15.8	112.8
Toluene	1.01	1.73	263	−70.7	18.4	116.9
Ethylbenzene	0.258	2.02	318	−81.0	18.6	111.5
Cyclohexane	0.117	−0.1	360	−94.8	25.3	114.7
Pentane	0.095	−2.0	400	−102.8	30.0	111.9
Hexane	0.020	0.0	440	−109.1	25.0	108.9
				Average	22.2 ± 5.5 (SD)	112.8 ± 2.4 (SD)

*Propylbenzene is omitted for lack of solubility data.
†Data of McAuliffe (29), except for benzene (30).
‡$\Delta H°$, ΔCp, and $\Delta S°$ are given for the transfer of the hydrocarbon from liquid hydrocarbon to H_2O. Data of Gill *et al.* (5) for the enthalpy of solution ($\Delta H°$) and the difference in heat capacity (ΔCp) between aqueous solution and liquid hydrocarbon.
§Standard entropy of solution, from Eq. 2.
¶Temperature of minimum solubility, where $\Delta H° = 0$; data of Gill *et al.* (5).
‖Temperature where $\Delta S° = 0$, calculated from $\Delta S°/\Delta Cp$ by Eq. 5, assuming ΔCp = constant.

Table 2. Calculation of T_s for benzene from data taken at various temperatures

T, °C	Solubility,* mol fraction $\times 10^{-4}$	$\Delta H°$,† kJ·mol^{-1}	$\Delta S°$,‡ J·deg^{-1}·mol^{-1}	$\Delta G°$,§ kJ·mol^{-1}	T_s,¶ °C
15	3.99	−0.15	−65.57	18.75	112.5
25	4.01	2.08	−58.05	19.39	112.8
30	4.09	3.16	−54.45	19.67	113.3
35	4.20	4.37	−50.47	19.92	112.5
				Average	112.8 ± 0.4 (SD)

*Data of Franks *et al.* (30) for the solubility of benzene in H_2O.
†Data of Gill *et al.* (5).
‡Calculated from Eq. **2**.
§From $\Delta G° = \Delta H° - T\Delta S°$.
¶Calculated from $\ln(T_s/T) = -\Delta S°/\Delta Cp$ with $\Delta Cp = 225$ J·deg^{-1}·mol^{-1} (Gill *et al.*, ref. 5).

Variation between hydrocarbons in the value of T_h is more than twice the variation in T_s (Table 1). The existence of T_h near 22°C may be a property shown only by larger hydrocarbon molecules. The transfer properties of liquified CH_4 and C_2H_6 have been estimated (31, 32); the results indicate that the temperatures of minimum solubility are well above room temperature.

The standard-state Gibbs energy of transfer in the range where ΔCp is constant can be obtained from values of $\Delta H°$, $\Delta S°$, and the relation $\Delta G = \Delta H - T\Delta S$. Table 2 compares values of $\Delta G°$, $\Delta H°$, and $\Delta S°$ for the transfer of benzene to H_2O at different temperatures in the range 15°C–35°C. Although $\Delta S°$ approaches zero with increasing temperature, $\Delta G°$ does not: instead, $\Delta G°$ increases with temperature. The data show that, although transfer of benzene from H_2O to liquid benzene is entirely entropy driven at 15.8°C, it becomes increasingly enthalpy driven as the temperature increases.

When $\Delta G°$ is calculated from Eqs. **5** and **6** with ΔCp = constant, $\Delta G°$ is found to increase steadily with increasing temperature. Nemethy and Scheraga (12) concluded earlier that the hydrophobic interaction becomes stronger with increasing temperature, as measured by the value of $\Delta G°$. They concluded that $\Delta G°$ reaches a maximum around 50°C–60°C but this conclusion was based on temperature coefficients of solubilities, which were known less accurately than the later values of $\Delta H°$ and ΔCp (5) determined calorimetrically. J. A. Schellman (personal communication) points out that it may be more appropriate to define the strength of the hydrophobic interaction by the solubility of a hydrocarbon in H_2O, which reaches a minimum at T_h, than by $\Delta G°$, which increases with temperature above T_h.

Application of the Hydrocarbon Model to Protein Folding

Values for $\Delta H°_{hyd}$ and $\Delta S°_{hyd}$ contributed by the hydrophobic interaction to $\Delta H°$ and $\Delta S°$ for protein unfolding can be computed from Eqs. **5** and **6**. The corresponding expression for $\Delta G°_{hyd}$ is

$$\Delta G°_{hyd} = \Delta Cp_{hyd}(T - T_h) + \Delta Cp_{hyd} T \ln(T_s/T). \quad [7]$$

Values for T_h (22°C = 295 K) and T_s (386 K) are provided by the hydrocarbon model, but the problem of assigning a value to ΔCp_{hyd} remains. One approach is to assume that the observed ΔCp for unfolding can be attributed entirely to the hydrophobic interaction. This approach allows values for $\Delta H°_{hyd}$, $\Delta S°_{hyd}$, and $\Delta G°_{hyd}$ to be calculated straightforwardly at temperatures for which ΔCp is constant. The results of applying this approach to Sturtevant's data (2) for hen lysozyme are shown in Table 3 and are discussed below.

Table 3. Thermodynamics of unfolding of hen lysozyme

T, °C	$\Delta H°$, kJ·mol^{-1}			$\Delta S°$, J·mol^{-1}·deg^{-1}			$\Delta G°$, kJ·mol^{-1}		
	Obs*	Hyd†	Res‡	Obs*	Hyd†	Res‡	Obs*	Hyd†	Res‡
10	137	−78	215	247	−2026	2273	67.4	495	−428
25	236	20	216	586	−1688	2274	60.7	523	−462
60	469	248	221	1318	−964	2282	27.2	569	−542
100	732	509	223	2067	−224	2291	−41.4	593	−634
	Average		219			2280			

*Observed value for unfolding of hen lysozyme at pH 7, taken from table 7 of Sturtevant (2).
†Estimated contribution of the hydrophobic interaction, calculated from Eqs. **5**, **6**, or **7** with T_h = 22°C, T_s = 386 K, and ΔCp = 6530 J·mol^{-1}·deg^{-1} [the observed value for hen lysozyme (2)].
‡Residual contribution to the observed change from sources other than the hydrophobic interaction.

A second approach is to assume that $\Delta S°_{res}$ can be obtained by extrapolation of $\Delta S°_{obs}$ to T_s, since $\Delta S°_{obs} = \Delta S°_{hyd} + \Delta S°_{res}$. This approach has already been used by Privalov (1), who extrapolated $\Delta S°_{obs}$ versus temperature. Since $\Delta S°_{hyd} = -\Delta Cp_{hyd} \ln(T_s/T)$, $\Delta S°_{obs}$ can be extrapolated against $\ln(T_s/T)$ and $\Delta S°_{res}$ can be found from the intercept at $T = T_s$. This plot is linear and yields $\Delta S°_{res} = 2290$ J·mol^{-1}·deg^{-1} (Fig. 1), in good agreement with the values shown in Table 3. The corollary of this second approach is to take $\Delta H°_{hyd} = 0$ at 22°C and to calculate the residual value $\Delta H°_{res}$ from $\Delta H°_{obs}$ at 22°C.

The results in Table 3 show the following properties. (*i*) $\Delta H°_{res}$ is independent of temperature and favors folding. At temperatures near 25°C, it makes the major contribution to $\Delta H°_{obs}$. (*ii*) At high temperatures (60°C and above) $\Delta H°_{hyd}$ makes the major contribution to $\Delta H°_{obs}$. Above 22°C, $\Delta H°_{hyd}$ favors folding. (*iii*) $\Delta S°_{res}$ is large, independent of temperature, and opposes folding, as expected if $\Delta S°_{res}$ is dominated by the change in conformational entropy on unfolding.

The per residue value for $\Delta S°_{res}$, from sources other than the hydrophobic interaction, is 2280/123 = 18.5 J·(mol of res)$^{-1}$·deg^{-1}, which is close to the value given by Privalov (1) from data for $\Delta S°_{obs}$ extrapolated versus temperature to 110°C. He points out that, if the per residue value of $\Delta S°_{res}$ is attributed entirely to the change in conformational entropy, it corresponds to an 8-fold increase in possible residue conformations upon unfolding. The hydrocarbon model provides an explanation for the intersection near 110°C found by Privalov (1) in the plots of specific entropy of unfolding against temperature. Such an intersection is expected if different proteins show the same value of $\Delta S°_{res}$ per residue and if the average residue weights of the different proteins are nearly the same. Because the proteins analyzed by Privalov differ in the number of S—S crosslinks, they are not expected to show identical changes in conformational entropy (per residue) on unfolding, as he points out (1).

Limitations of the Hydrocarbon Model

There are three major limitations on the approach presented here. The first is the much discussed problem of whether or not it is correct to use solvent transfer experiments as a model for the hydrophobic interaction in protein folding (20, 23–27, 33). As mentioned above, recent work (19) shows that the solvent transfer model does successfully relate the average extent of burial of different side chains to hydrophobicity measured on the Nozaki–Tanford scale. On the other hand, the solvent transfer model is unable to explain experiments on the pressure dependence of protein folding (34–36). The use of a liquid hydrocarbon as a model for the interior of a protein may seem inappropriate in view of the semipolar character of the protein interior. Note, however, that I consider here only the transfer of hydrocarbon molecules without H-bond donor or acceptor groups. Nozaki and

Tanford (10) consider transfer from water to ethanol, and in this way they take partial account of the H-bonding properties of amino acid side chains containing H-bond donor or acceptor groups.

The second limitation on the analysis given here is that it is valid only at temperatures where ΔCp is constant, and the measurements considered here (5) stop at 35°C. Nevertheless, ΔCp in protein unfolding experiments is found to be constant up to 80°C (1). The equations presented here are valid in the temperature range in which ΔCp is constant whether or not ΔCp decreases at high temperatures. Reasons for supposing that ΔCp may decrease at high temperatures are as follows [see also Shinoda and Fujihira (31)]. (*i*) Accurate calorimetric data for aliphatic amines over a broad temperature range show ΔCp decreasing at high temperatures (37). It is possible, however, that the polar amine group is responsible for this effect. (*ii*) A model for hydrophobic solvation has been presented (38) that fits the available data on aqueous solutions of inert gases and predicts that ΔCp decreases at high temperatures.

The third limitation on this approach is that the hydrocarbon model cannot be applied directly to protein unfolding experiments without making an assumption about ΔCp. One can assume either that the hydrophobic interaction entirely determines the value of ΔCp_{obs} (as in Table 3) or that ΔCp is constant and $\Delta S°_{obs}$ can be extrapolated to 113°C to give $\Delta S°_{res}$ (as in Fig. 1). The possibility that soft vibrations contribute to ΔCp for protein unfolding has been put forward (2, 3).

The nature of the hydrophobic interaction has not been discussed here except to consider whether or not it is legitimate to model it by a solvent transfer experiment. It remains a controversial subject: [cf. Lee (25, 26)]. As discussed above, the hydrocarbon model makes specific predictions about the contributions of the hydrophobic interaction to the thermodynamics of protein folding. It should be possible to test the model further and to see if refinement of it is warranted. I had hoped to find an explanation for the intersection point near 110°C observed by Privalov (1) in plots of the specific enthalpy of unfolding versus temperature for different proteins, but its connection with the hydrocarbon model is not apparent at this time.

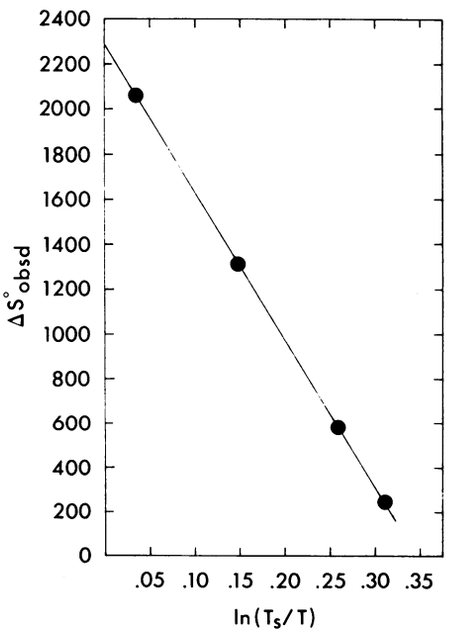

FIG. 1. Plot of observed entropy change on unfolding of hen lysozyme (2) versus $\ln(T_s/T)$, where T is the temperature in K and T_s is taken to be 386 K.

Several colleagues have criticized earlier drafts of this paper and have contributed to the development of the analysis given here. I would like to acknowledge in particular the discussion of Drs. T. E. Creighton, D. S. Eisenberg, S. J. Gill, W. Kauzmann, and J. A. Schellman, and to thank Carol Campbell for her help with the manuscript. This work was supported by Grant GM 19988-24 from the National Institutes of Health.

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