Temperature, Stability, and the Hydrophobic Interaction

John A. Schellman
Institute of Molecular Biology, University of Oregon, Eugene, Oregon 97403 USA

ABSTRACT Changes in free energy are normally used to track the effect of temperature on the stability of proteins and hydrophobic interactions. Use of this procedure on the aqueous solubility of hydrocarbons, a standard representation of the hydrophobic effect, leads to the conclusion that the hydrophobic effect increases in strength as the temperature is raised to ~140°C. Acceptance of this interpretation leads to a number of far-reaching conclusions that are at variance with the original conception of the hydrophobic effect and add considerably to the complexity of interpretation. There are two legitimate thermodynamic functions that can be used to look at stability as a function of temperature: the standard Gibbs free energy change, $\Delta\bar{G}°$, and $\Delta\bar{G}°/T$. The latter is proportional to the log of the equilibrium constant and is sometimes called the Massieu-Planck function. Arguments are presented for using $\Delta\bar{G}°/T$ rather than $\Delta\bar{G}°$ for variations in stability with temperature. This makes a considerable difference in the interpretation of the hydrophobic interaction, but makes little change in the stability profile of proteins. Protein unfolding and the aqueous solubility of benzene are given as examples. The contrast between protein unfolding and the hydration of nonpolar molecules provides a rough estimate of the contribution of other factors that stabilize and destabilize protein structure.

INTRODUCTION

This paper will address the problem of the hydrophobic interaction and its role in stabilizing proteins. The discussion will be at a broad thermodynamic level and will not make use of details of the structure of proteins or models for water or solvation. The aim is to clarify a few matters of principle, and because these are independent of a model, they are best dealt with when uncomplicated by more specific assumptions.

We will be concerned with a specialized class of processes with unusual thermodynamic characteristics. They are identified by changes in the sign of ΔH or ΔS as the temperature is varied and will be discussed more fully later. Two principal examples are protein unfolding and hydrophobic solvation:

$$\mathrm{N} \Leftrightarrow \mathrm{U} \qquad \Delta\bar{G}^{\circ}_{\mathrm{unf}} = \bar{G}^{\circ}_{\mathrm{u}} - \bar{G}^{\circ}_{\mathrm{n}} \qquad (1a)$$

$$\mathrm{A(np)} \Leftrightarrow \mathrm{A(aq)} \qquad \Delta\bar{G}^{\circ}_{\mathrm{tr}} = \bar{G}^{\circ}_{\mathrm{aq}} - \bar{G}^{\circ}_{\mathrm{np}} \qquad (1b)$$

where N and U represent the native and unfolded states of a protein, A represents an aliphatic or aromatic molecule, and np and aq stand for nonpolar and aqueous environments. At a given temperature and pressure, changes in Gibbs free energy (such as $\Delta\bar{G}^{\circ}_{\mathrm{tr}}$ or $\Delta\bar{G}^{\circ}_{\mathrm{unf}}$) are universally used to determine the direction and equilibrium of chemical reactions and physical transformations. In studies of proteins $\Delta\bar{G}^{\circ}_{\mathrm{unf}}$ is often referred to as the stability of the native form relative to the unfolded form. To maintain a parallel discussion we will use the same terminology for process 1b and refer to the positive quantity $\Delta\bar{G}^{\circ}_{\mathrm{tr}}$, the free energy of transfer, as the stability of A in a nonpolar environment relative to an aqueous one.

It is often of interest to determine $\Delta\bar{G}$ as a function of temperature to obtain information on the effect of T on stability. A prominent example of this is the determination of $\Delta\bar{G}^{\circ}_{\mathrm{unf}}$ of a protein over an extended temperature range, usually including an unfolding transition. But one must be careful in interpreting such data. The Gibbs free energy is the state function par excellence for conditions of constant temperature and pressure, but thermodynamics gives us no information on how to relate free energy changes at different temperatures to thermodynamic stabilities at different temperatures. (For example, is a protein with a $\Delta\bar{G}^{\circ}_{\mathrm{unf}}$ of 10 kJ at 373 K more stable than a protein with a $\Delta\bar{G}^{\circ}_{\mathrm{unf}}$ of 9 kJ at 273 K? We will suggest later that the answer is no, based on a choice of how the word "stability" is interpreted.) Nevertheless, ΔG versus T curves are routinely plotted and used for such concepts as the "temperature of maximum stability" or the temperature dependence of the strength of the hydrophobic interaction. The aim of the present paper is to show that ΔG versus T curves may lead to ambiguous conclusions, to present the reasons for this ambiguity, and to propose a rationale for an alternative treatment of the data. It will turn out that, for fortuitous reasons, the interpretation of the stability curves of proteins needs only a negligible correction, but that there is a significant change in the temperature dependence of the hydrophobic reaction.

Received for publication 18 April 1997 and in final form 12 September 1997.

Address reprint requests to Dr. John A. Schellman, Department of Molecular Biology, University of Oregon, Eugene, OR 97403. Tel.: 541-346-6093; Fax: 541-346-5891; E-mail: john@jas1.uoregon.edu.

0006-3495/97/12/2960/05 $2.00

THE TEMPERATURE DEPENDENCE OF THE SOLUBILITY OF HYDROPHOBIC MOLECULES IN WATER

Privalov and Gill (1988) have provided an unusually complete thermodynamic discussion of hydrocarbons in aqueous solution. They presented data and extrapolated temper-

ature curves for 10 different aliphatic and aromatic molecules and demonstrated the similar shape of the $\Delta\bar{G}_{tr}^{\circ}$ versus T curves of these molecules, which all indicate a maximum in the neighborhood of 400 K. Baldwin (1986) had already predicted the generality of this maximum based on an experimental generalization of Sturtevant (1977). On the other hand, the solubility of these substances has a minimum "near room temperature," an observation made by Kauzmann (1959) on a smaller sample in his first discussion of the hydrophobic interaction. We will make use of the benzene data as a representative example because of the completeness of the available experimental information, which includes the solubility data of Franks et al. (1963), the enthalpy determinations of Gill et al. (1975), and the heat capacity determinations of Makhatadze and Privalov (1988). The resulting free energy curve is shown in Fig. 1 *A*.

Privalov and Gill applied the canonical interpretation to Fig. 1 *A* and concluded that the process of transferring liquid hydrocarbons into water is most unfavorable at the temperature of the maximum, i.e., at ~400 K. This temperature is conventionally labeled T_s because it is the temperature at which the entropy change is zero. This type of analysis is standard practice in the field of protein stability. In the present instance this interpretation generates a number of concerns. If the transfer is most unfavorable at 400 K, why does the solubility increase from ~290 K to 400 K? If the main stabilization of proteins depends on the hydrophobic effect and if this increases in stability at high temperature, why do proteins unfold as the temperature is raised? Furthermore, because $\Delta S = 0$ when ΔG is at a maximum, this indicates that at its optimum temperature the hydrophobic effect is purely energetic. This relegates to a subordinate position decades of work and interpretation on the structure of water and its orientational and hydrogen bond properties. Thus the acceptance of ΔG versus T as a measure of temperature stability enforces a radical change in our conception of protein stabilization and should require very strong justification.

On the other hand, these anomalies do not arise if we use the solubility itself as the index of the strength of the hydrophobic interaction. The hydrophobic effect is at maximum in the neighborhood of 20°C. Its contribution to protein stability diminishes in strength as the temperature is raised or lowered relative to this temperature. The maximum in protein stability is then a direct reflection of the maximum in hydrophobic stabilization, but is displaced somewhat in temperature because of other stabilizing contributions. Recalling that for the solution of a pure liquid phase in a solvent, the equilibrium constant is the solubility itself (units of activity or concentration), this measure translates to using K or, better, $-\ln K$ as the stability index.

If it is to replace ΔG, an index of stability must be an extensive function of state which, like ΔG, is related by direct transformations to the first and second laws of thermodynamics. But this had already been established for $-\ln K$ many years ago. Before Gibbs' work, Massieu (1869a,b) developed a system of thermodynamics based on

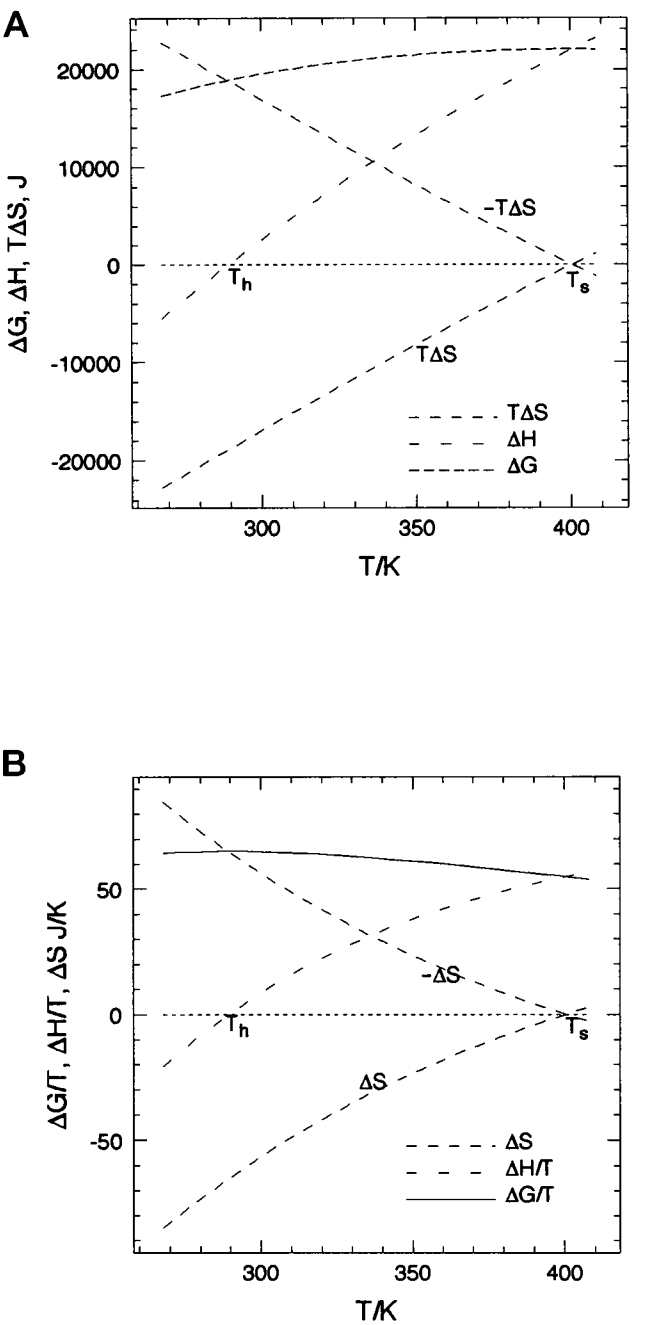

FIGURE 1 The thermodynamics of a solution of liquid benzene in water. (*A*) The Gibbs representation. This figure makes use of the data and model of Privalov and Gill (1988) and is almost identical to the solid curves in figure 12 of their paper. The maximum in $\Delta\bar{G}^{\circ}$ is at 403 K. Note that at T_h, $\Delta\bar{G}^{\circ} = -T\Delta S^{\circ}$, and at T_s, $\Delta\bar{G}^{\circ} = \Delta\bar{H}^{\circ}$. (*B*) The solubility representation. The maximum in $\Delta\bar{G}^{\circ}/T$ is at 289 K. The calculations were performed using data cited in the text, and following Privalov and Gill (1988), the experimental data were extrapolated to high temperatures, using their temperature-dependent ΔC_p.

entropy relations that is just as valid as the Gibbs system based on energy relations. In his system the function that plays the same role as the free energy is equivalent to $\Delta G/T$, i.e., to $-R \ln K$ for an equilibrium process. Clearly, this

function has all the predictive properties of ΔG itself at constant temperature, but provides an alternative interpretation when temperature is varied. Planck, in fact, made exclusive use of this function in preference to ΔG for the discussion of equilibrium in his early classic text on thermodynamics (Planck, 1945).

It might be thought that the temperature profiles of ΔG and $-R \ln K$ would vary little from one another, because they differ only by a factor that is linear in the temperature. This is often the case, but not for the class of processes we are discussing (high ΔC_p, and ΔH or ΔS crossing zero). The plot of $\Delta \bar{G}^\circ/T = -RT \ln S_o$ for benzene, where S_o is the solubility, is shown in Fig. 1 *B*. The maximum is at 289 K, and is labeled T_h, because at this temperature $\Delta H = 0$. It is our proposal that it is Fig. 1 *B* rather than Fig. 1 *A* that best describes the strength of the hydrophobic interaction as a function of T.

There is no suggestion of replacing ΔG by $\Delta G/T$ in general. Apart from a few special applications, the Gibbs system is simpler, more powerful, and more elegant than that of Massieu.

The above is certainly not a new idea. As is well known, intuitive solubility arguments were an important part of the earliest discussions of the hydrophobic effect (Frank and Evans, 1945; Kauzmann, 1959). What has been shown is that the solubility (or, in general, $-\ln K$) is on an equal thermodynamic footing with, but different from, ΔG when temperature is varied. Whether ΔG or $\Delta G/T$ is to be preferred depends on the kind of application one has in mind. If one is interested in stability or the likelihood or material yield of a process, $\Delta G/T$ is the function of choice because it is directly related to probability. This can be seen from its direct connection with the equilibrium constant, the partition function, and the exponent of the Boltzmann distribution. On the other hand, if the interest is in the generation of useful energy, such as electrical work or mechanical work (e.g., muscle action, expansion at constant pressure, etc.), then the process is best tracked by the ΔG versus T curve.

PROTEIN UNFOLDING

The arguments of the previous section should apply to the case of protein unfolding as well. Fig. 2 shows plots of $\Delta \bar{G}^\circ_{unf}$ and $\Delta \bar{G}^\circ_{unf}/T$ as functions of temperature. (To obtain data over the full temperature range indicated, it was necessary to destabilize the protein by the addition of 2 M guanidinium chloride. Extrapolation of such curves as a function of denaturant concentration indicates that the same general pattern also applies to the protein in aqueous solution but with higher stability.) The generality of a maximum in the free energy of unfolding of proteins goes back to the early work of Brandts (1969) and has recently been reviewed by Makhatadze and Privalov (1995). We see that there is little difference between the main features of the plots of $\Delta \bar{G}^\circ$ and $\Delta \bar{G}^\circ/T$. This is because both T_h, the position of the maximum in $\Delta \bar{G}^\circ$, and T_s, the position of the

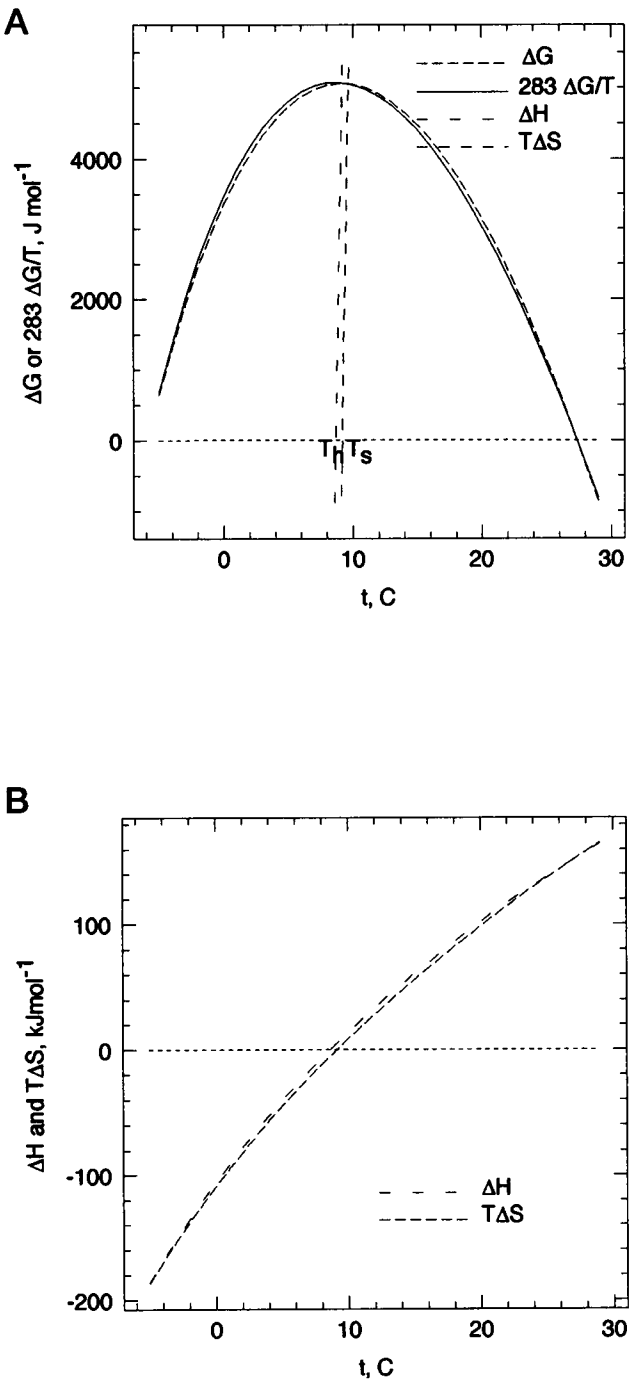

FIGURE 2 The thermodynamics of unfolding of T4 lysozyme in 2 M guanidinium chloride (data of G. Signor and W. Baase, personal communication). (*A*) Plots of $\Delta \bar{G}^\circ$, $\Delta \bar{H}^\circ$, $T\Delta \bar{S}^\circ$, and $\Delta \bar{G}^\circ/T$ as a function of temperature. $\Delta \bar{G}^\circ/T$ has been multiplied by the constant 283 K to bring it to the same scale as $\Delta \bar{G}^\circ$. The points where the curves for $\Delta \bar{H}^\circ$ and $T\Delta \bar{S}^\circ$ cross the temperature axis are defined as T_h and T_s, respectively, and are located at the maxima of $\Delta \bar{G}^\circ/T$ and $\Delta \bar{G}^\circ$. (*B*) Plots of $\Delta \bar{H}^\circ$ and $T\Delta \bar{S}^\circ$ as functions of temperature. At this scale the curve for $\Delta \bar{G}^\circ$ would lie almost flat on the abscissa, but is represented in the figure by the separation between the two curves. The points where the two curves meet are transition temperatures where $\Delta \bar{G}^\circ = 0$. We label the lower temperature as $T_{g'}$, and the higher (normal) one as T_g.

maximum in $\Delta\bar{G}°/T$, have been moved down in temperature and nearly coincide. T_h and T_s are separated by only 3° for the curves extrapolated to zero guanidinium chloride concentration. Because only modest accuracy is attainable with this type of experiment, this means that both methods fortuitously give the same qualitative result. There is no need to alter the standard practice of plotting $\Delta\bar{G}°$ as a function of T in discussing stability as a function of temperature. On the other hand, if the total curve for the protein is being decomposed into component contributions, then $\Delta\bar{G}°/T$ should be used, because this is the function that contributes directly to the probability

The origin of the contrast between the results for the hydrophobic effect and for unfolding thermodynamics of protein is discussed in the final section.

PROCESSES WITH A MAXIMUM IN ΔG (or $\Delta G/T$)

The transfer of hydrophobic groups to water and protein unfolding belong to a very special class of processes. They are characterized by a maximum in ΔG (or $\Delta G/T$) as a function of T and a large ΔC_p, which provides a strong negative curvature. (A minimum in ΔG (or $\Delta G/T$) can be converted to a maximum by reversing the direction of the process.) For convenience these processes will be called ΔG_{max} processes. In the case of protein unfolding, there are four characteristic temperatures, in order of ascending temperature:

1. $T_{g'}$, a low transition temperature at which $\Delta G = 0$,
2. T_h, a maximum in $\Delta G/T$ where $\Delta H = 0$,
3. T_s, a maximum in ΔG, where $\Delta S = 0$,
4. T_g, a high temperature transition temperature at which $\Delta G = 0$.

It is easy to show that if a process has a maximum in ΔG or in $\Delta G/T$ and if ΔC_p maintains a high negative value, then this process is predicted to possess all four of these characteristic temperatures, with T_s always greater than T_h, i.e., the maximum in ΔG is always at a higher T than the maximum in $\Delta G/T$. We note that high and low temperature unfolding is an intrinsic part of this set of properties. This collection of properties defines the special class of processes alluded to in the introductory paragraphs. It is rare that all four of these points can be observed experimentally. For proteins it is relatively easy to observe high temperature melting and the maxima at T_s and T_h, but special conditions and effort are required to observe the predicted low temperature melting. $T_{g'}$ is often located below the freezing point of water. In the case of the water solubility of hydrocarbon molecules, only the maximum in $\Delta G/T$ is observable. Privalov and Gill have estimated the position of T_s by extrapolation, but T_g and $T_{g'}$ (which can be calculated by extrapolation of the ΔC_p data) are far above and below, respectively, the temperature range at which liquid hydrocarbons and liquid water exist. The hydrophobic interaction remains unfavorable over all accessible temperatures.

ΔG_{max} processes can be entropy driven over large temperature ranges. For example, as shown in Fig. 1 *A* (or Fig. 1 *B*), $|T\Delta S| > |\Delta H|$ at all temperatures up to ~335 K. They are also the only processes that give qualitatively different results for temperature plots of ΔG and $\Delta G/T$. Both of our examples evidently stem from one kind of process, the immersion of nonpolar groups in water.

THE HYDROPHOBIC EFFECT AND PROTEIN STABILITY

For many years it was fashionable to consider the hydrophobic effect as totally dominating the stability of proteins. The opposite point of view appears to be gaining at present. One way to check on the relationship of two ΔG_{max} processes is by comparing the values of their characteristic temperatures. Table 1 presents a comparison of T_s and T_h for the unfolding of T4 lysozyme and a typical hydrophobic event: the passage of benzene from a nonpolar environment to water. There is a wide divergence in the characteristic temperatures. This is because the unfolding of a protein is a composite process involving contributions other than those coming from the hydrophobic reaction.

With some rough approximations, we can inquire into the thermodynamic character of these "other" contributions that produce such significant changes in T_s and T_h. We first assume that the hydrophobic effect is the main source of the large change in $\Delta\bar{C}_p^\circ$, which accompanies denaturation. There is evidence that this is an oversimplification (Makhatadze and Privalov, 1990; Privalov and Makhatadze, 1990), but moderate errors in this assumption should not affect the nature of the results, which will be qualitative. Because at 298 K, $\Delta\bar{C}_p^\circ$ for the unfolding of T4 lysozyme is ~29 times that for the aqueous solution of benzene, we consider that the hydrophobic contribution to the unfolding of T4 lysozyme is equivalent to the hydration of ~29 benzene molecules. For a protein that contains 164 residues, this does not appear to be unreasonable. The "other" contributions to the thermodynamics are then calculated by subtracting from the total via the formula

$$\Delta X(\text{other}) = \Delta X(\text{T4L, zero denaturant}) - 29\,\Delta X(\text{benzene} \rightarrow \text{aq})$$

where X is G, H, or S. We can use ΔG rather than $\Delta G/T$, because the comparison will be made at a fixed temperature.

TABLE 1 Comparison of T_s and T_h for protein and hydrophobic stability

	T_h/K	T_s/K
$C_6H_6(np) \rightarrow C_6H_6(aq)$	289	412
T4 lysozyme unfolding*	271	274

*Data extrapolated to zero guanidinium concentration.

The result of this calculation is that at 298 K

$$\begin{aligned}
\Delta\bar{G}^\circ \text{ (hydrophobic)} &\approx \text{kJ M}^{-1}\ (132\ \text{kcal} \cdot \text{M}^{-1}),\\
\Delta\bar{H}^\circ \text{ (other)} &\approx \text{kJ M}^{-1}\ (45\ \text{kcal} \cdot \text{M}^{-1}),\\
T/\Delta\bar{S}^\circ \text{ (other)} &\approx 720\ \text{kJ M}^{-1}\ (172\ \text{kcal} \cdot \text{M}^{-1}),\\
\Delta\bar{G}^\circ \text{ (other)} &\approx -530\ \text{kJ M}^{-1}\ (-126\ \text{kcal} \cdot \text{M}^{-1}).
\end{aligned}$$

It would be inappropriate to attach much value to the actual numbers, but the general picture is plausible. The "other" terms, by themselves, are destabilizing. About three-quarters of the stabilizing free energy comes from the hydrophobic interaction, and about one-quarter comes from other enthalpy effects. These are opposed by a large positive entropy of unfolding in addition to the hydrophobic entropy. Similar results would have been obtained if molecules other than benzene were used to represent the hydrophobic contribution. Many models have been proposed for "other" contributions. The point of the calculation is that one can go this far even before such models are considered. This is a much simpler picture of the hydrophobic interaction than is fashionable at the present time, but the simplicity arises from the alternative interpretation of the temperature dependence. When $\Delta G/T$ is plotted as a function of T for both protein unfolding and a typical hydrophobic transfer (solubility), the curves are very similar to one another, apart from a scaling factor. This is not true for ΔG versus T curves. With $-R$ ln K as the stability index, the profile of the hydrophobic effect can be seen "writ large" on the protein stability curve.

This paper is based on material presented at the symposium in honor of the retirement of Rufus Lumry, which was held in Kansas City in October 1990.

The data on T4 lysozyme were calculated from unpublished experiments of Giovanni Signor and Walter Baase. This paper benefitted from discussions with R. L. Baldwin and a careful reading by Charlotte Schellman.

This research was supported by National Institutes of Health grant GM 21095.

REFERENCES

Baldwin, R. L. 1986. Temperature dependence of the hydrophobic interaction. *Proc. Natl. Acad. Sci. USA.* 83:8069–72.

Brandts, J. F. 1969. Conformational transitions of proteins. *In* Structure and Stability of Biological Molecules. S. N. Timasheff and G. D. Fasman, editors. Dekker, New York. 213–290.

Frank, H. S., and M. W. Evans. 1945. Free volume and entropy in condensed systems. III Entropy in binary liquid mixtures; partial molal entropy in dilute solutions; structure and thermodynamics in aqueous electrolytes. *J. Chem. Phys.* 13:507–532.

Franks, F., M. Gent, and H. H. Johnson. 1963. The solubility of benzene in water. *J. Chem. Soc.* :2716–2723.

Gill, S. J., N. F. Nichols, and I. Wadso. 1975. Calorimetric determination of enthalpies of solution of slightly soluble liquids. I. Application to benzene in water. *J. Chem. Thermodyn.* 7:175–183.

Kauzmann, W. 1959. Some factors in the interpretation of protein denaturation. *Adv. Protein Chem.* 14:1–63.

Makhatadze, G. I., and P. L. Privalov. 1988. Partial specific heat capacity of benzene and of toluene in aqueous solution determined calorimetrically for a broad temperature range. *J. Chem. Thermodyn.* 20:405–412.

Makhatadze, G. I., and P. L. Privalov. 1990. Heat capacity effects of proteins. Partial molar heat capacity of individual amino acid residues in aqueous solutions: hydration effect. *J. Mol. Biol.* 213:375–384.

Makhatadze, G. I., and P. L. Privalov. 1995. Energetics of protein structure. *Adv. Protein Chem.* 47:307–425.

Massieu, M. F. 1869a. Sur les fonctions caractéristiques des divers fluides. *C. R. Acad. Sci.* LXIX:858–862.

Massieu, M. F. 1869b. Addition au précédent mémoire sur les fonctions caractéristiques. *C. R. Acad. Sci.* LXIX:1057–1061.

Planck, M. 1945. Treatise on Thermodynamics. Dover, New York.

Privalov, P. L., and S. J. Gill. 1988. Stability of protein structure and hydrophobic interaction. *Adv. Protein Chem.* 39:191–234.

Privalov, P. L., and G. I. Makhatadze. 1990. Heat capacity effects of proteins. II. Partial molar heat capacity of the unfolded polypeptide chain of proteins: protein unfolding effects. *J. Mol. Biol.* 213:385–391.

Sturtevant, J. M. 1977. Heat capacity and entropy changes in processes involving proteins. *Proc. Natl. Acad. Sci. USA.* 74:2236–2240.