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HEAT CAPACITY IN PROTEINS

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■ **Abstract** Heat capacity (Cp) is one of several major thermodynamic quantities commonly measured in proteins. With more than half a dozen definitions, it is the hardest of these quantities to understand in physical terms, but the richest in insight. There are many ramifications of observed Cp changes: The sign distinguishes apolar from polar solvation. It imparts a temperature (T) dependence to entropy and enthalpy that may change their signs, and which of them dominate. Protein unfolding usually has a positive ΔCp, producing a maximum in stability and sometimes cold denaturation. There are two heat capacity contributions, from hydration and protein-protein interactions; which dominates in folding and binding is an open question. Theoretical work to date has dealt mostly with the hydration term and can account, at least semi-quantitatively, for the major Cp-related features: the positive and negative hydration Cp of hydration for apolar and polar groups, respectively; the convergence of apolar group hydration entropy at T $\approx$ 112°C; the decrease in apolar hydration Cp with increasing T; and the T-maximum in protein stability and cold denaturation.

1. INTRODUCTION

Some of the earliest systematic calorimetric measurements were made by Benjamin Thompson in the 1780s, when he used the increase in temperature of a cask of water to measure the amount of heat produced by boring out cannon barrels. More than two centuries later, the sensitivity of calorimeters is probably a billion-fold higher. Heat changes produced by protein unfolding, protein association, ligand binding, and other protein reactions can now be measured routinely. The two principal instrument modes are differential scanning calorimetry (DSC), which measures sample heat capacity (Cp) with respect to a reference as a function of temperature, and isothermal titration calorimetry (ITC), which measures the heat uptake/evolution during a titration experiment. The third major tool is thermodynamic calorimetry, which does not use a calorimeter per se. The temperature dependence of an equilibrium constant, measured using any appropriate method (CD, fluorescence, NMR, etc.), is combined with the van't Hoff relations to obtain enthalpy and heat capacity changes. With these tools at the disposal

of biophysicists and biochemists, the output of heat capacity data on proteins has steadily increased. Most heat capacity data are collected at constant pressure, yielding Cp. Constant volume heat capacity (Cv) data are much rarer in the protein field. In addition, at 1 Atm PV (P = Pressure, V = Volume) changes in protein reactions are usually very small compared with kT (Thermal energy, where k is the Boltzmann constant, and T the absolute temperature). In this review, we refer almost exclusively to Cp and for brevity use enthalpy (H) and mean energy (E) interchangeably.

Cp is one of the five major thermodynamic quantities commonly tabulated in biophysical studies on proteins; the others are Gibbs free energy (G), enthalpy, entropy (S), and volume. Of course, these data are not an end in themselves, but they are measured with the aim of providing physical, mechanistic, even atomic level insight into how proteins fold, how they are stabilized, and how they function. In some respects, which we outline below, Cp is both the richest potential source of this insight and the hardest of the five thermodynamic quantities to understand in physical terms. Volume is by definition a physical quantity and straightforward to understand, although there are some subtleties in defining the volume of a protein in solution. Enthalpy is a direct measure of heat or energy, whereas entropy quantifies the "disorder" or number of configurations available to the system. Free energy has a direct relationship to a primary observable, the equilibrium constant K through $\Delta G = -kT \ln K$, which describes the balance between enthalpy and entropy. In our experience, however, heat capacity is less intuitively understood. Why should one protein or protein state be able to absorb more heat than another for the same increase in T? What is the physical origin of Cp differences? In this review, we first provide a short theoretical overview of heat capacity and then discuss some theoretical and experimental papers. Our goal is not to be exhaustive either in the theoretical overview or in the literature review, but rather to provide, through some background and simple models, physical intuition about Cp that can be used to parse experimental measurements; to indicate the many potential ramifications of Cp changes observed in the literature; and to focus on some experimental and theoretical studies that, in our view, elucidate physical origins and fundamental aspects of heat capacity changes in proteins. Space limitations preclude discussion of many studies of particular protein systems and tabulations of heat capacity data. Furthermore, our focus is on physical aspects rather than on applications.

2. THEORETICAL OVERVIEW OF HEAT CAPACITY

2.1. Definition of Heat Capacity

Heat capacity has more than half a dozen definitions, of which the first is synonymous with its name

$$Cp = \frac{dH}{dT}, \qquad 1.$$

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i.e., the increase in energy (heat) with temperature. The other three commonly used definitions are

$$Cp = T\frac{dS}{dT} = -T^2\frac{d^2G}{dT^2} = \frac{\langle \delta H^2 \rangle}{kT^2}, \qquad 2.$$

which are, respectively, the temperature dependence of the entropy, the second derivative or curvature of the free energy, and the mean squared fluctuation in energy scaled by kT^2. Equations 1 and 2 apply also to changes in entropy, enthalpy, and free energy by substituting ΔCp, ΔH, ΔS, and ΔG. Equations 1 and 2a together describe how the heat capacity is a measure of the temperature dependent form of entropy/enthalpy compensation. Because $G = H - TS$ and entropy and enthalpy change in the same direction, free energy has a much weaker dependence on temperature than either of its components has. Heat capacity expressed as the curvature of the free energy means that a positive (negative) change in heat capacity yields a downward (upward) curvature to ΔG, and potentially a maximum (minimum) in free energy with T if the accessible temperature range permits. At this extremum, it follows from $S = -dG/dT$ that the entropy change is zero. The fourth definition of Cp (Equation 2c) as the mean squared fluctuation in the enthalpy is, on the face of it, less obvious than the temperature derivative of the enthalpy, but it usually provides the easiest route to a physical interpretation. Because Cp can be defined in terms of the temperature derivative of both entropy and enthalpy, there is an entropic version of the fluctuation formula:

$$Cp = \frac{\langle \delta S^2 \rangle}{k}. \qquad 3.$$

All these definitions are equivalent at a statistical mechanical level. The configurational part of the all-important partition function is

$$Z = \int e^{-U(\mathbf{r})/kT} d\mathbf{r}, \qquad 4.$$

where U is the Hamiltonian or potential energy as a function of the coordinates $\mathbf{r}$. The probability of a particular state is

$$p(\mathbf{r}) = e^{-U(r)/kT}/Z. \qquad 5.$$

Then the entropy may be written in terms of the famous Boltzmann equation as

$$S/k = -\int p(\mathbf{r}) \ln p(\mathbf{r}) d\mathbf{r} = -\langle \ln p(\mathbf{r}) \rangle, \qquad 6.$$

where $\langle\rangle$ indicates an ensemble average. To extend this to heat capacity, we use the statistical mechanical result that the temperature dependence of the ensemble average of a quantity X is given by

$$\frac{d\langle X \rangle}{dT} = \frac{1}{kT^2}\langle XU \rangle - \langle X \rangle \langle U \rangle = \frac{1}{kT^2}\langle \delta X \delta U \rangle, \qquad 7.$$

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the covariance of its fluctuation with the energy fluctuation. Combined with Equations 2a and 5 we obtain the statistical mechanical expression for the heat capacity

$$Cp/k = \langle[\ln p(\mathrm{r})]^2\rangle - \langle[\ln p(\mathrm{r})]\rangle^2 = \langle[\delta \ln p(r)]^2\rangle, \quad 8.$$

as the mean squared fluctuation in log probability (compare also Equations 3 and 6). We note that using X = U in Equation 7 allows us to derive the aforementioned identity

$$Cp = \frac{dH}{dT} = \frac{\langle \delta H^2\rangle}{kT^2}, \quad 9.$$

where $\langle \mathrm{U}\rangle = \mathrm{E} \approx \mathrm{H}$. Equation 9 is an example of a general relationship in statistical mechanics between a susceptibility of some quantity and the magnitude of the square of the fluctuations in that quantity at equilibrium (1). Lastly, we can use the statistical mechanics relationships to obtain our final expression for the heat capacity

$$Cp = \frac{\langle \delta H \delta S\rangle}{kT}, \quad 10.$$

as the covariance in enthalpy-entropy fluctuations that gives formal expression to the concept of Cp as a measure of entropy-enthalpy compensation. Interestingly, because G = H − TS, Equations 2, 3, and 10 tell us that the total fluctuation in free energy is identically zero (2),

$$\begin{aligned}\langle \delta G^2\rangle &= \langle(\delta H - T\delta S)(\delta H - T\delta S)\rangle \\ &= \langle \delta H^2\rangle + T^2\langle \delta S^2\rangle - 2T\langle \delta H \delta S\rangle = 0. \end{aligned} \quad 11.$$

Which of the seven expressions for heat capacity, Equations 1–3, 8, and 10, one should use depends on several criteria. For the experimentalist, these criteria include how the heat capacity is being measured and what is being deduced from experimental measurements. For the theoretician aiming to calculate heat capacities, these criteria include what theory is being used, and what are the relative numerical difficulties inherent in the different expressions.

Integration of Equations 1 and 2 with the assumption of constant heat capacity leads to the very useful modified Gibbs-Helmholtz, or integrated van't Hoff Equation

$$\Delta H(T) = \Delta H(T^{ref}) + \Delta Cp(T - T^{ref}), \quad 12a.$$

$$\Delta S(T) = \Delta S(T^{ref}) + \Delta Cp \ln(T/T^{ref}), \quad 12b.$$

$$\begin{aligned}\Delta G(T) &= \Delta H(T^{ref}) - T\Delta S(T^{ref}) \\ &\quad + \Delta Cp[(T - T^{ref}) - T\ln(T/T^{ref})], \end{aligned} \quad 12c.$$

which gives the enthalpy, entropy, and free energy at a particular temperature in

terms of entropy, enthalpy, and heat capacity at a second conveniently chosen reference temperature T^{ref}. This Equation is commonly used either to extract enthalpy, entropy, and heat capacity changes from a temperature-dependent series of free energies or equilibrium constants (thermodynamic calorimetry), or to extrapolate protein free energies, stabilities, etc., to various temperatures given values of enthalpy, entropy, and heat capacity at the reference temperature. Many analyses of protein data assume a temperature invariant heat capacity, an assumption that often, but not always, leads to little error (3). However, more extended experimental data sets do provide information on the temperature dependence of the heat capacity (4, 5), and theoretical analyses of the hydrophobic effect are now addressing this higher-order thermodynamic effect (6, 7). Assume a linear dependence of ΔCp on temperature with a coefficient D, $\Delta Cp = \Delta Cp^{ref} + D(T - T^{ref})$. Substituting into Equations 1 and 2a, and integrating with respect to T gives the next highest order form of the integrated van't Hoff Equation

$$\begin{aligned}\Delta G(T) &= \Delta H(T^{ref}) - T\Delta S(T^{ref}) + \Delta C_p^{ref}[(T - T^{ref}) - T\ln(T/T^{ref})] \\ &\quad + D[(T - T^{ref})^2/2 - T(T - T^{ref}) + TT^{ref}\ln(T/T^{ref})]. \end{aligned} \quad 13.$$

Higher-order forms are easily derived. For example, Brandts (8) found that best fit to Chymotrypsin denaturation data required a T-dependent Cp model of the form $\Delta Cp = -2BT - 6CT^2$, but this order of van't Hoff analysis is the exception rather than the rule due to lack of suitable data.

2.2. Simple Models for Interpreting Heat Capacity

Simple thermodynamic models are rarely applicable to protein data when applied literally, but they are often useful for building up intuition about the underlying physical chemistry. The simplest models for looking at heat capacity are the harmonic oscillator and the two-energy-level model. In particular, the two-energy-level model is rich enough to demonstrate most of the physical effects necessary for a qualitative understanding of heat capacity. For a harmonic oscillator with frequency ν, the heat capacity is (9)

$$Cp = \frac{(h\nu/2kT)^2}{\sinh^2(h\nu/2kT)}, \quad 14.$$

where h is Planck's constant. For a two-energy-level model, where the two states are separated by an energy of ΔU, the heat capacity is easily evaluated using Equation 2c as

$$Cp = \frac{\Delta U^2 e^{-\Delta U/kT}}{kT^2(1 + e^{-\Delta U/kT})^2} = \frac{\Delta U^2}{kT^2} p_0 p_1, \quad 15.$$

where p_0 and p_1 are the probabilities of the system being in the lower and upper states, respectively.

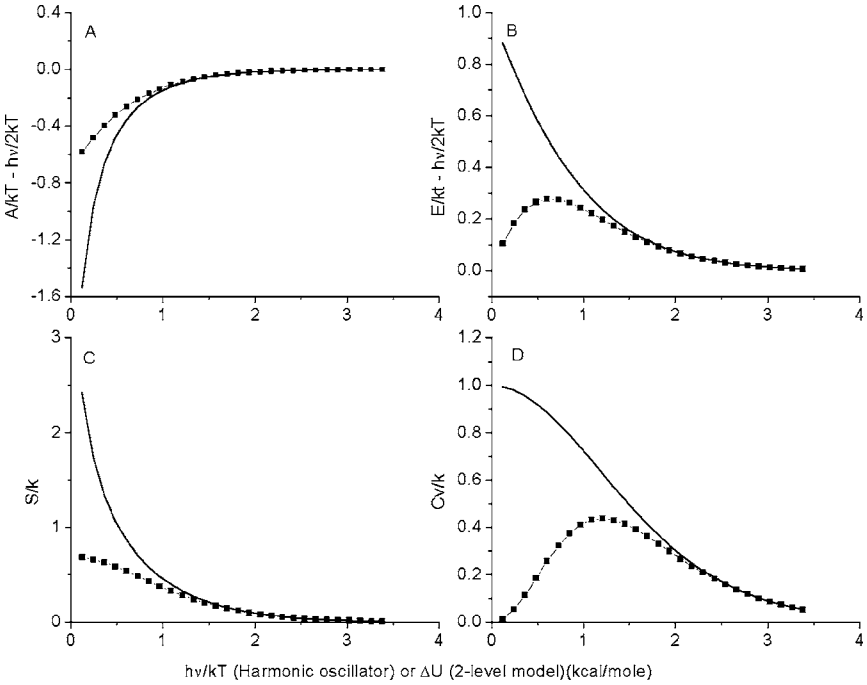

Figure 1 Free energy, energy, entropy, and heat capacity for harmonic oscillator (—) and two-energy-level model (ν).

Figure 1 plots the free energy, enthalpy, entropy, and heat capacity for these two simple models at T = 298 K, where we have set $\Delta U = h\nu/kT$ (A and E for the harmonic oscillator model exclude the zero point energy $h\nu/2kT$). At high-frequency/energy gap, the harmonic oscillator and two-state models converge. In this region, the behavior of the harmonic oscillator is dominated by the occupation probability of the two lowest energy levels at $h\nu/2kT$ and $3h\nu/2kT$, i.e., it is effectively a two-energy-level model. Several important features are apparent from these plots. First, although the entropy and heat capacity are both related to how 'extensively' the system samples its different states, there is no monotonic relationship between them. For the two-state model, the maximum entropy occurs when the system spends equal time in both states, but here the heat capacity is zero, because fluctuations between these two states produce no energy difference. For the two-level model, the maximum in Cp is best understood in terms of its enthalpy fluctuation definition. For a low energy gap, fluctuation between the states is large (the p_0p_1 term in Equation 15), but the resulting energy fluctuation (the ΔU^2 term) is small. With a high energy gap, the reverse is true. A critical energy gap of about 2.36 kT produces the maximum effect because of the trade-off between these two factors.

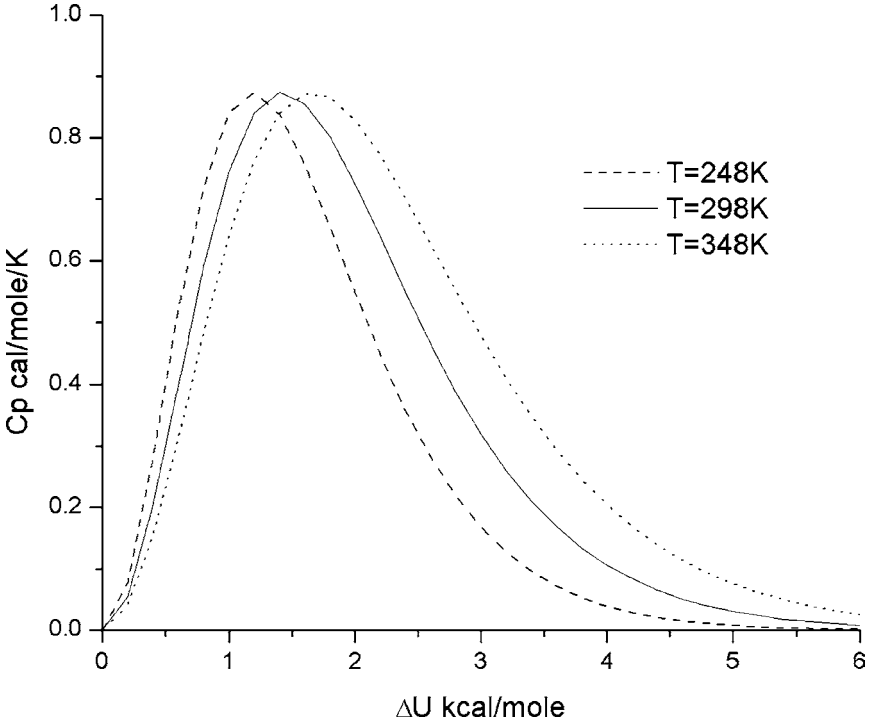

Figure 2 Effect of temperature on heat capacity in a two-energy-level system.

Figures 2 and 3 show the effect of temperature on the heat capacity in the two-energy-level model. Increasing T simply has the effect of scaling the energy axis uniformly. However, the direction of the effect on Cp depends on whether we are above or below the critical energy gap value. With larger gaps, increasing the temperature increases Cp. With smaller gaps, increasing T decreases Cp. Thus depending on where the system is relative to the critical energy gap value and the accessible temperature range, Cp may appear to increase monotonically, decrease monotonically, or exhibit a maximum (Figure 3).

In Figure 4, the effect on the heat capacity of increasing the degeneracy of the upper level to N = 2, 4, 8, 16, etc., is plotted. Here the more general expression for Cp in the two-energy-level model is

$$Cp = \frac{\Delta U^2 N e^{-\Delta U/kT}}{kT^2(1 + Ne^{-\Delta U/kT})^2} = \frac{\Delta U^2}{kT^2} p_0 p_1, \qquad 16.$$

where now p_1 is the probability of occupying any of the upper energy levels. For large energy gaps, Cp increases almost linearly with N, hence the qualitative description of Cp as a measure of the number of thermally accessible high energy states. For a low energy gap the situation is more complex. With increasing N, Cp

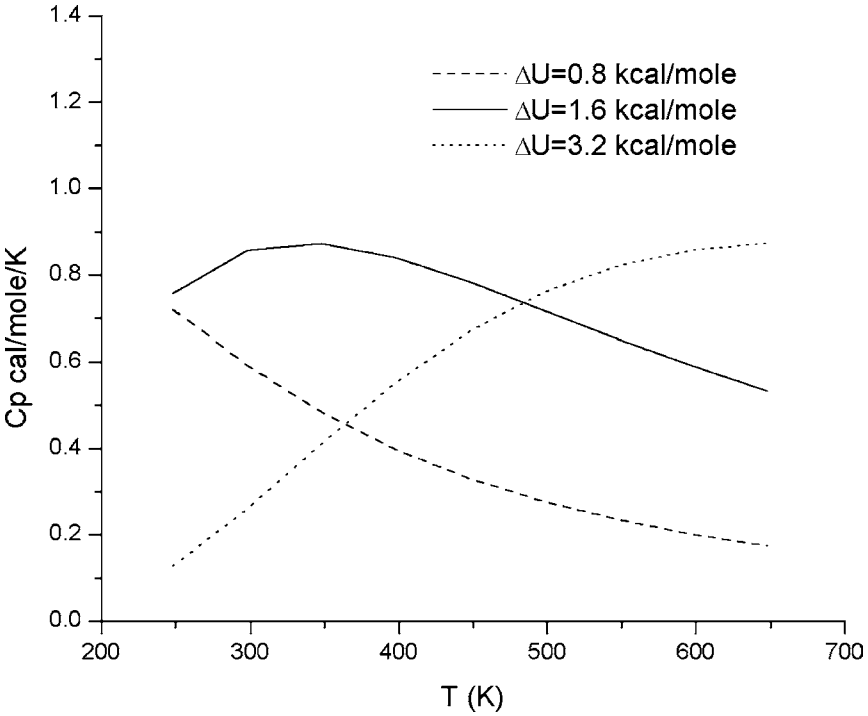

Figure 3 Temperature profile of Cp for a two-energy-level system.

increases then decreases. This again is best understood in terms of the enthalpy fluctuation definition. As the degeneracy (entropy) of the higher energy state is increased, at first Cp increases as the factor p_0p_1 increases. Eventually as N increases, the system spends most of its time in the upper energy states, $p_0 \ll 1$, and the energy fluctuation decreases. With increasing N, both the position and the height of the maximum in Cp increases. From this one might say, using the language of entropy-enthalpy compensation, that the maximum Cp is achieved when the effect of degeneracy and energy gap balance, i.e., when there is the right balance of a "low energy, low entropy" ground state, and a "high entropy, high energy" upper state.

To summarize the points emerging from simple models of heat capacity, the magnitude of Cp is determined by a balance between fluctuations of the systems between different states, which is large when their energy gap is small, and the energy changes occurring upon transition between these states, which is large when the energy gaps are large. The nonmonotonic dependence of Cp on energy gap, temperature, and energy level degeneracy makes interpretation of Cp changes more difficult than that of entropy, for example. In this regard, it is usually easier to think of Cp in terms of energy fluctuations rather than of temperature derivatives of H, S,

4 Jan 2005 3:28 AR AR241-PC56-18.tex XMLPublish^{SM}(2004/02/24) P1: IKH
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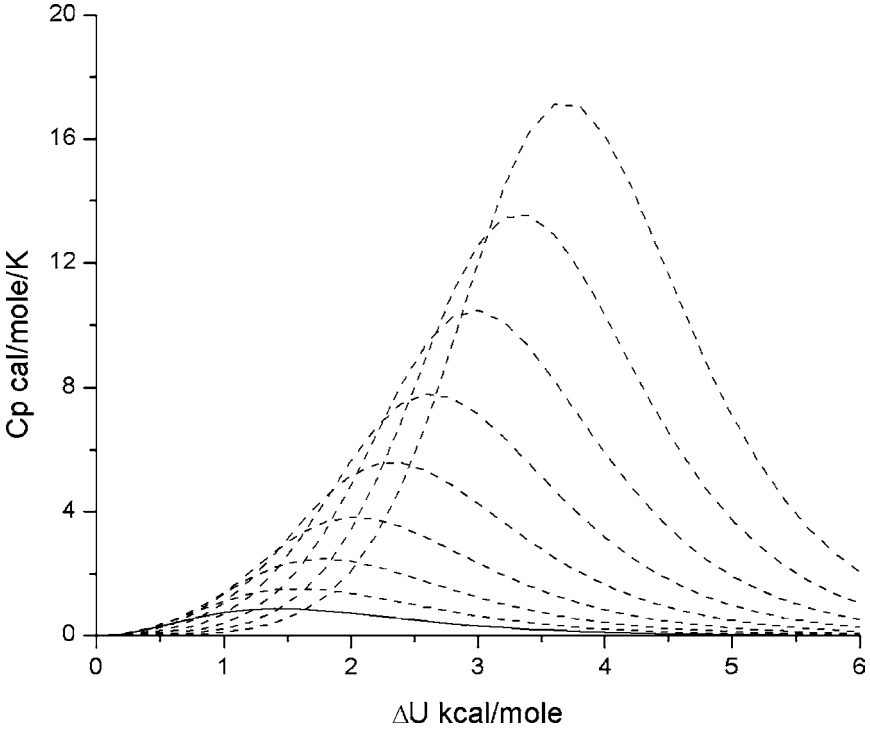

Figure 4 Effect of upper energy level degeneracy, N, on heat capacity. *Solid line*: N = 1. *Dashed lines* indicate successive doubling of N to 2, 4, 8, 16, 32, 64, 128, and 256.

or the other definitions of Cp. We can also identify two general regimes: a regime where the energy gap(s) between the most probable states are large, characterized by an increase in Cp with temperature, and a small energy gap regime where Cp decreases with increasing T. Experimental determination of the dependence of Cp on T can thus provide some general information on the protein energy landscape. The reader is referred to the elegant work of Poland (10, 11) for a more detailed exposition of the relationship between heat capacity and protein energy landscapes.

3. SOME IMPLICATIONS OF OBSERVED Cp CHANGES IN PROTEINS

The multiple definitions for heat capacity probably make for a richer set of consequences than for any of the other four major thermodynamic quantities—G, H, S, and V—usually measured for proteins. Some of the more intriguing

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consequences are summarized below and discussed in more detail in separate sections.

i. The sign of ΔCp distinguishes apolar (+) from polar (–) solvation (3, 4, 12, 13), in contrast to ΔS and ΔH of hydration, which are both negative for apolar and polar groups. Indeed, a positive ΔCp, rather than negative ΔS, is now considered the signature of the hydrophobic effect. Thus, from Equations 1 and 2a, polar and apolar groups buried in protein folding and binding impart different temperature dependences to the entropy and enthalpy of these processes, to the extent that, depending on the mix of apolar/polar groups involved, a process may switch from being entropy driven at one temperature to enthalpy driven at another, or dominated by apolar interactions at one temperature and polar interactions at another.

ii. Solvation of polar groups found in proteins is characterized by both negative ΔCp and ΔS at room temperature (14, 15). Therefore one can infer from Equation 2a that the entropy of hydration becomes more negative as the temperature is raised, i.e., that, at least in this temperature range, there is increased structuring of water by the solute at higher temperature. This is indeed counterintuitive.

iii. Globular protein unfolding usually has a positive ΔCp. From Equation 2b one can infer that the stability versus temperature profile has an inverted U-shape. Indeed, many proteins have a maximum in stability, often close to their normal ambient temperature. In addition to the expected decrease in stability with increasing temperature (the familiar melting transition), the downward curve in stability imparted by a positive ΔCp implies that proteins become less stable at low temperature and can even denature at low enough temperatures (3, 16, 17). The temperature-stability profile, and thus the heat capacity of unfolding also, has important implications for the mechanism of stabilization of thermophilic and hyperthermophilic proteins that function at temperatures of 60–120°C (18–20).

iv. Base sequence-specific binding of proteins to DNA is usually accompanied by a very large decrease in Cp, whereas nonspecific binding is not (21–24). This difference is increasingly seen as the thermodynamic signature of sequence-specific recognition. The large decrease in enthalpy fluctuations produced by sequence-specific binding, deduced from Equation 2c, is largely unexplained, but it may reflect large changes in DNA dynamics.

4. Cp CHANGES AND MEASUREMENT OF PROTEIN ENTHALPY

Although Cp changes in proteins are themselves of considerable interest, they can also complicate the measurement of enthalpy. Enthalpy changes can be measured directly by calorimetric techniques such as ITC for binding and DSC for melting.

One can also obtain a calorimetric measurement of ΔCp, for example, from ITC measurements at different temperatures using Equation 1. The alternative approach is to obtain ΔH and ΔCp from the temperature dependence of the equilibrium constant or ΔG, through the van't Hoff equation for enthalpy

$$\Delta H = \frac{\partial \Delta G/T}{d1/T}, \qquad 17.$$

and fitting to the integrated van't Hoff equation (Equation 12c) for ΔCp. Differences between calorimetric and van't Hoff measurements of enthalpy have been consistently observed for a number of protein systems (25, 26) to the extent that theoretical justifications for such differences were proposed, including radical reinterpretations of DSC curves (27) and questioning of the van't Hoff Equation (28). Attempts to provide theoretical reasons for the discrepancies have focused on the statistical mechanical definition and physical interpretation of heat capacity. Equation 17 says that, in principle, the enthalpy can be obtained from just the slope of ΔG/T. In practice, reliable determination of the slope requires measurement over an appreciable temperature range, and one must therefore account for the temperature dependence of ΔH imparted by any heat capacity change. Thus, ΔH is usually obtained from Equation 12c simultaneously with ΔCp by fitting. Chaires (29) has analyzed in depth correlations in errors between ΔCp and ΔH inherent in this fitting that account in large part for spurious differences in calorimetric and van't Hoff enthalpies. This analysis also emphasizes the need for many more data points for reliable fitting of ΔG versus T than are typically used. In addition, a comparison of Equations 12c and 13 shows that neglect of the temperature dependence of Cp itself will likely result in further systematic deviation of the van't Hoff enthalpy from the calorimetric value. Moreover, even more temperature measurements should be used for van't Hoff fitting. Other authors agree with the assessment that differences in the two estimates result from measurement/fitting inadequacies, and vigorously rebut any theoretical justification for the discrepancies (30, 31).

5. LINKED EQUILIBRIA AND HEAT CAPACITY CHANGES

In an influential paper, Eftink & Biltonen laid out the implications of linked equilibria for the measurement of thermodynamic parameters on proteins (32). In summarizing the part of their analysis relevant to heat capacity, consider a protein with two conformations A and B in equilibrium described by the constant $K_{ab} = [B]/[A]$, and a ligand L that can only bind the B-form with affinity constant $K_L = [BL]/[B][L]$. The net binding equilibrium is given by

$$K_{net} = \frac{[BL]}{[L]([A]+[B])} = \frac{[BL]}{[L][B](1+1/K_{ab})} = K_L f_b, \qquad 18.$$

where f_b is the fraction of the protein in the B-form in the absence of ligand. Taking $\Delta G_{net} = -kT \ln K_{net}$ and using Equation 2b, the expression for the heat

capacity is

$$\Delta Cp^{net} = \Delta Cp^{L} + \Delta Cp^{ab} f_a - \frac{\Delta H_{ab}^2}{kT^2} f_a f_b, \qquad 19.$$

where $f_{ac} = 1 - f_b$ is the fraction of the protein in the A-form in the absence of ligand. The first term in Equation 19 is the intrinsic heat capacity of ligand binding. The second term is the intrinsic heat capacity for converting the fraction of protein in the A-form to the B-form so that L can bind. The third term has no connection with the intrinsic heat capacities of either the protein or its interaction with the ligand. It arises purely from the link in protein conformational equilibrium with the binding. In the absence of ligand, the protein fluctuates between two forms with different energy, but when bound to the ligand it is constrained to the B-form, resulting in a loss of this heat capacity contribution (note the analogous form of this term to Equation 15). Such a contribution to heat capacity is often referred to as apparent (i.e., it is apparently not what the experimentalist was hoping to measure!), although it is as real and as measurable as both the intrinsic contributions. Adopting the statistical mechanical perspective, we can see that this contribution arises as the system fluctuates between the "states" A+L, B+L, and BL, each with a different average energy, while "intrinsic" Cp contributions arise from fluctuations in energy levels within the states A, B, or L. All Cp contributions are the same in the sense that they simply reflect fluctuations between states of different energy.

An important point emerging from this analysis is that if the linked equilibrium is of the type where the ligand binds predominantly to one form (mandatory coupling in the language of Eftink & Biltonen), then this heat capacity contribution is always negative irrespective of which conformation (A or B) has the lower energy. It is also present even if the intrinsic heat capacity changes are zero. It is therefore interesting that this term has the right sign to explain in part the unexpectedly large negative heat capacity changes that accompany base sequence–specific binding of proteins to DNA (21–24). This explanation assumes a protein binding–linked perturbation of some conformational equilibrium in the DNA. (If the observed Cp effect was positive one could immediately rule out this kind of explanation.)

The fluctuation of a protein between two states is also responsible for the peak in Cp seen in DSC melt curves (33) (Figure 5). The excess or conformational fluctuation heat capacity is given by $\Delta H_{ND}^2 f_N f_D / kT^2$, where ΔH_{ND} is the difference in enthalpy between, and f_N, f_D are the fractions of, the native and denatured states. Both states are appreciably populated during the melting transition. In this case, integration of the excess Cp over T is used to extract the unfolding enthalpy.

When imparting a heat capacity to a process, linked equilibrium effects can complicate extraction of enthalpies by van't Hoff analysis and produce the apparent discrepancy with calorimetric values discussed above. In a systematic evaluation of this effect, Horn et al. (34, 35) have shown, using some of the same protein systems previously thought to show systematic differences, that within experimental error van't Hoff and calorimetric enthalpy estimates agree. Their analysis also describes

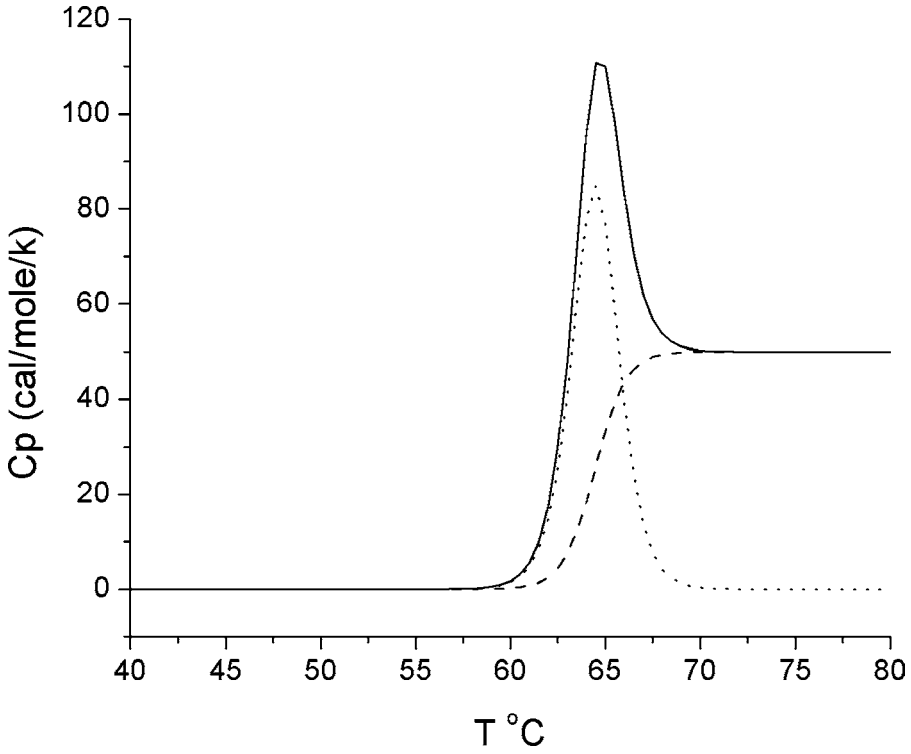

Figure 5 Model DSC trace for protein melting, generated using $\Delta H = 80$ kcal/mol, $\Delta S = 0.2$ kcal/mol/K, and $\Delta Cp = 50$ cal/mol/K for unfolding at $T = 298$ K, and Equation 12. $\Delta Cp^{fluc} = \Delta H^2 f_N f_D / kT$ (···), $\Delta Cp^{ND} = f_D \Delta Cp$ (- - -), and Cp^{tot} (—).

contributions to the putative discrepancies from linkage to proton exchange with buffer and protein-ion binding. These discrepancies are particularly insidious, as the proton and ion equilibria are usually invisible to the techniques commonly used to measure protein binding and unfolding. In summary, careful analyses of calorimetric-van't Hoff discrepancies of the type described in this and the preceding sections show that they can be attributed to measurement artifacts or incorrect assumptions in the model of analysis, and indeed there is no sound theoretical reason to expect them to differ (30, 31).

6. SEPARATION OF HEAT CAPACITY COMPONENTS

In analysis of the thermodynamics of complex systems such as proteins, a widespread and extremely useful practice is to divide net changes in G, H, S or Cp into components reflecting either different physical contributions (e.g., electrostatic,

4 Jan 2005 3:28 AR AR241-PC56-18.tex XMLPublish^{SM}(2004/02/24) P1: IKH
AR REVIEWS IN ADVANCE10.1146/annurev.physchem.56.092503.141202

van der Waals, etc.) or different group contributions. Thus if the potential function describing the system is a sum of terms

$$U = U_1 + \ldots U_i \ldots + U_N, \qquad 20.$$

one can express the mean energy in terms of ensemble averages (indicated by $\langle\rangle$)

$$\begin{aligned} E &= \langle U_1 + \ldots U_i \ldots + U_N \rangle = \langle U_1 \rangle + \ldots \langle U_i \rangle \ldots + \langle U_N \rangle \\ &= E_1 + \ldots E_i \ldots + E_N, \end{aligned} \qquad 21.$$

i.e., as a sum of corresponding terms. The ability to unambiguously separate mean energy or enthalpy terms, as indicated by Equation 21, is generally accepted (36). This does not mean that the terms are independent, in the sense that switching off or modifying a term U_i (e.g., through mutations in vitro, or through some free energy perturbation process in silico) would result in different ensemble averaging, as total energy is always used to compute Boltzmann weight, resulting in a different value for $\langle U_j \rangle$. Questions have been raised about the separation of other thermodynamics terms such as free energy and entropy (37, 38). Using the definition given in Equation 1, one can extend the formal separation of components to Cp

$$Cp = d\langle U \rangle / dT = d\langle U_1 \rangle / dT + \ldots \langle U_N \rangle / dT = Cp_1 + Cp_N. \qquad 22.$$

Interestingly, using the enthalpy fluctuation definition of Cp, this implies that the component partition of Equation 22 automatically partitions the correlation in fluctuations between different enthalpy terms, e.g., for a two-component partition

$$kT^2Cp = \langle \delta H^2 \rangle = \langle (\delta H_1 + H_2)^2 \rangle = \langle \delta H_1 \rangle^2 + \langle \delta H_2 \rangle^2 + 2\langle \delta H_1 \delta H_2 \rangle \equiv Cp_1 + Cp_2. \qquad 23.$$

Analogous partitioning of cross terms of free energy has been shown to be important in the formal separation of ΔG components (39–41). In fact, this connection demonstrates yet another important aspect of Cp. The formal separation of components of enthalpy, heat capacity, and higher-order T-derivatives of Cp has been used to provide theoretical justification for the widespread partitioning of free energy and entropy components (39, 42).

On an experimental level, the measurement of Cp changes for hydration of model compounds and amino acids has been crucial in separating, analyzing, and interpreting Cp changes for protein unfolding (4, 5, 14, 15, 43, 44). These studies have confirmed the usefulness of group additivity and transferability—which in turn rely on separability—of heat capacity components.

7. GROUP ADDITIVITY, HEAT CAPACITY, AND TEMPERATURE CONVERGENCE OF ENTROPY AND ENTHALPY CHANGES

Starting with the findings of Sturtevant (45) that in certain solvation processes the ratio $\Delta S/\Delta Cp$ is approximately constant at 25°C, Baldwin made the key observation that a linear relationship between ΔS and ΔCp implied convergence of entropy values for hydrophobic group solvation at a unique temperature, $T^s \approx 112$°C (13). This behavior was initially observed for homologous series of model solutes containing hydrophobic groups, such as inert gases and linear alkanes.

To understand this convergence behavior, consider such a series of compounds where the entropy change is linearly related to the heat capacity change, as $\Delta S = a_s \Delta Cp + b_s$ at a given temperature T^{ref}. Substituting into the van't Hoff relation Equation 12b, for a given compound i

$$\Delta S^i = b_s + \Delta Cp^i[a_s + \ln(T/T^{ref})]. \quad 24.$$

At the convergence temperature of $T^s = T^{ref} e^{-a_s}$, the entropy change is $\Delta S^i = b_s$, i.e., it is identical for all compounds in the series. Experimental data show that for hydrophobic solutes such as inert gases and linear alkanes, b_s is also close to zero. Similarly, if $\Delta H = a_h \Delta Cp + b_h$, then

$$\Delta H^i = b_h + \Delta Cp^i[a_h + (T - T^{ref})], \quad 25.$$

and at $T^h = T^{ref} - a_h$, $\Delta H^i = b_h$. Expressed as plots of ΔS or ΔH versus T the convergence behavior described by Equation 24, 25 produces a series of lines, one for each compound, that cross at a single temperature (3). For homologous solutes, group additivity or scaling behavior provides the simplest explanation for this behavior. As homologous groups are added to a compound, or as a homogenous solute such as a linear alkane or inert gas is increased in size, one expects a linear change in all the usual extensive thermodynamic hydration quantities,

$$\Delta X^i = p_X + q_X Q^i, \quad 26.$$

where $\Delta X = \Delta S$, ΔH, ΔCp, etc., and p_X and q_X are coefficients describing the linear behavior of quantity X. Q^i represents the size of the i^{th} solute in terms of a physical dimension or number of homologous groups as appropriate. Eliminating Q^i between pair of such linear size or scaling equations leads straightforwardly to linear relationships between Cp and S or H, and hence to convergence behavior for small solutes. Such scaling is the basis for explanations of convergence behavior from several studies of solutes (15, 16, 46, 47). In addition, as Murphy and coworkers have pointed out, the coefficients *a* and *b* are themselves valuable in that they enable the contribution of the constant to be separated out from the varying parts of a homologous solute series (12, 16).

Given the additivity/scaling explanation of convergence for homologous solutes, it was remarkable that convergence behavior was subsequently found for

unfolding a set of small globular proteins, for both enthalpy and entropy, when normalized by the number of residues (12). Moreover, for entropy, T^s was close to that of hydrophobic solutes. This surprising result has sparked considerable interest and ongoing debate on the dependence of the hydrophobic effect on temperature and the role of hydrophobic stabilization in proteins at physiological and higher temperatures (13, 14, 46–52). To highlight just a few issues that have emerged, consider the two observations that T^s is similar for hydrophobic solutes and these proteins, and that for the former, b_s is close to zero (i.e., at about 112°C the entropy of apolar hydration is close to zero). First, this shows that, due to the large positive hydration Cp characteristic of hydrophobic solvation, close to and above T^s, the hydrophobic effect is dominated by unfavorable enthalpy, not by unfavorable entropy. Second, it implies a universal behavior for the hydration entropy of hydrophobic solutes. Third, it raises the possibility that by measuring or calculating the entropy of protein unfolding at T^s and then extrapolating back to ambient temperatures using the van't Hoff relations, one can cleanly separate out one contribution (apolar hydration entropy) to protein stability. This is a rare and tantalizing possibility for thermodynamic measurements on such complex systems as proteins. This kind of extrapolation and dissection has been used to good effect in the parameterization of empirical energy functions for proteins (53–55).

The dominant role of hydrophobic interactions in protein stabilization was a common theme in the different explanations proposed for entropy and enthalpy convergence behavior in proteins referenced above. However, in an extensive analysis of protein unfolding thermodynamics that includes many more proteins, Robertson & Murphy have shown that the evidence for convergence behavior in ΔS and ΔH of unfolding is not compelling, i.e., plots of these quantities against ΔCp of unfolding show little evidence of linearity (16). Interestingly, the thermodynamics of hydrophobic hydration has now been invoked to explain the *scatter* in the $\Delta S/\Delta Cp$ plots (52). Thus, as a large group, proteins do not act as a set of homologous compounds (when normalized by their size) but smaller, more related sets of proteins may.

8. CONTRIBUTIONS TO Cp IN PROTEINS

An influential study by Sturtevant (45) listed most of the possible sources of Cp changes in protein unfolding (ΔCp^{unfold}) and binding; these include the hydration of hydrophobic groups, hydrogen bonding, electrostatics, and protein conformational entropy, vibrational terms, and changes in equilibrium (i.e., the linked equilibrium effect described above). More than 25 years later, this remains predominantly a list. The exception is the polar and apolar hydration contributions, which have been fairly accurately quantified for protein unfolding mostly due to systematic measurements on model compounds and peptides (4, 5, 14, 15, 43, 44). The magnitude, importance, and even the sign of the other terms remain uncertain. Reasons for this uncertainty include absence of suitable model systems, difficulty

4 Jan 2005 3:28 AR AR241-PC56-18.tex XMLPublish^{SM}(2004/02/24) P1: IKH
AR REVIEWS IN ADVANCE10.1146/annurev.physchem.56.092503.141202

in extracting specific contributions from measured net Cp changes, and the inherent difficulties in simulating or developing theoretical models for a higher-order derivative/fluctuation quantity like Cp in molecular detail for large molecules such as proteins. Most current work has therefore addressed the contributions to protein Cp changes with a much broader division: into hydration versus nonhydration, i.e., protein terms. To put this into the framework of the separability of Cp components described by Equation 22, the net enthalpy change in a protein unfolding or binding reaction can be divided up as

$$\Delta H = \Delta H^{\text{protein-protein}} + \Delta H^{\text{protein-solvent}} + \Delta H^{\text{solvent-solvent}}, \qquad 27.$$

with corresponding ΔCp contributions,

$$\begin{aligned} \Delta Cp &= \Delta Cp^{\text{protein-protein}} + (\Delta Cp^{\text{protein-solvent}} + \Delta Cp^{\text{solvent-solvent}}) \\ &= \Delta Cp^{\text{protein-protein}} + \Delta Cp^{\text{hydration}} \end{aligned}$$

The solvent-solvent term arises because of protein-solvent interactions, and both involve solvent so they can be grouped into a single hydration term. The relative size of these two terms is not known in general, but from a molecular dynamics study of Xenon in water (56), the solvent-solvent contribution to Cp is about an order of magnitude higher than the solute-solvent interaction, which is consistent with the general view that for hydrophobic compounds, the water-water interaction is the dominant term. Debate about the relative importance of Cp contributions, summarized below, centers around whether the Cp increase upon protein unfolding can mostly or completely be accounted for by hydration effects or whether there are significant internal protein conformations, i.e., what are the relative sizes of $\Delta Cp^{\text{protein-protein}}$ and $\Delta Cp^{\text{hydration}}$.

8.1. Is Protein Unfolding Cp in Proteins Dominated by Hydration or by Protein-Protein Interactions?

For protein unfolding, hydration effects are clearly significant; early evidence showed that hydration is the major effect (57). The strongest current evidence is that one can account for $\Delta Cp^{\text{unfold}}$ for many proteins by adding up hydration contributions from individual groups (58–61). Indeed, the prevalent use of area models (see below) to analyze protein Cp data is based on this assumption. However, the protein data themselves are insufficient to clinch the argument; Robertson & Murphy's analysis shows that the data can be equally well fit by a number of area-based hydration models (16). This variability may also reflect overestimation of how unfolded the protein is in the denatured state (61–63), which factors into the correct calculation of area changes. Also, recent measurement of changes in $\Delta Cp^{\text{unfold}}$ due to point mutations shows that the direction is consistent with the hydration effect but sometimes fivefold larger (64). Assessment of the nonhydration contributions to $\Delta Cp^{\text{unfold}}$ is difficult, but Dadarlat & Post (65) found that $\Delta Cp^{\text{unfold}}$ increased with compressibility of the native state protein. This increase

4 Jan 2005 3:28 AR AR241-PC56-18.tex XMLPublish^{SM}(2004/02/24) P1: IKH
AR REVIEWS IN ADVANCE10.1146/annurev.physchem.56.092503.141202

implies that the protein chain contribution is not dominant, otherwise one would expect the opposite correlation. Liang & Dill (66) found that larger proteins are more loosely packed than smaller proteins, and that ΔCp^{unfold} decreases as packing increases; however, when normalized by chain length, ΔCp^{unfold} is independent of packing density, which implies that van der Waals interactions are not a dominant contribution.

Recent experimental and theoretical studies of Cp alpha helix-coil transition (67, 68) represent an interesting but relative rare excursion into the field of protein heat capacities: an intermediate size system between model solutes—which have little potential for nonhydration contributions to Cp—and the more complex fully sized globular proteins. Both simulation and experiment found a negative ΔCp for helix unfolding, around -32 J/mol K for the DSC/CD study, and about -60 J/molK, at 273 K, dropping to zero at 383 K for the simulations. The opposite sign of ΔCp^{unfold} compared with larger proteins has been attributed by Richardson & Makhatadze to the dominant role of polar group hydration upon unfolding; The helix, unlike a protein, has no hydrophobic core (67), a finding again consistent with a dominant hydration term and a small contribution from the protein chain. Indeed, application of the area model for hydration Cp by Richardson & Makhatadze gave a very similar value to their measurement, -30 Jmol^{-1} K^{-1}.

Contrary evidence to the effect that the protein makes a major contribution to ΔCp^{unfold} comes from a variety of sources, mostly indirect, as attempts to calculate net Cp using atomic level models of proteins are virtually unknown. An exception is recent work of Lazaridis & Karplus (62) using a simplified treatment of solvent with an all atom representation of the protein. In this work, models of the denatured state of the C12 protein were generated using molecular dynamics simulations. Their calculation of ΔCp^{unfold} includes a contribution from the temperature dependence of the protein conformational distribution. A value of $\Delta Cp^{unfold} = 0.525$ kcal/mol K was obtained. This is of the right magnitude and sign, but an underestimate of the experimental value. This underestimate was attributed to limitations in sampling and the hydration model. The major conclusion from this work was that a large component of ΔCp^{unfold} comes from fluctuations of nonbonded interactions within the protein, i.e., the $\Delta Cp^{protein-protein}$ term. The compactness of their model of the denatured state compared with an extended conformation also resulted in a smaller contribution from the exposure of nonpolar groups than would be predicted by area-based models.

Cooper et al. (69) used a less detailed, lattice model for protein Cp and found large effects due to the networks of weak interactions in cooperative protein systems. This work also suggested that for close-fitting protein-protein interactions, there is a significant Cp contribution due to changes in zero point energy, a quantum effect. However, there is no experimental system to date for testing this prediction.

Deep & Ahluwalia (70) have applied a disulfide bond correction to area-based models of Cp change. They found an empirical relationship in terms of the number of S–S bonds n: $\Delta\Delta Cp = 23.5–4.23n$. The correction was used to calculate ΔCp^{unfold} for eight proteins and shown to correlate better than previous

4 Jan 2005 3:28 AR AR241-PC56-18.tex XMLPublish^{SM}(2004/02/24) P1: IKH
AR REVIEWS IN ADVANCE10.1146/annurev.physchem.56.092503.141202

estimates. However, this improvement could be interpreted either as a better treatment of area hydration or as evidence of an effect of S–S bonds on the protein conformational contribution to ΔCp^{unfold}.

Although the contribution of DNA to protein-DNA binding Cp changes is beyond the scope of this review, we note that for specific base protein-DNA binding where coupled protein folding and binding is not involved, e.g., BamHI (23, 24), one cannot account for the large negative Cp changes with any reasonable hydration model. Thus, binding-related damping of DNA internal fluctuations very likely contributes significantly to Cp. However, it is still an open question as to whether protein conformational fluctuations, or any other $\Delta Cp^{protein-protein}$ term, is comparable to the $\Delta Cp^{hydration}$ term for folding and protein-protein binding.

9. THEORETICAL MODELS FOR Cp CONTRIBUTIONS IN PROTEINS

We can broadly identify two heat capacity contributions in proteins, $\Delta Cp^{protein-protein}$ and $\Delta Cp^{hydration}$. What can be said about the physical origins, in molecular and atomic terms, of each of these? The experiments have given us mostly thermodynamic data, and from thermodynamics one cannot infer mechanisms. Thus the answer lies in what physical models, or simulation methodologies, are available to calculate Cp contributions and compare with experiment. As mentioned above, there are almost no atomic level treatments of heat capacity at the level of whole proteins, and there are unlikely to be any in the near future. Simulation technology has advanced to the point where free energy changes can be obtained for proteins, but with considerable computational effort and usually for rather small changes, i.e., point residue mutations. Entropy can be extracted from such simulations for similar size changes, but with even more effort. Thus to extract, for example, a higher-order fluctuation/derivative quantity like Cp, and for a large-scale change such as unfolding, is difficulty squared. Atomic detail theoretical models and simulations of heat capacity relevant to proteins are thus almost entirely confined to treatment of the hydration term, usually studied on protein constituents or model compounds. The aim has been to account for the major Cp-related features of hydration:

i. The positive Cp of hydration for apolar groups;
ii. The negative Cp of hydration for polar groups;
iii. The convergence of apolar group hydration at $T \approx 112^{\circ}C$;
iv. For apolar groups the switch from negative entropy, enthalpy of hydration at room temperature to positive entropy, enthalpy of apolar hydration above the convergence temperature (this regime is still relevant to the biology of protein stability because proteins from hyperthermophiles function at these temperatures);
v. The decrease in apolar hydration Cp with increasing temperature; and

vi. The temperature maximum in stability that most proteins exhibit, and the cold-induced denaturation that some proteins can be made to undergo.

An additional important goal has been to develop practical but accurate models for use on large protein and DNA systems to calculate $\Delta Cp^{hydration}$ for binding and unfolding.

9.1. Area-Based Models for Hydration Cp

One of the simplest, but most widely used, models for calculating hydration heat capacity in proteins is the solvent-accessible surface area model. Careful measurements on model compound and protein data (4, 5, 14, 15, 43, 44) show unequivocally that hydration of apolar and polar groups is accompanied by increases and decreases in Cp, respectively. For the model compound data, these changes are to a good approximation proportional to the changes in apolar and polar solvent-accessible area, ΔA_n and ΔA_p. Similar relations apply to the protein data, where Makhatadze & Privalov (4, 5) analyzed Cp contributions from backbone and side chains. They found nonpolar $\Delta Cp^{hydration}$ is positive at low temperature, and decreases with increasing temperature, whereas for polar groups it is negative at low temperature. $\Delta Cp^{hydration}$ of side chains correlated well with surface area. These and other studies led to the area model hydration heat capacity equation

$$\Delta Cp^{hydration} = C_n \Delta A_n + C_p \Delta A_p.$$

This equation is easy to apply to protein systems, although, as mentioned above, calculation of unfolded state areas has uncertainties. This equation is also the basis for a general and extremely versatile total protein energy function used to study conformational aspects of proteins (54, 71, 72). However, depending on the data sets used to derive the coefficients, a variety of values for C_n and C_p have been obtained (Table 1). In many applications, these give very similar results (64), albeit with variations. In the most extensive analysis of protein data to date, Robertson & Murphy pointed out that the Cp data can be fit equally well with a number of area models (owing to the variability and complexity of the proteins, and attendant Cp

TABLE 1 Area coefficients for hydration heat capacity equation[a]

Source	Data set	C_n	C_p
Spolar et al. (59)	12 proteins	1.34	−0.59
Murphy & Friere (101)	Cyclic dipeptides	1.88	−1.09
Myers et al. (61)	26 proteins	1.17	−0.38
Makhatadze & Privalov (58)	20 proteins	2.14	−0.88
Robertson & Murphy (16)	49 proteins	0.66	0.52
Sharp & Madan (73)	Nucleic acid fragments	0.71	0.71

[a]For the equation $\Delta Cp^{hydration} = C_n \Delta A_n + C_p \Delta A_p$, values in J/mol/K/Å^2.

4 Jan 2005 3:28 AR AR241-PC56-18.tex XMLPublish^{SM}(2004/02/24) P1: IKH
AR REVIEWS IN ADVANCE10.1146/annurev.physchem.56.092503.141202

measurement uncertainties). This points to the limitation in using protein data alone and the importance of using model compound data (16). These protein coefficients have also been used, for want of any alternative, to compute the contribution to ΔCp^{bind} from burial of DNA surface by proteins in an attempt to explain large base sequence-specific decreases in Cp (60). However, more recent work on nucleic acid model compounds, which also provides preliminary coefficients for the area model (Table 1) (73), shows that hydration of nucleic acids is quite different, and that use of protein values will lead to large errors in $\Delta Cp^{hydration}$. Finally, these area models, though useful, are entirely empirical, and shed no light on why the hydration Cps of polar and apolar groups have the sign and magnitude they do.

9.2. Two-State Models for Hydration Cp

Extending an earlier two-state model (74), Muller (75) developed a simple but influential two-water-state model to explain the increase in Cp upon hydrating apolar groups. The two states are made and broken water H-bonds, which are in equilibrium. H-bond breaking is characterized by positive enthalpy and entropy changes, changes that are different in bulk water and in the solute hydration shell. The model thus needs four thermodynamic parameters, which are obtained from thermodynamic data on water and fitting. The result satisfactorily reproduces the positive $\Delta Cp^{hydration}$ for apolar solute solvation, and even the observed decrease in $\Delta Cp^{hydration}$ with increasing T. The model has subsequently been generalized (76). Referring to additional experimental rationales for treating water as a mixture of two of more distinct states (77), Silverstein et al. recently refined the model further (6), using better data on water. This refinement has improved the fit to the temperature dependence of $\Delta Cp^{hydration}$ of inert gases. In the context of a two-state model, an interesting inference that can be drawn from the observed decrease of $\Delta Cp^{hydration}$ with increasing T is that hydrophobic solvation at room temperature occurs in a low energy gap regime (Figure 3).

However, with both original and refined parameter values, the Muller model gives a positive entropy and enthalpy of solvation of apolar groups at room temperature, opposite to experiment. Muller's original intent was to explain Cp changes with his model. The original paper apparently shows the correct direction of S and H changes, but there is an error in both entropy and enthalpy expressions, although not with the Cp expression.

Bakk et al. (78, 79) have made an interesting extension to the two-state model for Cp hydration, adding an extra term from immobilization of water by H bonded around polar groups to treat the polar contribution too. They have applied this extension to proteins, where it produces ΔG^{unfold} versus T profiles with magnitude and curvature similar to those of protein data, exhibiting both melting and cold denaturation behavior.

9.3. Statistical Mechanical Models for Hydrophobic Solvation

Statistical mechanical and atomic level simulation studies of solute hydration are too numerous to summarize here, and we focus on a subset that

4 Jan 2005 3:28 AR AR241-PC56-18.tex XMLPublish^{SM}(2004/02/24) P1: IKH
AR REVIEWS IN ADVANCE10.1146/annurev.physchem.56.092503.141202

specifically addresses the increase (decrease) in Cp upon apolar (polar) solute hydration.

Most of the statistical mechanical studies on apolar solute hydration to date have focused on simplified, spherical solute representations. An early model of this type for hydrophobic solvation is scaled particle theory (SPT). This theory estimates the thermodynamic work to create a spherical solute-size cavity subject to avoiding any solvent overlap. In its unmodified form, this work would be purely entropic and thus not directly able to model, e.g., the temperature dependence of solvation enthalpy. However, by applying principles of enthalpy/entropy compensation, Graziano & Lee (47, 80) have extended the model. The model produces a positive heat capacity for hydrophobic solvation, and the correct entropy convergence behavior. In this model, the positive heat capacity and entropy convergence arise because of a linear relationship of entropy terms with temperature. However, SPTs do not provide a molecular picture of the local structure of water and how it might be perturbed around solutes.

There has been a sustained development of statistical mechanical models for the hydrophobic effect, dating back to the influential Pratt-Chandler theory (81), and culminating in recent models based on information theory and Gaussian fluctuation (7, 46, 52, 82–87). Like SPT models, these focus on the energetics of forming a spherical cavity in water, which is a prerequisite for inserting the solute. Unlike SPT models, these models do not assume the water is hard sphere; they use more detailed solvent structural information, specifically the pair correlation function, and can account for weak solute-solvent interactions (84). These theories successfully reproduce experimental chemical potential (free energy) data for spherical hydrophobic solutes such as inert gases, e.g., methane. They also accurately model the observed decrease in entropy, the entropy convergence behavior, and the curvature of the excess chemical potential versus T, i.e., they predict a positive heat capacity of hydration. For the information theory models, variation in fluctuation in solvent density with temperature is weak (46), which presumably reflects some underlying entropy-enthalpy compensation.

A feature common to these models is that the temperature dependence of the free energy of solute insertion comes almost entirely from the curvature of the factor $T\rho^2$ where ρ is the water density at the vapor-water coexistence curve (46, 52). The density at different temperatures is taken from tabulated experimental values, not calculated from the models. In addition, details about local water structure and orientation effects appear only implicitly, via the pair correlation function (82). Because Cp essentially comes from the experimental dependence of water density, these models do not provide a physical picture of the increase in heat capacity in terms of water's molecular structure. It is also claimed that information theory models do not correctly predict Cp versus T (88), although they accurately determine the sign, but this assertion is under debate (7, 89). It should be emphasized that calculating the T-dependence of Cp, which is a third derivative thermodynamic quantity, is probably a challenge for any level of theory; calculating the right sign of the temperature dependence is a already a major advance.

Another approach to explain apolar hydration Cp at molecular resolution of water structure uses a two-dimensional water model, the so-called Mercedes-Benz model, which has three equally spaced H-bonding arms. The advantage of a 2-D model is that sampling can be much more thorough, a crucial requirement for precise Cp calculations. The model reproduces many features of water (89), including the increase in Cp for hydrophobic solvation. This latter increase arises from a balance between pair-wise and three-body (perhaps orientational?) entropy terms that have different temperature dependences.

The statistical mechanical theories discussed above have to date addressed only hydrophobic solute solvation. Thus they do not explain one of the more puzzling and counterintuitive effects: the decrease in Cp upon solvation of polar groups. As previously pointed out, combined with the negative solvation entropy of polar groups, this decrease implies that the hydration entropy becomes more negative, i.e., that at least at moderate temperatures (circa 25–40°C), water is more structured by the solute as T is raised! To date, the most comprehensive physical picture of the effects of hydration heat capacity comes from a combination of the random network model [which treats liquid water as patchworks of continuously distorted H-bonds (90)] with atomic level simulations (73, 91–97). This approach uses an all-atom model of water shown to reproduce many features of water [TIP4P (98)]. Parameters are not adjusted specifically for Cp calculations, nor is there any a priori assumption about two-state water, and there is explicit recognition of H-bonding and angular structure. A wide variety of size, shape, and chemical types of solutes has been examined with this approach, ranging from ions, aromatic compounds and urea, hydrophobes such as butane, to amino acid and nucleic acid fragments. In this model, the most important effect of solutes on water structure for Cp changes is distortions in the angular structure of water. Bulk water has a continuous but bimodal distribution of water-water H-bond angles. Apolar solutes shift the distribution to low angles, whereas polar groups shift it to high angles (Figure 6). Both distribution changes have the effect of lowering the entropy because they make the distribution less uniform. However, the apolar solutes increase the water heat capacity as low-angle-geometry water pairs have a larger energy fluctuation than high-angle pairs have (97). Polar solutes decrease the heat capacity because they deplete the low-angle population and augment the high-angle population, which has smaller energy fluctuations. Fluctuations in water energy increase with decreasing angle because the waters interact more strongly, magnifying the effect of fluctuations in liquid structure. The changes in water structure seen in this model are also supported by FTIR spectroscopy studies of solutions (99). This model provides a unified explanation for apolar and polar hydration Cp changes in terms of local water structure changes at the molecular level.

An intriguing consequence of Cp changes in proteins is the phenomenon of cold denaturation. At the level of thermodynamic explanations, cold denaturation occurs because ΔG^{unfold} versus T for proteins has a downward curvature, which in turn is because ΔCp^{unfold} is positive (Equation 2). Accepting the evidence that it is positive primarily because of exposure of hydrophobic groups, cold

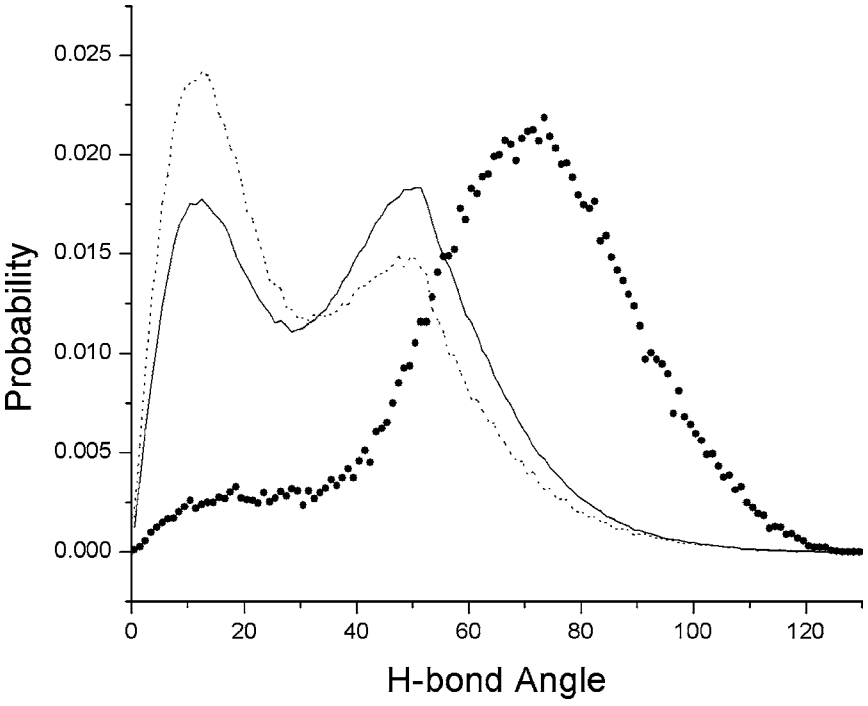

Figure 6 Water-angle probability distributions from the random network-TIP3P water simulation model for Cp. Distributions for bulk water (—), water in the first hydration shells of methane (· · · · ·), and K^+ (ν).

denaturation is explained at this level by any model that has a positive $\Delta Cp^{hydration}$ for apolar solutes, which includes all the different statistical mechanical models just discussed. This is not so satisfactory if one is seeking an explanation in terms of molecular level changes in water structure, and there have been several explanations put forth.

Privalov & Gill in their early comprehensive reviews of the phenomenon (3, 17) stated that cold denaturation was caused by destabilization of the hydrophobic interaction by cold water, although they were unable to describe the changes in water structure that were responsible. This view is consistent with the recent elegant determination of the structure of a cold denatured protein. The cold denatured form appears to fall apart in a noncooperative, subdomain-type fashion, exactly as might be expected from a weakening of tertiary, hydrophobic driven interactions (100). This is in contrast to heat-denatured forms of proteins where the more uniform effect of heat destabilization acts on both secondary and tertiary interactions to produce something more random and coil-like. An explanation for cold denaturation along the lines suggested by Privalov & Gill has recently been proposed by Chandler (83), based on the theory of hydrophobicity propounded by

4 Jan 2005 3:28 AR AR241-PC56-18.tex XMLPublish^{SM}(2004/02/24) P1: IKH
AR REVIEWS IN ADVANCE10.1146/annurev.physchem.56.092503.141202

Lum et al. (82). In this theory water density near a hydrophobic surface is depleted until it is vapor-like. This depletion is unfavorable, hence driving hydrophobic surface burial. Lowering T makes it harder to form this vapor-like layer (as one moves away from the vapor-water coexistence curve), thereby weakening the hydrophobic stabilization. This model is controversial because the depletion zone is significantly reduced when even weak solute-solvent attraction terms (such as the van der Waals interaction) are added to the theory (84). It is also not clear how much this depletion layer would persist around a heterogeneous protein-water interface, where any apolar surface is never far from a strongly solvent-interacting polar group. In the model of Sharp and coworker for Cp changes, hydrophobic groups promote low-angle water pair configurations, which have a more favorable energy of interaction and a larger energy fluctuation (92, 93, 97). In this model, as the temperature is lowered, the entropy penalty for forming lower-angle geometries is weakened, the favorable interaction increases so the penalty for hydrating apolar groups is reduced, and there is an overall weakening of the hydrophobic stabilization, resulting in cold denaturation.

The existence of at least two completely different proposals for the molecular basis of cold denaturation, one based on solvent density effects, the other on solvent angular effects [and there are other more controversial proposals, e.g., see (27)] is indicative of the deep nature of the problem and how much remains to be elucidated about changes in protein heat capacity.

10. CONCLUDING REMARKS

Of all the major thermodynamic variables measured for proteins, heat capacity is the one with the most diverse set of definitions and the richest set of implications for protein folding and binding. There are many ramifications of observed Cp changes. The sign distinguishes apolar from polar solvation. It imparts a temperature dependence to entropy and enthalpy that may change their signs and determine which of them will dominate. Protein unfolding usually has a positive ΔCp, producing a maximum in stability and sometimes cold denaturation. Cp is also the quantity where even the most basic questions are still unanswered. What are the molecular, physical origins of Cp? What are the major contributions? What are their relative magnitudes? What are their signs? Two general sources of heat capacity change have been identified, from hydration (protein solvent, and accompanying solvent-solvent interactions) and protein-protein interactions; it is an open question as to which source dominates in folding and binding. At this point the solvation contribution is well characterized experimentally, particularly for small model compounds and amino acid analogs. Theoretical work to date has dealt mostly with the hydration term and can account, at least qualitatively, for the major Cp-related features. These include the positive and negative hydration Cp of hydration for apolar and polar groups, respectively; the convergence of apolar group hydration entropy at T $\approx$ 112°C; the decrease in apolar hydration Cp

with increasing T; and the T maximum in protein stability and cold denaturation. Relatively little can be said in terms of mechanism or precise numbers, either experimentally or theoretically, about the protein contribution. This ambiguity leaves plenty of interesting work to be done.

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