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Relationships between the temperature dependence of solvent denaturation and the denaturant dependence of protein stability curves☆

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Abstract

We have used a simple binding model to consider how the thermodynamics of denaturant–protein interactions might influence the shape of protein stability curves (free energy change as a function of temperature), and how these effects translate into a temperature dependence of the apparent *m*-value (sensitivity of unfolding free energy to denaturant). We find that for an exothermic binding reaction with no binding heat capacity increment, increasing denaturant concentrations produces an apparent increase in curvature in the protein stability curve, giving rise to an increase in the heat capacity increment of unfolding. Similar increases are seen if the binding heat capacity increment is taken as positive. However, for a negative binding heat capacity increment, increasing denaturant concentrations decreases the curvature of the stability curve, giving rise to a decrease in the heat capacity of unfolding. At very high denaturant concentrations (above which the heat capacity of denaturation becomes negative) the stability curve becomes dimpled, showing two separate maxima rather than one. These three models result in very different temperature dependencies of apparent *m*-values. For urea-induced unfolding of the ankyrin-domain of the Drosophila Notch protein, we find a dependence of experimental *m*-values on temperature that is similar to that produced by a negative binding heat capacity increment. This temperature dependence is consistent with the observed decrease in heat capacity of unfolding as denaturant is added.

Keywords: Solvent denaturation; Heat capacity; Protein stability; Denaturants; Thermodynamics; Notch

☆ Dedicated to John Schellman for his contributions in the areas of protein stability and solvent denaturation.

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1. Introduction

One of the most important representations of the thermodynamic stability of globular proteins is the 'protein stability curve' [1–4]. In this depiction, free energy of unfolding (ΔG_u^0) is plotted as a function of temperature. In addition to providing values for the stability, as represented by ΔG_u^0, the shape of the stability curve is connected to other thermodynamic quantities [1,2]. The slope of the stability curve is equal to the negative of the unfolding entropy (ΔS_u^0), and is related to the unfolding enthalpy (ΔH_u^0) by combination with ΔG_u^0. The curvature of the stability curve is directly related to $\Delta C_{p,u}$[1]. For all proteins where stability curves have been determined, curvature is negative, indicating a positive $\Delta C_{p,u}$ for unfolding. This positive $\Delta C_{p,u}$ gives the stability curve a shape much like an inverted parabola. The positive value of $\Delta C_{p,u}$ is consistent with ideas relating exposure of nonpolar surface area to water on unfolding [5–9]. Given the sensitivity of stability curves to ΔH_u^0, ΔS_u^0, and $\Delta C_{p,u}$, these curves provide a detailed picture of the thermodynamics of protein stability. Recently, protein stability curves have been used to depict the origins of enhanced thermostability in proteins derived from thermophilic organisms [10–13].

Protein stability curves can be estimated in a variety of ways. For a few proteins of modest stability, both high- and low-temperature denaturation can be detected in aqueous solution [14–16], allowing an accurate determination of $\Delta C_{p,u}$ as well as ΔH_u^0, ΔS_u^0, and ΔG_u^0 simply by following the unfolding equilibrium constant as a function of temperature. However, most proteins are too stable to reveal low-temperature unfolding, and the derivative parameters must be measured by other methods. For proteins that undergo reversible thermal unfolding transitions, direct calorimetric estimates of $\Delta C_{p,u}$, ΔH_u^0, ΔS_u^0, and ΔG_u^0 can be made, and from these parameters, the stability curve can be constructed [4].

Another method to access both the high- and low-temperature transitions for proteins of high stability is to measure thermal denaturation curves in the presence of low concentrations of denaturant [17–24]. While this approach allows direct estimation of $\Delta C_{p,u}$, ΔH_u^0, ΔS_u^0, these parameters and the resulting stability curve apply to unfolding in the presence of denaturant. Projection of the measured stability curve to denaturant-free solutions requires extrapolation of $\Delta C_{p,u}$, ΔH_u^0, ΔS_u^0, in addition to ΔG_u^0 as a function of denaturant. Whereas extrapolation of ΔG_u^0 as a function of denaturant has been thoroughly investigated from an experimental [25–31] and theoretical perspective [32–37], the means to extrapolate the other parameters, especially $\Delta C_{p,u}$, has not been adequately determined.

A variation of the denaturant-based method described above, which is becoming increasingly popular for determining stability curves [11,12,20,21,38,39], does not require the denature dependence of $\Delta C_{p,u}$, ΔH_u^0, and ΔS_u^0, to be known. In this method, originally used by Pace and Laurents [38], stability is estimated as a function of temperature through a series of isothermal solvent denaturation curves at different temperatures. At each temperature, ΔG_u^0 is extrapolated to zero denaturant concentration, providing points that lie on the stability curve in the absence of denaturant. These points can then be fitted to an equation for the stability curve, to yield values for $\Delta C_{p,u}$, ΔH_u^0, ΔS_u^0. In principal, by extrapolating the ΔG_u^0 values to non-zero denaturant concentrations, this method can be used to determine ΔH_u^0, ΔS_u^0, and $\Delta C_{p,u}$ as a function of denaturant concentration, although the form of the expression used to extrapolate ΔG_u^0 puts restrictions on the form of the denaturant dependence of these derivative parameters.

Here we investigate the ways in which denaturant might effect the shape of the protein stability curve and its underlying thermodynamic parameters. We have done this through representation of the thermodynamics of denaturant–protein interaction using a simple binding model [26,32]. We find that the denaturant dependence of $\Delta C_{p,u}$ depends on the enthalpy of denaturant binding (ΔH_b^0) as well as the heat capacity of binding ($\Delta C_{p,b}$). The changes in the shapes of the stability curves are clearly related to the temperature dependence of *m*-values, which we are able to

[1] The exact relationship is that $\Delta C_{p,u}$ is equal to the negative of the product of temperature and the second derivative of ΔG_u^0 with respect to temperature.

approximate using the binding model. We present data on the two-state urea-induced unfolding of an ankyrin domain of the Drosophila Notch receptor that are consistent with a negative heat capacity increment for urea-protein interaction, and thus, a decrease in heat capacity of unfolding with increasing denaturant concentration, and a maximum in the temperature dependence of the *m*-value for unfolding.

2. Experimental

2.1. Materials

A recombinant polypeptide containing the ankyrin repeats of the Notch receptor was expressed and purified as described previously [39]. This protein contains seven ankyrin repeats, from residues 1902 to 2148 of the Drosophila Notch receptor [40]. In addition, both cysteines have been replaced with serines to enhance long term stability and reversible unfolding, and the polypeptide contains an N-terminal hexahistidine tag to facilitate purification. Ultrapure urea was obtained from ICN (Aurora, OH).

2.2. CD unfolding studies

Urea-induced unfolding transitions were acquired on an Aviv 62A DS spectropolarimeter (Aviv Associates, Lakewood, NJ) equipped with a thermoelectric cell holder. Circular dichroism was monitored at 222 nm using a 1-cm cuvette. Automated urea titrations (at 293 K and above) were performed using a computer-controlled Hamilton Microlab 500 titrator as described previously [39].

Manual urea titrations (at 288 K and below) were performed by preparing 2.0 ml samples containing 2–5 μM protein in 25 mM Tris–HCl pH 8.0, 150 mM NaCl and urea at a concentration between 0 and 6 M. Samples were mixed and allowed to equilibrate for 2.5 h in a water bath at the experimental temperature. Samples were transferred to the thermostated cell holder and were equilibrated for an additional 5 min. Following equilibration, circular dichroism 222 nm was averaged over 60 s.

Thermodynamic parameters for unfolding of the Notch ankyrin domain were estimated by fitting an equation relating the observed CD signal to the populations of native and denatured proteins using the non-linear least-squares fitting algorithm of the program Kaleidegraph (Synergy Software, Reading, PA). In this equation, baselines are allowed to vary linearly with denaturant concentration. Free energy of unfolding was treated as a linear function of denaturant [25,27].

3. Results

3.1. Connecting a thermodynamic model for the temperature dependence of denaturant interactions to protein folding

To examine the effects of denaturant on the shapes of protein stability curves, a model is needed that accounts for the temperature dependence of the effect of denaturant interactions on proteins. The most frequently used relationship to describe the effect of denaturant on protein stability is a simple, empirical equation where unfolding free energy is treated as a linear function of denaturant concentration

$$\Delta G^0_u(x) = \Delta G^0_{H_2O} - m[x] \quad (1)$$

where $\Delta G^0_{H_2O}$ is unfolding free energy in the absence of denaturant, and $[x]$ is used to represent denaturant molarity [25,27]. The term *m* represents the effectiveness of the denaturant in destabilizing the protein and is considered to be independent of denaturant concentration in the linear treatment. Although this relationship has the advantage of simplicity (and as a result, a minimum number of parameters), and although it gives an adequate description of unfolding of most proteins for which the model has been critically tested [28,31,41], it has the disadvantage that the denaturant dependence is not in a form that is easily amenable to thermodynamic dissection. Although the *m*-value can be connected to other thermodynamic parameters of unfolding through differentiation, connecting it to thermodynamic parameters for denaturant interaction such as enthalpy, entropy, and heat capacity is not straightforward.

In contrast, the simple binding model for solvent denaturation [26,32] provides a direct way to analyze the effects of ΔH_b^0, ΔS_b^0, and $\Delta C_{p,b}$ on protein stability and its temperature dependence. In this model, unfolding free energy is a logarithmic function of denaturant activity:

$$\Delta G^0(a_x) = \Delta G^0_{H_2O} - \Delta nRT\ln(1 + ka_x) \quad (2)$$

The denaturant interaction is governed by two parameters: Δn, the difference in the number of denaturant binding sites between the denatured and native states, and k, a microscopic binding constant for denaturant to binding sites on the protein. In the binding model, denaturant concentration is typically represented as a dimensionless activity on the molar concentration scale. However, at the low denaturant concentrations considered here ($<$ 3 M), the two quantities ($[x]$ and a_x) are linearly related to within the accuracy of the conversion, particularly for urea[2]. As the data analyzed here involve urea denaturation, we will approximate activity using molarity. This approximation simplifies comparison of Eqs. (1) and (2), but aside from scaling k for urea by approximately 0.92, does not significantly distort Eq. (2).

Representation of denaturant interaction with the binding model has several disadvantages. First, it relies on the unlikely assumption that denaturant–protein interactions have the same affinity at all sites. Although the complexity associated with relaxing this approximation can be represented analytically [35,43], this heterogeneity is not easy to observe experimentally. Second, the binding model has two parameters describing the denaturant–protein interaction (Δn, k) instead of one (m), and since denaturant–protein interactions appear to be quite weak [27,44], these two parameters cannot be uniquely determined [27,30]. Third, the effect of denaturants on protein stability is treated as an interaction for which simple mass action between protein and denaturant applies [34], and omits effects mediated by water. Improvements to this treatment have been made that include changes in water activity as a result of high concentrations of denaturant [35,45].

Despite these limitations, the binding model is simple and it allows thermodynamic treatment of the effect of denaturant on protein stability. The mass-action origin of the binding model permits the denaturant–protein interaction to be represented (through the parameter k) in terms of enthalpy, entropy, and the heat capacity increment of the denaturant–protein interaction [44], and thus allows the temperature dependence of the denaturant effect on protein stability to be examined:

$$\begin{aligned} k &= e^{-[\Delta G_b{}^0]/RT} \\ &= e^{-[\Delta H_b{}^0 - T\Delta S_b{}^0]/RT} \\ &= e^{-[\Delta H_b(T_r) - T\Delta S_b(T_r) + \Delta C_{p,b}\{T - T_r - T\ln(T/T_r)\}]/RT} \end{aligned} \quad (3)$$

The subscript b is used to indicate a change associated with denaturant binding, as opposed to unfolding. The value of the reference temperature T_r, which is specified as a limit of integration, is arbitrary. In the last line of Eq. (3), $\Delta C_{p,b}$ is assumed to be temperature independent.

3.2. The effect of denaturant on protein stability curves

The temperature dependence of protein stability can be written as

$$\begin{aligned} \Delta G^0_u = {} & \Delta H^0_u(T_m) + \Delta C_{p,u}(T - T_m) \\ & - T\Delta S^0_u(T_m) - T\Delta C_{p,u}\ln\left(\frac{T}{T_m}\right) \end{aligned} \quad (4)$$

where $\Delta C_{p,u}$ is assumed to be independent of temperature [1]. T_m is the temperature at which native and denatured states have the same populations, and thus the same Gibbs free energies. In principle, this relationship can be evaluated in the presence of an arbitrary, fixed denaturant concentration, as long as $\Delta C_{p,u}$ remains temperature-independent. However, we will use Eq. (4) as a means to evaluate the temperature dependence of stability in the absence of denaturant, i.e. as an explicit temperature dependent $\Delta G^0_{H_2O}$ in Eq. (2). Combining these two equations gives

[2] Using the standard values relating urea activity and molarity at 25 °C [42], linear regression between the two quantities yields the relationship $a_{urea} = -0.005 + 0.92562$ (urea), with a linear correlation coefficient of 0.9998. Thus, although the activity coefficient for urea deviates slightly from unity, it is constant within error. For guanidine HCl, deviation from linearity is noticeable, although from 0 to 3 M, a linear correlation coefficient of 0.985 is obtained in linear regression between activity and molarity.

Table 1
Parameters used in simulations of protein stability curves and m'-values

		Simulation 1	Simulation 2	Simulation 3
$\Delta C_{p,u}$ [a]	(kJ mol^{-1} K^{-1})	9.75	9.75	9.75
$\Delta H_u^0(T_m)$ [b]	(kJ mol^{-1})	439	439	439
$\Delta S_u^0(T_m)$ [b]	(kJ mol^{-1} K^{-1})	1.33	1.33	1.33
T_m [b]	(K)	333	333	333
$\Delta C_{p,b}$	(kJ mol^{-1} K^{-1})	0	−0.84	+0.28
Δn [c]		132	132	132
$\Delta H_b^0(T_r)$ [c]	(kJ mol^{-1})	−9.0	−9.0	−9.0
$\Delta S_b^0(T_r)$ [c]	(kJ mol^{-1} K^{-1})	−0.054	−0.054	−0.054
T_r [c]	(K)	N/A	298	298

All protein unfolding parameters were derived from empirical correlations to protein size [46,47]. Parameters were calculated for a hypothetical protein of 150 residues, representative of a typical globular protein.

[a] $\Delta C_{p,u}$ was estimated from Myers et al. [47] for a 150 residue protein.

[b] $\Delta H_u^0(T_m)$, $\Delta S_u^0(T_m)$, and T_m were estimated from Robertson and Murphy [46].

[c] All denaturant-binding parameters were estimated from Makhatadze and Privalov [44]. Δn was estimated by constructing a linear relation between measured values of Δn for urea on three proteins studied in [44] and the chain length of these proteins. Δn was then solved from this linear relation for a 150 residue chain. T_r was taken as the center of the temperature range from which ΔH_b^0 and ΔS_b^0 were estimated in [44].

$$\Delta G^0(x) = f(T)_{H_2O} - g(x,T),$$

where

$$f(T)_{H_2O} = \Delta H^0_{u,H_2O}(T_m) - T\Delta S^0_{u,H_2O}(T_m) + \Delta C_{p,u,H_2O}\left\{T - T_m - T\ln\left(\frac{T}{T_m}\right)\right\} \quad g(x,T) = \Delta nRT\ln\left(1 + [x]\, e^{-[\Delta H_b(T_r) - T\Delta S_b(T_r) + \Delta C_{p,b}\{T - T_r - T\ln(T/T_r)\}]/RT}\right) \quad (5)$$

The function $f(T)_{H_2O}$ gives the protein stability curve (Eq. (4)) evaluated in the absence of denaturant, and the function $g(x, T)$ is the second term in Eq. (2) (again, substituting molarity for activity), with an explicit temperature dependence for k (Eq. (3)).

Although, Eq. (5) contains the assumptions of the binding model as well as the assumptions that both binding and unfolding heat capacity increments are insensitive to temperature, it provides a thermodynamically reasonable expression to evaluate how protein stability curves can be affected by the presence of denaturant. We have used Eq. (5) to simulate stability curves at different denaturant concentrations for a hypothetical protein with 'typical' unfolding thermodynamics (Table 1). Values for unfolding enthalpy and entropy in the absence of denaturant were estimated from a study by Robertson and Murphy [46] of the dependence of these quantities on protein size, and a value for the unfolding heat capacity increment was taken from a similar study by Myers et al. [47]. Values for enthalpy and entropy of denaturant binding were taken from a calorimetric study of protein–urea interactions by Makhatadze and Privalov [44]. According to this study, denaturant binding is enthalpically favored (exothermic), but entropically disfavored, at 298 K (the temperature taken as T_r in Eq. (3) and Eq. (5) [44]). This picture is in qualitative agreement with several other studies [48,49]. However, these studies do not give a consistent estimate of the heat capacity increment of denaturant binding, $\Delta C_{p,b}$, even at a qualitative level. As this parameter seems likely to influence the shape of protein stability curves, we have investigated the effects of different values of $\Delta C_{p,b}$ on protein stability curves (Table 1). In what we will refer to as 'simulation 1', we have set $\Delta C_{p,b}$ equal to zero, whereas in 'simulation 2' and 'simulation 3', we have assumed a negative and positive value of $\Delta C_{p,b}$, respectively.

Using thermodynamic values listed for simulation 1 (Table 1, $\Delta C_{p,b} = 0$), we have evaluated Eq. (5) as a function of temperature at different fixed denaturant concentrations (Fig. 1A). As denaturant concentration is increased, the stability curve shifts down. This effect is most pronounced at low

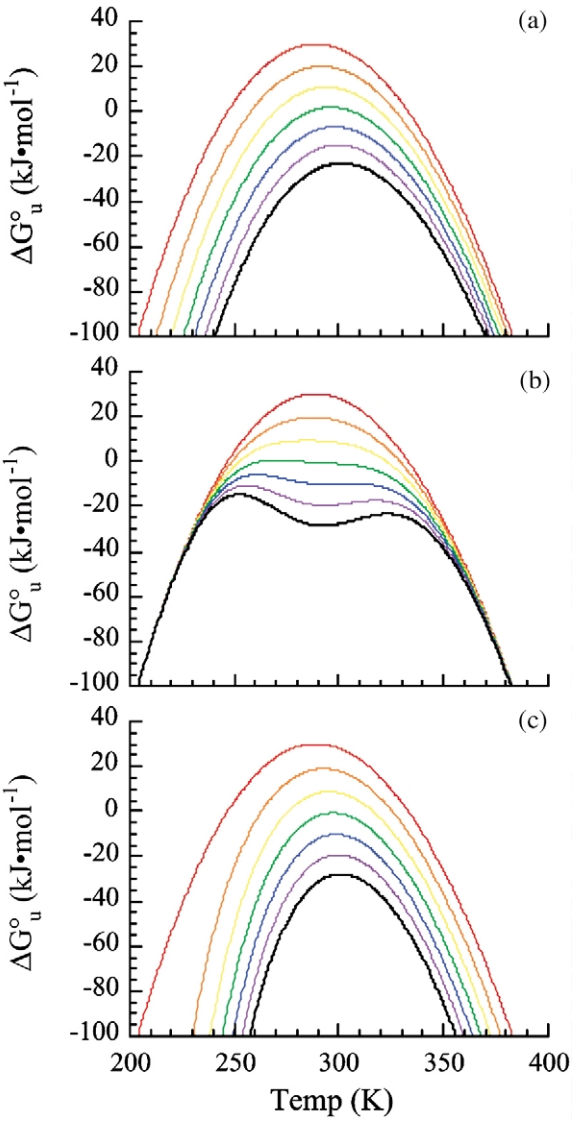

Fig. 1. Possible effects of denaturant on protein stability curves. The binding model was used to calculate protein stability as a function of temperature at different denaturant concentrations using Eq. (5). (A) A zero heat capacity increment of denaturant binding, (B) a negative heat capacity increment of binding, and (C) a positive heat capacity increment of binding. Other parameters are given in Table 1 (simulations 1, 2, and 3 correspond to panels A, B, and C, respectively). For all simulations, denaturant concentration increases from top (0.0 M) to bottom curves (3.0 M) on 0.5 M increments.

temperatures, where the stability curves fan out. In addition, the curvature in the stability curves appears to increase with denaturant concentration, consistent with an increase in heat capacity of unfolding ($\Delta C_{p,u}$) as denaturant concentration is increased. This was confirmed by fitting an equation analogous to Eq. (4) to stability curves simulated at each denaturant concentration: fitted values of $\Delta C_{p,u}$ increased with increasing denaturant concentrations, although the quality of the fits decreased as denaturant concentration increased. As the heat capacity increment of denaturant binding was set to zero in this simulation, the increase in $\Delta C_{p,u}$ with denaturant concentration must be a result of increased enthalpy fluctuations resulting from the weak, exothermic denaturant–protein interactions. Since more denaturant is modeled to bind to the denatured state than to the native state by virtue of an increase in site number in the denatured state, the partial occupancy of binding sites leads to a greater enthalpy fluctuation for the denatured state than for the native state, and thus, leads to a greater heat capacity for the denatured state [6,50]. The observation that the stability curves deviate at low temperature and converge at high temperatures is a result of the exothermic denaturant–protein interaction: at low temperatures the equilibrium constant for denaturant binding gets larger (tighter binding), and thus, the denaturant becomes more strongly destabilizing. The observation that Eq. (4) fits poorly to the calculated stability curves in the presence of denaturant indicates that $\Delta C_{p,u}$ is no longer independent of temperature.

Very different changes to the shape of the stability curve result from modeling $\Delta C_{p,b}$ as negative (Table 1, simulation 2). As denaturant concentration increases, the stability curves become flattened at their peaks, but are unaffected at high and low temperatures (Fig. 1B). At high denaturant concentrations, the stability curves actually invert, showing a minimum in between two separate maxima. Over the temperature range where the stability curves flatten, the curvature clearly decreases, changing sign (from negative to positive) approximately 1.5 M denaturant (Fig. 1B),

consistent with a negative heat capacity of unfolding at high denaturant concentrations. Since at high and low temperatures the stability curves converge, it is clear that the curvature, and thus $\Delta C_{p,u}$, become temperature dependent in high denaturant concentrations. The apparent decrease in $\Delta C_{p,u}$ with denaturant concentration can be explained as a decrease in the mean heat capacity of the denatured state due to excess binding, since in this simulation, denaturant binding is modeled to decrease the overall heat capacity. The observation that stability curves converge at high and low denaturant concentrations can be attributed to the effects of $\Delta C_{p,b}$ on binding affinity. Modeled with a negative heat capacity increment of binding, the affinity gets weaker both at high and low temperatures, just as the stability of proteins decreases at both high and low temperatures.[3]

Not surprisingly, the stability curve is affected oppositely as a result of modeling $\Delta C_{p,b}$ as positive (Table 1, simulation 3). As denaturant concentration increases, stability curves shift down, although this shift becomes more pronounced at both high and low temperatures (Fig. 1C). This downward shift at extremes of temperature results in an increased curvature, and thus, an increase in $\Delta C_{p,u}$. The apparent increase in $\Delta C_{p,u}$ with denaturant concentration can be explained as an increase in the mean heat capacity of the denatured state due to excess binding, since in this simulation, denaturant binding is modeled to increase the overall heat capacity. The observation that stability curves fan out at both high and low denaturant concentrations can again be attributed to the effects of $\Delta C_{p,b}$ on binding affinity, with affinity increasing at both high and low temperatures.

3.3. Calculation of apparent m-values and their temperature dependence

Using the denaturant binding model, the effects of denaturant on protein stability vary with temperature, as is apparent in the three simulated families of stability curves (Fig. 1). Since stability curves are plotted at equal denaturant concentration intervals (of 0.5 M) in all three panels of Fig. 1, the spacing between each curve is closely related to the rate of change of unfolding free energy with molar denaturant concentration, that is

$$\Delta G^0_{u,[x]_j} - \Delta G^0_{u,[x]_i} \approx \left([x]_j - [x]_i\right)\left(\frac{\partial \Delta G^0_u}{\partial [x]}\right)_T \approx -0.5 \times m \quad (6)$$

where the difference on the left-hand-side is the spacing between stability curves i and j, and $[x]_i$ and $[x]_j$ are the corresponding denaturant concentrations. Eq. (6) is approximate, because the binding model is used to generate the denaturant effect on the stability curves in Fig. 1, resulting in a denaturant dependence to the derivative on the right-hand-side. Given that denaturant binding is far from sativation at concentrations considered here, the variation of unfolding free energy with respect to denaturant is nearly linear, and thus the approximation above is reasonable (see below). In light of Eq. (6), the variation in spacing between stability curves in Fig. 1 at different temperatures suggests that experimental *m*-values should be sensitive to temperature, assuming the model for denaturant interaction above. In simulation 1 ($\Delta C_{p,b} = 0$, Fig. 1A), denaturant brings about a greater spacing between protein stability curves at low temperature than at high temperature, suggesting that *m*-values in this simulation should be greater at low temperature than at high temperature. This decrease has been observed previously by DeKoster and Robertson [24]. In contrast, in simulation 2 ($\Delta C_{p,b} = -0.84$ kJ mol^{-1} K^{-1}, Fig. 1B), denaturant brings about a greater spacing between protein stability curves at intermediate temperatures, suggesting that *m*-values in this simulation should reach a maximum at intermediate temperatures.

The temperature dependence of the sensitivity of unfolding to denaturant concentration (or in approximate terms, the temperature dependence of the *m*-value) can be evaluated more precisely by explicitly determining the partial derivative in Eq. (6) above, where unfolding free energy is given in Eq. (5):

[3] In this analogy, denaturant–protein complex is equivalent to native protein in that the heat capacity is lower than the unbound, and unfolded forms, respectively.

$$\left(\frac{\partial \Delta G_u^0}{\partial [x]}\right)_T = \left(\frac{\partial f(T)_{H20}}{\partial [x]}\right)_T - \left(\frac{\partial g(x,T)}{\partial [x]}\right)_T$$
$$= 0 - \Delta nRT\left(\frac{\partial \ln\{1+k[x]\}}{\partial [x]}\right)_T$$
$$= -\Delta nRT\frac{k}{1+k[x]} \equiv -m'. \quad (7)$$

In the last line of Eq. (7), we have used the symbol m' to represent the derivative of unfolding free energy with denaturant concentration to distinguish this quantity from the more familiar m-value of the linear model, which is not a function of denaturant concentration.

The temperature dependence of the m'-value can be evaluated by substituting for k in Eq. (7) using the third line of Eq. (3), for each of the parameter sets in the three simulations (Table 1). However, to obtain a numerical value for m', a denaturant concentration must be selected. The most appropriate choice of denaturant concentration in terms of matching experimentally determined m-values is that on which the unfolding transition is centered, i.e. C_m. Substituting C_m for $[x]$ in Eq. (7) to evaluate the temperature dependence of m' introduces the complication that the numerical value of C_m changes with temperature, as protein stability varies. This variation can be explicitly accounted for using the fact that at C_m, $\Delta G_u^0 = 0$, which permits Eq. (5) to be rearranged to give

$$f(T)_{H_2O} = g(C_m,T) = \Delta nRT\ln(1+kC_m) \quad (8a)$$
$$(1+kC_m) = \exp^{(f(T)_{H_2O}/\Delta nRT)} \quad (8b)$$
$$C_m = \frac{1}{k}\left(\exp^{(f(T)_{H_2O}/\Delta nRT)} - 1\right) \quad (8c)$$

Substituting Eq. (8c) into $[x]$ of Eq. (7) gives a value for m' at C_m of

$$m' = \frac{\Delta nkRT}{\exp^{(f(T)_{H_2O}/\Delta nRT)}} \quad (9)$$

where $f(T)_{H_2O}$ is as in Eq. (5). This relation for m' is plotted as a function of temperature, for the three parameter sets (Table 1) in Fig. 2. Like the denaturant dependencies of the stability curves in Fig. 1, different values of $\Delta C_{p,b}$ give rise to very different temperature dependencies for m'-values.

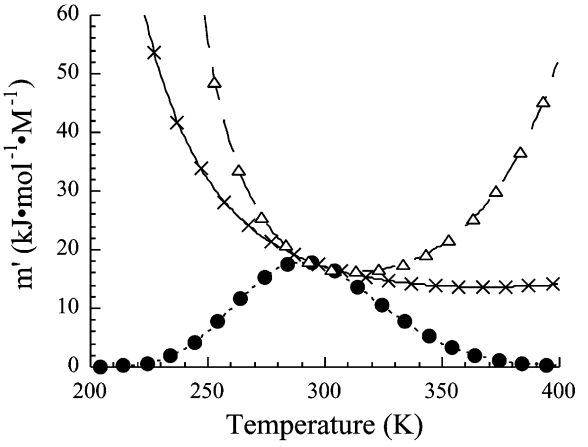

Fig. 2. The effect of temperature on m'-values for the three different binding heat capacity increments considered here: $\Delta C_{p,b} = 0$ (×'s); $\Delta C_{p,b} = -0.84$ kJ mol^{-1} K^{-1} (●'s); $\Delta C_{p,b} = +0.28$ kJ mol^{-1} K^{-1} (Δ's). Values for m' were calculated by evaluating Eq. (7) at the denaturant concentration equal to the midpoint of the unfolding transition (C_m, Eq. (9)), using parameters listed in Table 1.

If there is no heat capacity increment of binding (×'s in Fig. 2), m' decreases sharply with temperature at low temperatures, but becomes relatively insensitive to temperature as temperature increases. If, however, $\Delta C_{p,b}$ is less than zero (●'s in Fig. 2), m' reaches a maximum (approximately 290 K for the parameter set in simulation 2), and goes to zero at high and low temperatures. If instead $\Delta C_{p,b}$ is greater than zero, then the temperature dependence of m' becomes inverted, showing a minimum (approximately 320 K for the parameter set in simulation 3), and increasing sharply both with increasing and decreasing temperatures (Δ's in Fig. 2).

3.4. Temperature dependence of denaturant interactions in unfolding of the ankyrin repeats of the Notch protein

To investigate whether the denaturant dependence of $\Delta C_{p,u}$ and the temperature dependence of the m-value can be experimentally observed, we have measured the urea-induced unfolding of the ankyrin-repeat domain of Drosophila Notch over a range of temperatures. The Notch ankyrin domain is well-suited for these studies because it

has been shown to unfold in a two-state reaction [39,51], and because it is relatively large (268 residues) compared with most proteins that show equilibrium two-state unfolding [47]. The large size of the Notch ankyrin domain results in a large *m*-value and $\Delta C_{p,u}$, facilitating accurate measurement of these quantities, and allowing small fractional changes in these quantities to be detected.

Urea denaturation curves of a construct containing seven ankyrin repeats from the Drosophila Notch protein were obtained from 273 to 313 K. Denaturation curves were analyzed at each temperature with the linear extrapolation method (Eq. (1)) to examine the effect of temperature on the *m*-value. To assess the reproducibility of trends in the data and fitted parameters, we measured two independent sets of urea denaturation curves over the above temperature range. For both data sets, the *m*-values for the Notch ankyrin domain show a clear temperature dependence, which appears to be non-linear (Fig. 3). At low temperature, the *m*-values appear to be at or near a maximum, whereas at higher temperatures, the *m*-values decrease with temperature. This temperature dependence resembles that seen in simulation 2, where a negative $\Delta C_{p,b}$ produces a maximum in the *m*′-value, with downward concavity. Consistent with this observation, fitting Eq. (9) independently to the two sets of *m*-values (solid and dashed lines, Fig. 3) gives $\Delta C_{p,b}$ values of -0.16 and -0.30 kJ mol^{-1} K^{-1}. Although there are clear deviations of the data from the fitted model, and although the fitted $\Delta C_{p,b}$ values differ by nearly twofold, the match between the overall shape of the two sets of *m*-values suggests that the interaction of urea with this protein is accompanied by a decrease in heat capacity. Based on the results of simulation 2 above, this suggests a decrease in $\Delta C_{p,u}$ with increasing urea concentration.

To examine the effects of increasing denaturant on the shape of the stability curve and value of $\Delta C_{p,u}$ for the Notch ankyrin domain, extrapolated ΔG_u^0 values were extrapolated to different urea concentrations and were plotted as a function of temperature (Fig. 4A). ΔG_u^0 values at common urea concentrations were then fitted using Eq. (4) (solid lines, Fig. 4A), providing an estimate of $\Delta C_{p,u}$ at each urea concentration (Fig. 4B). For

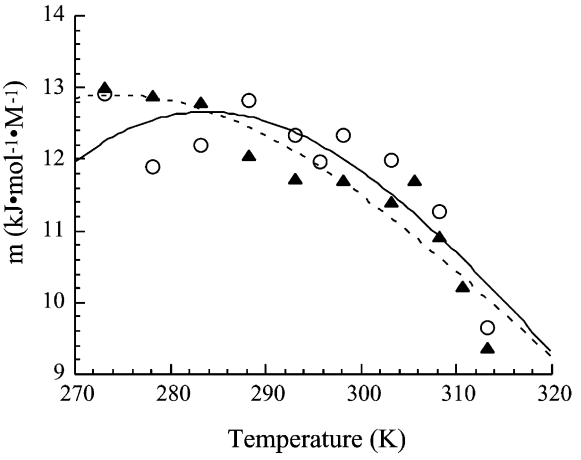

Fig. 3. The temperature dependence of the *m*-value for unfolding of the Drosophila Notch ankyrin domain. *m*-values were estimated from the fitting Eq. (1) to urea unfolding transitions at different temperatures. The two different symbols indicate two different independent data sets (see text). The two solid lines result from fitting Eq. (9) to each of the two data sets independently, adjusting $\Delta C_{p,b}$, ΔH_b^0, and Δn in the fitting procedure. ΔS_{bind} was fixed at the value in Table 1. Protein stability parameters (i.e. those in $f(T)_{H_2O}$) were taken from parameters resulting from fitting Eq. (4) to $\Delta G_{H_2O}^0$ as a function of temperature in the absence of urea (top curve, Fig. 4A). The fitted values of $\Delta C_{p,b}$ are -0.16 kJ mol^{-1} K^{-1} (triangles, dashed line) and -0.30 kJ mol^{-1} K^{-1} (circles, solid line).

ΔG_u^0 values extrapolated to higher urea concentrations, the stability curves connecting them display decreased curvature (Fig. 4A), consistent with a decrease in $\Delta C_{p,u}$ with increasing urea concentration. Indeed, fitted $\Delta C_{p,u}$ values decrease substantially with increasing urea concentrations (Fig. 4B). Although there seems to be a very strong linear correlation between $\Delta C_{p,u}$ and urea concentration, this is likely a result of the use of a linear model to project the stability curves to different denaturant concentrations, rather than evidence supporting linear extrapolation of $\Delta C_{p,u}$. The result of linear extrapolation of ΔG_u^0 is to stretch (or compress) stability curves, which should change curvature by changing scale. Because the scale of the stability curve is forced to change linearly with denaturant, its curvature (and thus $\Delta C_{p,u}$) should also be forced to vary linearly with denaturant.

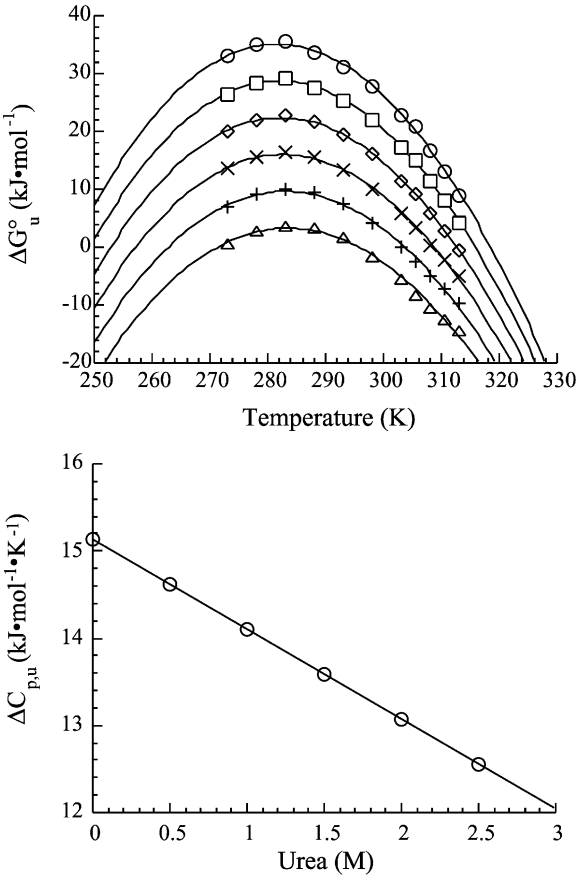

Fig. 4. Protein stability curves for the Drosophila Notch ankyrin repeats extrapolated to different urea concentrations (A), and apparent denaturant dependence of ΔC_p (B). In (A), urea concentrations are (○), 0 M; (□), 0.5 M; (◇), 1.0 M; (×), 1.5 M; (+), 2.0 M; (Δ), 2.5 M.

4. Discussion

4.1. Effects of denaturant on protein stability curves and heat capacities of unfolding

Extrapolation of solvent denaturation data to water at different temperatures is becoming a standard method to obtain protein stability curves and to compare the folding energetics of different proteins [11,12,20,21,38,39]. This method has been used to dissect the origins of thermostability in proteins from bacteria that thrive at high temperature, where changes in heat capacity of unfolding appears to be critical for extending stability to high temperatures [11,12]. Thus, understanding how $\Delta C_{p,u}$ depends on denaturant is important not only for understanding the physical chemistry of solvent–protein interactions, it is important for understanding the basis of experimental assessments of adaptation in thermophiles.

Since the large heat capacity increments that accompany protein unfolding are often attributed to hydration of nonpolar groups upon unfolding [5–9], it might be expected that the effect of denaturants on $\Delta C_{p,u}$ could be rationalized by (if not anticipated from) the effect of denaturants on energetics related to hydrophobic interactions. To the extent that denaturants increase the solubility of nonpolar side chains [52,53] and thus weaken the hydrophobic effect, $\Delta C_{p,u}$ may be expected to decrease with increasing denaturant concentration. However, to the extent that denaturants interact favorably with polar groups [54], $\Delta C_{p,u}$ may be expected to increase with denaturant concentration as a result of the increased structure resulting from the interaction between denaturants and polar backbone and side-chain groups. The simple binding model we have used in our simulations demonstrates this clearly. When the denaturant–protein interaction is treated as exothermic, as would be expected if favorable interactions are made between denaturant and polar groups on the protein [44], the apparent $\Delta C_{p,u}$ increases with increasing denaturant even in the absence of a binding heat capacity increment (simulation 1). Since the interaction of both urea and guanidine with proteins has been shown to be exothermic [44,49], there should be some tendency for $\Delta C_{p,u}$ to increase with both denaturants. To get a decrease in $\Delta C_{p,u}$ with increasing denaturant concentrations, our simulations require a negative binding heat capacity increment to offset the contribution of the exothermic binding. The observation that in simulation 1, $\Delta C_{p,u}$ changes in the absence of a heat capacity increment of binding highlights the fact that $\Delta C_{p,u}$ can change as a result of equilibrium fluctuations [6,50], in this case resulting from denaturant–protein interactions. Although this mechanism of perturbing $\Delta C_{p,u}$ from its value in the absence of denaturant differs from mechanisms that are intrinsic to the protein itself (such as excitation of low frequency vibrations), it provides

a real contribution to the heat capacity in the mixed solvent system, and is directly relevant to experiments combining temperature and denaturant variations to protein stability.

For the ankyrin repeat domain of the Notch receptor, stability curves change their shape as urea is added. Rather than simply shifting up or down, they decrease their curvature as urea concentration is increased (Fig. 4A). This has two consequences. First, the decrease in curvature results in a decrease in $\Delta C_{p,u}$ as urea concentration is increased. This decrease in $\Delta C_{p,u}$ with urea is consistent with a negative heat capacity increment of interaction between urea and the Notch ankyrin domain. Second, the decrease in curvature in stability curves as urea is added produces changes in *m*-values as temperature changes.

4.2. Effects of temperature on m-values

As discussed above, changes in the shape of the stability curve in different denaturant concentrations are related to changes in *m*-value with temperature. Although *m*-value, as defined in the linear equation for solvent denaturant (Eq. (1)) is a phenomenological quantity, it represents the degree to which the denaturant favorably interacts with the denatured state compared to the native state in terms of free energy [34]. This free energy of interaction must be determined by enthalpy, entropy, or both. If the interaction entropy is non-zero, the free energy of interaction will change with temperature, and as a result, so will the *m*-value for solvent denaturation. Thus, it is quite reasonable to expect *m*-values to be temperature-dependent, though it is difficult to extend this analysis without a specific model in which the thermodynamic parameters of interaction can be expressed.

We have chosen to use a simple binding model to evaluate the denaturant dependence of stability curves, and to investigate how these curves depend on the thermodynamic parameters of denaturant–protein interaction. This also allows the temperature dependence of the *m*-value to be considered. However, there is an obvious difficulty in this comparison: the linear and binding equations are orthogonal functions of denaturant concentration, and cannot be simultaneously satisfied, except at one or two denaturant concentrations. Thus an approximation must be made that allows the denaturant dependence from the binding model to be compared with the *m*-value of Eq. (1).

Several approximations have been described that facilitate this comparison. First, it is widely recognized that the denaturant–protein interaction is weak enough that very little deviation from linearity is expected when the binding model is evaluated over the range of denaturant concentrations where protein stability can be measured [34]. The range over which the binding model appears linear in denaturant concentration is $0 \leqslant x \ll k^{-1}$ [30]. Estimates of k for urea range from 0.14 [27,30] (association constant on a molar activity scale) to approximately 0.061 [44], restricting the linear range to well below 7.7–17.7 M urea. In analyses restricted to low denaturant concentrations, both the linear and binding equations are roughly linear in denaturant concentration, and a comparison of slopes yields the approximate relationship

$$m \approx \Delta nkRT \tag{10}$$

The term of the right-hand side of Eq. (10) is simply the coefficient associated with the linear term in a Taylor's expansion of Eq. (2) approximately $[x] = 0$ M.

Another more accurate approximation that relates the *m*-value of the linear equation to predictions of the binding model, which was used by DeKoster and Robertson [24], is a two-step procedure in which the binding model (Eq. (2)) is used to calculate free energies of unfolding over a narrow range of denaturant centered on C_m, and then these data are fitted by a line whose slope is then taken as an approximate *m*-value. This method is useful for evaluating *m*-values if binding parameters and their temperature dependencies are either known or assumed, and provides a route to approximating *m*-values as a function of temperature. However, this method does not provide an explicit functional dependence of m on T, and thus, it cannot be used, for example, to fit binding parameters to experimentally determined *m*-values.

Instead, we have related experimentally determined *m*-values to the parameters of the binding model by differentiation of the linear and binding Eq. (1) and Eq. (2), respectively, with respect to

denaturant concentration (again, we substitute molarity for activity [2]). These two differentiations give

$$\left(\frac{\partial \Delta G_u^0}{\partial [x]}\right) = -m \quad \text{(linear model)}$$
$$\left(\frac{\partial \Delta G_u^0}{\partial [x]}\right) = -\Delta nRT\frac{k}{1+k[x]} \equiv -m' \text{(binding model)} \quad (11)$$

In the binding model, as denaturant concentrations approach k^{-1}, the sensitivity of ΔG_u^0 to denaturant begins to deviate from the value given by Eq. (10), although to good approximation, ΔG_u^0 is still *locally* approximately linear in denaturant concentration. Thus the method used here of comparing derivatives, rather than evaluation of the leading term in the Taylor's expansion of Eq. (2) about $[x]=0$ M, takes advantage of this local linearity without requiring ΔG_u^0 to be linear from 0 M denaturant to C_m. Since experimental *m*-values are determined locally around C_m, *m*-values can be considered to be a good experimental approximation to Eq. (9). Thus, in fitting experimental *m*-values with Eq. (9), our approximation boils down to assuming that a linear fit around the midpoint of the unfolding transition gives a reasonable estimate of the slope of ΔG_u^0 versus denaturant concentration, that is, we assume that $m \approx m'$ at C_m.

In our three simulations, we find that the value of the heat capacity increment of denaturant binding greatly influences the shape of the m'-value temperature profiles (Fig. 2). Depending on whether $\Delta C_{p,b}$ is positive (Δ's), negative (●'s), or zero (×'s), we find that m' displays a minimum, a maximum, or a monotonic decrease with temperature, respectively. Thus, provided that experimental *m*-values can be measured accurately over a broad range of temperatures, their temperature dependence should contain information about the sign of the heat capacity increment of denaturant interaction.

For the ankyrin repeats of Notch, we find that the *m*-value for urea decreases with temperature in a nonlinear way (Fig. 3), as expected from the effect of urea on the shape of the stability curve for this protein (Fig. 4). The *m*-values are relatively insensitive to temperature at low temperatures, but become increasingly temperature sensitive as temperature is increased. This temperature dependence is consistent with a negative heat capacity increment of binding. Indeed, fitting Eq. (9) to our measured *m*-values (making the assumption that $m \approx m'$) yields negative $\Delta C_{p,b}$ values. As Δn cannot be determined accurately (but rather $\Delta n\, k$, [30]), and since the fitted values of $\Delta C_{p,b}$ differ by nearly twofold in our two data sets, this value should not be taken literally. However, the sign of this fitted parameter, which should not be dependent on the indeterminacy of Δn and k, appears to be robust.

4.3. Heat capacities of model compounds in water and urea solutions

If heat capacity increments of protein unfolding decrease in urea solutions, consistent with a negative heat capacity of binding of urea to protein, a heat capacity decrease may be expected to accompany the transfer of model compounds from water to urea solutions. In a study of the heat capacities of nonpolar amino acids and glycine in water and in 6 M urea, Kresheck and Benjamin [55] found significant decreases in C_p upon transfer of nonpolar groups from water to 6 M urea. In a study of transfer of *t*-butanol and tetrabutyl ammonium bromide from water to urea solutions, de Visser et al. found a decrease in heat capacity of transfer with increasing urea concentrations [56], but a slight increase in heat capacity for transfer of sodium chloride. In studies of transfer of both polar and nonpolar amino acids from water to 7 M urea, Enea et al. found a heat capacity decrease upon transfer of nonpolar groups, a heat capacity increase upon transfer of polar groups, and no change in heat capacity upon transfer of the backbone [57]. However, in a study of transfer of polyglycine from water to urea solutions, Hakin et al. found an increase in heat capacity with increasing urea concentrations [58].

Taken as a whole, this collection of studies suggests that nonpolar groups have a negative ΔC_p for transfer from water to urea, ionic groups have a positive ΔC_p for transfer, and the backbone has a ΔC_p of transfer that ranges from small [57]

to positive [58]. This picture is consistent with that from high-precision studies of the heats of transfer of cyclic dipeptides from water to urea solutions [48], where it was found that transfer of nonpolar groups to urea is enthalpically disfavored, but transfer of polar groups is enthalpically favored. Interpretation of these results as disruption of solvent structure around nonpolar groups, and formation of structure around polar groups suggests a lower heat capacity for nonpolar groups in urea solutions, and a higher heat capacity for polar groups.

Since most nonpolar groups increase their solvent accessibility on protein unfolding, the contribution of these groups to the heat capacity of unfolding should decrease as urea concentration increases. Buried ionic groups would offset this effect, however since most ionic groups are exposed in both the native and denatured states, these groups should contribute little to the heat capacity of unfolding. Thus, the model compound studies suggest that $\Delta C_{p,u}$ should decrease with increasing urea concentrations, as we see for the ankyrin domain of Drosophila Notch.

4.4. Comparison to denaturant effects in other protein folding reactions

Several studies have examined the effects of denaturant on ΔC_p, and likewise, the effects of temperature on the sensitivity of protein stability to denaturants. Kresheck and Benjamin found that the heat capacity of folded ovalbumin in water is the same, within error, as that of unfolded ovalbumin in 6 M urea [55]. As pointed out by the authors, unfolded ovalbumin would be expected to have a significantly larger heat capacity than that for folded ovalbumin in water. Thus, the similarity of the heat capacity of native ovalbumin in water to denatured ovalbumin in 6 M urea suggest that the denatured form decreases its heat capacity as denaturant is added.

In a study of the guanidine dependence of the denaturation enthalpy and heat capacity of unfolding of lysozyme, Pfeil and Privalov found that calorimetrically determined $\Delta C_{p,u}$ values are relatively insensitive to guanidine [49]. In a subsequent calorimetric study, Makhatadze and Privalov found that $\Delta C_{p,u}$ values for unfolding of RNase A and lysozyme increase in increasing concentrations of guanidine, but show little variation with urea concentration [44].

In a study of the guanidine dependence of the heat capacity of unfolding of Barstar, Agashe and Udgaonkar [20] used spectroscopic methods to determine $\Delta C_{p,u}$ from thermally induced heat- and cold-denaturations at different guanidine concentrations. $\Delta C_{p,u}$ values extracted from individual thermal denaturations did not vary significantly with guanidine. Likewise, *m*-values determined from individual guanidine unfolding transitions did not vary significantly with temperature [20]. However, by analyzing thermal unfolding at different guanidine concentrations using an equation analogous to Eq. (4) above, and then plotting ΔG_u^0 at various temperatures as a function of guanidine, slopes were found (analogous to *m*-values[4]) that show a subtle variation with temperature. This variation is as in simulation 3 of the present analysis (Fig. 3), consistent with a small increase in $\Delta C_{p,u}$ upon addition of guanidine. In a recent study of the temperature dependence of guanidine *m*-values of thermophilic and mesophilic RNases H, Hollien and Marqusee found an increase in *m*-value with temperature for the mesophilic enzyme, however, the *m*-value of the thermophilic enzyme showed no discernable temperature dependence [11].

In a study of the combined effects of urea and temperature on the stability of HPr, a small bacterial protein, Nicholson and Scholtz found $\Delta C_{p,u}$ to decrease with increasing urea concentrations [21]. Like the analysis of Agashe and Udgaonkar, the temperature dependence of the *m*-value could not be seen simply by analyzing individual solvent denaturation curves at different temperatures, but required global analysis of the data using a treatment in which $\Delta C_{p,u}$ was treated as linear in denaturant concentration. In contrast to what was seen for HPr, Johnson and Fersht found $\Delta C_{p,u}$ to increase with increasing concentrations of urea [23]. In an analysis of individual urea-induced

[4] Note, however, that in Ref. [20], the *m*-values presented differ in sign from those considered here.

Table 2
Literature values for the temperature dependence of *m*-values, and for the denaturant dependence of $\Delta C_{p,u}$

Protein	Denaturant	Method[a]	$\left(\frac{\partial \Delta C_{p,u}}{\partial [x]}\right)_T$	$\left(\frac{\partial m}{\partial T}\right)_x$	Reference
Ovalbumin	Urea	C	<0		[55]
Rnase A	Urea	C, T, *x*	≈ 0		[44]
Lysozyme	Urea	C, T, *x*	≈ 0		[44]
Ovomucoid 3rd domain	Urea		≈ 0	<0	[60]
Barnase	Urea	C, T	>0		[23]
HPr	Urea	S, T, *x*	<0	≈ 0	[21]
Notch ankyrin repeats	Urea	S, *x*	<0	Concave down	This study
Rnase T1	Urea	S, *x*		≈ 0	[59]
Lysozyme	Guanidine	C, T	≈ 0		[49]
T4 lysozyme	Guanidine	S, *x*		≈ 0	[19]
Rnase A	Guanidine	C, T, *x*	>0		[44]
Lysozyme	Guanidine	C, T, *x*	>0		[44]
Rnase A	Guanidine	S, *x*		<0	[24]
Ovomucoid 3rd domain	Guanidine		≈ 0	<0	[60]
Barstar	Guanidine	S, T, *x*	>0	Concave up	[20]
E coli Rnase H	Guanidine	S, *x*		>0	[11]
T. Thermophilus Rnase H	Guanidine	S, *x*		≈ 0	[11]

Empty table cells indicate that the parameter was not determined in the study; ≈ 0 indicates that sensitivity of the parameter to the perturbant was examined, but did not vary within error.

[a] C, calorimetry; S, spectroscopy; T, thermal denaturation in fixed denaturant concentrations; *x*, isothermal solvent denaturation at fixed temperatures.

unfolding curves of RNase T1, Hu et al. found *m*-values from to be insensitive to temperature [59].

In a study of the effects of both urea and guanidine on the thermal unfolding of RNase A, DeKoster and Robertson found a modest, approximately linear decrease in the *m*-value for guanidine with increasing temperatures from analysis of thermal unfolding at different fixed guanidine concentrations, however, the *m*-value for urea showed no discernable temperature dependence [24]. Swint and Robertson observed a small decrease in both the guanidine and urea *m*-values, with increasing temperature, for denaturation of turkey ovomucoid 3rd domain ([60], see Fig. 4 therein), although $\Delta C_{p,u}$ appeared to be insensitive to these denaturants, within error.

The studies described above show a variety of different temperature dependencies of *m*-values, and a variety of denaturant dependencies of $\Delta C_{p,u}$. These results are summarized in Table 2. Although there are no inviolable rules, there are some trends. Most notably, although there are cases for both urea and guanidine where $\Delta C_{p,u}$ did not vary with denaturant concentration, (within the limits of detection), in cases where significant variation was seen, $\Delta C_{p,u}$ always increased with guanidine concentration, whereas it always decreased with urea concentration with the exception of Barnase [23]. In addition, in all cases where urea *m*-values showed significant variation with temperature, *m*-values for urea decreased with temperature. In contrast, *m*-values for guanidine showed both increases and decreases with temperature. Thus, the temperature dependence of the urea *m*-value and the urea dependence of $\Delta C_{p,u}$ seen here for the Notch ankyrin domain are consistent with effects of urea on other several other proteins.

In many of the studies listed in Table 2, temperature dependencies of *m*-values were not discernable, nor were denaturant dependencies of $\Delta C_{p,u}$. In this regard, it should be kept in mind that these parameters are quite difficult to measure, because these dependencies are analogous to second-order cross-derivatives of protein stability. Determination of *m*-value involves evaluating the slope of a denaturation curve, and determination

of whether an *m*-value is sensitive to temperature involves evaluation of a slope of this slope, at the least. Likewise, evaluation of $\Delta C_{p,u}$ from stability data requires determination of the second derivative of stability with respect to temperature, and determination of the denaturant sensitivity of $\Delta C_{p,u}$ involves detection of the slope of this second derivative versus denaturant. The apparent insensitivities of *m*-values and $\Delta C_{p,u}$ of some proteins to temperature and denaturant, respectively, should be interpreted with these experimental limitations in mind.

4.5. Summary

For the Drosophila Notch ankyrin domain, the *m*-value decreases with temperature with a downward concavity, and as a result, $\Delta C_{p,u}$ decreases with denaturant concentration. Simple simulations using a binding model indicate that this behavior is consistent with a negative heat capacity increment in denaturant–protein interaction. This negative heat capacity increment is in turn consistent with model compound studies showing urea to decrease the heat capacities of hydrated nonpolar groups, and with several studies of the effect of urea and temperature on stability.

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