Protein Stability as a Function of Denaturant Concentration: The Thermal Stability of Barnase in the Presence of Urea

Christopher M. Johnson and Alan R. Fersht*

Cambridge Centre for Protein Engineering, Hills Road, Cambridge CB2 2QH, England, U.K.

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ABSTRACT: The conventional procedure for analyzing urea denaturation curves assumes that the free energy of unfolding ($\Delta G_{U\text{-}F}$) is linearly related to [urea] that is, $\Delta G_{U\text{-}F} = \Delta G^{H_2O}_{U\text{-}F} - m$[urea], where m is a constant, specific for each protein, and $\Delta G^{H_2O}_{U\text{-}F}$ is the free energy of unfolding in water. This relationship can be measured directly, however, over only a small concentration range of approximately ±0.8 M urea around the midpoint of the unfolding transition. A nagging discrepancy (1.6 kcal mol^{-1}) between $\Delta G^{H_2O}_{U\text{-}F}$ at 298 K of barnase extrapolated from such an equation and the equivalent value obtained from thermal unfolding measurements has stimulated a re-evaluation of the equation. Differential scanning calorimetric measurements have been made of the thermal unfolding of barnase in the presence of concentrations of urea between 0 and 4.5 M, the midpoint of the unfolding transition at 298 K, to test the denaturation equation over a wide range of [urea]. Values for $\Delta G_{U\text{-}F}$ at 298 K ($\Delta G^{298}_{U\text{-}F}$) for each concentration of urea were extrapolated from the calorimetrically measured enthalpies and the denaturational heat capacity change (ΔC_p^d) measured for that concentration of urea. A plot of $\Delta G^{298}_{U\text{-}F}$ against [urea] deviates systematically from linearity and fits better the equation: $\Delta G^{298}_{U\text{-}F} = 10.5 \pm 0.08 - ((2.65 \pm 0.05) \times$ [urea]) + $((0.08 \pm 0.01) \times$ [urea]^2) kcal mol^{-1}. The curvature in the plot leads to apparent values of m that increase when measurements are made at lower concentrations of urea. This could account for increases in m at low values of pH or in destabilized mutants since the protein denatures at lower concentrations of urea. It has been shown previously that small curvature in the free energy of unfolding versus [urea] leads to negligible errors in measurements of $\Delta\Delta G_{U\text{-}F}$, the change in free energy of unfolding on mutation, providing that the curvature is similar for all mutants. The calorimetrically measured enthalpies of unfolding are decreased in the presence of urea while ΔC_p^d is increased. Both of these observations are consistent with an overall exothermic interaction between urea and protein with a net increase on unfolding.

A rational approach to protein engineering and design requires a fundamental thermodynamic description of protein systems at a molecular level. One useful technique in this area is the comparison of the kinetic and equilibrium free energies for the reversible unfolding of proteins to analyze the structures of transition and intermediate states (Fersht et al., 1992). The use of chemical denaturants such as urea and guanidine hydrochloride to alter the free energies of unfolding ($\Delta G_{U\text{-}F}$) has become a central element of these studies. Despite the widespread use of denaturants to provide quantitative data for $\Delta G_{U\text{-}F}$, the mode of action of these agents is not understood. It is not clear if they act by direct binding or interaction with the protein or indirectly through effects on the solvent (Schellman, 1987). There is also some conjecture as to whether these denaturants have a linear or non-linear effect on $\Delta G_{U\text{-}F}$, and several models exist for the extrapolation of measured stabilities to the value in water (Pace, 1986). A general problem in analyzing models is that the equilibrium constant for unfolding in the presence of denaturant can be measured only in the range of ca 0.1−10, which is equivalent to a stability of 1.4 to −1.4 kcal mol^{-1} at 298 K. Since most proteins have a free energy of unfolding of 5−15 kcal mol^{-1}, the behavior in the long extrapolation of $\Delta G_{U\text{-}F}$ to the absence of denaturant cannot be examined without the inclusion of a second destabilizing factor. The use of a linear extrapolation, in which $\Delta G_{U\text{-}F}$ is related to the denaturant concentration by a constant term, the m-value ($\partial\Delta G_{U\text{-}F}/\partial$[urea]), is the simplest method. Alternative models are based on the free energies of transfer for peptides and amino acid from water to denaturant or assume direct binding of the denaturant to these groups (Tanford, 1970). This results in a slight curvature in the free energy profile such that the stability in the absence of denaturant, $\Delta G^{H_2O}_{U\text{-}F}$, is larger than that indicated from linear extrapolation. A linear dependence of $\Delta G_{U\text{-}F}$ on [urea] has some support from theoretical models (Alonso & Dill, 1991; Schellman, 1978; 1987), but experimental evidence, such as the value of $\Delta G^{H_2O}_{U\text{-}F}$ being independent of chemical or physical (pH, heat, etc.) denaturant used, is patchy and generally compares measurements made under different conditions [Pace (1986) and references therein]. The models based on denaturant binding are supported by some reports of curvature in plots of $\Delta G_{U\text{-}F}$ against [urea] (Shortle et al., 1989) and by direct calorimetric measurement of the interaction of denaturants with proteins which can be described assuming denaturant binding at similar independent sites (Makhatadze & Privalov, 1992). In practice, the simplicity of a linear extrapolation model has resulted in a general

* To whom correspondence should be addressed.

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acceptance of this method for the analysis of equilibrium unfolding data (Pace, 1986).

The dependence of equilibrium unfolding energies on denaturant concentration has been examined using the additional destabilizing effects of mutation (Matouschek et al., 1994; Shortle et al., 1988; 1989), temperature (Hu et al., 1992; Santoro & Bolen, 1992), and pH (Pace et al., 1992, 1990). We have examined the effects of mutations as part of a detailed characterization of the folding pathway of the ribonuclease barnase from *Bacillus amyloliquefaciens*. The urea-mediated unfolding of the wild-type protein and a wide range of mutants has been studied extensively using equilibrium and kinetic techniques (Matouschek et al., 1992; Serrano et al., 1992). Kinetic measurements of the rate constant of unfolding, reflecting the free energy between the folded and main transition states, have revealed a small dependence on urea concentration (Matouschek et al., 1994). The *m*-values for equilibrium unfolding measured in over 160 barnase mutants were also found to vary with [urea]. Since the unfolding of each mutant is measured over a different range of [urea], this suggests that the overall unfolding free energy might not be a linear function of denaturant concentration. However, this analysis was limited to the range of mutant stabilities available and by the low accuracy of *m*-values determined at lower urea concentrations. The urea-induced equilibrium unfolding of barnase, as well as ribonuclease A and T1, has also been studied as a function of pH (Pace et al., 1992, 1990). In each case, a marked variation in the *m*-value was observed at different pH values. The changes in *m*-value were interpreted in terms of increasing accessibility of the unfolded state to solvent with decreasing pH. However, the unfolding at each pH was measured over a different range of urea concentrations, and therefore, the *m*-value variation could also result from a nonlinearity of unfolding free energy with denaturant concentration.

The use of temperature to destabilize proteins to urea unfolding has some advantages. It is quite easy to measure thermal unfolding in the presence of a fixed concentration of urea and the corresponding equilibrium urea denaturation at various temperatures. In addition, calorimetric measurements have quantified the heat effects of denaturant interaction with proteins, and these are large (Makhatadze & Privalov, 1992). However, rather surprisingly, the combination of temperature and denaturants in stability studies has not been employed widely (Hu et al., 1992; Santoro & Bolen, 1992).

The thermal unfolding of barnase has a large enthalpy change and is highly reversible which has allowed a detailed analysis based on equilibrium thermodynamics (Griko et al., 1994; Martinez et al., 1994). However, the stability of barnase at 298 K determined from thermal unfolding measurements is larger by some 1.6 kcal mol^{-1} than the value obtained from urea equilibrium unfolding (Clarke & Fersht, 1993; Matouschek et al., 1994). Prompted by this apparent discrepancy and our initial observations of the curvature in the unfolding kinetics and the *m*-value variation in mutants, we have examined the thermal unfolding of barnase in the presence of urea at concentrations up to 4.5 M urea (the denaturant midpoint for isothermal measurement at 298 K). These studies show that the *m*-value for urea equilibrium unfolding is dependent on denaturant concentration and the consequential variation in the unfolding free energy profile is sufficient to account for the apparent difference in stabilities determined from thermal and urea unfolding measurements.

MATERIALS AND METHODS

Water was purified to 18 MΩ resistance using an Elgastat UHP system. Wild-type barnase was purified as previously described (Kellis et al., 1989), dialyzed against water, and stored frozen under liquid nitrogen. All buffers were prepared using Analar grade reagents and stored at 4 °C. Urea solutions were prepared from ultrapure material (Bethesda Research Laboratories) and stored frozen at −20 °C in small aliquots. Buffers were used at 50 mM based on formic acid/sodium formate for pH 2.85−4.25, acetic acid/sodium acetate for pH 4.25−5.5, and MES (2-(*N*-morpholino)ethanesulfonic acid) for pH 6.3. Ionic strength (*I*) was maintained at 50 mM using KCl.

Samples for thermal unfolding experiments were prepared by weighing appropriate aliquots from concentrated stocks of buffer, protein, and 8 M urea assuming a specific gravity of 1.118 g mL^{-1} for the denaturant. These samples were equilibrated for approximately 1 h at 4 °C before use. Some samples without urea were prepared by dialysis against several changes of buffer at 4 °C. In all cases, these yielded identical results to samples prepared directly by weighing. Preparation of samples containing urea from concentrated stocks was considered more accurate than dialysis as it avoided the problems of concentration changes resulting from solvent evaporation and the potential formation of cyanates that are known to form from the decomposition of urea (Stark, 1965).

Differential scanning calorimetry (DSC) measurements were performed using a Microcal MC-2D instrument using a nominal scan rate of 60 K h^{-1}, unless otherwise indicated, using a concentration of barnase of approximately 0.5 mg mL^{-1}. The exact concentration was determined from absorbance measurements using an extinction coefficient of 27 364 cm^{-1} M^{-1} L^{-1}. Samples for DSC analysis were degassed under vacuum for 2−3 min with gentle stirring. During scans, the sample and reference were held under 2 atm N_2 pressure to suppress further bubble formation. Before rescanning, the samples were cooled *in situ* for 50 min. DSC thermograms were analyzed as previously described (Jackson et al., 1993; Matouschek et al., 1994) according to a single non-two-state transition model in which the T_m, calorimetric enthalpy (ΔH_{cal}), and van't Hoff enthalpy (ΔH_{vh}), are fitted independently. Each DSC scan was fitted separately at least five times using different buffer base lines recorded before and after the barnase measurement, and the values presented are the average of these. The spread of values observed in repeated fits of the same data was always less than ±3% of the average value. The sources and magnitudes of the errors in the T_m and the two enthalpy values determined from a series of eight DSC measurements made under the same conditions have been discussed (Matouschek et al., 1994). Errors in the determination of the T_m (mean = 324.5 K) were found to be negligible with a spread of ±0.1 K in values and a standard error of ±0.03 K. The observed spread in the values of ΔH_{cal} of ±4 kcal mol^{-1} (mean = 128.6 kcal mol^{-1} ±3%) and ΔH_{vh} of ±2 kcal mol^{-1} (mean = 128.4 kcal mol^{-1}, ±1.5%) represents the maximum estimate of

error for a single determination of the enthalpy. The standard errors for the mean values are again about 4 times lower than the spread. In the presence of urea or at lower pH, the stability of barnase is much reduced such that the percentage errors on the enthalpy values are probably increased as the pretransition base line becomes shorter and the smaller enthalpy of unfolding means that the thermal transitions are broader. Therefore, we consider that a fixed error of ±4 and ±2 kcal mol^{-1} on ΔH_{cal} and ΔH_{vh} values, respectively, is most appropriate to the data in this study and represents the largest estimate of error on these values.

Thermal unfolding of barnase appears to follow closely a two-state equilibrium between folded and unfolded states under these experimental conditions so that ΔH_{cal} and ΔH_{vh} values should be identical (see Results). We have opted to use ΔH_{vh} for all analysis since this enthalpy, being independent of measured protein concentration and DSC cell volume, has a smaller overall error than ΔH_{cal}. An analysis based on ΔH_{cal} yields qualitatively similar results, although the small changes detected would require more data to be statistically significant.

Thermal unfolding was monitored by circular dichroism (CD) at 230 nm using a Jasco J-720 spectropolarimeter interfaced to a Neslab water bath. Samples were scanned at a heating rate of 50 K h^{-1} in a 0.1 cm path length cell. The concentration of barnase was the same as that used in DSC. Thermal transitions were analyzed as described previously (Oliveberg et al., 1994) using a ΔC_p^d appropriate to the concentration of urea used (see Results). Calculations of T_m and ΔH_{vh} were found, however, to be essentially independent of the value of ΔC_p^d used between 1600 and 2600 cal mol^{-1} K^{-1}. The errors in T_m from analysis of CD transitions are comparable to those from DSC measurement. However, ΔH_{vh} values determined are subject to a considerable error which we estimate at about ±10% over the temperature range examined (this study; Oliveberg et al., 1994).

Fluorescence spectra were recorded using an Aminco Bowman series 2 spectrofluorimeter. Excitation was at 280 nm with a 4 nm band-pass. The temperature was monitored with a thermocouple located in the sample solution above the instrument light beam. The sample chamber was purged with nitrogen at about 10 L min^{-1} to prevent condensation on the optical surfaces at temperatures <0 °C.

RESULTS

Thermal Unfolding of Barnase in the Presence of Urea. A typical DSC unfolding endotherm and a CD unfolding transition for the thermal denaturation of wild-type barnase are shown in Figure 1. The thermal unfolding is completely reversible as measured by both techniques and under all the conditions of urea and pH used in this study. A ratio of ΔH_{vh} to ΔH_{cal} equal to 1 (1.00 ± 0.04, $n = 45$) was observed by DSC, as has been previously found in the absence of urea (Griko et al., 1994; Martinez et al., 1994; Matouschek et al., 1994), and is characteristic of a two-state equilibrium with only folded and unfolded species significantly populated during the time scale of measurement (Privalov, 1986). The complete reversibility of unfolding and the constancy of the T_m and enthalpies on rescanning also indicate that the formation of cyanates from the decomposition of urea at the elevated temperatures required for unfolding is either insignificant or does not have any effect on the stability of barnase.

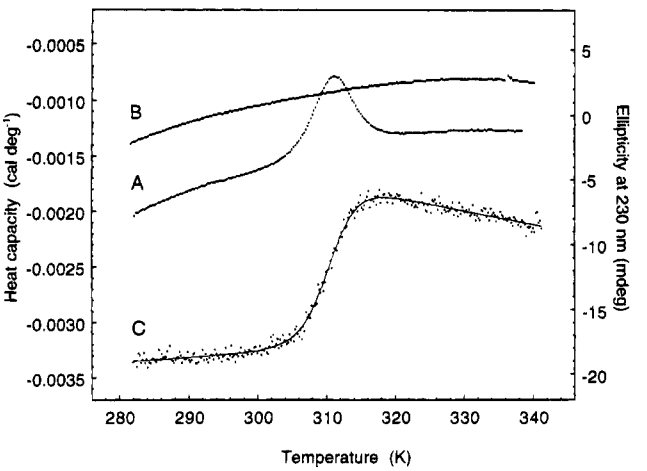

FIGURE 1: Thermal unfolding of barnase in 50 mM MES buffer, pH 6.3, and in the presence of 3 M urea. The heat capacity of barnase (trace A) was measured using DSC with a base line (trace B) indicated for comparison. Analysis of the DSC data gives a T_m of 310.9 ± 0.01 K, a ΔH_{cal} of 98.4 ± 0.2 kcal mol^{-1}, and a ΔH_{vh} of 98.1 ± 0.3 kcal mol^{-1}. The ellipticity at 230 nm (trace C) was measured as described in Materials and Methods, and the fit to this transition (solid line) gives a T_m of 310.5 ± 0.1 K and a ΔH_{vh} of 93.4 ± 2.7 kcal mol^{-1}. Standard errors quoted are from a single fit to the experimental data.

Thermal Unfolding in the Presence of Urea at pH 6.3. The T_m and ΔH_{vh} for the thermal unfolding of wild-type barnase in 50 mM MES buffer, pH 6.3, determined from DSC and CD measurements change in parallel with [urea] (Figure 2A). Consequently, the ΔH_{vh} of unfolding varies linearly with T_m (Figure 2B). The variation of ΔH_{vh} with temperature in the absence of urea as the pH changes between 2.85 and 4.75 is also plotted in Figure 2B. These data were recorded in acetate and formate buffer systems where changes in protonation of the protein during unfolding do not distort the enthalpy of unfolding since the enthalpies for ionization of these buffers (ΔH_i) and the analogous groups titrating in the protein are very small (Cooper & Johnson, 1994). In contrast, MES has a significant ΔH_i (3.5 kcal mol^{-1} at 298 K). The higher values of ΔH_{vh} found in this buffer in the absence of urea than those at similar T_m in acetate buffer ($\Delta\Delta H_{vh}^{MES\text{-}acetate}$ approximately 5 kcal mol^{-1}) suggest that there may be changes in protonation of the protein during unfolding. Interestingly, the stability of barnase monitored by urea equilibrium is at a maximum and fairly constant over the pH range 5.5−6.5, which is not consistent with such a large protonation effect (Pace et al., 1992). One explanation for this difference could be that the ΔH_i of MES is temperature dependent and larger at the T_m of unfolding.

It is evident from the data in Figure 2B that the enthalpy of unfolding in the presence of urea is less than that observed in aqueous buffer alone at any temperature of comparison. This effect is seen most clearly when the data are offset to intercept in the absence of urea (i.e., removing the $\Delta\Delta H_{vh}^{MES\text{-}acetate}$). The decrease in ΔH_{vh} is greatest at high urea concentrations and/or low T_m of unfolding. This effect is consistent with an exothermic interaction between urea and protein (Makhatadze & Privalov, 1992), such that at higher urea concentrations and lower T_m the number of urea

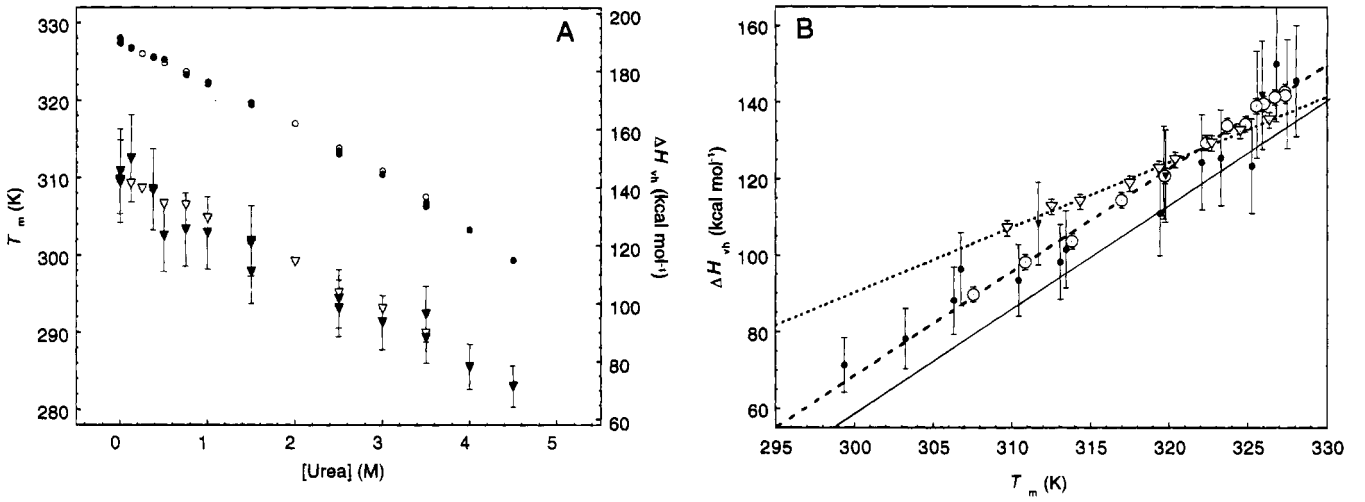

FIGURE 2: Dependence of T_m and ΔH_{vh} on urea concentration for the thermal unfolding of wild-type barnase. Error bars are included for ΔH_{vh}, but for T_m the errors are smaller than the symbols used. (A) Dependence of T_m (circles) and ΔH_{vh} (triangles) on urea concentration determined by DSC (open symbols) and CD (filled symbols) measurement in 50 mM MES buffer, pH 6.3. (B) Temperature dependence of ΔH_{vh} in the presence (circles) and absence (triangles) of urea. Measurements in the presence of 0−4.5 M urea were made in 50 mM MES buffer, pH 6.3, using DSC (open circles) and CD (filled circles). The dashed line represents the linear regression of these data using weighted errors and has a slope of 2.71 ± 0.08 kcal mol^{-1} K^{-1}. The solid line has the same slope but is offset to intersect with data recorded in formate and acetate buffers to indicate the probable $\Delta\Delta H_{vh}$ when buffer protonation effects are eliminated (see text). Measurements in the absence of urea were made in 50 mM formate and acetate buffer between pH 2.85 and 4.75 at I = 50 mM using DSC (open triangles) and CD (filled triangles). The dotted line represents the linear regression of these data using weighted errors and has a slope of 1.71 ± 0.13 kcal mol^{-1} K^{-1}.

molecules interacting with the protein upon unfolding ($\Delta n = n_{unfolded} - n_{folded}$) is increasing

$$\Delta H_{app} = \Delta H_{prot} + (\Delta n \Delta H_{urea}) \quad (1)$$

where ΔH_{app} is the measured ΔH_{vh} or ΔH_{cal} in the presence of urea, ΔH_{prot} is the enthalpy of unfolding of the protein, and ΔH_{urea} is the average enthalpy of interaction with the protein for each urea molecule. It appears that the effects of urea and temperature on the exothermic $\Delta n \Delta H_{urea}$ term are linear, or combine to yield a linear function, over the range examined. This confirms direct calorimetric measurements which have shown that the heat effect of urea interaction with folded and unfolded proteins is essentially linear with each of these parameters (Makhatadze & Privalov, 1992).

The heat capacity change for protein unfolding (ΔC_p^d) is defined from the Kirchoff equation as

$$\Delta C_p^d = \delta \Delta H / \delta T_m \quad (2)$$

In the presence of urea, this will become an apparent term ($\Delta C_p^d{}_{app}$) relating the change in ΔH_{app} with T_m. Equation 2 has been used extensively to give a reliable measure of the ΔC_p^d for the unfolding of proteins (Privalov, 1979). A ΔC_p^d of 1.71 ± 0.13 kcal mol^{-1} K^{-1} is obtained for the unfolding of barnase in the absence of urea (Figure 2B), which is in good agreement with values for barnase determined by DSC (Griko et al., 1994; Martinez et al., 1994). The larger variation of ΔH_{app} with T_m in the presence of urea (2.71 kcal mol^{-1} K^{-1}; Figure 2B) is not $\Delta C_p^d{}_{app}$ for barnase since changes in this enthalpy contain contributions from the variation in both temperature and [urea] [see Pfeil and Privalov (1976) for a more detailed discussion].

Thermal Unfolding in the Presence of Fixed Concentrations of Urea. The variation of the ΔH_{app} of unfolding with T_m in the presence of fixed concentrations of urea is required to determine the value of $\Delta C_p^d{}_{app}$. In this case, only the variations with temperature of the ΔH_{prot} and $\Delta n \Delta H_{urea}$ terms in eq 1 are measured. The thermal unfolding of barnase was examined between pH 2.9 and 4.8 at fixed concentrations of 0, 1, 2, and 3 M urea (Figure 3). Clearly, there is a small but significant increase in the temperature dependence of ΔH_{vh} with increasing concentrations of urea such that $\Delta C_p^d{}_{app}$ increases with denaturant concentration as shown in Figure 4.

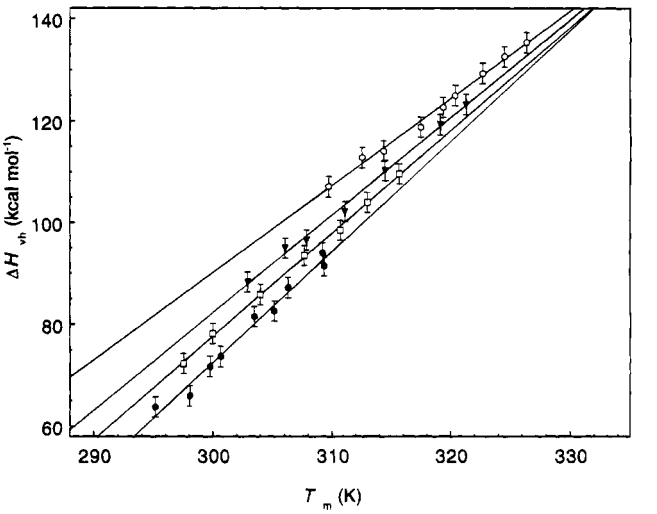

FIGURE 3: Temperature dependence of ΔH_{vh} determined by DSC for wild-type barnase in the presence of 0, 1, 2, and 3 M urea. DSC measurements were made in formate and acetate buffers between pH 2.85 and 4.75 at I = 50 mM. The solid lines represent the linear regression of ΔH_{vh} values measured in the presence of 0 M (open circles), 1 M (filled triangles), 2 M (open squares), and 3 M (filled circles) urea. These have slopes of 1.71 ± 0.13, 1.91 ± 0.12, 2.02 ± 0.12, and 2.17 ± 0.14 kcal mol^{-1} K^{-1}, respectively.

The values of ΔC_p^d or $\Delta C_p^d{}_{app}$ can also be measured directly from each DSC endotherm, but this value is determined with a much higher error because of uncertainties

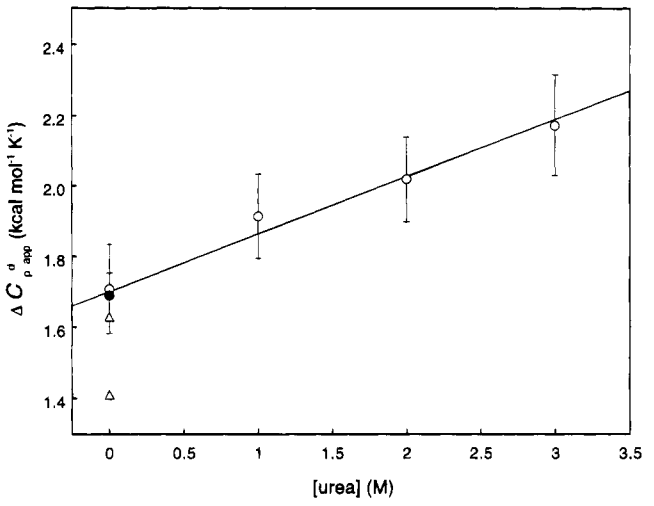

FIGURE 4: Dependence of $\Delta C_p^{d}{}_{app}$ for wild-type barnase on urea concentration. Values for $\Delta C_p^{d}{}_{app}$ (open circles) were determined from linear regression of the data in Figure 3. A value of ΔC_p^{d} determined in 50 mM acetate or formate buffer, pH 2.1−4.9, and in the presence of 100 mM KCl is included at 0 M urea (closed circle, unpublished work). Linear regression of these data weighted according to their error yields the relationship $\Delta C_p^{d}{}_{app} = 1.70 \pm 0.05 + ((0.16 \pm 0.04) \times [\text{urea}])$ kcal mol^{-1} K^{-1}. Values of ΔC_p^{d} for wild-type barnase determined elsewhere are included for comparison (open triangles) (Griko et al., 1994; Martinez et al., 1994).

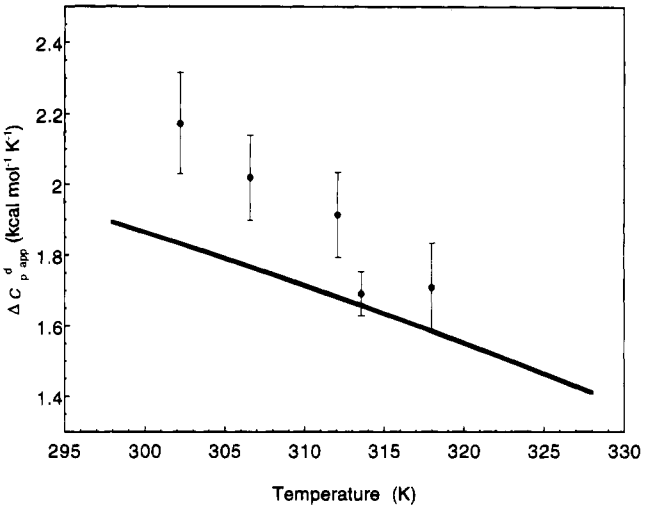

FIGURE 5: Temperature dependence of ΔC_p^{d} for barnase. The calculated temperature dependence of ΔC_p^{d} is indicated by the solid line (Martinez et al., 1994), and the values of $\Delta C_p^{d}{}_{app}$ or ΔC_p^{d} observed in the presence and absence of urea (filled circles) are plotted at the midtemperature of the ΔH_{vh} versus T_m plots from which they are derived.

in extrapolation of the heat capacity of the folded and unfolded states into the transition region. Further, the apparent temperature variation of the heat capacity of these states differs to such an extent that the ΔC_p^{d} should apparently become smaller at higher T_m and eventually invert sign. Such changes in ΔC_p^{d} are not observed when the T_m is higher, for example, because of an increase in pH. This suggests some uncertainty in the ΔC_p^{d} value observed directly from the endotherm. Individual DSC scans show that the directly observed ΔC_p^{d} is generally larger at higher concentrations of urea, in agreement with the pattern observed in Figure 4. However, a similar but smaller trend was also observed in the absence of urea when the T_m of barnase was decreased at low pH.

Effect of Urea and Temperature on ΔC_p^{d}. The large positive ΔC_p that is observed when proteins unfold has been attributed mainly to the exposure of nonpolar groups in the unfolded state as well as more minor contributions (~10−20% of the ΔC_p^{d}) resulting from increases in the configurational entropy of this state (Privalov & Makhatadze, 1990; Sturtevant, 1977). It has been noted that the ΔC_p^{d} of barnase is unusually large, which may indicate that it has an extensive core structure exposed to solvent upon unfolding (Martinez et al., 1994).

The increases in the ΔC_p^{d} of barnase in the presence of urea can be predicted from the observation that the interaction of this denaturant with proteins is exothermic (Makhatadze & Privalov, 1992). Since more urea will interact with proteins at higher concentrations of denaturant, then at any one temperature of comparison the exothermic term superimposed on the intrinsic endothermic ΔH_{prot} of unfolding will be larger (eq 1). At a fixed concentration of urea, the exothermic urea term will be variable since the interaction of denaturant will be reduced at the higher temperature of unfolding. Combining these changes as a function of temperature will mean that the ΔC_p^{d} term, $\Delta C_p^{d}{}_{app}$, will be increased (eqs 1 and 2). The increases in ΔC_p^{d} in the presence of urea and guanidine hydrochloride have been demonstrated directly for several proteins by DSC and isothermal calorimetric titration (Griko & Privalov, 1992; Makhatadze & Privalov, 1992; Pfeil & Privalov, 1976). Some studies have failed to show a clear dependence of ΔC_p^{d} on denaturant, but these have been based on an indirect determination of ΔC_p^{d} and only consider a narrow concentration range (Brandts & Hunt, 1967; Pace & Laurents, 1989). The magnitude of the change in ΔC_p^{d} expected in the presence of urea can be calculated from published data obtained from calorimetric titration (Makhatadze & Privalov, 1992) by considering both the difference in heat effects from titration of folded and unfolded protein with denaturant, ΔQ ($\Delta Q = \Delta Q_{unfolded} - \Delta Q_{folded}$), and the temperature dependence of this effect. Using these authors' data for ribonuclease A, we expect the increment in ΔC_p^{d} of unfolding to be about 0.06 kcal mol^{-1} K^{-1} per mol of urea. This value is less than the change of 0.16 kcal mol^{-1} K^{-1} M^{-1} observed in Figure 4.

It is becoming clear that ΔC_p^{d} may itself have some degree of temperature dependence, and it has been suggested that this follows a broad bell-shaped function with a maximal and roughly constant value around 40 °C (Privalov & Makhatadze, 1990). The observed value of ΔC_p^{d} may depend, therefore, on the temperature of measurement or, in the case of ΔC_p^{d} determined from eq 2, the temperature range of a series of ΔH_m measurements, since the linear fit to these data will be a tangent to the curved ΔC_p^{d} function centered around the midpoint. Preliminary unpublished data (C. Johnson and A. Fersht) indicate that the formula describing this temperature dependence for barnase, which has been calculated using the amino acid sequence and the observed heat capacity of the folded state (Martinez et al., 1994), may describe this variation quite well.

The predicted variation in ΔC_p^{d} with temperature is shown in Figure 5 with the ΔC_p^{d} values determined in the presence of urea plotted at their appropriate midtemperature of measurement. The observed ΔC_p^{d} values are still significantly larger than those predicted by the temperature dependence, indicating additional effects from the presence

of the urea. Indeed, the variation in ΔC_p^d per mol of urea from temperature effects is approximately 0.08 kcal mol^{-1} K^{-1} M^{-1} (since the temperature range over which measurements can be made is reduced by about 5 K/mol of urea) and that predicted from calorimetric measurement of urea interaction is approximately 0.06 kcal mol^{-1} K^{-1} M^{-1} as discussed above. Therefore, the combination of these corrections actually predicts a urea dependence for $\Delta C_p^d{}_{app}$ which is quite close to the observed value of 0.16 kcal mol^{-1} K^{-1} M^{-1} in Figure 4.

Extrapolation of Thermal Unfolding Data. The free energy of unfolding of barnase at 298 K can be calculated using standard thermodynamic relationships:

$$\Delta G_{U\text{-}F}^{298} = [\Delta H_m + \Delta C_p^d(298 - T_m)] - 298[\Delta S_m + \Delta C_p^d(\ln 298/T_m)] \quad (3)$$

where T_m is the mid-point of thermal unfolding and ΔH_m and ΔS_m are the observed enthalpy and entropy of unfolding at the T_m. ΔS_m is given by $\Delta H_m/T_m$ since at this temperature $\Delta G_{U\text{-}F} = 0$. The errors in extrapolating $\Delta G_{U\text{-}F}$ to other temperatures depend on the intrinsic errors in ΔH_m and ΔC_p^d as well as the length of the extrapolation. It has been shown previously that for a 25 K extrapolation from 323 K the error in $\Delta G_{U\text{-}F}$ is given by

$$\delta\Delta G = [1 - (T/T_m)]\delta\Delta H_m + [(T - T_m) - T\ln(T/T_m)]\delta\Delta C_p^d \quad (4)$$

$$\delta\Delta G \approx 0.08\delta\Delta H_m - \delta\Delta C_p^d \text{ kcal mol}^{-1} \quad (5)$$

(Matouschek et al., 1994). This may be scaled for smaller temperature extrapolations. In the presence of urea, both ΔH_m and ΔC_p^d are apparent terms with errors of ±2 kcal mol^{-1} and approximately ±10% of the measured value (Figure 4), respectively. Therefore, it is possible to calculate $\Delta G_{U\text{-}F}^{298}$ for barnase at any concentration of urea and pH with a realistic estimate of the error in this value.

If the ΔC_p^d term is also temperature dependent then this variation will be included initially in the observed $\Delta C_p^d{}_{app}$ values in the presence of urea since each value is determined over a different temperature range. However, extrapolation to 298 K will not include any further temperature dependence in the ΔC_p^d term. The effect of this additional variable will be greatest for the longest extrapolations in temperature, i.e., the data recorded in the absence of urea. The effect of using the formula for a temperature dependent ΔC_p^d on the extrapolation of this data is to increase the $\Delta G_{U\text{-}F}^{298}$ by about 0.15 kcal mol^{-1} or 1.4% of the value in the absence of urea (see Discussion). For shorter extrapolations, such as from data recorded in 3 M urea, the effect is minimal. Because of these small errors and in the absence of published experimental evidence for a temperature dependent ΔC_p^d in barnase, we have not included this additional variable in our extrapolations.

Cold Unfolding of Barnase. The phenomenon of cold unfolding of proteins is well documented although the number of systems in which it can be directly observed is limited (Privalov, 1990). The temperature at which proteins are denatured by cooling is determined by the relationship:

$$T_c = T_m^2/[2(\Delta H_m/\Delta C_p^d) + T_m] \quad (6)$$

where T_c is the cold unfolding midpoint and ΔH_m is the enthalpy of unfolding at the T_m (ΔH_{cal} or ΔH_{vh}) (Privalov et al., 1986). Consequently, proteins with a small ΔH_m and/or a large ΔC_p^d are expected to exhibit cold unfolding at higher temperatures. The effects of urea on the thermal unfolding of barnase are to decrease the T_m (Figure 2A), to reduce the ΔH_m (Figure 2b), and to increase the ΔC_p^d (Figure 4). The values of these parameters extrapolated to concentrations of urea in the 5−6 M range indicate that cold unfolding of wild-type barnase should be observable at around 258 K (−15 °C) in MES buffer, pH 6.3. However, the cold and heat unfolding transitions overlap extensively, and maximal stability, which occurs at around 276 K, results only in a fractional occupancy of the folded state.

DSC thermograms measured in the presence of 5.4 and 6 M urea are shown in Figure 6, panels A and B, respectively. The increases in excess heat capacity observed at temperatures below 273 K are consistent with some form of cold unfolding. Further, these data follow quite closely the calorimetric behavior predicted from the extrapolation of data at lower [urea] and are less consistent with the behavior expected when the value of ΔC_p^d observed in the absence of urea is used. This is particularly evident in Figure 6A where both the increase in excess heat capacity and the temperature of maximal stability are modeled using only the urea dependent value of ΔC_p^d.

It is not possible to extend the range of DSC measurement to lower temperatures because of the hazard of freezing the solutions in the calorimetric cells. Nevertheless, there is some indication that if these scans are extrapolated the magnitude of the increase in excess heat capacity upon cooling is not as large as that observed during the heat unfolding transition. This might indicate that the cold unfolded state is much more structured than the denatured state achieved at higher temperature. It could also be the result of a downward curvature in the heat capacity function of the unfolded state at lower temperatures. Such curvature has been reported for the denatured states of a number of proteins (Privalov et al., 1989), and a consequence of this effect is that the overall ΔC_p^d will show a temperature dependence. Further effects from the slow kinetics of unfolding and refolding at low temperatures relative to the instrumental scan rate may also be important in determining the shape and magnitude of the cold unfolding transition observed.

Cold unfolding was also monitored by following the intrinsic fluorescence spectra of barnase in 50 mM MES buffer, pH 6.3, in the presence of 6 M urea. These changes are complicated by the strong temperature dependencies of fluorescence intensity and the λ_{max} of the spectra (Demchenko, 1986). Nevertheless, a small but detectable increase in λ_{max} was observed upon cooling the sample below 276 K which is consistent with unfolding of the sample (data not shown). The λ_{max} also increased when heated, and its value at 276 K indicated about 50% occupancy of the folded state when compared with the values for folded and unfolded barnase at 298 K (Sancho & Fersht, 1992). These observations are again consistent with the simulated stability profile for barnase using extrapolated values of ΔH_m and ΔC_p^d.

DISCUSSION

Stability of Barnase in the Presence of Urea. The stabilities of wild-type barnase and over 160 of its mutants

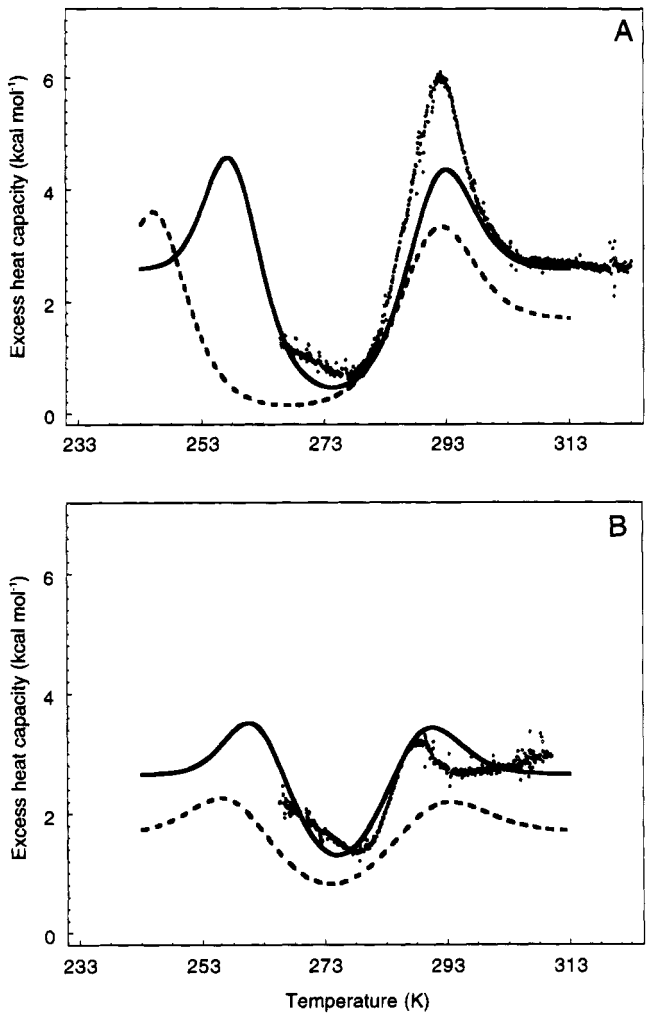

FIGURE 6: DSC thermograms for wild-type barnase in the presence of (A) 5.4 M urea and (B) 6 M urea. DSC data (open circles) were recorded at a scan rate of 30 K h^{-1} and then corrected by subtraction of instrumental base lines recorded in the presence of the appropriate concentration of urea and normalized to molar excess heat capacity. Simulations of the excess heat capacity profiles were made using a two-state unfolding model in which the heat capacities of the folded state, which is arbitrarily set at 0, and unfolded state have no temperature dependence. The parameters used were obtained by extrapolation of the data observed in 50 mM MES buffer, pH 6.3, at lower urea concentrations. (A) 5.4 M urea, T_m = 288 K, ΔH_m = 35.3 kcal mol^{-1}, and ΔC_p^d = 2.58 (solid line) or 1.7 (dotted line) kcal mol^{-1} K^{-1}. (B) 6 M urea, T_m = 277 K, ΔH_m = 5.3 kcal mol^{-1}, and ΔC_p^d = 2.68 (solid line) or 1.7 (dotted line) kcal mol^{-1} K^{-1}. The DSC data were aligned with the heat capacity of the heat unfolded state of these simulations using the higher ΔC_p^d (solid line) in each case since this is the only state which is fully populated in the measurable temperature range.

have been measured by equilibrium unfolding in the presence of urea at 298 K in 50 mM MES buffer pH 6.3 (Matouschek et al., 1992; Serrano et al., 1992). The linear dependence of protein stability on [urea] which is observed in the transition region is generally assumed to extend over the entire concentration range (Pace, 1986), i.e.,

$$\Delta G_{U\text{-}F} = \Delta G_{U\text{-}F}^{H_2O} - m[\text{urea}] \quad (7)$$

where $\Delta G_{U\text{-}F}$ is the stability at any concentration of urea, $\Delta G_{U\text{-}F}^{H_2O}$ is the stability in the absence of denaturant, and m is the constant of proportionality equal to $\partial\Delta G_{U\text{-}F}/\partial[\text{urea}]$. These measurements have been made repeatedly for wild-type barnase to give a mean value for the midpoint of the unfolding transition ($[\text{urea}]_{50\%}$) of 4.58 M and an m-value of 1.92 kcal mol^{-1} M^{-1} (Clarke & Fersht, 1993). These values give $\Delta G_{U\text{-}F}^{H_2O}$ = 8.8 kcal mol^{-1} on extrapolation.

We have examined here the thermal stability of barnase in 50 mM MES buffer, pH 6.3, at concentrations of urea below 4.5 M in the region which is not accessible to equilibrium unfolding measured by urea denaturation at 298 K. Values of $\Delta G_{U\text{-}F}^{298}$ in this range are shown in Figure 7 with errors calculated as described earlier. The stability in the absence of urea is 10.4 kcal mol^{-1} which is significantly higher than the value expected from the linear extrapolation of urea equilibrium data indicated by the dashed line in Figure 7. This value is consistent, however, with a number of other measurements of the stability of wild-type barnase determined by thermal denaturation (Griko et al., 1994; Martinez et al., 1994; Matouschek et al., 1994; Oliveberg et al., 1994). Closer examination of the values of $\Delta G_{U\text{-}F}^{298}$ in Figure 7 reveals a small curvature in the stability profile. Because of this apparent nonlinearity, the data in Figure 7 were fitted to a second-order polynomial: $\Delta G_{U\text{-}F}^{298} = 10.5 \pm 0.08 - ((2.65 \pm 0.05) \times [\text{urea}]) + ((0.08 \pm 0.01) \times [\text{urea}]^2)$ kcal mol^{-1}. The deviations from this fit (residuals) are relatively random, whereas the residuals for the linear fit deviate systematically (Figure 7). The intercept of the polynomial fit at $\Delta G_{U\text{-}F} = 0$ is 4.57 M urea, and the gradient is 1.92 kcal mol^{-1} M^{-1} at this concentration. These values are in excellent agreement with those observed during equilibrium unfolding at 298 K. In contrast, the linear fit to the data has intercept of 4.59 M urea but a gradient of 2.19 kcal mol^{-1} M^{-1}.

It is also possible to fit the values of $\Delta G_{U\text{-}F}^{298}$ to an equation assuming binding of urea to the folded and unfolded states of a protein as first suggested by Tanford (1970). This is of the form:

$$\Delta G_{U\text{-}F} = \Delta G_{U\text{-}F}^{H_2O} - \Delta nRT \ln(1 + aK_{ass}) \quad (8)$$

where Δn is the number of urea molecules binding upon unfolding, a is the denaturant activity, and K_{ass} is the average association constant at each site. Values of $\Delta G_{U\text{-}F}^{H_2O} = 10.5 \pm 0.08$ kcal mol^{-1}, $\Delta n = 53 \pm 6$, and $K_{ass} = 0.088 \pm 0.012$ M^{-1} are obtained from fitting the data in Figure 7. These values are consistent with estimates of K_{ass} and values of Δn based on the total number of residues and the aromatic component of barnase (Pace, 1986). This agreement cannot, however, be taken as evidence for the validity of a binding model because of the small degree of curvature and the large errors in the derived binding parameters.

The derivative of the polynomial fit to the $\Delta G_{U\text{-}F}^{298}$ profile in Figure 7 ($\partial\Delta G_{U\text{-}F}/\partial[\text{urea}]$) predicts a dependence of the equilibrium m-value on [urea]. The range over which this can be measured in the equilibrium unfolding of barnase is the $[\text{urea}]_{50\%} \pm 0.8$ M. However, $\Delta G_{U\text{-}F}^{H_2O}$ can be varied in a number of ways including mutation (Matouschek et al., 1994), pH (Pace et al., 1992), and temperature (M. Long, and A. Fersht, unpublished data), thereby revealing any variation in m-value over the different range of [urea] required for unfolding. The m-values observed in these studies are plotted in Figure 8 with the predicted dependence of this parameter on [urea] derived from the polynomial fit in Figure 7. There is reasonable agreement between these

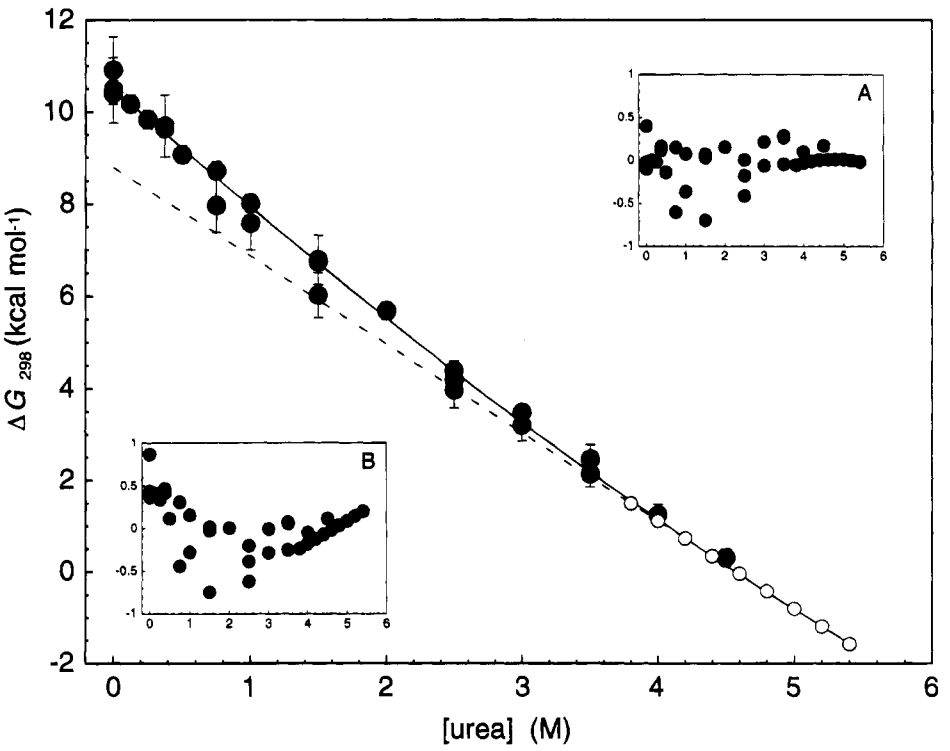

FIGURE 7: Urea dependence of $\Delta G^{298}_{U\text{-}F}$ for barnase in 50 mM MES buffer, pH 6.3. Values of $\Delta G^{298}_{U\text{-}F}$ from DSC and CD measurements (solid circles) were calculated as described in the text. Values of $\Delta G^{298}_{U\text{-}F}$ from urea equilibrium unfolding (open circles) were calculated from the $[\text{urea}]_{50\%} = 4.58$ M for a distance of ±0.8 M at intervals of 0.2 M and the *m*-value = 1.92 kcal mol^{-1} M^{-1}. The combined data can be fitted to a second-order polynomial of the form $\Delta G^{298}_{U\text{-}F} = 10.5 \pm 0.08 - ((2.65 \pm 0.05) \times [\text{urea}]) + ((0.08 \pm 0.01) \times [\text{urea}]^2)$ kcal mol^{-1} (solid line) and the linear function $\Delta G^{298}_{U\text{-}F} = 10.1 \pm 0.06 - ((2.19 \pm 0.01) \times [\text{urea}])$ kcal mol^{-1} (not shown). Residuals of the data to these two fits are indicated in inserts A and B, respectively. Linear extrapolation from the urea equilibrium data is indicated by the dashed line.

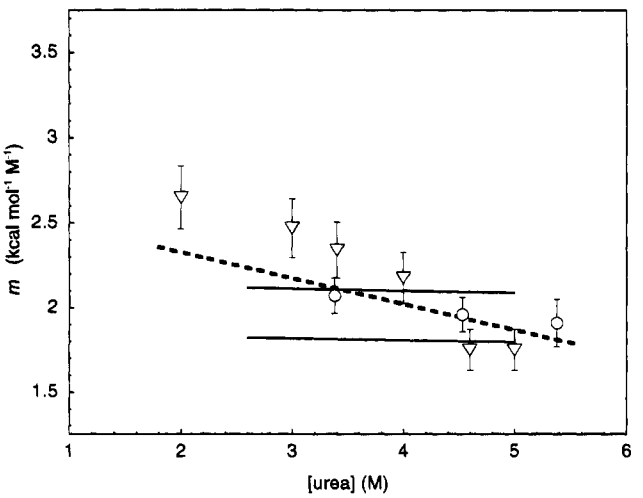

FIGURE 8: Urea dependence of the *m*-value determined by equilibrium unfolding at 298 K. The derivative of the polynomial fit in Figure 7 (dashed line) is plotted with the *m*-values determined for over 160 mutants of barnase (the upper and lower limits of the fit to these data are indicated with solid lines; Matouschek et al., 1994) and for wild-type barnase with *m*-values determined at different temperatures (open circles; M. Long and A. Fersht, unpublished data) and different pH values (open triangles; Pace et al., 1992). All data are plotted at the measured value of $[\text{urea}]_{50\%}$.

data, and this is additional support for the stability of barnase being nonlinearly related to [urea].

The logarithm of the rate constant for unfolding (k_u) of wild-type barnase and numerous mutants, which reflects the difference in energy between the folded state and the main transition state, also follows a nonlinear function with [urea] (Matouschek et al., 1994). The slight curvature in plots of log k_u versus [urea] leads to an increase in the value of the activation energy for unfolding when extrapolated to water of about 1 kcal mol^{-1}, compared with the value obtained from linear extrapolation. The values of $\Delta G_{U\text{-}F}$ of three destabilized mutants of staphylococcal nuclease also appear to be a nonlinear function of [urea] at very low concentrations of denaturant (Shortle et al., 1989), but the curvature is downward, rather than the upward deviation observed here.

The observation of deviations in the plots of free energy versus [urea] implies that this denaturant is a more effective destabilizing agent at low concentrations. This would be expected in a system where protein and denaturant interact in some way unless the association constant for this process is zero (i.e., no interaction). While the exact mechanism of action of chemical denaturants is not known, it is clear from structural (Pike & Acharya, 1994) and calorimetric (Makhatadze & Privalov, 1992) studies that there is interaction or "binding" to the folded and unfolded states of proteins. Indeed, the small degree of curvature in values of $\Delta G^{298}_{U\text{-}F}$ with [urea] observed in Figure 7 is exactly what has been predicted from isothermal calorimetric titration measurements (Makhatadze & Privalov, 1992).

ΔC_p^d and Extrapolated Values of $\Delta G^{298}_{U\text{-}F}$. The curvature in plots of $\Delta G^{298}_{U\text{-}F}$ against [urea] would be increased if ΔC_p^d is temperature dependent. As considered earlier, the values of $\Delta G^{298}_{U\text{-}F}$ at low [urea] will be increased by ≤0.15 kcal mol^{-1} while those at higher [urea] will be unaffected. Nevertheless, the values of $\Delta G^{298}_{U\text{-}F}$ that are calculated using the urea dependent $\Delta C_p{}^d{}_{app}$ term derived from Figure 4 (with or without the additional temperature dependence of $\Delta C_p{}^d$) are actually very similar to stabilities calculated using a $\Delta C_p{}^d$

of 1.7 kcal mol^{-1} K^{-1}, i.e., the value observed in the absence of urea (data not shown). This is because the longest extrapolations in temperature (data at low [urea]) use a $\Delta C_p{}^d{}_{app}$ close to 1.7 kcal mol^{-1} K^{-1}, whereas when $\Delta C_p{}^d{}_{app}$ is around 2.5 kcal mol^{-1} K^{-1} (data at high [urea]) the extrapolation required to 298 K is short. The urea dependence of $\Delta C_p{}^d{}_{app}$ does contribute slightly, however, to the curvature in the $\Delta G^{298}_{U\text{-}F}$ profile. The observations of the cold unfolding of barnase indicate that the increase in the $\Delta C_p{}^d{}_{app}$ term can be extrapolated from the data in Figure 4 to higher urea concentrations and does occur at pH 6.3.

The temperature dependence of ΔH_{vh} in the presence of different concentrations of urea has a slope of around 2.7 kcal mol^{-1} K^{-1} (Figure 2B). As already noted, this is not the $\Delta C_p{}^d$ or $\Delta C_p{}^d{}_{app}$ of unfolding since both temperature and [denaturant] are variables. However, a value derived in this way has been used in a thermodynamic study on the thermal unfolding of oxidized thioredoxin in the presence of guanidine hydrochloride (Santoro & Bolen, 1992). These authors measured enthalpy values in the presence and absence of denaturant and derived a "$\Delta C_p{}^d$" which they use to correct thermal unfolding data to give $\Delta G^{298}_{U\text{-}F}$. The agreement between these values and stabilities extrapolated from isothermal urea equilibrium unfolding was good, and this was considered evidence in support of a linear extrapolation model. Interestingly, when the same procedure is used on the data reported here for barnase (i.e., "$\Delta C_p{}^d$" = 2.7 kcal mol^{-1} K^{-1}), we also obtain a linear function which intercepts with a $\Delta G^{H_2O}_{U\text{-}F}$ very close to the value of 8.8 kcal mol^{-1} obtained from urea equilibrium experiments. Although the reasons for this apparent agreement are not clear, it is incorrect to use this $\Delta C_p{}^d$ value for data recorded in the absence of urea where it is well established that $\Delta C_p{}^d$ is actually around 1.7 kcal mol^{-1} K^{-1}.

Effects of pH on the Equilibrium m-Value. Changes in the *m*-value for the urea equilibrium unfolding of different ribonucleases as a function of pH have been interpreted as an effect of increasing intramolecular charge repulsion on the residual interactions in the unfolded state of these proteins (Pace et al., 1992, 1990). In earlier work on the unfolding of myoglobin using guanidine hydrochloride, similar changes were considered to be a function of [denaturant] (Pace & Vanderburg, 1979). In barnase, the curvature in the unfolding kinetics and the changes in equilibrium *m*-value with mutation and temperature have all been observed at pH 6.3 (Matouschek et al., 1994). Further, the data presented in Figure 3 can be used to calculate $\Delta G^{298}_{U\text{-}F}$ at each value of pH examined for [urea] between 0 and 3 M. It is not possible to observe any curvature in these plots over this smaller range of denaturant concentration and with limited data points. However, the *m*-values obtained from the linear regression to these data sets are essentially identical (2.31 ± 0.05 kcal mol^{-1} M^{-1}, n = 8) and no different from that observed in MES buffer, pH 6.3, over the same range of urea concentration (2.4 kcal mol^{-1} M^{-1}). Thus, the dependence of the free energy of unfolding of barnase on [urea] appears to be independent of pH within the range examined. This finding does not exclude pH-mediated effects which may be observed during equilibrium unfolding since the combination of thermal denaturation and pH destabilization used here produces the unfolded state of barnase only above 298 K. However, the effect of temperature on the unfolded state of proteins seems to be a reduction in their hydrodynamic volume, induced by a net increase in the magnitude of hydrophobic interactions (Privalov et al., 1989). This "squeezing" phenomenon might lead to an underestimate of the Δn term for urea (eq 1) since the unfolded state is always obtained at a higher temperature than that at which binding to the folded state is occurring. If this is significant, it might lead to a decrease in the net interaction of urea upon unfolding at higher temperatures (i.e., low [urea]) and thus cause an underestimate of the curvature in the stability profile. This may explain why the *m*-value variation observed by Pace and co-workers at 298 K is greater than that predicted from our thermal unfolding measurements (Figure 8).

Consequences for the φ-Value Analysis of Barnase. The folding pathway of barnase has been mapped in considerable detail using protein engineering and the ϕ-value analysis approach developed in our laboratory (Fersht, 1993; Fersht et al., 1992). In these studies, the differences in energy levels on the folding pathway are compared following mutation. The effects of the urea-mediated curvature in kinetic measurements of k_u on this analysis have already been considered in some detail (Matouschek et al., 1994). It has been shown that the deviation from linearity does not have a significant effect providing, as appears to be the case, the degree of curvature is similar in all mutants examined. Similarly, the small dependence of the equilibrium *m*-value in mutants on [urea] observed between 2 and 5 M does not affect the measurement of the differences in stabilities ($\Delta\Delta G_{U\text{-}F}$) between wild-type and mutants as shown by a mathematical analysis and verified by experiment, comparing $\Delta\Delta G_{U\text{-}F}$ measured by DSC and equilibrium urea denaturation (Matouschek et al., 1994). The analysis assumed a similar curvature in plots of $\Delta G_{U\text{-}F}$ against [urea] for all mutants. This assumption would seem likely in view of the weak nature of the interaction between urea and protein and the conservative nature of most mutations examined.

The only quantity on the folding pathway of barnase that is affected by the curvature in plots of $\Delta G_{U\text{-}F}$ against [urea] is the free energy of the folding intermediate (or collection of intermediates) relative to the unfolded state ($\Delta G^{H_2O}_{U\text{-}I}$). Fortuitously, the original value used (3.2 kcal mol^{-1}; Matouschek et al., 1990) is very close to the best estimates ($\Delta G^{H_2O}_{U\text{-}I}$ = 2.8 kcal mol^{-1}) derived from the data here and from kinetic studies (Matouschek et al., 1994). This results from some compensating effects during the analysis of data. The value of $\Delta G^{H_2O}_{U\text{-}F}$ of barnase was originally estimated from measurements of urea denaturation assuming that the fluorescence of native and denatured barnase does not change with [urea] (i.e., "constant base lines"). This gave values of 2.23 kcal mol^{-1} M^{-1} for the *m*-value and 10.2 kcal mol^{-1} for $\Delta G^{H_2O}_{U\text{-}F}$ (Serrano et al., 1990; Matouschek et al., 1990). The improved procedure of fitting the curves to equations that allow the fluorescence of the native and denatured states to change linearly with [urea] (Pace et al., 1990) gives the lower values of 1.92 kcal mol^{-1} M^{-1} and 8.8 kcal mol^{-1}, respectively (Pace et al., 1992; Clarke & Fersht, 1993).

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