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Thermodynamics of Transient Conformations in the Folding Pathway of Barnase: Reorganization of the Folding Intermediate at low pH

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ABSTRACT: New classes of small proteins have recently beenfound that refold rapidly with two-state kinetics from a substantially unfolded conformation ("U") and without the accumulation of a folding intermediate. Barnase, on the other hand, is representative of a class of proteins that display multistate kinetics and refold from a partly structured conformation, a folding intermediate (I). The accumulation of I on the folding pathway of barnase is highly dependent on the experimental conditions: a transition from multistate to two-state folding behavior can be induced simply by changing the reaction conditions away from physiological, i.e., elevated temperatures, high concentration of denaturant, or low pH. We argue that the change in folding behavior results from the denatured state changing under different conditions. The denatured state seems compact and partly structured at conditions that favor folding but is disorganized at denaturing conditions. At physiological pH and temperature, the denatured state (D^{phys}) is the folding intermediate because it is the most stable of the denatured conformations, i.e., $D^{phys} \equiv I$. At high temperature or [urea], however, D^{phys} becomes destabilized relative to less structured denatured states ("U"). Kinetics under these extreme conditions is two-state because the refolding reaction is from "U" to the native state with no significant accumulation of D^{phys} ($\equiv$I) which is here a high-energy intermediate. The two-state behavior at low pH results from a different cause. The acid-denatured state of barnase (D^{acid}) is not as unfolded as "U" but energetically similar to D^{phys} ($\equiv$I). It appears that protonation of D^{phys} has only marginal effects on its stability, so that the protonated form of D^{phys} constitutes the acid-denatured state at equilibrium. The energetic similarity between D^{phys} and D^{acid} gives rise to two-state kinetics at low pH, although the refolding is from a compact denatured state throughout the pH range. Protonation of D^{phys} to give D^{acid} causes the structure to become more disorganized and hydrated. The heat capacity of D^{phys} ($\equiv$I) at pH 6.3 is in between that of "U" and the native protein. We suggest that protonation of folding intermediates disrupts their structural integrity and allows isoenergetic reorganizations that increase the solvation of charged residues. Such protonated and reorganized folding intermediates may then constitute the molten globules, which are compact denatured states that are sometimes observed at equilibrium at low pH and high ionic strength. Under all experimental conditions, the heat capacity of the major transition state is close to that of the native protein. This, together with its titration properties, shows that the transition state is an expanded form of the native state with a weakened but poorly hydrated hydrophobic core, and with disrupted surface regions.

The folding of a disordered polypeptide (D)[1] chain into the ordered structure of the native protein (N) under equilibrium conditions is typically an apparent two-state process, $D \rightleftarrows N$, which is accompanied by significant changes in enthalpy, entropy, and heat capacity (Privalov, 1992). When the solvent-denatured protein is rapidly mixed into refolding conditions, however, it appears to undergo a very rapid structural condensation [cf. Dill and Shortle (1991)]. Hence, the observed refolding process takes place from a denatured species that is different from the unfolded state observed in high concentrations of denaturants. This compact denatured state, or actually the family of conformations in the preequilibrium, is usually referred to as a folding intermediate, $D \rightleftarrows I \rightleftarrows N$ (Schmid & Baldwin, 1979; Kuwajima *et al.*, 1985, 1991; Roder *et al.*, 1988; Matouschek *et al.*, 1990; Udgaonkar & Baldwin, 1990; Briggs & Roder, 1992; Chaffotte *et al.*, 1992; Lu & Dahlquist, 1992; Radford *et al.*, 1992; Svensson *et al.*, 1995). It must be realized that folding intermediates in this context represent the denatured protein under physiological conditions, i.e., $I \equiv D^{phys}$. Other forms of folding intermediates are native-like conformations with an incompatible isomerization around the prolines (Kiefhaber *et al.*, 1992; Schreiber & Fersht, 1993) or "off-route" conformations that are geometrically restricted by disulfide bridges (Kim & Baldwin, 1990; Creighton, 1992). Under some conditions, in particular at low pH and high ionic strength, compact denatured states can also be observed at equilibrium (Kuwajima, 1989; Ptitsyn, 1992). Since it is generally believed that these so called molten-globule states represent (protonated) forms of folding intermediates (or D^{phys}) (Kuwajima, 1989; Ptitsyn *et al.*, 1990), they have been subject to a large number of studies [see Ptitsyn (1992) and references therein]. Although very little is known about their energetics, the structure of molten globules appears compact and fluctuating, with a significant amount of secondary structure but no fixed tertiary interactions. There have been reports on the importance of hydrophobic interactions in

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[1] Denatured conformations are generally denoted D; these could be either compact or substantially unfolded, "U". When we refer to a particular denatured state, this is indicated by a superscript. For example, the urea denatured state is D^{urea} ("U").

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molten globules (Dobson, 1992; Barrick & Baldwin, 1993), and the presence of nonhydrated hydrophobic elements is further supported by findings of relatively low heat capacities for the molten globules of α-lactalbumin (Griko *et al.*, 1994) and apomyoglobin (Nishii *et al.*, 1994)—the heat capacity (C_p) is related to the excess heat required to melt shells of ordered water molecules which form around apolar groups of the protein and is proportional to the solvent exposure of hydrophobic residues (Privalov & Makhatadze, 1990). In other cases, however, the heat capacity of the molten globule has been found to be similar to that of the denatured state (Privalov & Makhatadze, 1990; Yutani *et al.*, 1992). The reports suggest that either the molten globules constitute a heterogeneous group of protein conformations, or that the various D^{phys} states are perturbed differently by protonation (charge repulsion).

The transition state for unfolding (‡) is the most structured transient conformation on the folding pathway, immediately preceding the native state. Theoretical studies suggest that the transition state is an expanded form of the native state, in which the core remains nonhydrated but has lost a significant amount of van der Waals contacts (Shakhnovich & Finkelstein, 1989). The swelling of the transition state, however, is believed to be insufficient to allow rotational isomers of the residues (Shakhnovich & Finkelstein, 1989). From these assumptions it has been calculated that the unfolding process should be predominantly enthalpically controlled whereas folding is predominantly entropically controlled (Shakhnovich & Finkelstein, 1989). The model is in agreement with experimental results on lysosyme which show that the heat capacity of the transition state is similar to that of the native state (Sugihara & Segawa, 1984) and with mutational analysis of the transition state of barnase which reveals a weakened hydrophobic core as well as significant tertiary interactions (Matouschek *et al.*, 1989; Serrano *et al.*, 1992).

In the present study, we investigate changes in thermodynamics and heat capacity for the increasingly consolidated conformations in the folding pathway of barnase, $D \rightleftarrows I \rightleftarrows \ddagger \rightleftarrows N$, and provide novel information about their structural characteristics. In particular, we focus on the importance and stability of the folding intermediate and show how this can be related to denatured conformations observed at equilibrium. The study is carried out at pH values between 0.2 and 6.3, and at temperatures between 10 and 70 °C, and is complementary to an accompanying report on changes in the titration properties upon refolding (Oliveberg & Fersht, 1996).

EXPERIMENTAL PROCEDURES

Materials. Protein and buffers were prepared according to Oliveberg and Fersht (1996). The fluorescent probe 1-anilino-naphthalene-8-sulfonate (ANS) was purchased from Molecular Probes (Bethesda) and used as an indicator of solvent-exposed hydrophobic clusters (Semisotnov *et al.*, 1991).

Kinetic Measurements. The time-resolved changes in far-UV ellipticity were measured using a prototype stopped-flow CD instrument from Applied Photophysics with a dead-time of about 5 ms. Barnase was denatured in 32 mM HCl and mixed 1:1 (vol/vol) with MES refolding buffer (7 mM acidic form and 93 mM sodium salt) to a final pH of 6.3. The protein concentration was chosen so that the absorbance after mixing was 0.8−0.9. Conventional stopped-flow experiments following fluorescence changes and data analysis procedures were performed as described (Oliveberg & Fersht, 1996).

Pressure Jump and Temperature Jump. The pressure-jump set-up is a home-built, modified version of the apparatus developed by Dr H. Gutfreund (personal communication), equipped with self-sealing windows and a system of valves to reduce pressure waves in the hydraulics. The cell volume is 1.5 mL, and its content is thermostated with an external water bath which maintains the temperature within ±0.1 °C. In a typical experiment, the pressure was increased to 700 bar for 100 s and released to 1 bar within 60 μs. For the temperature-jump experiments a commercial instrument from DIA-LOG (Germany) was used. The rise time for this instrument, which depends on the experimental conditions, was 2−8 μs. Protein relaxation processes were monitored either by fluorescence (excitation at 280 nm and emission collected at wavelengths greater than 315 and 335 nm, using cut-off filters) or by absorbance between 220 and 240 nm. The protein concentration was varied between 1 and 80 μM. The buffers were 50−200 mM glycine/HCl (pH 1.5−3.0), 50−200 mM sodium formate/formic acid (pH 2.7−4.2), 50−200 mM sodium acetate/acetic acid (pH 3.7−5.3), and 50 mM 2-(*N*-morpholino)ethanesulfonic acid (MES) (pH 6.3). The ionic strength was controlled by KCl.

RESULTS

It is demonstrated in the accompanying paper (Oliveberg & Fersht, 1996), that the folding pathway of barnase can be described by

Scheme 1

$$D \overset{\text{fast}}{\rightleftarrows} I \underset{k_u}{\overset{k_f}{\rightleftarrows}} N$$

where k_f is the refolding rate constant which represents the free-energy difference between the folding intermediate (I) and the major transition state (‡), and k_u is the unfolding rate constant which represents the free-energy difference between the native protein (N) and the major transition state (‡). The interconversion between D and I is fast and takes place in the dead-time of the stopped-flow instrument.

The experimentally observed refolding rate constant is denoted k_f^{obs}, and the experimentally observed unfolding rate constant k_u^{obs}. Under all conditions the observed rate constant, k^{obs} (i.e., either k_f^{obs} or k_u^{obs}), is the sum of the forward and reverse constants according to

$$k^{obs} = k_f^{app} + k_u = Ck_f + k_u \tag{1}$$

where k_f^{app} is the apparent refolding rate constant which is related to k_f by the occupancy of I, $C = [I]/([I] + [D])$, in the preequilibrium ($D \rightleftarrows I$). It follows from eq 1 that $k_f^{obs} \approx k_f^{app}$ under folding conditions where $k_f^{app} \gg k_u$, and $k_f^{obs} \approx k_u$ under denaturing conditions where $k_f^{app} \ll k_u$. In the unfolding transition region where $k_f^{app} \approx k_u$, $k_f^{obs} = k_f^{app} + k_u$.

Temperature Dependence of the Refolding Kinetics. The temperature dependence of the observed refolding rate constant (k_f^{obs}) at pH 6.3 and $\mu = 50$ mM is shown in Figure

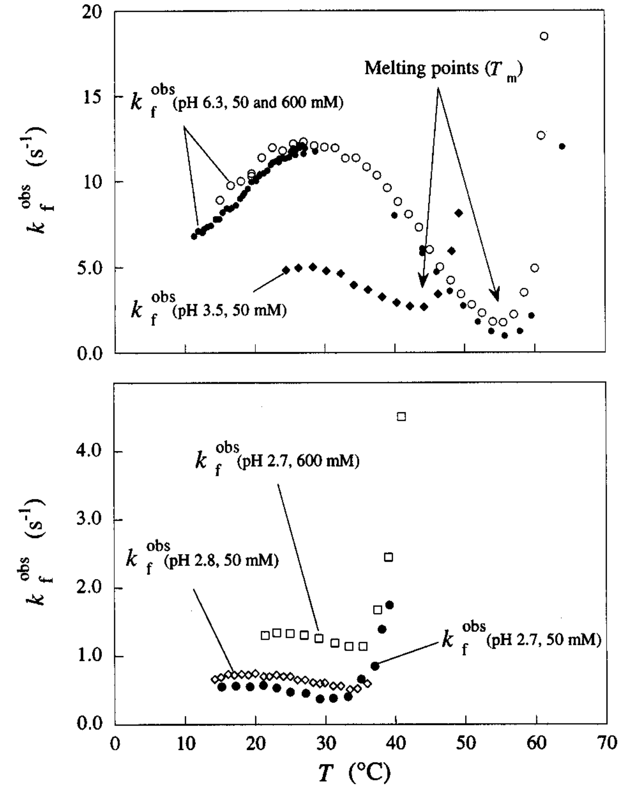

FIGURE 1: Temperature dependence of the observed refolding kinetics versus pH and ionic strength (μ). At all temperatures k_f^{obs} is the sum of the (apparent) refolding rate constant k_f^{app} and the unfolding rate constant k_u (eq 1). The minima of k_f^{obs} correspond to the midpoints for the thermal unfolding transitions (T_m), and at these temperatures $k_f^{obs} \approx k_u$. At temperatures below the transition midpoints $k_f^{obs} \approx k_f^{app}$, and at temperatures above the transition midpoints $k_f^{obs} \approx k_u$. (Top panel) (○ and ●). Refolding rate constants at pH 6.3, $\mu = 50$ mM and $\mu = 600$ mM, respectively [k_f^{obs} (pH 6.3, 50 and 600 mM)]. (◆) Refolding rate constant at pH 3.5 and $\mu = 50$ mM [k_f^{obs} (pH 3.5, 50 mM)]. (Lower panel) (◇) Refolding rate constant at pH 2.8 and $\mu = 50$ mM [k_f^{obs} (pH 2.8, 50 mM)]; (●) at pH 2.7 and $\mu = 50$ mM [k_f^{obs} (pH 2.7, 50 mM)]; and (□) at pH 2.7 and $\mu = 600$ mM [k_f^{obs} (pH 6.3, 600 mM)].

1.[2] The refolding process reveals a complex temperature dependence that can be divided into three regions [cf. Oliveberg *et al.* (1995a)]. At lower temperatures, 10−30 °C, k_f^{obs} displays a small increase with increasing temperature. At room temperature, the increase starts to level off, and k_f^{obs} reaches a maximum of about 13 s^{-1} around 30 °C. Above 30 °C, k_f^{obs} decreases with increasing temperatures. Around 55 °C, at the midpoint for the thermal transition, k_f^{obs} shows a minimum of less than 2 s^{-1}, and at still higher temperatures its value displays a rapid exponential increase. According to eq 1, the minimum and subsequent increase of k_f^{obs} are associated with the contribution from the unfolding rate constant (k_u), which becomes the predominant component of k_f^{obs} under denaturing conditions.

At low pH values, the minimum in the plot of k_f^{obs} against temperature occurs at lower temperatures, which here corresponds to the pH-induced decrease in T_m. The maximum of k_f^{obs}, however, is less dependent on pH and remains around room temperature (Figure 1). The effect of salt on this maximum is relatively small, although it seems more pronounced at low pH values (Figure 1). The results are analyzed in more detail in the Discussion.

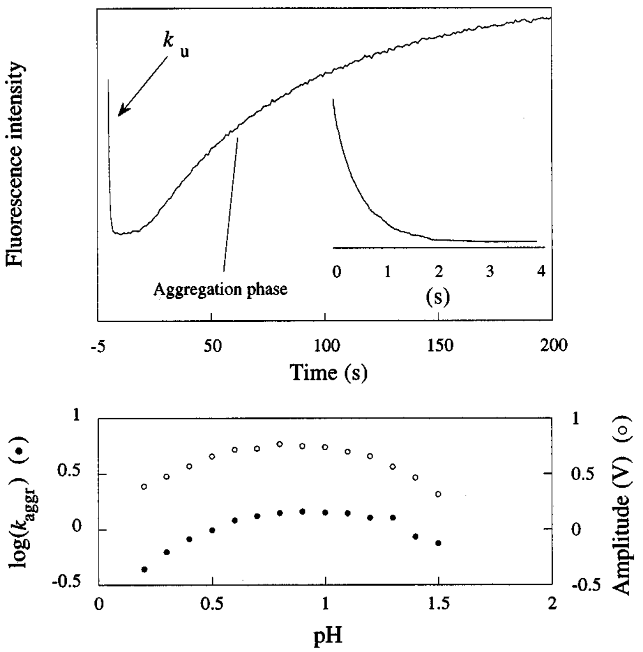

FIGURE 2: Kinetics of aggregation/association following unfolding at $\mu = 600$ mM. The rate constant has units of s^{-1}. The top panel shows the time course of the major unfolding reaction over 200 s, with the inset showing the first 5 s of the same trace. The initial decrease in fluorescence corresponds to unfolding of the native protein (k_u), and the slower recovery of the fluorescence is associated with aggregation/association of the denatured state. When fitted to an exponential function, the rate constant (k_{aggr}) and extent of this reaction are at a maximum just below the unfolding transition and decrease at lower pH values. The lower panel shows k_{aggr} (●) and the amplitude of the aggregation/association phase (○).

Unfolding Rate Constant and Its Temperature Dependence As Determined by Urea Unfolding at pH 6.3. The unfolding rate constant (k_u) under conditions of maximum stability was determined by stopped-flow at urea concentrations between 4 and 8 M and subsequently extrapolated to pure water using a nonlinear regression fit of a quadratic expression [eq 7 in Oliveberg and Fersht (1996)]. The temperature dependence of k_u^0 (the unfolding rate constant in pure water) was then determined by these urea-extrapolation experiments performed at a series of temperatures ranging from 15 to 35 °C (unpublished data, this laboratory).

Comparative Studies of the Denatured State and the Folding Intermediate. The analysis of kinetic and thermodynamic data in this and the accompanying paper suggests that the folding intermediate under some conditions constitutes the acid-denatured species (see Discussion). To test this result, we have performed a series of comparative measurements of the acid/thermally denatured state and the folding intermediate, to see whether they show the same properties.

Probing the Denatured State and the Folding Intermediate: 1. pH and Temperature Dependence of Salt-Induced Aggregation Processes of the Acid-Denatured State. At high concentrations of salt (i.e., at $\mu \geq 500$ mM), the acid-denatured protein was found to undergo a slow aggregation process. In a typical unfolding experiment, these events are seen as a fluorescence increase, which follows the much faster unfolding transition (Figure 2). The aggregation events can be followed also by light scattering at 300−500 nm, but

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[2] Abbreviation: μ, ionic strength.

this method of detection turned out to be less sensitive: under conditions where only very small changes are detected by fluorescence, no changes are observed by light scattering. An explanation may be that under mildly aggregating conditions the protein clusters are very small and soluble, perhaps being dimers. It can be seen in Figure 2 that the major aggregation phase is preceded by a distinct lag time. If this lag is neglected, however, a single exponential function can be precisely fitted to the major phase. Although this treatment of data may not be very strict, it gives a qualitative idea about the extent and kinetics of these events. The rate constants and amplitudes of aggregation obtained by this procedure are plotted versus pH in Figure 2. In general, the rate of aggregation as well as its amplitude reaches a maximum around pH 0.9 and decreases at lower pH values to become negligible finally at pH 0.2.

Similar to the pH dependence, the aggregation/association reaches a maximum at temperatures just above the thermal transition and then disappears at higher temperatures: At pH 1.5, the aggregation maximum occurs around 25 °C and disappears when the temperature is raised above 50 °C; at pH 0.2, the aggregation is suppressed above 30 °C (data not shown). It should be noted that, in contrast to the observed aggregation/association behavior, protein aggregation which is due to intermolecular hydrophobic interactions is predicted to show an increase at higher temperatures as the hydrophobic effect becomes more pronounced (Privalov & Gill, 1988). The aggregation/association is comparatively slow and does not affect the initial unfolding kinetics (cf. ANS experiments below).

Probing the Denatured State and the Folding Intermediate: 2. Effects of ANS. In all experiments, ANS was included in the syringes containing the refolding/unfolding buffer in order not to perturb the initial state of the protein, i.e., to avoid second-order effects such as polymerization and aggregation processes. Acid-mediated unfolding in the presence of ANS is accompanied by a decrease in fluorescence intensity above 400 nm. Conversely, the refolding kinetics shows an increase in fluorescence intensity. Under all conditions used, the time course of the second order ANS binding is rate limited by the observed unfolding/refolding reactions of the protein [cf. Semisotnov *et al.* (1991)]. The aggregation of the denatured protein is drastically enhanced by ANS; the slow increase in fluorescence associated with the aggregation is always detected by the ANS emission, even under conditions of low ionic strength. Since equilibrium experiments show that acid-denatured barnase does not precipitate at low ionic strength in the absence of ANS, it appears that ANS induces precipitation/aggregation of the denatured state, irrespective of ionic strength. Consequently, equilibrium-titration experiments in the presence of ANS will report on the properties of the clusters/aggregates and cannot be used to gain information about the denatured monomer. The ANS-binding properties of the acid-denatured monomer can be studied only by the burst-phase kinetics preceding the aggregation events. The top panel of Figure 3 shows the pH dependence of the unfolding rate constant at μ = 600 mM in the presence of ANS (obtained from the ANS emission), compared with the unfolding rate constant obtained in the absence of ANS (from Figure 2 in the accompanying paper). The two plots coincide precisely, showing that ANS does not affect the activation barrier for unfolding or significantly change the equilibrium between I and N. The lower panel of Figure 3 shows the corresponding fluorescence amplitudes. The denaturation curves described by the amplitude plots appear not to be coincident. The difference, however, cannot be related to non-two-state behavior in the transition region, since it is likely to be caused by the strong pH dependence of the fluorescence associated with the denatured protein, i.e., sloping baselines [cf. appendix in Dill and Shortle (1991)]. However, it is apparent that the ANS emission of the denatured monomer is highest under conditions where it aggregates (cf. Figure 2).

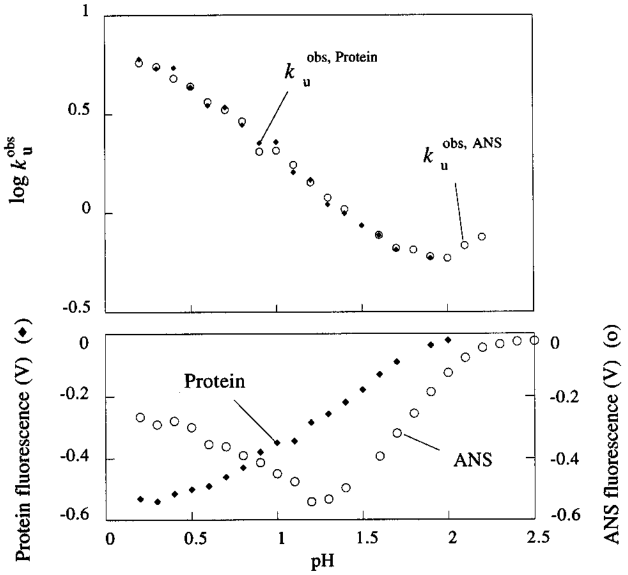

FIGURE 3: Kinetic data from acid unfolding of barnase at $\mu = 0.6$ M in the presence and absence of ANS. The rate constant has units of s^{-1}. The upper panel shows the rate constant obtained from ANS fluorescence above 400 nm, $k_u^{obs,ANS}$ (○), in comparison with the rate constants obtained from protein fluorescence in the absence of the hydrophobic probe, $k_u^{obs,Protein}$ (◆). The lower panel shows the corresponding unfolding amplitudes. In order to minimize contributions from the pH dependence of the intrinsic ANS fluorescence, the ANS amplitudes (○) are normalized to the fluorescence intensity at $t = 0$. The procedure does not significantly change the shape or position of the plot but reduces the data scatter.

The temperature dependence of ANS fluorescence upon transient unfolding at pH 1.5 is shown in Figure 4; the amplitude of ANS binding starts to decrease above 25−30 °C and becomes undetectable at temperatures higher than 45 °C. Also in this case the binding of ANS to the denatured monomer is most pronounced under aggregating conditions.

The refolding experiments were done to investigate the binding of ANS to the folding intermediate. The amplitude associated with expulsion of ANS upon refolding shows a maximum around 20 °C and decreases at higher temperatures. The decrease in amplitude observed below 20 °C is due to refolding of the denatured protein at pH 1.5. In a control experiment, the ANS fluorescence in the absence of protein shows very small variations with temperature. It must be pointed out, however, that the control experiment constitutes a poor measure of the intrinsic temperature dependence of ANS in the presence of denatured protein, and that any fluorophore is expected to show an decreased emission at higher temperatures.

In summary, the acid-denatured monomer binds ANS under conditions where it is found to aggregate/associate, and these are the conditions where the protonated form of the folding intermediate is expected to be significantly

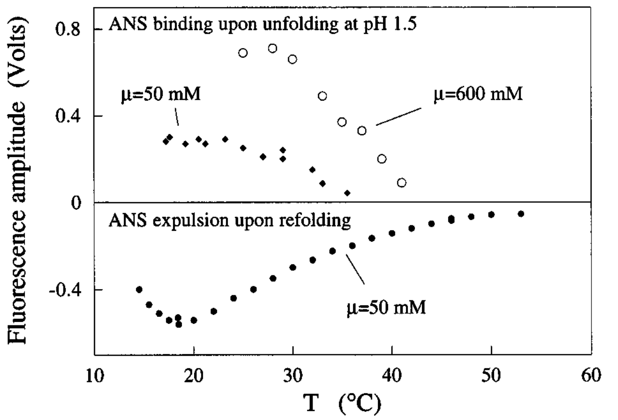

FIGURE 4: Temperature dependence of the fluorescence change associated with ANS binding to the acid-denatured monomer of barnase. The positive amplitudes represent ANS binding upon jumping from pH 6.3 to unfolding conditions at pH 1.5, $\mu = 50$ mM (◆) and $\mu = 600$ mM (○). Correspondingly, the negative amplitudes show the expulsion of ANS when jumping from pH 1.5 to refolding conditions at pH 6.3, $\mu = 50$ mM (○). It can be noticed that the acid-denatured state and the folding intermediate appear to bind ANS to a similar extent. It may also be concluded that the acid-denatured state and the folding intermediate undergo an unfolding transition (to a less ANS binding form) above room temperature.

populated at equilibrium (see Discussion). In the refolding reaction, the folding intermediate binds ANS to the same extent as the acid-denatured monomer (cf., amplitudes in Figure 4).

Probing the Denatured State and the Folding Intermediate: 3. The CD Spectra. The rate constant for the refolding process as monitored by far-UV CD is in good agreement with fluorescence data. The refolding experiments were performed by jumping the protein from pH 1.5 to 6.3, monitoring at wavelengths between 215 and 250 nm. The following parameters are of particular interest: (i) the value of the *differential molar CD extinction coefficient* ($\Delta\epsilon$) immediately after mixing $[\Delta\epsilon(t = 0)]$, which represents the CD spectrum of the denatured protein under refolding conditions ($D^{phys} \equiv I$), and (ii) the value of $\Delta\epsilon$ after 200 s when the system has reached equilibrium $[\Delta\epsilon(t = 200\ s)]$, which represents the CD spectrum of the refolded, native conformation. Figure 5 shows $[\Delta\epsilon(t = 0)]$ and $[\Delta\epsilon(t = 200\ s)]$ plotted versus the wavelength. The values obtained for $\Delta\epsilon(t = 0)$ are inconsistent with the CD spectrum of the fully denatured state (pH 1.5 and 5 M of urea) but resemble more the spectrum of the acid- and thermally denatured protein. The values of $\Delta\epsilon(t = 200\ s)$ fit well with the far-UV spectrum of native barnase. The deviations between kinetic and equilibrium data in Figure 5 are probably within the experimental error.

DISCUSSION

In the accompanying paper, we investigate the effect of pH on the folding pathway at 25 °C, and in this complementary study we include the temperature dependence of these effects: the titration data rely critically on stabilities extrapolated from elevated temperatures, and we demonstrate that pH effects on their own can be misleading. The following discussion focuses mainly on three issues: (1) the transition from multistate to two-state folding behavior as a function of temperature and pH; (2) the thermodynamic and electrostatic properties of the folding intermediate (I); and (3) the thermodynamic and electrostatic properties of the major transition state (‡). An important implication from the present study is that the equilibrium between the denatured state and the folding intermediate ($D \rightleftarrows I$) is only marginally affected by pH, so that the protonated form of I could be populated at equilibrium under acid conditions. To test this, we have as far as possible tried to characterize and compare the properties of the acid denatured state(s) and the folding intermediate.

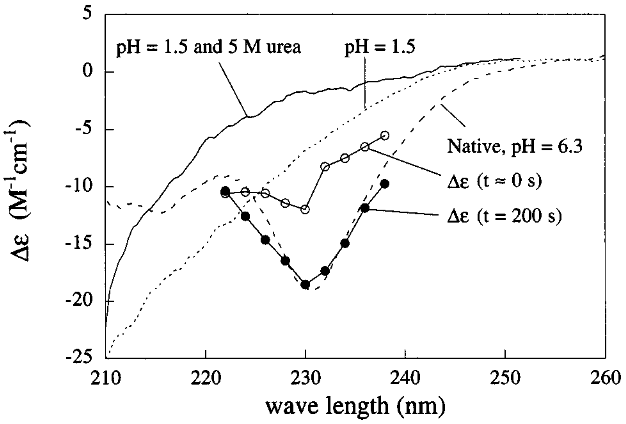

FIGURE 5: Far-UV CD spectra of barnase at pH 6.3 and 25 °C from equilibrium and kinetic experiments. $\Delta\epsilon$, the differential molar CD extinction coefficient, is calculated from the absorbance difference between right-hand and left-hand circular-polarized light. (○) CD spectrum of barnase immediately after jumping the acid denatured state from pH 1.5 to 6.3, $\Delta\epsilon(t = 0)$. (●) CD spectrum obtained 200 s after initiation of the refolding reaction, $\Delta\epsilon(t = 200\ s)$. Each value represent an average of 16 experiments. The broken line is the CD spectrum of native barnase, and the dotted line is that of its acid-denatured state at pH 1.5.

Transition from Multistate to Two-State Folding Behavior. The transition from multistate ($D \rightleftarrows I \rightleftarrows N$), where D and I are in rapid equilibrium and the observed reaction is $I \rightarrow N$, to two-state ($D \rightleftarrows N$) folding behavior can be induced at a fixed concentration of denaturant by simply changing the temperature.[3] With reference to Figure 6, it can be seen that below ~50 °C the observed refolding rate constant $[k_f^{obs}(T)]$ is lower than that calculated from the free-energy difference between D and ‡ $[k_{D\to N}^{calc}(T)]$. This suggests that the observed refolding reaction under these conditions takes place from a conformation that is more stable than D: i.e., D collapses rapidly to a more stable form (in the dead-time of the stopped-flow instrument) and thereby increases the

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[3] The analysis of the temperature data is analogous to that in the accompanying paper and relies on a comparison of the temperature dependence of the observed refolding rate constant $[k_f^{obs}(T)$, eq 1] with that expected from the free-energy difference between the denatured state and the major transition state $[k_{D\to N}^{calc}(T)]$ (cf. Figures 7 and 9, and eq 8 in accompanying paper); $k_{D\to N}^{calc}(T)$ is calculated from the unfolding rate constant $[k_u(T)]$ and the equilibrium constant for unfolding $[K_{D\text{-}N}(T)]$ according to $k_{D\to N}^{calc}(T) = k_u(T)/K_{D\text{-}N}(T)$. $K_{D\text{-}N}(T)$ is derived from experimental data using

$$-RT \ln K_{D-N}(T) = \Delta H_{D-N}(T_m)(1 - T/T_m) - \Delta C_p[(T_m - T) + T \ln (T/T_m)] \quad (3)$$

where $T_m = 328$ K is the temperature for the transition midpoint, $\Delta H_{D\text{-}N}(T_m) = 120$ kcal/mol, and $\Delta C_p = 1880$ cal/(mol·K) [the thermodynamic data and eq 3 are obtained from Oliveberg *et al.* (1994)]. The temperature dependence of $k_u(T)$ was obtained from $k_f^{obs}(T)$ at temperatures above the transition region and urea-jump experiments (Figure 6).

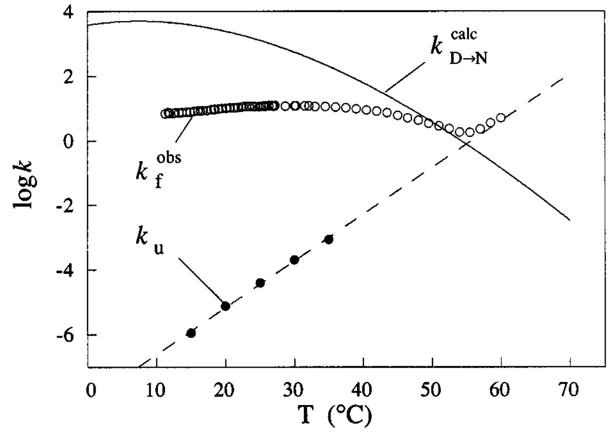

FIGURE 6: Temperature dependence of the unfolding rate constant (●) and the observed refolding rate constant (○) of barnase at pH 6.3, showing the deviation from two-state behavior as a function of temperature. The rate constant has units of s^{-1}. The solid line, which represents the refolding rate constant expected from a two-state folding process [$k^{\text{calc}}_{\text{D}\to\text{N}}(T)$, Figure 7, accompanying paper], coincides with the observed refolding rate constant [$k^{\text{obs}}_{\text{f}}(T)$] in the transition region but becomes faster than $k^{\text{obs}}_{\text{f}}(T)$ at lower temperatures. The broken line represents the unfolding rate constant extrapolated into the transition region at 55 °C. Data points obtained above 60 °C (cf. Figure 1) have been left out since the refolding amplitudes at these temperatures are too small to allow accurate determination of $k^{\text{obs}}_{\text{f}}(T)$.

activation barrier for observed refolding reaction (Matouschek et al., 1990; Oliveberg & Fersht, 1996). It should be noticed that the collapsed form of D [normally referred to as the folding intermediate (I), Scheme 1)] is the most stable denatured conformation under physiological conditions ($\text{I} \rightleftarrows \text{N}$) and, hence, constitutes the physiologically relevant denatured state, $\text{I} \equiv \text{D}^{\text{phys}}$ (Figure 7). At higher temperatures, the stability of I decreases and the difference between $k^{\text{calc}}_{\text{D}\to\text{N}}(T)$ and $k^{\text{obs}}_{\text{f}}(T)$ becomes smaller (cf. Figure 9 in the accompanying paper). Above ~50 °C, where $k^{\text{calc}}_{\text{D}\to\text{N}}(T) \approx k^{\text{obs}}_{\text{f}}(T)$, I has become less stable than D, and the refolding reaction takes place from D. In other words, at low temperatures the rapid preequilibrium ($\text{D} \rightleftarrows \text{I}$) is shifted toward I and the refolding reaction proceeds $\text{D} \to \text{I} \to \text{N}$, where $\text{D} \to \text{I}$ takes place in the dead-time of the experiment and $\text{I} \to \text{N}$ is the observed reaction, and at high temperatures the preequilibrium is shifted toward D and the reaction proceeds $\text{D} \to \text{N}$ (Figure 7).

Thermal Stability of the Folding Intermediate Gives Further Insight into its Titration Properties. The apparent stability of I relative to D at 25 °C ($\Delta G^{\text{app}}_{\text{D-I}}$) is obtained from the difference between log $k^{\text{calc}}_{\text{D}\to\text{N}}$(25 °C) and log $k^{\text{obs}}_{\text{f}}$(25 °C) according to

$$\Delta G^{\text{app}}_{\text{D-I}}(25\ °\text{C}) = \Delta G_{\text{D-}\ddagger}(25\ °\text{C}) - \Delta G_{\text{I-}\ddagger}(25\ °\text{C}) = 2.3RT[\log k^{\text{calc}}_{\text{D}\to\text{N}}(25\ °\text{C}) - \log k^{\text{obs}}_{\text{f}}(25\ °\text{C})] \quad (4)$$

where the free-energy differences and the rate constants are related by the transition-state theory, $\log k_{\text{u}}(T) = \log k_{\text{B}}T/h - \Delta G^{\ddagger}(T)/2.3RT$ [cf. the analogous analysis versus pH in the accompanying paper (Oliveberg & Fersht, 1996)]. The value obtained for $\Delta G^{\text{app}}_{\text{D-I}}$ at pH 6.3 and 25 °C is about 2.8 kcal/mol (Table 1).

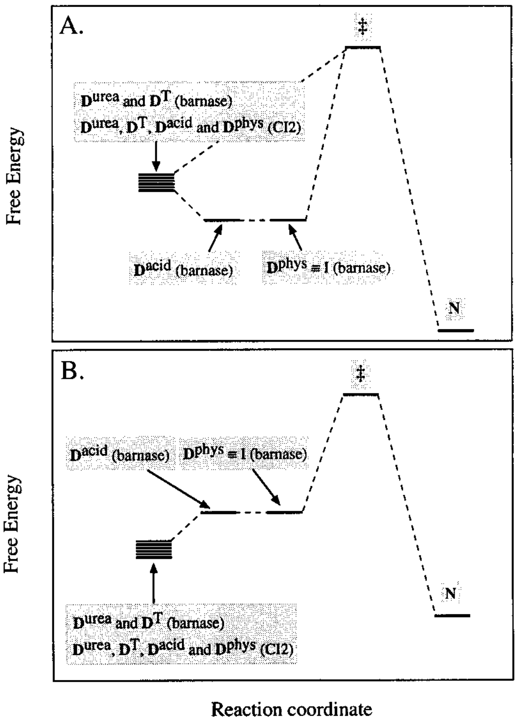

FIGURE 7: Free-energy diagram illustrating the various denatured conformations of barnase and CI2. D^{urea}, the urea-denatured state. D^{T}, the thermally denatured state. D^{acid}, the acid-denatured state. D^{phys}, the denatured state under physiological conditions. ‡, the major transition state. N, the native protein. With barnase, D^{urea} and D^{T} represent substantially unfolded conformations ("U"), whereas D^{acid} and D^{phys} show some degree of structure. With CI2, the denatured conformation is substantially unfolded ("U") at all conditions. (Panel A) D^{urea} and D^{T} of barnase extrapolated to physiological conditions are less stable than the partly structured D^{phys} (≡I). When jumping from elevated temperature or high [urea] to physiological conditions, the denatured conformation collapses rapidly ($\leq 1\ \mu$s) into D^{phys}, the folding intermediate. Consequently, the free-energy difference between N and D^{phys}, $\Delta G_{\text{I-N}} \approx 8$ kcal/mol (obtained from the kinetics), is smaller than the free-energy difference between N and the more unfolded conformations D^{urea} or D^{T}, $\Delta G_{\text{D-N}} \approx 10.5$ kcal/mol (obtained from stability measurements at high T or [urea]). The difference between $\Delta G_{\text{I-N}}$ and $\Delta G_{\text{D-N}}$ constitutes the evidence for multistate folding behavior and reflects the stability of D^{phys} relative to D^{urea} and D^{T}. The acid-denatured state (D^{acid}), however, appears more stable than D^{urea} and D^{T}, and if this is used as the reference for the "unfolded" state, the protein will display two-state kinetics although it refolds from a compact denatured state, i.e., $\Delta G_{\text{I-N}} \approx \Delta G_{\text{D}^{\text{acid}}\text{-N}}$. With CI2, on the other hand, the denatured state does not collapse significantly at physiological conditions but refolds directly from a substantially unfolded conformation. Since $\text{D}^{\text{urea}} \approx \text{D}^{\text{T}} \approx \text{D}^{\text{acid}} \approx \text{D}^{\text{phys}}$, the stability obtained by the kinetics under physiological conditions corresponds to that obtained by equilibrium techniques at extreme conditions. (Panel B) At elevated temperatures or high [urea], the compact D^{phys} of barnase is less stable than the more unfolded conformations. Hence, D^{phys} is here a high-energy intermediate which does not significantly accumulate in the refolding process. At extreme conditions of low pH, however, the free-energy of the various denatured states is similar to that in panel A: the protein refolds with two-state kinetics from a compact denatured state D^{acid} which is energetically similar to D^{phys}. Despite the energetic similarity, D^{acid} and D^{phys} appear to have distinct structural differences: D^{acid} shows few fixed interactions and is likely to be extensively hydrated, whereas D^{phys} contains a significant amount of structure and is poorly hydrated. Both D^{acid} and D^{phys} start to unfold above 30 °C. The refolding of CI2 at extreme conditions take place from a denatured conformation similar to that at physiological conditions.

It can be concluded from the deviation between $k^{\text{calc}}_{\text{D}\to\text{N}}(T)$ and $k^{\text{obs}}_{\text{f}}(T)$ (Figures 6 and 7) that I (or any distribution of folding intermediates in the preequilibrium) reaches a maximum occupancy below 30 °C and gradually "unfolds" at higher temperatures. At $T_{\text{m}} \approx 55$ °C the system

Table 1: Thermodynamic Parameters for the Conformations Identified on the Folding Pathway of Barnase[a]

	ΔC_p [cal/(mol·K)]	ΔH (kcal/mol)	ΔS [cal/(mol·K)]	ΔG (kcal/mol)
N	1880[b]	84.5[b]	249[b]	10.2[b]
‡	1500[c]	27[c]	134[c]	−13[c]
I	1200[d]	31[d]	94[d]	2.8[d]
D	0	0	0	0

[a] The values are at pH 6.3, $\mu = 50$ mM, and 25 °C. All values are given relative to those of the thermally denatured state extrapolated to 25 °C, i.e., the free energy of the denatured state minus that of the more structured conformation. [b] The values are obtained from thermal denaturation experiments (Oliveberg *et al.*, 1994). [c] The values are derived by subtracting the parameters obtained from the temperature dependence of unfolding kinetics from those obtained by thermal denaturation experiments. Alternatively, these values can be obtained from $k_{\mathrm{D\rightarrow N}}^{\mathrm{calc,pH6.3}}$ in Figure 8 according to eq 6. [d] The values are derived from those of ‡ and the refolding kinetics in Figure 8 ($k_{\mathrm{f}}^{\mathrm{pH6.3}}$, eq 6).

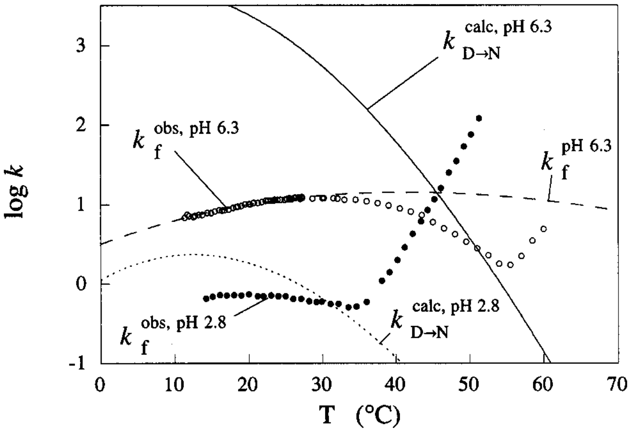

FIGURE 8: Temperature dependence of the rate constant for refolding from the transient intermediate. The rate constant has units of s^{-1}. The observed refolding rate constant ($k_{\mathrm{f}}^{\mathrm{obs}}$) at pH 6.3 (○) and pH 2.8 (●). The kinetic data at pH 2.8 include unfolding rate constants obtained by acid jump experiment, pH 6.3 → pH 2.8. The dashed line shows the temperature dependence of the rate constant associated with refolding from the transient intermediate at pH 6.3 ($k_{\mathrm{f}}^{\mathrm{pH6.3}}$) (see Figure 7, accompanying paper) and the solid line the rate constant expected for refolding from the fully denatured state at pH 6.3 ($k_{\mathrm{D\rightarrow N}}^{\mathrm{calc,pH6.3}}$). The two lines intersect at 47 °C where the free energy of the intermediate equals that of the denatured state. At pH 2.8, the rate constant predicted from a two-state behaviour (dotted line, $k_{\mathrm{D\rightarrow N}}^{\mathrm{calc,pH2.8}}$) does not deviate from the observed kinetics as much as observed at pH 6.3.

converges to two-state behavior, since at this temperature the components of the preequilibrium constitute the denatured state (D) in the thermal unfolding transition. It is important to realize, however, that D at this temperature may not represent the fully unfolded state but could still constitute a distribution of partly consolidated conformations that unfold further at higher temperatures [cf. model in Griko *et al.* (1994)].

Under acid conditions, where barnase approaches two-state behavior at room temperature, the refolding kinetics behave somewhat differently from that observed at pH 6.3: the difference between $k_{\mathrm{D\rightarrow N}}^{\mathrm{calc}}(T)$ and $k_{\mathrm{f}}^{\mathrm{obs}}(T)$ increases only marginally with decreasing temperature and remains constant (or even decreases) below ~15 °C (Figure 8). There are two possible explanations for this. The most straightforward is that, at low pH, the occupancy of the intermediate does not continue to increase with decreasing temperatures but reaches a maximum just below the transition region. Interestingly, the highest value of $\Delta G_{\mathrm{D\text{-}I}}^{\mathrm{app}}$, which is ~0.3 kcal/mol at 15 °C (eq 4), is only a fraction of that observed at pH 6.3 and corresponds to at most 20% occupancy of the intermediate in Scheme 1. A behavior such as this may at first seem unlikely but can be understood in terms of cold unfolding of a marginally stable conformation with a heat capacity lower than that for the unfolded state (Privalov *et al.*, 1986; Nishii *et al.*, 1994; see further discussion below).

The alternative (and more attractive) explanation is that, at low pH, the difference between $k_{\mathrm{D\rightarrow N}}^{\mathrm{calc}}(T)$ and $k_{\mathrm{f}}^{\mathrm{obs}}(T)$ does not reflect directly the stability of I but is a consequence of the experimental procedure: if the preequilibrium ($\mathrm{D} \rightleftarrows \mathrm{I}$) shows a pronounced dependence on temperature but is only marginally affected by pH, then the protein may refold from the same denatured conformation (say I) throughout the pH range, providing that the temperature is kept constant. Interestingly, the two-state behavior observed at low pH and 25 °C (Figure 9) is then not a consequence of I being depopulated in the preequilibrium but of the stability measurements being carried out at temperatures where I, and not D, constitutes the denatured state (Figure 10). These measurements will yield the equilibrium constant between N and I [$K_{\mathrm{I\text{-}N}}(T)$], and, consequently, the refolding rate constant calculated from $K_{\mathrm{I\text{-}N}}(T)k_{\mathrm{u}}$ will relate to the free-energy difference between I and ‡. This will result in two-state behavior since $k_{\mathrm{I\rightarrow N}}^{\mathrm{calc}}(T) \equiv k_{\mathrm{f}}(T)$ (cf. Figure 7 in accompanying paper). In other words, the system will show two-state behavior as long as the denatured state of the thermal unfolding transition is the same as the ground state for the refolding reaction, even if this happens to be a partly structured conformation. Consistent with this idea, the deviation from two-state behavior starts to occur above pH 2.7 at $\mu = 50$ mM and above pH 2.4 at $\mu = 600$ mM, both of which represent the pH at which T_{m} is 33 °C (Figure 9). As seen in Figure 8, 33 °C is also the temperature where the preequilibrium starts to shift from the intermediate to the denatured state at pH 6.3 (cf. the phase diagram in Figure 10). On this basis, it may be concluded that I, or actually its protonated form, is the predominant denatured species at equilibrium at temperatures below 33 °C, irrespectively of the pH. A factor that may well contribute to the incomplete unfolding of I ($\equiv \mathrm{D}^{\mathrm{phys}}$) with acid is the anamolous titration properties of the more unfolded conformations. For example, the thermally denatured state shows carboxylate pK_{A} values which are on average 0.4 units lower than those of model compounds (Oliveberg *et al.*, 1995b). If the pK_{A} values of more unfolded conformations are similar to those of I, i.e., titrate in a similar way, the destabilization by acid will be small [cf. Oliveberg *et al.* (1994)].

It should be noted that the latter interpretation does not exclude that the preequilibrium is also affected by cold unfolding and/or pH. In fact, recent studies on mutant proteins imply that a single buried salt bridge is maintained in the acid form of I, and that this to some extent destabilizes I at low pH (M.O. and A.R.F., unpublished results). However, the general interpretation of a weak pH dependence of I is still valid and exemplifies well how temperature-induced changes of the preequilibrium can perturb the analysis of stability and titration data. Also, it accounts for the ambiguous pH dependence of the kinetics relating to the folding intermediate [see accompanying paper (Oliveberg & Fersht, 1996)]. It must be realized, however, that although

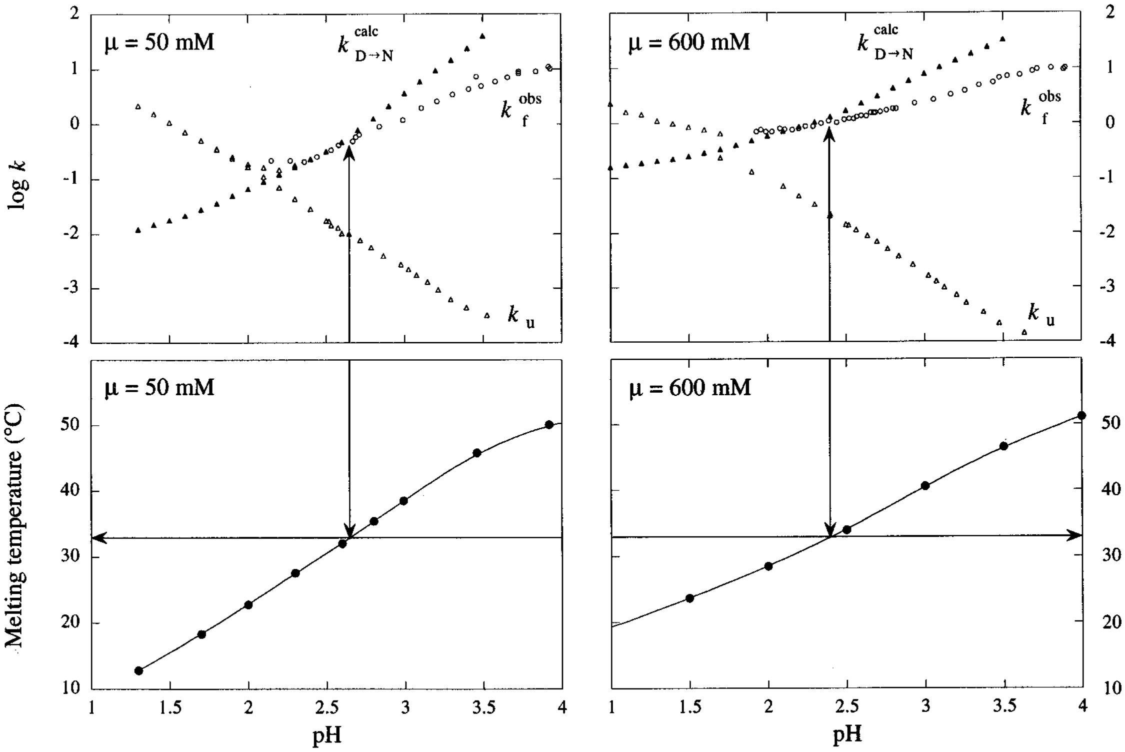

FIGURE 9: Comparison of the observed rate constants for refolding (k_f^{obs}) (○) and those calculated for a two-state transition ($k^{calc}_{D\to N}$) (▲) versus pH. The rate constant has units of s^{-1}. $k^{calc}_{D\to N}$ is calculated from the unfolding rate constant (k_u) (△) and the value of $K_{D\text{-}N}$ (eq 2). $k^{calc}_{D\to N}$ and k_f^{obs} converge and exhibit two-state behavior at pH values where the melting temperature of the native protein is below 33 °C. The top panels show the pH dependence of the kinetics at $\mu = 50$ mM and $\mu = 600$ mM (adapted from Figure 9 in the accompanying paper). The lower panels show the temperature of the midpoint for the thermal transition of barnase at $\mu = 50$ mM and $\mu = 600$ mM. At 33 °C, the unfolding transition for native barnase takes place around pH 2.7 at $\mu = 50$ mM and around pH 2.4, at $\mu = 600$ mM. Interestingly, these are the pH-values below which the kinetics show a two-state unfolding transition at 25 °C. Along with other observations, the result suggests that, irrespective of the pH, thermal transitions which take place below 33 °C produce a denatured species which is equivalent to the folding intermediate (I = D^{phys}).

the protonated form of I is energetically indistinguishable from the folding intermediate at pH 6.3, it may not have the same structure.

Are Molten-Globule Conformations Protonated and Reorganized Forms of Folding Intermediates? Since characterization of the acid-denatured state by NMR reveals only very few fixed interactions (Arcus *et al.*, 1994) whereas the folding intermediate at pH 6.3 has considerable structure as revealed by protein engineering (Matouschek *et al.*, 1992), it is apparent that the protonation of I causes structural reorganization. It has been observed that specific ionic interactions within the protein contribute little to the stability, i.e., if both partners of a buried salt link are substituted isosterically with hydrophobic moieties, this will not significantly destabilize the protein (Hendsch & Tidor, 1994). With partly structured conformations like folding intermediates, it is possible that protonation of a salt link will cause the loose structure to reorganize isoenergetically in order to better solvate the hydrophilic residues and to fill any resulting cavity with hydrophobic groups. This will lead to disruption of several tertiary interactions and a redistribution of charged and polar residues to the surface of the protein. The core of the protonated folding intermediate will then ultimately consist of a dense but "molten" cluster of hydrophobic residues, consistent with the properties typically observed for molten globule states (Ptitsyn, 1992). The only direct relation between folding intermediates and molten globules has been observed with apomyoglobin (Jennings & Wright, 1993). Here, the folding intermediate has the same pattern of protected NH groups as the equilibrium molten globule, hence, suggesting that these two conformations have similar structure. Interestingly, the study of Jennings and Wright refers to the *deprotonated* "molten globule" at pH 4.2 and the folding intermediate at pH 6.1 which, according to above, are predicted to be the same species.

Comparison of the Properties of the Acid-Denatured State and the Folding Intermediate. (i) The Aggregation Behavior. In several other studies, partially structured molten-globule states are found to be "sticky" and susceptible to aggregation (Kuwajima, 1989; Ptitsyn, 1992). In this study, the observed aggregation/association behavior follows precisely the predicted occupancy of the protonated intermediate: the aggregation/association is most pronounced around room temperature, starts to decrease above 35 °C, and disappears finally above 50 °C. This is in good agreement with recent NMR data which show that the acid-denatured state contains more structure at 30 °C than at ~60 °C (Arcus *et al.*, 1995). According to the small ionic-strength dependence of the refolding kinetics (Figure 1), the presence of salt has only a marginal effect on the D $\rightleftarrows$ I equilibrium. However, the salt decreases intermolecular repulsion and may, hence, promote aggregation/association of denatured monomers.

(ii) ANS and Fluorescence Data. The changes in fluorescence associated with expulsion and binding of ANS imply that the denatured protein (D and I), irrespective of the pH, binds ANS at temperatures below ~30 °C, where it is significantly compact, but not above ~50 °C, where it is substantially unfolded (Figures 4 and 10).

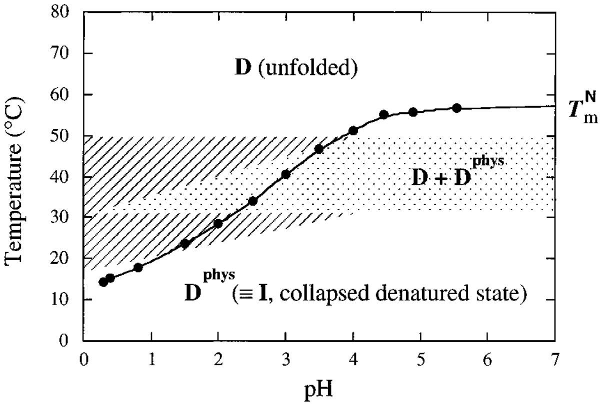

FIGURE 10: Phase diagram which shows the most stable denatured conformations of barnase versus pH and temperature. The data points connected by a solid black line show the melting temperature for the native protein (T_m^N): denatured conformations which are shown above T_m^N can be isolated under equilibrium conditions, whereas the denatured conformations which are shown below T_m^N can be observed only in the kinetic preequilibrium of the refolding reaction. D denotes the denatured species which is obtained by thermal unfolding experiments at pH 6.3. D^{phys} denotes the denatured species in the kinetic preequilibrium at pH 6.3 and 25 °C, which constitutes the most stable denatured conformation under physiological conditions, i.e., the folding intermediate. The dotted area indicates conditions where both D and D^{phys} are populated, i.e., the D $\rightleftarrows$ D^{phys} transition region. The striped areas represent the uncertainty of the phase interfaces; the uncertainty of the nature of denatured state in these regions is mainly due to unclear or contradicting experimental observations but reflects to some extent variations of the denatured conformation with ionic strength. It is suggested that the structure of D^{phys} undergoes an iso-energetic reorganisation as D^{phys} becomes protonated below pH 4, and that the protonated form of D^{phys} may constitute a so-called molten globule conformation.

In this context, it can be mentioned that the change in the intrinsic fluorescence of the protein upon refolding from pH 1.5 to 6.3 increases significantly between 30 and 45 °C, although, generally, the overall fluorescence is expected to decrease with increasing temperatures (data not shown). This observation is consistent with a gradual shift of the preequilibrium, from a fluorescent partially structured conformation around 25 °C toward a less fluorescent and more unfolded state at higher temperatures.

(iii) CD Spectra of the Acid-Denatured State and the Folding Intermediate. The far-UV CD spectrum of the components in the preequilibrium at pH 6.3 is similar to that of the acid-denatured state (Figure 5). Why the thermally denatured state also has an equivalent spectrum is less clear. A possible explanation is provided by Privalov *et al.* (1989), who suggest that the denatured state acquires a higher degree of compactness at elevated temperatures because of the increased hydrophobic effect. That the thermally denatured state is compact is also supported by its anomalous titration properties (Oliveberg et al., 1995b). The CD properties may then be a consequence of the gradual thermal unfolding of the intermediate being compensated by an increasingly collapsed thermally denatured state.

Investigation of the I $\rightleftarrows$ D Transition by Pressure-Jump and Temperature-Jump Experiments. In an attempt to resolve the rapid interconversion between D and I, fluorescence and absorbance monitored rapid-relaxation experiments were carried out under conditions where both D and I are expected to be present at equilibrium (cf. Figure 10).

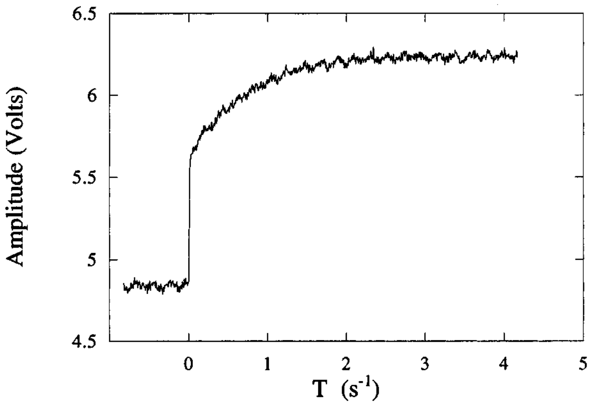

FIGURE 11: Example of the refolding relaxation as observed by pressure-jump experiments under acid conditions at 25 °C. The dead-time of the experiment is ~60 μs. The initial rapid increase (step) in fluorescence follows the time course of the pressure release and corresponds well to the pressure effect on the fluorescence. The step is followed by a slower increase in fluorescence which shows the refolding over the main transition state ‡. Pressure-jump experiments were carried out systematically at conditions where both D and D^{phys} are predicted to be populated at equilibrium, i.e., between pH 1 and 4 and between 10 and 50 °C (see phase diagram in Figure 10). However, no rapid phase corresponding to the interconversion of D and D^{phys} can be detected.

By affecting the water structure, the elevated pressures obtained in a pressure-jump experiment will favor the more tightly hydrated anionic forms of the buffer molecules, causing a decrease in pH. Upon rapid release of the pressure, the perturbed pH will relax back to its original value, shifting the protein equilibrium toward more folded conformations. Pressure jumps from 700 to 1 bar and with a dead-time of 60 μs were performed in the transition regions as well as under denaturing conditions, at a variety of pH values, urea concentrations, and temperatures. The results, however, show only relaxations over the main transition state (‡), and no additional fast phase associated with the D $\rightleftarrows$ I relaxation can be resolved (Figure 11).

Since the D $\rightleftarrows$ I equilibrium displays a pronounced temperature dependence, we carried out temperature-jump experiments under a series of conditions, in particular at low pH and 20−40 °C, where a D $\rightleftarrows$ I relaxation is predicted from the kinetics. As with the pressure jumps, however, the only observed relaxation is the slow interconversion over the major transition state (Martin Long and A.R.F., data not shown).

The fact that no rapid phases are observed suggests that the relaxation of the preequilibrium is too fast to be resolved and takes place in the submicrosecond time scale or that the intermediate and the denatured state are spectroscopically indistinguishable. The latter explanation, however, is made unlikely by the observed temperature dependence of the refolding amplitudes; these imply that the fluorescence of the intermediate is higher than that of more unfolded conformations. In addition, data on mutants suggest that the structure of the intermediate retains significant interactions with the main fluorophore Trp 71 in hydrophobic core 3 (Matouschek *et al.*, 1992).

Heat Capacities of the Major Transition State and the Folding Intermediate. The heat capacity, C_p [cal/(mol·K)], of a protein is defined as the energy required to raise the temperature of a protein−water solution 1 K relative to that of pure water. It is generally assumed that the main component of C_p is not caused by the protein itself but by the extra heat required to gradually melt pockets of ordered water molecules forming the interface between exposed hydrophobic residues and the solvent (Sturtevant, 1977). Accordingly, the heat capacity of the folded state of the protein is less than that of its fully hydrated denatured conformation.

A difference in heat capacity between any two protein conformations, say A and B, gives rise to a temperature dependence of $\Delta H_{\mathrm{A\text{-}B}}$ and $\Delta S_{\mathrm{A\text{-}B}}$ according to

$$\Delta C_p^{\mathrm{A-B}} = \frac{\delta(\Delta H_{\mathrm{A-B}})}{\delta T} = T\frac{\delta(\Delta S_{\mathrm{A-B}})}{\delta T} \qquad (5)$$

which is most clearly reflected as a curved temperature dependence of $\Delta G_{\mathrm{A\text{-}B}}$ (the Gibbs−Helmholtz relation). Equation 5 can be directly incorporated with the transition-state theory [eqs 1 and 2 in the accompanying paper (Oliveberg & Fersht, 1995)] to give

$$\begin{aligned}\log k = \log\left(\frac{k_{\mathrm{B}}T}{h}\right) - \frac{1}{2.3RT}[\Delta H^{\ddagger}(\mathrm{pH}) - T\Delta S^{\ddagger}(\mathrm{pH})] = \\ \log\left(\frac{k_{\mathrm{B}}T}{h}\right) - \frac{1}{2.3RT}[\Delta H^{\ddagger}(T_0) + \Delta C_p^{\ddagger}(T - T_0) - \\ T\Delta S^{\ddagger}(T_0) + T\Delta C_p^{\ddagger}\ln(T/T_0)] \quad (6)\end{aligned}$$

where $\Delta C_p^{\ddagger}$ is the activation heat capacity, i.e., the change in heat capacity between the ground state and the transition state [cf. Chen *et al.* (1989)]. When $\Delta C_p^{\ddagger} \neq 0$, the Eyring plot is no longer linear but displays a curvature.

The apparently linear Eyring plots of the unfolding rate constant (Figure 6 in this paper and Figure 3 in the accompanying paper) reveal that the change in heat capacity between the native state and the major transition state ($\Delta C_p^{\ddagger\text{-N}}$) is relatively small. Accordingly, the main change in heat capacity upon unfolding takes place between D and ‡. The large difference in C_p between D and ‡ is manifested in the strong curvature of the calculated refolding rate constant $k_{\mathrm{D\rightarrow N}}^{\mathrm{calc}}(T)$ which, when fitted to eq 6, yields a value of $\Delta C_p^{\ddagger} \approx 1500$ cal/(mol•K) (Figure 8 and Table 1). The total change in heat capacity upon unfolding ($\Delta C_p^{\mathrm{D\text{-}N}}$) is about 1880 cal/(mol•K) (Oliveberg et al., 1994). Since changes in heat capacity reflect mainly exposure of nonpolar side chains to water, the values of $\Delta C_p^{\mathrm{D}\text{-}\ddagger}$ and $\Delta C_p^{\mathrm{D\text{-}N}}$ suggest that the exposure of hydrophobic residues in the transition state remains similar to that in the native protein. In other words, the partial loosening up of the structure in the transition state for unfolding appears to involve only a small increase in the exposure of nonpolar groups, and does not greatly alter the overall hydration shell of the protein. As a comparison, the ratio of $\Delta C_p^{D\text{-}\ddagger}$ and $\Delta C_p^{\mathrm{D\text{-}N}}$ is ∼0.8 (Figure 12), which agrees well with the value for "relative exposure of hydrophobic residues" in ‡, calculated from the *m* values for denaturation with urea (see Figure 11 in the accompanying paper). The enthalpy difference between N and ‡ ($H_{\mathrm{N}} - H_{\ddagger}$) is ~ 57.5 kcal/mol (eq 6, Table 1, and Figure 12), and the entropy difference between N and ‡ ($S_{\mathrm{N}} - S_{\ddagger}$) is ∼115 cal/(mol•K) (eq 6, Table 1, and Figure 12).

Analogously, the heat capacity of the transient intermediate at pH 6.3 relative to that of the transition state is obtained from the curvature of $k_{\mathrm{f}}(T)$ (Figure 8) since this is determined by the change in free energy between I and ‡. Because of variations in the preequilibrium (D $\rightleftarrows$ I), the temperature dependence of $k_{\mathrm{f}}(T)$ can be obtained from the observed refolding rate constant [$k_{\mathrm{f}}^{obs}(T)$] under conditions only where the preequilibrium is substantially shifted toward I and, hence, its occupancy (C) is close to one, i.e., $k_{\mathrm{f}}^{obs}(T) \approx Ck_{\mathrm{f}}(T)$ (cf. eq 1). Fitting eq 6 to $k_{\mathrm{f}}^{obs}(T)$ at temperatures below 25 °C, where $\Delta G_{\mathrm{D\text{-}I}}$ is at least 2.5 kcal/mol, $C > 0.99$, and $k_{\mathrm{f}}^{obs}(T) \approx k_{\mathrm{f}}(T)$, yields a heat-capacity change of 289 ± 54 cal/(mol•K) between I and ‡ (Figure 8 and Table 1). The enthalpy of the intermediate relative to the denatured state $\Delta H_{\mathrm{D\text{-}I}}$ is derived from $\Delta H_{\mathrm{D}\text{-}\ddagger}$, which yields a value of 31 kcal/mol (Table 1). In the same way, $\Delta S_{\mathrm{D\text{-}I}}$ is determined to 94 cal/(mol•K). It would have been interesting to see how the heat capacity and the thermodynamics of I change as it becomes protonated at lower pH. At this stage, however, the uncertainties about the data obtained at low pH do not allow such a detailed analysis.

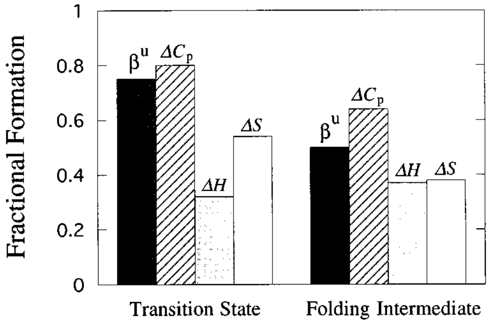

FIGURE 12: Degree of formation (reaction coordinates) of the transition state and the folding intermediate, as determined by their thermodynamic characteristics: ΔC_p, ΔH, and ΔS. For comparison, we have included the corresponding reaction coordinate determined by urea denaturation experiments (β^{u}) which is believed to reflect the degree of solvent exposure of hydrophobic residues. As predicted, this shows good agreement with the relative change in ΔC_p which is also related to the exposure of hydrophobic surface area. There is only a small change in enthalpic interactions and total entropy between the intermediate and the transition state, and from a thermodynamic point of view these two conformations appear quite similar: the important difference may be the transition state is more dehydrated and contains a more extensive network of intramolecular charge interactions.

Although the values for the intermediate listed in Table 1 may be subject to a considerable error because of the limited temperature range used for the fit of $k_{\mathrm{f}}(T)$, it is evident that the heat capacity of the intermediate is significantly lower than that of the denatured state; the degree of hydrophobic exposure in I (reaction coordinate) is $\Delta C_p^{\mathrm{D\text{-}I}}/\Delta C_p^{\mathrm{D\text{-}N}} = 0.64$ (cf. 0.8 for ‡, Figure 12). The result suggests that the structure of the intermediate at pH 6.3 only partially involves exposure of nonpolar groups to water and is consistent with the high content of structure determined previously for the transient intermediate by the ϕ-value analysis (Matouschek et al., 1992).

Interestingly, the high value of $\Delta C_p^{\mathrm{D\text{-}I}}$ in combination with small changes in enthalpy upon unfolding of the intermediate predicts "cold unfolding" of the intermediate at relatively high temperatures (Privalov et al., 1986; Nishii et al., 1994). In particular, at low pH or in the presence of other denaturants, where the stability and $\Delta H_{\mathrm{D\text{-}I}}$ may be even further reduced, cold destabilization could start to occur at room temperature. Cold unfolding may thus constitute an important factor in the energetics of poorly hydrated denatured conformations and could lead to intermediate states occurring only in a narrow temperature range.

Thermally Induced Changes in the D $\rightleftarrows$ I Equilibrium May Perturb Estimates of $\Delta C_p^{\text{D-N}}$. The change in heat capacity upon denaturation of barnase ($\Delta C_p^{\text{D-N}}$) has previously been determined to be 1400−1880 cal/(mol·K) (Griko *et al.*, 1994; Martinez *et al.*, 1994; Oliveberg *et al.*, 1994). If calculated per residue, this value appears considerably higher than the heat capacities observed for other small monomeric proteins (Makhatadze & Privalov, 1990). It has been put forward that the (thermally) denatured state of barnase is unusually extended and, therefore, more hydrated than other proteins (Martinez *et al.*, 1994). In view of the results in this study, however, it may be suggested that the value of $\Delta C_p^{\text{D-N}}$, as determined from the temperature dependence of $\Delta H_{\text{D-N}}$, is an overestimate: the enthalpy derived from thermal transitions taking place at temperatures below ∼45 °C represents unfolding to the intermediate and is, hence, up to 30 kcal/mol lower than $\Delta H_{\text{D-N}}$ (cf. Table 1).

CONCLUSIONS

There are four identifiable conformations in the folding pathway of barnase: the denatured state (D), the folding intermediate (I), the major transition state (‡), and the native state (N); D $\rightleftarrows$ I $\rightleftarrows$ ‡ $\rightleftarrows$ N.

The major transition state (‡) is an expanded form of the native protein: parts of its structure appears compact and native-like whereas its surface regions are significantly loosened and solvated. The overall hydration of ‡ is similar to that of the native protein ($\Delta C_p^{\text{D-‡}}/\Delta C_p^{\text{D-N}} = 0.8$), i.e., ‡ is a "dry" conformation with a poorly hydrated interior. The transition state contains a few strong ionic interactions, which are manifested in highly perturbed pK_A values (≤ 2) for some if its carboxylate groups. However, the electrostatic interactions which are localized in the surface regions of the native protein are not consolidated in ‡. The disruption of N into ‡ involves a 68% loss of enthalpy and a 46% gain in entropy (Table 1).

The folding intermediate (I) constitutes the most stable denatured conformation of barnase under physiological conditions (I $\equiv$ D^{phys}) and is 2−3 kcal/mol more stable than the thermally denatured state (D) at pH 6.3 and 25 °C. The equilibrium between D and I shows little dependence on pH, and it appears that I cannot be completely unfolded by acid. As a consequence, the protonated form of I may be populated at equilibrium at low pH and constitute part of the acid-denatured protein (Figure 10). It must be realized, however, that the present approach is based on kinetic and thermodynamic data, and, hence, the denatured conformation distinguished as the protonated folding intermediate means a protein state energetically equivalent to I at pH 6.3. The energetic similarity, however, may not be directly linked to a specific spatial conformation: it is possible that protonation of I induces significant structural rearrangements (disruption of salt links followed by solvation of charged residues) that are energetically compensated for by an increased overall compactness, or changes in the hydration. This could explain why NMR studies of the acid denatured state at pH 1.9 and 30 °C (Arcus et al., 1994) reveal much less structure than implied for the energetically equivalent intermediate at pH 6.3. Along these lines, it may be speculated that protonated forms of folding intermediates constitute the, so-called, molten globule conformations which are sometimes observed at low pH and high ionic strength.

The equilibrium between D and I shows a pronounced dependence on temperature, and at pH 6.3 the transition midpoint between D and I takes place around 47 °C. Around and below this temperature, barnase displays multistate folding behavior and refolds via transient accumulation of I, whereas above ∼50 °C I does not accumulate in the refolding process and the protein displays two-state behavior. This shows that the presence of a folding intermediate is not synonymous with a certain type of folding mechanism, and that a change from multistate to two-state behavior can be induced simply by changing the experimental conditions. Hence, multistate and two-state folding behavior may well reflect a common folding mechanism.

The interconversion between D and I is fast and takes place in a submicrosecond time scale. This implies that any cooperative transition between the two conformations proceeds over a relatively low transition state, or that D and I represent the end points in a continuum of denatured conformations whose average properties display activationless changes with denaturant.

The heat capacity of I at pH 6.3 is significantly lower than that for D, $\Delta C_p^{\text{D-I}}/\Delta C_p^{\text{D-N}} = 0.64$. Together with the finding that I displays no clear perturbation of its pK_A values, this suggests that I is sufficiently expanded and hydrated to reduce electrostatic forces but still contains poorly hydrated clusters of hydrophobic residues. The conversion of N into I is accompanied by a 63% loss of enthalpy and a 62% gain in entropy (Table 1).

To this end, we are now directing mutational and structural studies to understand better the relationship between structure and energetics under conditions where the protein adopts partly structured, and possibly fluctuating, denatured conformations.

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