Movement of the Position of the Transition State in Protein Folding

Andreas Matouschek,[‡] Daniel E. Otzen,[§] Laura S. Itzhaki, Sophie E. Jackson, and Alan R. Fersht*

MRC Unit for Protein Function and Design and Cambridge Centre for Protein Engineering, University Chemical Laboratory, Lensfield Road, Cambridge CB2 1EW, U.K.

Received June 6, 1995; Revised Manuscript Received August 17, 1995[⊗]

ABSTRACT: Hammond behavior, in which two neighboring states move closer to each other along the reaction coordinate as the energy difference between them becomes smaller, has previously been observed for the transition state of unfolding of barnase. Here, we report Hammond behavior for the small protein chymotrypsin inhibitor 2 (CI2), which folds and unfolds via a single rate-determining transition state and simple two-state kinetics. Mutants have been generated along the entire sequence of the protein and the kinetics of folding and unfolding measured as a function of concentration of denaturant. The transition state was found to move progressively closer to the folded state on destabilization of the protein by mutation. Different regions of CI2 all show a similar sensitivity to changes in the energy of the transition state. This is in contrast to the behavior of barnase on mutation for which the position of the transition state for its unfolding is sensitive to mutation in some regions, especially in its major α-helix, but not in others. The transition state for the folding and unfolding of CI2 resembles an expanded version of the folded state and is formed in a concerted manner, in contrast to that for barnase, in which some regions of structure are fully formed and others fully unfolded. The reason for the general sensitivity of the position of the transition state of CI2 to mutation is presumably the relatively uniform degree of structure formation in the transition state and the concerted nature of its formation. *Hammond behavior was also observed for both CI2 and barnase when the temperature and denaturation conditions of the unfolding reaction are altered.* For both proteins, the position of the transition state of protein folding is more sensitive to changes in energy than is observed for the changes of covalent bonds in organic chemistry and enzyme catalysis. The finding of Hammond behavior for two proteins suggests that it is a general phenomenon in protein folding. The movement of the transition state on changing the conditions does have implications in the interpretation of computer simulations of protein folding done under extreme conditions.

The position of the transition state of protein folding can be moved along the reaction coordinate by mutagenesis (Matouschek & Fersht, 1993; Matthews & Fersht, 1995). If the difference in energy between the transition and folded state is reduced by making mutations in either the major α-helix or the main hydrophobic core of the ribonuclease barnase, the two states approach each other on the reaction coordinate according to Hammond (1955) behavior, which is well established in classical organic chemistry. In this paper, we show that this movement of the transition state is more general in protein folding. Hammond behavior is here observed for a protein other than barnase and can be produced by means other than mutagenesis. We compare the sensitivity to changes in energy of the transition state of protein folding, which involves changes in just noncovalent interactions, with that of the transition state in simple organic reactions and enzyme catalysis, which involves covalent bond changes, and find that the transition state of folding is the more sensitive.

Barley chymotrypsin inhibitor 2 (CI2) is a small protein with a molecular mass of 7300 Da. Its folding and unfolding pathways have been characterized extensively by protein engineering (Jackson & Fersht, 1991; Jackson *et al.*, 1993a,b; Otzen *et al.*, 1994). The folding of CI2 was the first example found of a classical two-state transition in which only the native and denatured states are significantly populated both in kinetic experiments and at equilibrium (Jackson & Fersht, 1991). The structure of the rate-limiting transition state has been mapped from kinetic and equilibrium measurements of the folding and unfolding of mutants and shown to resemble an expanded form of the folded structure (Otzen *et al.*, 1994). Here, we show that mutations in different regions of CI2, namely, in two hydrophobic cores, the helix, the β-sheet, and loop and turn regions, lead to a relative movement of the positions on the reaction coordinate of the rate-limiting transition state and the folded state.

The energy difference between the transition state and the folded state can also be changed by variation of the reaction temperature and solvent conditions. The unfolding rate constants are increased and the energy difference between the ground and transition state reduced by increasing the temperature or by increasing the denaturant concentration. In both cases, we observe a movement of the transition state along the reaction coordinate for barnase and CI2.

The position of intermediates and transition states on the reaction coordinate of chemical and biochemical processes can frequently be determined by the application of linear free energy relationships (LFERs) (Warshel *et al.*, 1994). The most commonly applied LFER is the Brønsted relationship where changes in the equilibrium constant of a reaction are related to the change in the rate constants. The slope β

* To whom correspondence should be addressed.

[‡] Present address: Biocenter, University of Basel, Klingelbergstrasse 70, 4056 Basel, Switzerland.

[§] Supported by a predoctoral grant from the Danish Natural Sciences Research Council. Present address: Enzyme Function, Novo Nordisk A/S, Novo Allé, DK-2880 Bagsvaerd, Denmark.

[⊗] Abstract published in *Advance ACS Abstracts,* September 15, 1995.

0006-2960/95/0434-13656$09.00/0

of the observed linear relationship between the free energy of activation and the equilibrium free energy is an approximate measure of the position of the transition state on the reaction coordinate. In protein folding experiments, an estimate of β for the rate-limiting transition state [which we call β_T, with the subscript T in recognition of Tanford (1968, 1970), who introduced much of the relevant theory] can be obtained from the dependencies on the denaturant concentration of the free energy of activation for unfolding ($\Delta G_{\ddagger\text{-F}}$) and the equilibrium free energy of unfolding ($\Delta G_{\text{U-F}}$) (Matouschek & Fersht, 1993):

$$G_{\text{U-F}} = \Delta G_{\text{U-F}}^{\text{H}_2\text{O}} - m_{\text{U-F}}[\text{denaturant}] \quad (1)$$

$$G_{\ddagger\text{-F}} = \Delta G_{\ddagger\text{-F}}^{\text{H}_2\text{O}} - m_{\ddagger\text{-F}}[\text{denaturant}] \quad (2)$$

$$\beta_T = \frac{m_{\ddagger\text{-F}}}{m_{\text{U-F}}} \quad (3)$$

where $\Delta G_{\ddagger\text{-F}}^{\text{H}_2\text{O}}$ is the free energy of activation for unfolding in water and $\Delta G_{\text{U-F}}^{\text{H}_2\text{O}}$ is the equilibrium free energy of unfolding in water. $m_{\text{U-F}}$ and $m_{\ddagger\text{-F}}$ are constants of proportionality for the dependence of free energy changes on denaturant concentration. Equations 1 and 2 hold over small ranges of [denaturant] but are definitely curved over extended ranges of [urea] for the unfolding of barnase (Matouschek *et al.*, 1994; Johnson & Fersht, 1995). It is thus better to define $m_{\text{U-F}} = \partial\Delta G_{\text{U-F}}/\partial[\text{denaturant}]$ and $m_{\ddagger\text{-F}} = \partial\Delta G_{\ddagger\text{-F}}/\partial[\text{denaturant}]$ over the ranges of [denaturant] employed.

We have shown previously how β_T for the transition state of folding in barnase is affected by mutations by constructing plots of β_T versus $\Delta\Delta G_{\ddagger\text{-F}}$, which we call Hammond plots (Matouschek & Fersht, 1993). For the set of mutations in barnase that we have analyzed, the relationship is roughly linear, and we can define a new variable, γ, as the mean slope of the Hammond plot over the observed range

$$\gamma = \frac{\partial\beta_T}{\partial\Delta G_{\ddagger\text{-F}}} \quad (4)$$

γ is a measure of the sensitivity of the position of the transition state on the reaction coordinate to changes in energy so that

$$\beta_T = \beta_o + \gamma\Delta\Delta G_{\ddagger\text{-F}} \quad (5)$$

The calculation of β_T from the ratios of $m_{\ddagger\text{-F}}$ and $m_{\text{U-F}}$ for proteins for which $m_{\ddagger\text{-F}}$ and $m_{\text{U-F}}$ vary with [denaturant] should ideally use values of $m_{\text{U-F}}$ that are measured over the same range of [denaturant]. It is not easy to measure $m_{\text{U-F}}$ outside a narrow range of [denaturant], and the demonstration of curvature in plots of $\Delta G_{\text{U-F}}$ versus [urea] for barnase required a combination of differential scanning calorimetry and urea-mediated denaturation (Johnson & Fersht, 1995). However, the important variable when examining a change of β_T on mutation is $m_{\ddagger\text{-F}}$ since it is found experimentally and expected theoretically that values of $m_{\text{U-F}}$ change relatively little, if at all, on mutation of CI2 and barnase (Serrano *et al.*, 1992a).[1] The use of a value $m_{\text{U-F}}$ made at a somewhat different concentration of denaturant leads to a small error in β_T which does not lead to a gross change in interpretation of data. (In any case, β_T is only a very rough measure of the position of the transition state on the reaction coordinate, and it is the changes in β_T that are of major interest here.) What is important, however, is to compare the values of $m_{\ddagger\text{-F}}$ that are made over the same ranges of [denaturant] for the different mutants.

For mutations in the main hydrophobic core of barnase we found that $\beta_o = 0.31 \pm 0.01$ and $\gamma = 0.02 \pm 0.01$ mol/kcal, and for mutations in the major α-helix $\beta_o = 0.31 \pm 0.01$ and $\gamma = 0.036 \pm 0.03$ mol/kcal for unfolding in 7.25 M urea at 25 °C (Matouschek & Fersht, 1993; Matthews & Fersht, 1995).

MATERIALS AND METHODS

All the mutations discussed here have been described (Serrano *et al.*, 1992; Jackson *et al.*, 1993c; Otzen *et al.*, 1994). The proteins were expressed and purified as described.

The rate constants of protein unfolding and refolding were measured as described (Matouschek *et al.*, 1989, 1990; Jackson & Fersht, 1991; Jackson *et al.*, 1993b). Unfolding was initiated by rapidly diluting one volume of protein (~20 μM) in 50 mM MES, pH 6.3, with 10 volumes of concentrated denaturant solution containing 50 mM MES, pH 6.3. This results in a final urea concentration between 3.5 and 9 M in the case of barnase and in final guanidinium chloride (GdmCl) concentrations between 3 and 7 M for CI2. The lowest denaturant concentration was chosen to result in at least 98% unfolded protein. In most cases, the experiments were performed at 25 °C. For wild-type barnase, they were repeated at 15, 20, 30, and 35 °C and for wild-type CI2 at 17, 21, 29, and 32 °C. For CI2, refolding experiments were also performed. In a pH-jump experiment, 20 μM CI2 was acid-denatured by adding HCl to 20 mM, which lowers the pH of the solution to 1.7. The solution was rapidly diluted with an equal volume of strongly buffered solution to give a final pH of 6.3. The final GdmCl concentration was varied between 0 and 0.6 M. Under these conditions, the protein becomes at least 99% folded. Unfolding was followed by monitoring the intrinsic fluorescence of barnase or CI2 using a Perkin Elmer MPF 44 B fluorescence spectrophotometer fitted with a stopped flow mixing head (bandwidths 5−10 nm; excitation and emission wavelengths as in the equilibrium experiments). Refolding kinetics was measured using a thermostatted Applied Photophysics Stopped-Flow Spectrophotometer Model SF 17MV. Excitation was at 280 nm, and emission at wavelengths greater than 315 nm was collected via a cut-off filter.

Unfolding kinetics were analyzed according to eq 2. At a given denaturant concentration, the changes upon mutation in the difference in free energy between the rate-limiting transition state and the folded state, $\Delta\Delta G_{\ddagger\text{-F}}$, can be determined from the rates of protein unfolding (Matouschek *et al.*, 1989; Jackson *et al.*, 1993c):

$$\Delta\Delta G_{\ddagger\text{-F}} = -RT \ln \frac{k_u'}{k_u} \quad (6)$$

where the prime indicates mutant. Unfolding data are used in the linear range of the plots of log k_u versus [denaturant],

[1] There is a wide variation of m values found for staphylococcal nuclease, but Creighton and Shortle (1994) have shown that there is a compact folding intermediate present under the denaturing conditions used for determining m for this protein, and so the values of m were not measured under two-state conditions.

i.e., 5.5−9 M urea in the case of barnase and 5−7 M GdmCl for CI2, and $\Delta\Delta G_{‡\text{-}F}$ are determined at an average denaturant concentration of 7.25 M urea for barnase and 6 M GdmCl for CI2.

Since the folding of CI2 appears to follow classical two state behavior, the refolding kinetics can also be analyzed, using

$$\Delta G_{‡-U} = \Delta G^{H_2O}_{‡-U} - m_{‡-U}[\text{denaturant}] \quad (7)$$

Plots of log k_f versus [GdmCl] appear perfectly linear for all mutants in the range 0−0.6 M. These linear plots are thus used directly to obtain the variables k_f and $m_{‡\text{-}U}$. Values of $\Delta G_{‡\text{-}U}$ were calculated at 0.3 M GdmCl.

The change upon mutation of the difference in free energy between transition state and unfolded state, $\Delta\Delta G_{‡\text{-}U}$, can be determined from the refolding rate constants (Jackson *et al.*, 1993c) as follows:

$$\Delta\Delta G_{‡-U} = -RT \ln \frac{k_f}{k_f'} \quad (8)$$

The stability of the folded form of the proteins relative to the unfolded form was determined by solvent-induced equilibrium denaturation monitored by fluorescence spectroscopy (Serrano *et al.*, 1992; Matouschek *et al.*, 1994; Jackson *et al.*, 1993a). The data were analyzed as described (Matouschek *et al.*, 1994) and the free energy of denaturation, $\Delta G_{U\text{-}F}$, its dependence on the denaturant concentration, $m_{U\text{-}F}$, and the destabilization energy upon mutation relative to wild-type protein, $\Delta\Delta G_{U\text{-}F}$, determined. Experimentally, $m_{U\text{-}F}$ is determined with an error of 5−10%. For barnase, $m_{U\text{-}F}$ has been measured for 166 mutants (Matouschek *et al.*, 1994), and so we use an average value $m_{U\text{-}F}$ = 1.92 ± 0.02 kcal mol^{-2}, based on an average of all the individual m values. This has been justified previously (Serrano *et al.*, 1992a; Matouschek & Fersht, 1993). For CI2, we use an average value $m_{U\text{-}F}$ = 1.93 ± 0.03 kcal mol^{-1} M^{-1}, based on an average of the individual $m_{U\text{-}F}$ values of the 76 mutants measured.

RESULTS

Relationship between γ for Folding and Unfolding. The slope of the Hammond plots can be measured in two directions: for unfolding, γ_U is the slope of $m_{‡\text{-}F}/m_{U\text{-}F}$ versus $\Delta G_{‡\text{-}F}$ (see eq 4); and for folding, γ_F is the slope of $m_{‡\text{-}U}/m_{U\text{-}F}$ versus $\Delta G_{‡\text{-}U}$. Now, the movement of the transition state on mutation should be opposite and equal when measured from either direction of folding or unfolding, with $\partial(m_{‡\text{-}F}/m_{U\text{-}F}) = -\partial(m_{‡\text{-}U}/m_{U\text{-}F})$. The relative values of γ_F and γ_U depend therefore on the relative changes of $\Delta\Delta G_{‡\text{-}F}$ and $\Delta\Delta G_{‡\text{-}U}$ on mutation. We find that, for CI2, on average Φ_U = 0.7 (where $\Phi_U = \Delta\Delta G_{‡\text{-}F}/\Delta\Delta G_{U\text{-}F}$) so that $\Delta\Delta G_{‡\text{-}F}/\Delta\Delta G_{‡\text{-}U}$ = 0.7/0.3, i.e., 2.3:1. Therefore, on average, γ_F should be ~2.3γ_U.

Unfolding. There is a significant linear correlation between $m_{‡\text{-}F}/m_{U\text{-}F}$ and $\Delta\Delta G_{‡\text{-}F}$ at 6 M GdmCl for all mutations of CI2 plotted together (Figure 1A) with slope γ_U = 0.05. The correlation is observed also when individual regions of CI2 are analyzed separately (Figure 1B,C). The correlations are summarized in Table 1.

Refolding. A linear relationship between $m_{‡\text{-}U}/m_{U\text{-}F}$ and $\Delta\Delta G_{‡\text{-}U}$ at 0 M GdmCl is also observed for all mutations of CI2 plotted together (Figure 2 and Table 2) with slope γ_F = 0.14. This is close to the expected value of 2.3γ_U. There is a very poor correlation for the α-helix, however, with a correlation coefficient of only 0.22. But, the span of values of $\Delta\Delta G_{‡\text{-}U}$ is only 1 kcal mol^{-1}, which is half that of the other plots, and so the errors are magnified.

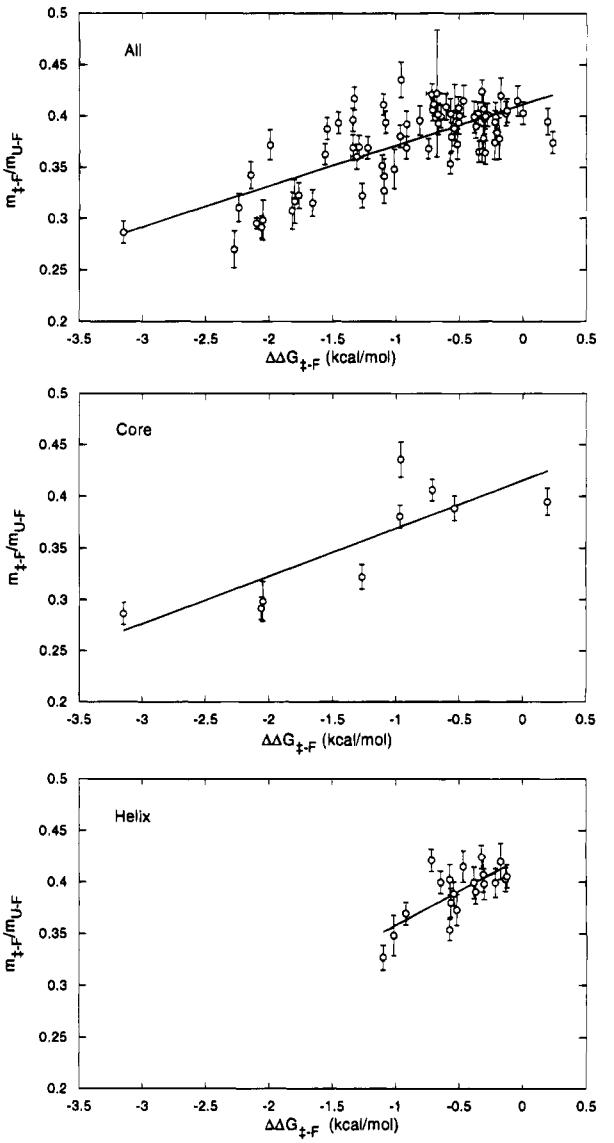

FIGURE 1: Hammond plots for mutations of CI2 from unfolding data. Plots of $m_{‡\text{-}F}/m_{U\text{-}F}$ versus $\Delta\Delta G_{‡\text{-}F}$ for all mutations in the protein, for mutations in the main hydrophobic core, and for mutations in the α-helix. The average value of $m_{U\text{-}F}$ (1.93 ± 0.03 kcal mol^{-1} M^{-1}) is used throughout. $\Delta\Delta G_{‡\text{-}F}$ is calculated at 6 M GdmCl.

Temperature Dependence: Eyring Plots. The energy difference between the transition state and the folded state was altered by changing the temperature at which the unfolding experiments are performed. The energy of activation, $\Delta G_{‡\text{-}F}$, decreases with increasing temperature. The enthalpy and entropy of activation can be determined from Eyring plots, that is plots of $\ln(k_u/T)$ vs $1/T$ (Figure 3A,B). According to transition state theory, the slope of Eyring plots is $-\Delta H_{‡\text{-}F}/R$ and the Y-axis intercept is $-\ln(k_B/h) + \Delta S_{‡\text{-}F}/R$, where $\Delta H_{‡\text{-}F}$ is the enthalpy of activation, $\Delta S_{‡\text{-}F}$ the entropy of activation, R the gas constant, k_B the Boltzmann constant, and h the Planck constant. The unfolding rate constants of wild-type barnase at 7.25 M urea have a positive enthalpy and entropy of activation: $\Delta H_{‡\text{-}F}$ = 38.3 ± 0.003 kcal mol^{-1},

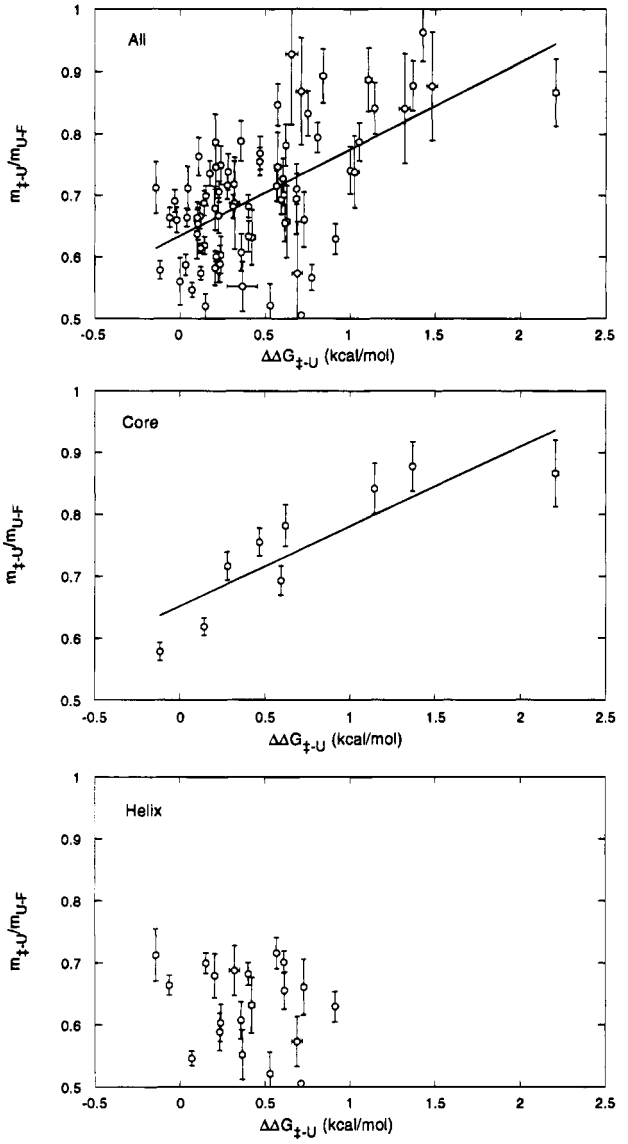

FIGURE 2: Hammond plots for mutations of CI2 from refolding data. Plots of $m_{‡-U}/m_{U-F}$ versus $\Delta\Delta G_{‡-U}$ for all mutations in the protein, for mutations in the main hydrophobic core, and for mutations in the α-helix. The average value of m_{U-F} (1.93 ± 0.03 kcal mol^{-1} M^{-1}) is used throughout. $\Delta\Delta G_{‡-U}$ is calculated at 0.3 M GdmCl.

$\Delta S_{‡-F} = 67 \pm 0.9$ cal K^{-1} mol^{-1}. $m_{‡-F}$ is strongly dependent on temperature whereas m_{U-F} is much less so. The values of $\Delta\Delta G_{‡-F}$, $m_{‡-F}$, m_{U-F}, and $m_{‡-F}/m_{U-F}$ are listed in Table 3. $\Delta\Delta G_{‡-F}$ and $m_{‡-F}/m_{U-F}$ show a clear correlation (Figure 4A; correlation coefficient $r = 0.998$, intercept, 0.311 ± 0.003, slope −0.076 ± 0.005 mol kcal^{-1}). For CI2, m_{U-F} is constant within error over the range of temperatures measured (17−32 °C). Therefore, the average value determined for 25 °C is used in the calculations. A similar behavior is observed for the unfolding of CI2. The enthalpy of activation is 40.1 ± 1.7 kcal mol^{-1}, the entropy of activation 55 ± 2 cal K^{-1} mol^{-1}. The values of $\Delta\Delta G_{‡-F}$ and $m_{‡-F}$ and $m_{‡-F}/m_{U-F}$ are listed in Table 3. Again, $\Delta\Delta G_{‡-F}$ and $m_{‡-F}/m_{U-F}$ show a clear correlation (Figure 4B; $r = 0.91$, intercept, 0.373 ± 0.003, slope 0.07 ± 0.02 mol kcal^{-1}).

DISCUSSION

We have shown previously that the position on the reaction coordinate of the transition state of folding and unfolding

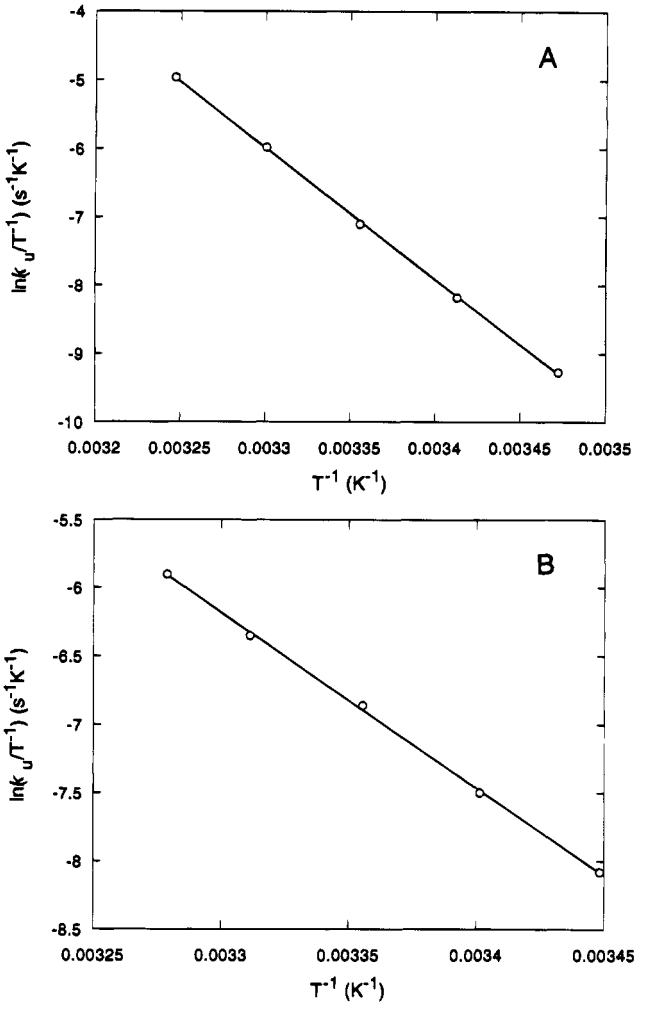

FIGURE 3: Eyring plot [$\ln(k_u/T)$ vs $1/T$] for (A) barnase and (B) CI2.

of the small ribonuclease barnase is sensitive to mutation (Matouschek & Fersht, 1993). The more the energy difference between the folded and the transition state is reduced, the more the two states resemble each other structurally. Some regions of structure were sensitive to mutation, but not others. These experiments have now been performed on a second protein, chymotrypsin inhibitor 2 (CI2), which is structurally unrelated to barnase, and we have made similar observations. For CI2, however, the position of the transition state on the reaction coordinate changes when mutations are introduced in the different parts of the protein (Figure 1). The movement of the transition state is proportional to the change in the energy difference between transition state and ground state. The transition state of CI2 appears more sensitive to changes in $\Delta\Delta G_{‡-F}$ caused by mutations than that of barnase is to mutations in its core and similar to the sensitivity of barnase to mutations in its major α-helix. The reason for the somewhat increased sensitivity could be the smaller size of CI2 and the fact that its transition state is less compact and more exposed to solvent as shown in the difference in β_0 (Tanford, 1968) ($\beta_0 = 0.41$ for CI2 and $\beta_0 = 0.31$ for barnase from unfolding kinetics); β_0 is a measure of the fractional exposure of the transition state relative to the unfolded state (Tanford, 1968). The different regions of CI2 show a similar sensitivity to mutations in terms of γ. The formation of the transition state for folding of CI2 is a highly concerted process in which tertiary and secondary

Table 1: Correlations between $m_{\ddagger\text{-}F}/m_{U\text{-}F}$ and $\Delta\Delta G_{\ddagger\text{-}F}$ from Unfolding Kinetics at $\langle[GdmCl]\rangle = 6$ M[a]

position in CI2	number of mutants	intercept β_o	slope γ (mol/kcal)	correlation coefficient r
whole protein	77	0.41 ± 0.005	0.05 ± 0.004	0.73
main core	9	0.42 ± 0.02	0.05 ± 0.01	0.82
hydrophobic patch[b]	17	0.44 ± 0.02	0.06 ± 0.01	0.77
helix (except residues forming part of main core)	20	0.42 ± 0.01	0.07 ± 0.02	0.72
β-sheet	15	0.39 ± 0.01	0.04 ± 0.01	0.10
loop and turns	16	0.41 ± 0.01	0.05 ± 0.01	0.79

[a] Based on an average value of $m_{U\text{-}F}$ (1.93 ± 0.03 kcal mol^{-1} M^{-1}). There is no significant difference in the quality of the correlation when individual values of $m_{U\text{-}F}$ are employed. [b] Residues Leu 51, Val 57, and Phe 69.

Table 2: Correlations between $m_{\ddagger\text{-}U}/m_{U\text{-}F}$ and $\Delta\Delta G_{\ddagger\text{-}U}$ from Refolding Kinetics at $\langle[GdmCl]\rangle = 0.3$ M[a]

position in CI2	number of mutants	intercept β_o	slope γ (mol/kcal)	correlation coefficient r
whole protein	73	0.63 ± 0.01	0.14 ± 0.02	0.59
main core	9	0.65 ± 0.03	0.13 ± 0.03	0.87
hydrophobic patch[b]	17	0.68 ± 0.03	0.13 ± 0.04	0.66
helix (except residues forming part of main core)	20	0.65 ± 0.03	−0.05 ± 0.05	0.22
β-sheet	15	0.60 ± 0.03	0.27 ± 0.06	0.77
loop and turns	12	0.64 ± 0.03	0.34 ± 0.10	0.75

[a] From pH-jump studies (0−0.6 M GdmCl) and based on an average value of $m_{U\text{-}F}$ (1.93 ± 0.03 kcal mol^{-1} M^{-1}). There is no significant difference in the quality of the correlation when individual values of $m_{U\text{-}F}$ are employed. [b] Residues Leu 51, Val 57, and Phe 69.

Table 3: Effect of Temperature on the Unfolding of Barnase and CI2[a]

	T (°C)	$\Delta\Delta G_{\ddagger\text{-}F}$ (kcal/mol)	$m_{\ddagger\text{-}F}$ (kcal mol^{-1} M^{-1})	$m_{U\text{-}F}$ (kcal mol^{-1} M^{-1})	$m_{\ddagger\text{-}F}/m_{U\text{-}F}$
barnase	15	-0.65	0.69 ± 0.02	1.90 ± 0.03	0.36 ± 0.01
	20	-0.31	0.65 ± 0.01		
	25	0.00	0.61 ± 0.03	1.98 ± 0.06	0.31 ± 0.02
	30	0.36	0.55 ± 0.01		
	35	0.70	0.54 ± 0.01	2.08 ± 0.01	0.26 ± 0.01
CI2	17	-0.24	0.76 ± 0.01		0.39 ± 0.01
	21	-0.15	0.73 ± 0.00		0.38 ± 0.01
	25	0.00	0.73 ± 0.01		0.38 ± 0.01
	29	0.05	0.70 ± 0.01		0.36 ± 0.01
	32	0.14	0.71 ± 0.01		0.37 ± 0.01

[a] T is the temperature at which the unfolding experiments were performed. $\Delta\Delta G_{\ddagger\text{-}F}$ is the change in free energy of activation for unfolding relative to the free energy of activation for 25 °C as calculated from transition state theory from the unfolding rate constants at 6 M GdmCl for CI2 and 7.25 M urea for barnase assuming that the transmission coefficient is independent of temperature. $m_{\ddagger\text{-}F}$ is defined by eq 3 and calculated at 6 M GdmCl for CI2 and 7.25 M urea for barnase. For CI2, $m_{\ddagger\text{-}F}/m_{U\text{-}F}$ is calculated using the average wild-type $m_{U\text{-}F}$ value of 1.93 ± 0.03 kcal mol^{-1} M^{-1}, since we find that $m_{U\text{-}F}$ does not significantly change over the temperature range.

elements of structure are formed in parallel (Otzen *et al.*, 1994), unlike that of barnase which has some regions fully formed and others fully disordered (Fersht, 1993). These structural properties are presumably the basis of why CI2 has a rather uniform response to mutation and barnase does not.

If the folding of CI2 follows a two-state model, the change in the position on the reaction coordinate of the transition state relative to the folded state should also be proportional to the change in energy difference between the transition state and the unfolded state. This is indeed the case. The β_o values for unfolding and refolding add up to 1, because the fractional change in exposure from the unfolded state to the transition state and from the transition state to the folded state add up to the overall change from the unfolded protein to the folded protein. The slopes of the Hammond plots, γ, measured in the directions of folding and unfolding are in

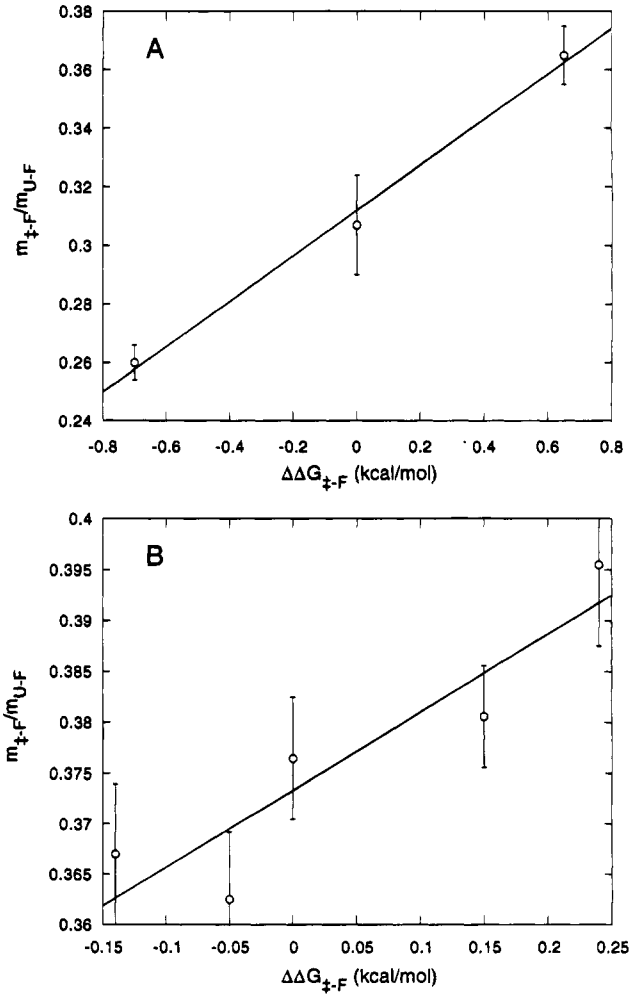

FIGURE 4: Hammond plot for barnase and CI2 where changes in $\Delta\Delta G_{\ddagger\text{-}U}$ are induced by variation of temperature.

the ratios expected from the values of $\Delta\Delta G_{\ddagger\text{-}F}$ and $\Delta\Delta G_{\ddagger\text{-}U}$, and thus the sensitivity of the position of the transition state on the reaction coordinate to changes in energy is the same when measured in the two directions. The ratio of

$\Delta\Delta G_{\ddagger\text{-F}}/\Delta\Delta G_{\ddagger\text{-U}}$ is 2.5, and so the measurements from the unfolding kinetics have more than twice the span on the x-axis compared with the measurements from the refolding kinetics, and the refolding data have only 0.4 times the slope. The mutations in the α-helix appear to be an exception. However, the data from the refolding measurements of α-helix mutants are unreliable because of the small range of $\Delta\Delta G_{\ddagger\text{-U}}$.

Differences in energy between the transition and ground states can be produced by means other than mutagenesis. For example, the rate of unfolding can also be increased and thus $\Delta G_{\ddagger\text{-F}}$ decreased by raising the temperature. For both CI2 and barnase this also leads to a movement of the transition state. The transition state appears to be more sensitive to changes in $\Delta\Delta G_{\ddagger\text{-F}}$ caused by variation of temperature than to those caused by mutagenesis in the hydrophobic core of barnase. [A more detailed analysis of the temperature dependence of β_T for barnase on temperature shows a more complex behavior over extreme ranges of [denaturant] (M. T. Oliveberg, Y.-J. Tan, P. Dalby, and A.R.F., unpublished).]

The rate constants of unfolding and refolding are also sensitive to the denaturant concentration. The higher the concentration of urea or GdmCl, the faster the protein unfolds and the slower it refolds. Thus, we expect the transition state to move toward the folded state on the reaction coordinate and further from the unfolded state as the denaturant concentration is increased. This could manifest itself as a decrease in $m_{\ddagger\text{-F}}$ with increasing denaturant concentrations, and a downward curvature in plots of log k_u against [denaturant] is seen (Matouschek & Fersht, 1993; Matouschek *et al.*, 1994). Our refolding data do not cover a sufficiently broad range of [denaturant] to allow us to ascertain whether or not there is a consistent degree of curvature in plots of log k_f against [denaturant], and so the observed curvature could result from other factors.

A very useful tool for the structural analysis of protein folding pathways is the Φ_F value [$=\Delta\Delta G_{\ddagger\text{-U}}/\Delta\Delta G_{\text{F-U}}$, where $\Delta\Delta G_{\text{F-U}}$ is the change on mutation in free energy of folding and $\Delta\Delta G_{\ddagger\text{-U}}$ the change in activation energy of folding (Matouschek *et al.*, 1989, 1990; Fersht, 1995)]. The value of Φ_F is an index of formation of structure, 0 meaning no structure formation, and 1 complete formation. Hammond behavior of proteins is also reflected in this parameter. For example, the Φ_F values for interactions within the major α-helix in barnase (residues 6, 8, 15, 16, 17, and 18) change with the urea concentration for which they are determined [measured from values of Φ_U; see Matthews and Fersht (1995) for individual values]. The Φ_F values determined for 0 M urea for residues in the major helix in the transition state are high, but less than 1, indicating that there is significant formation of structure but that it is somewhat weakened compared with the folded protein. In 7.25 M urea, the Φ_F values are closer to 1, and the structure of the helix in the transition state is closer to that in the native protein. This is expected as the structure of the transition state moves closer to that of the folded structure with increasing urea concentrations and unfolding rates (see above). However, not all mutations lead to Hammond behavior. Previously, we have shown how mutations in certain parts of the protein have no effect on the position of the transition state on the reaction coordinate (Matouschek & Fersht, 1993). Mutations can also have effects opposite to that expected by the Hammond postulate and show "anti-Hammond" behavior (Matthews & Fersht, 1995).

Linear free energy relationships have been constructed for a long time in organic chemistry. When the energy difference between the transition state and the ground state is changed over a very wide range of energies, deviations from linearity in Brønsted plots can also be observed for simple organic reactions (Jencks, 1969). The magnitude of this deviation depends on the sensitivity of the states on the reaction pathway to changes in structure of the reactants or experimental conditions. The relationship between the curvature in Brønsted plots and the slope of Hammond plots is given by

$$\frac{\partial^2 \Delta G_{\ddagger-\text{F}}}{(\partial \Delta G_{\text{U}-\text{F}})^2} = \gamma\beta \qquad (9)$$

It is interesting to compare the sensitivity of the transition state of simple organic reactions to changes in the energy difference between ground and transition state ($\Delta G_{\ddagger}$) with that of the transition state of protein folding. In organic chemistry, it is common to observe linear Brønsted plots over 7 kcal/mol of $\Delta\Delta G^{\ddagger}$, and thus the position of the transition state appears to be rather insensitive to changes in energy compared with those observed in protein folding. The main difference between the two experimental systems is that, generally, in organic chemistry changes in covalent bonds are observed, whereas the protein folding process only involves changes in noncovalent interactions or interactions with partially covalent character such as hydrogen bonds. Also, the structural changes in organic chemistry are usually localized to a few bonds whereas, in protein folding, the structural changes are spread over the whole macromolecule.

We can also compare the transition state in protein folding to that in enzyme catalysis. Brønsted plots were constructed for mutations in the active site of the tyrosyl tRNA-synthetase with changes of up to 3.0 kcal/mol in $\Delta G^{\ddagger}$ (Avis & Fersht, 1993). No deviation from linearity could be observed. In protein folding, on the other hand, we would expect β to change considerably, by 0.06 from 0.31 to 0.25 over the same range of $\Delta\Delta G^{\ddagger}$ caused by mutations in the main hydrophobic core of barnase and by 0.11 from 0.30 to 0.19 for mutations in the major α-helix in barnase. Enzymes catalyze changes in the covalent structure of their substrates, but the interactions of the substrate with the protein are noncovalent or of partially covalent nature. The changes in structure are frequently located around the active site of the protein. The transition state in enzyme catalysis, in this example, is also less sensitive to changes in $\Delta G^{\ddagger}$ than protein folding.

In conclusion, we show that Hammond behavior is observed in protein folding for two structurally unrelated proteins. It can be produced by mutagenesis or by changes in experimental conditions such as denaturant concentration and temperature. This can explain the observed deviations from ideal behavior such as curved plots of log k_u against [denaturant] and changes in the slopes of these plots with mutation (Matouschek *et al.*, 1994). The smaller protein with the less structured and more concertedly formed transition state appears to be more sensitive to changes in energy than the larger. For both proteins, however, the position of the transition state of protein folding is more sensitive to changes in energy than observed in organic chemistry and enzyme

catalysis. Hammond behavior should be taken into consideration on any extrapolation of theoretical or practical measurements over wide ranges of conditions.

ACKNOWLEDGMENT

We thank Martin Long for performing unfolding experiments on wild-type barnase at different temperatures.

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BI951272V