Folding of Chymotrypsin Inhibitor 2. 2. Influence of Proline Isomerization on the Folding Kinetics and Thermodynamic Characterization of the Transition State of Folding

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ABSTRACT: The refolding of chymotrypsin inhibitor 2 (CI2) is, at least, a triphasic process. The rate constants are 53 s^{-1} for the major phase (77% of the total amplitude) and 0.43 and 0.024 s^{-1} for the slower phases (23% of the total amplitude) at 25 °C and pH 6.3. The multiphase nature of the refolding reaction results from heterogeneity in the denatured state because of proline isomerization. The fast phase corresponds to the refolding of the fraction of protein that has all its prolines in a native trans conformation in the denatured state. It is not catalyzed by peptidyl-prolyl isomerase. The rate-limiting step of folding for the slower phases, however, is proline isomerization, and they are both catalyzed by peptidyl-prolyl isomerase. The slowest phase has properties consistent with a process involving proline isomerization in a denatured state. In particular, the activation enthalpy is large, 16 kcal mol^{-1} K^{-1}, and the rate is independent of guanidinium chloride concentration ([GdnHCl]). In comparison, the intermediate phase shows properties consistent with a process involving proline isomerization in a partially structured state. The activation enthalpy is small, 8 kcal mol^{-1} K^{-1}, and the rate has a strong dependence on [GdnHCl]. Temperature dependences of the rate constants for unfolding and for the fast refolding phase, both in the absence and in the presence of GdnHCl, were used to characterize the thermodynamic nature of the transition state and its relative exposure to solvent. The Eyring plot for unfolding is linear, indicating that there is relatively little change in heat capacity between native state and transition state. The Eyring plot for refolding, however, shows significant curvature and shows there is a substantial change in heat capacity between the denatured state and the transition state. In addition, the change in enthalpy and entropy calculated for unfolding are 37.7 kcal mol^{-1} and 50.6 cal mol^{-1} K^{-1}, respectively, and for refolding are 11.92 kcal mol^{-1} and −10.4 cal mol^{-1} K^{-1}, respectively. The transition state of folding for CI2 appears to be a high-energy form of the native state in which stabilizing interactions, present in the native state, are broken. Loss of such interactions seems to be accompanied by a partial opening of the hydrophobic core.

We have shown that chymotrypsin inhibitor 2 is a good example of a simple model system for studying protein folding (Jackson & Fersht, 1991). CI2 is a small monomeric protein that does not contain any disulfide bonds or cis prolines. Although small, it has significant secondary and tertiary structure that has been well defined by both the crystal structure (McPhalen & James, 1987) and the NMR solution structure (Clore et al., 1987a,b). In addition, the gene encoding for the protein has been cloned and can be expressed at high levels in *Escherichia coli* (Longstaff et al., 1990). Equilibrium studies have shown that CI2 undergoes a reversible cooperative two-state transition between native and denatured states that can be induced by various denaturants including GdnHCl, acid, and heat. There is a large fluorescence change on denaturation that can be used to follow folding and that has been shown to correspond to a global denaturation of the protein. In addition, the kinetics of unfolding and refolding have been investigated and also shown to follow a two-state model of folding, where there is no accumulation of intermediates either on the unfolding or refolding pathway (Jackson & Fersht, 1991). With such a system it is possible to measure the thermodynamic parameters of folding very accurately.

In this paper, we present a detailed characterization of the refolding reaction. With stopped-flow techniques, refolding can be studied by either [GdnHCl] or pH-jump experiments. In both cases, the refolding reaction is found to be triphasic with the three phases well-resolved at 25 °C and at low concentrations of denaturant. This allows the rate constants and amplitudes for all three phases to be measured accurately. The intermediate and slow phase are characterized by a study of their temperature dependence, [GdnHCl] dependence, and catalysis by the enzyme peptidyl-prolyl isomerase. From these data, we show that the multiphasic nature of the refolding reaction is due to a heterogeneous population in the denatured state. We present evidence that both the intermediate and slow phase correspond to slow rate-limiting isomerizations about proline bonds.

The fast phase is shown to correspond to the refolding of the fraction of protein that has all its prolines in a native conformation in the denatured state and, therefore, to a rate-limiting step involving folding of the protein and not proline isomerization. The fast phase of refolding and the unfolding reaction are studied to characterize the transition state of folding. The principle of microscopic reversibility states that, under the same conditions at equilibrium, the transition state for folding is the same as the transition state for unfolding. Whereas the fast phase corresponding to folding of the protein can be investigated under native conditions, unfolding has to be studied in denaturing conditions. However, the unfolding data can be extrapolated to native conditions because of the linear relationship between the change in free energy between the transition state and the native state and

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the concentration of denaturant. In this way, both unfolding and refolding data are used to characterize the transition state for folding under native conditions. In addition, the solvent accessibility of the transition state can be studied by temperature-dependence studies in the presence of denaturant. Both the thermodynamic data and the solvent accessibility data are used to characterize the transition state of folding for CI2.

Experimental Procedures

Materials

The buffer used in the experiments with peptidyl–prolyl isomerase was Tris-HCl purchased from Sigma. The peptidyl–prolyl isomerase used was recombinant human cyclophilin and was a generous gift from Sandoz Pharma Ltd., Basel, Switzerland. All other materials used were as described in the preceding paper (Jackson & Fersht, 1991).

Equipment and General Procedures

[GdnHCl] Jump Experiments. Reactions were followed with a Perkin-Elmer MPF-44B fluorescence spectrophotometer equipped with a rapid mixing head as described in the preceding paper (Jackson & Fersht, 1991). The observation cell and reservoir syringes were thermostated separately with two water baths, and the temperature of each was monitored with an Edale instrument thermometer. Temperatures were kept within ±0.1 °C. Data were acquired and analyzed as described in the preceding paper (Jackson & Fersht, 1991).

pH-Jump Experiments. An Applied Photophysics stopped-flow spectrophotometer model SF 17MV was used to monitor the reaction as described in the preceding paper (Jackson & Fersht, 1991). The temperature of the cell and reservoir syringes was maintained by thermostating with a Grant LTD6 water bath. Temperatures were maintained to ±0.1 °C with an internal temperature probe in the stopped-flow apparatus that had previously been calibrated against an Edale instrument thermometer. The data were acquired and analyzed as described in the preceding paper (Jackson & Fersht, 1991).

Characterization of the Intermediate and Slow Phase of Refolding

Temperature Dependence. The protein was initially acid denatured by lowering the pH of the solution to 1.7 by addition of 5 M HCl. At this pH, it was found that the protein is completely denatured as determined by fluorescence spectroscopy. The subsequent addition of GdnHCl makes no further difference to the fluorescence spectrum. The protein was refolded by rapid mixing (1:1) with a strongly buffered MES solution at high pH. The final pH was 6.3.

GdnHCl Dependence. GdnHCl dependence of the intermediate and slow phase was studied by using pH-jump experiments as described above. In order to minimize mixing artifacts, which result from the mixing of high molarity solutions with low molarity solutions, GdnHCl was present in both the denaturing and renaturing buffers at equal concentration.

Catalysis by Peptidyl–Prolyl Isomerase. Catalysis of the intermediate and slow phase of refolding by PPI was studied by using pH-jump experiments. The protein was denatured by the addition of 5 M HCl to pH 1.7. The protein was rapidly refolded by mixing (1:1) with a strongly buffered renaturing solution. The renaturing solution was buffered with Tris-HCl, and the final pH was 8.0. PPI was added to the renaturing buffer; final concentrations after mixing were between 0 and 0.75 μM. The PPI concentration was determined spectrophotometrically using an extinction coefficient A_{280} = 0.44 for a concentration of 1 mg mL^{-1} (Liu et al., 1990). The denaturing solution was the same throughout the experiment, ensuring that the concentration of denatured protein did not vary. This is important because the denatured protein is the substrate for PPI and the rate of catalysis may be dependent upon the substrate concentration. CI2 was approximately 10 μM after mixing. pH-jump experiments were used instead of [GdnHCl]-jump experiments because of the sensitivity of PPI to even small amounts of GdnHCl (Lang et al., 1987). A final pH of 8.0 was used as this is the optimal pH of catalysis for this enzyme (Lang et al., 1987).

Table I: Activation Enthalpies, ΔH_f^*, and Activation Entropies, ΔS_f^*, for the Proline-Dependent Refolding Phases of CI2

	ΔH_f^* (kcal mol^{-1})	ΔS_f^* (cal mol^{-1} K^{-1})
intermediate phase[a]	7.8 ± 0.8	−12 ± 2
slow phase[b]	16.0 ± 0.5	−34 ± 3

[a] Calculated from the Eyring plots for the intermediate phase (Figure 2A) with eq 1. [b] Calculated from the Eyring plot from the slow phase (Figure 2B) with eq 1. Refolding conditions were 0 M GdnHCl, pH 6.3.

Characterization of the Unfolding Reaction and of the Fast Phase of Refolding

[GdnHCl] Jump Experiments. The experiments were performed as described in the preceding paper (Jackson & Fersht, 1991).

pH-Jump Experiments. For unfolding, native protein, weakly buffered at pH 6.3 with 10 mM MES, was jumped into a strongly buffered citrate solution, at acidic pH, under which conditions the protein was denatured. The temperature dependence of the fast phase of refolding was studied as described above for the temperature dependence of the intermediate and slow phase of refolding.

Results

Characterization of the Intermediate and Slow Phases of Refolding

The refolding of CI2, by either [GdnHCl] jump or by pH-jump experiments, is a triphasic process with the three phases well resolved at 25 °C and at low concentrations of denaturant. Figure 1A shows the fast phase of refolding, and Figure 1B shows the two slower phases of refolding at 25 °C, in 0 M GdnHCl, pH 6.3. Under these conditions, the rate constants for the fast, intermediate, and slow phases are 53, 0.43, and 0.024 s^{-1}, respectively.

Temperature Dependence. The temperature dependence of the three refolding phases of CI2 was studied over the range 21–29 °C by pH-jump experiments. Eyring plots [ln (k_f/T) vs $1/T$], where k_f is the rate of folding of a particular phase, for both the intermediate and slow phase are linear over the temperature range investigated. The activation parameters for folding can be calculated from the Eyring plot, by using eq 1, obtained from transition-state theory (Laidler, 1950),

$$\ln (k_f/T) = \ln (k_B/h) + \Delta S_f^*/R - \Delta H_f^*/RT \quad (1)$$

where k_f is the rate of folding, ΔS_f^* is the activation entropy of folding, ΔH_f^* is the activation enthalpy of folding, and h and k_B are Planck's and Boltzmann's constants, respectively. Table I summarizes the results for the intermediate and slow phase. The temperature dependence of the fast phase is discussed in more detail later.

The equilibrium constants for the formation of the intermediate and slow refolding species of CI2 can be calculated from the relative amplitudes of the three phases, assuming that

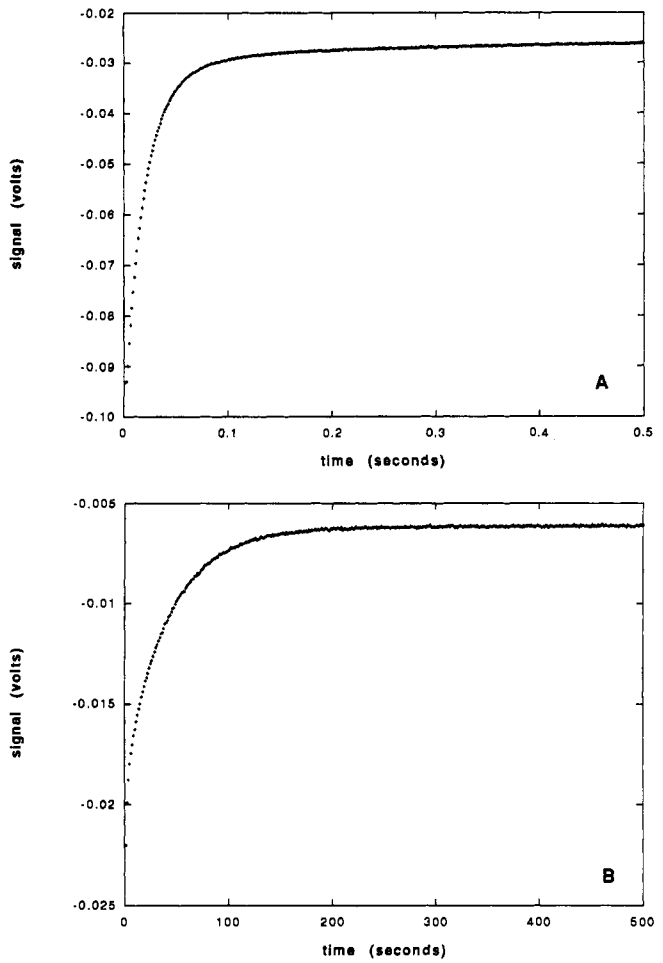

FIGURE 1: Refolding of CI2 measured by pH-jump experiment, at 0 M GdnHCl, pH 6.3, 25 °C, monitored by fluorescence using an Applied Photophysics SF 17MV stopped-flow spectrophotometer, with an excitation wavelength of 280 nm and cut-off filter below 335 nm. Both traces are the average of at least three separate runs. (A) Fast phase of refolding of CI2 over a time scale of 0–500 ms. The fast phase has a rate constant of 53 s^{-1} and a relative amplitude of 77%. (B) Two slow phases of refolding of CI2 over a time scale of 1–500 s. The fast phase is over before the first time point is taken. These phases have rate constants of 0.043 and 0.002 s^{-1} and relative amplitudes of 8% and 15%, respectively.

the three phases are the result of heterogeneity in the denatured state, i.e., the fast refolding species corresponds to the fraction of denatured protein with all its prolines in a trans conformation and the intermediate and slow refolding species correspond to the fraction of denatured protein with a proline in a cis conformation, and that the fluorescence change is directly proportional to the concentration of folding species in the denatured state. $\Delta H(T)$, the enthalpy change for the formation of the slow refolding species, can then be calculated from the temperature dependence of the equilibrium constant by using the van't Hoff relationship

$$\Delta H(T) = -R[\partial \ln K/\partial(1/T)] = RT^2(\partial \ln K/\partial T) \quad (2)$$

The relative amplitudes of the fast, intermediate and slow refolding phases change very little over the temperature range studied, and accurate values for $\Delta H(T)$ cannot therefore be measured. However, such data indicate that $\Delta H(T)$ is very small, probably less than 1 kcal mol^{-1}.

[GdnHCl] Dependence. The [GdnHCl] dependence of the three refolding phases was studied over the concentration range 0–1.25 M. All three phases show a linear dependence of ln k_f on [GdnHCl] (Figure 2). The rate constants for both the fast and intermediate phases show a strong dependence on

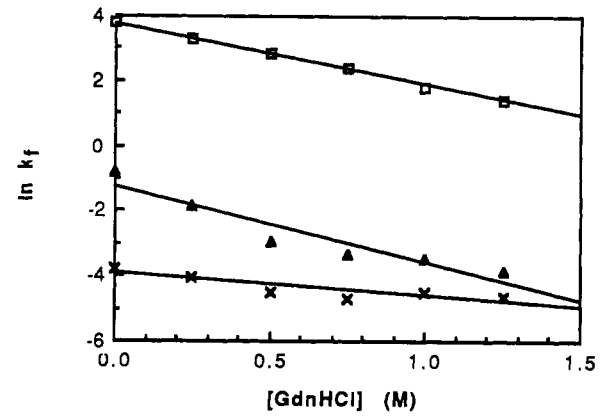

FIGURE 2: [GdnHCl] dependence of the natural logarithm of the rate constant, ln k_f, for the fast (□), intermediate (▲), and slow (×) refolding phases of CI2, pH 6.3, at 25 °C.

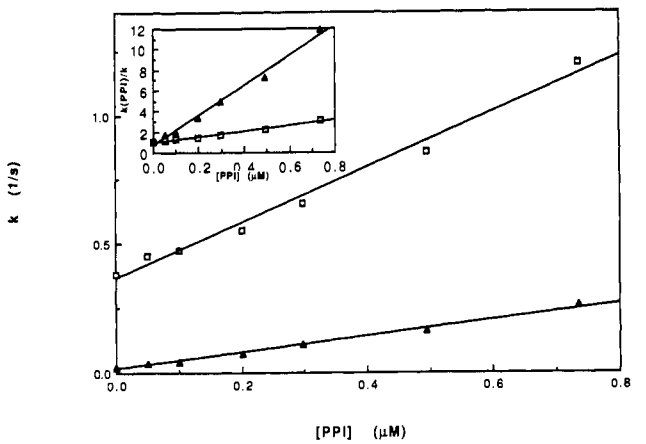

FIGURE 3: Catalysis of folding of the intermediate (□) and slow (▲) refolding species of CI2 by peptidyl–prolyl isomerase, 0 M GdnHCl, pH 8.0, at 25 °C. (Insert) The data were transformed to show the ratio of the catalyzed rate to the uncatalyzed rate, $k_f(PPI)/k_f$, for the intermediate (□) and slow (▲) refolding species of CI2.

[GdnHCl], and, in both cases, the rate decreases with increasing [GdnHCl]. The rate constant for the slow phase, however, is almost independent of the final GdnHCl concentration.

Catalysis by Peptidyl–Prolyl Isomerase. Peptidyl–prolyl isomerase has been found to catalyze the cis–trans isomerization of peptidyl proline bonds in both oligopeptides and proteins (Fischer et al., 1984; Lang et al., 1987; Lin et al., 1988). PPI catalyzes the intermediate and slow phases but does not affect the rate of the fast phase. PPI does not affect the relative amplitudes of the three species. Figure 3 shows the effect of PPI on the absolute rates of the intermediate and slow phases. There is a linear dependence between the rate of catalysis and the PPI concentration. The ratio of the catalyzed rate to the uncatalyzed rate, $k_f(PPI)/k_f$, shows the different degrees of catalysis of PPI on the two phases (Figure 3, insert). Under the reaction conditions, 1 μM PPI catalyzes the intermediate phase approximately 3-fold and the slow phase approximately 15-fold.

Characterization of the Transition State of Folding

Temperature Dependence of Unfolding. Unfolding, studied by [GdnHCl] jump experiments, is observed to be monophasic and can be fitted to a single exponential (Jackson & Fersht, 1991). The rates of unfolding, k_u, were measured at seven different final [GdnHCl] and at five different temperatures. The rate of unfolding in water was calculated by extrapolation of the linear plots of ln k_u versus [GdnHCl] to 0 M GdnHCl

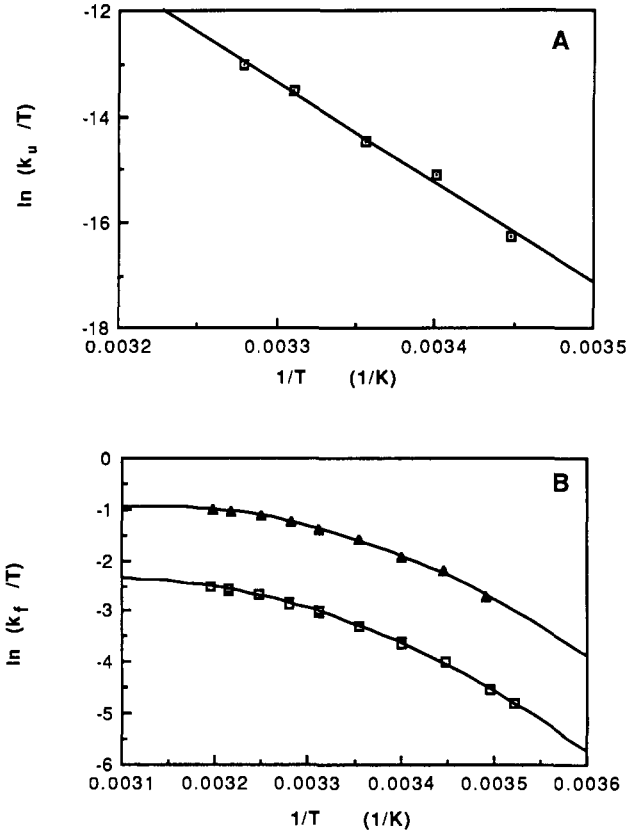

FIGURE 4: (A) Eyring plot of the temperature dependence of the unfolding rate constant in water, $k_u(H_2O)$, pH 6.3, calculated from extrapolation of the linear plots of ln k_u against [GdnHCl] to 0 M GdnHCl, at each temperature. Activation parameters calculated from the Eyring plot according to eq 3 are shown in Table II. (B) Eyring plot of the temperature dependence of the rate constant for refolding (fast phase) measured by pH-jump experiments, at 0 M GdnHCl, pH 6.3 (▲) and at 1.5 M GdnHCl, pH 6.3 (□). The solid curves are the best fit of the data to eq 4. Activation parameters calculated according to eq 4 are given in Table II.

at each temperature. No curvature in the Eyring plot [ln (k_u/T) vs $1/T$] is observed over the temperature range 19–32 °C (Figure 4A). It was not possible to extend the temperature range by using [GdnHCl] jump experiments, but unfolding could be studied over a wider temperature range by pH-jump experiments using an Applied Photophysics SMV-17 stopped-flow spectrophotometer. Native protein, weakly buffered at pH 6.3 with 10 mM MES, was jumped into denaturing acidic conditions by using a strongly buffered citrate solution. The rate of unfolding under these conditions was measured between 17 and 40 °C. No curvature was observed over this temperature range (data not shown). It is inferred from the linearity of the Eyring plot for unfolding that $\Delta C_{pu}^{\ddagger}$, the change in heat capacity between the native and transition state, is small. The data can then be fitted directly to eq 3, obtained

$$\ln (k_u/T) = \ln (k_B/h) + \Delta S_u^{\ddagger}/R - \Delta H_u^{\ddagger}/RT \quad (3)$$

from transition-state theory (Laidler, 1950), where the subscript u represents unfolding. With these values $\Delta G_u^{\ddagger}$, the free energy of activation of unfolding, may be calculated at any temperature, assuming $\Delta H_u^{\ddagger}$ and $\Delta S_u^{\ddagger}$ are independent of temperature. This assumption is valid over short temperature ranges because $\Delta C_{pu}^{\ddagger}$ is small. The activation parameters for unfolding in water are given in Table II.

Temperature Dependence of the Fast Refolding Phase. The temperature dependence of the fast refolding phase was determined by pH-jump experiments over the temperature range 13–40 °C. There is significant curvature in the Eyring plot for the temperature dependence of the refolding rate in water (0 M GdnHCl, pH 6.3) over this temperature range (Figure 4B).

Table II: Activation Parameters for Unfolding and for the Major Fast Refolding Phase of CI2

	$\Delta C_p^{\ddagger}$ (kcal mol^{-1} K^{-1})	$\Delta H^{\ddagger}$ (kcal mol^{-1})	$\Delta S^{\ddagger}$ (cal mol^{-1} K^{-1})	$\Delta G^{\ddagger}$ (25 °C) (kcal mol^{-1})
unfolding[a]	+0.20 ● 0.10[b]	+37.7 ± 1.6	+50.6 ● 5.2	+22.60
refolding[c]	−0.59 ± 0.06	+11.9 ● 0.3	−10.4 ± 0.8	+15.02

[a] Calculated from the Eyring plot for unfolding (Figure 5A) according to eq 3. [b] Calculated from the equilibrium change in heat capacity, ΔC_p, and the change in heat capacity on folding, $\Delta C_{pf}^{\ddagger}$, by using $\Delta C_p = \Delta C_{pu}^{\ddagger} + \Delta C_{pf}^{\ddagger}$ and not directly from the Eyring plot for unfolding. [c] Calculated from the Eyring plot for the fast phase of refolding (Figure 5b) according to eq 4. Both sets of data are obtained at 0 M GdnHCl, pH 6.3, 25 °C.

To ensure that the curvature observed is real and not an artifact of the experimental conditions (see Discussion) the rate constants were measured both by increasing the temperature from 13 to 40 °C and then by decreasing the temperature from 40 to 13 °C. No hysteresis was observed. In addition, 1.5 M GdnHCl was added to both denaturing and renaturing buffer to destabilize any residual structure that may be present due to the acidic conditions. The curvature is as significant as that observed in the absence of denaturant (Figure 4B), suggesting that curvature is not an artifact of the acid denaturation of the protein.

Pohl (1968a,b) and Segawa and Sugihara (1984) have observed Arrhenius plots of ln k_u that are linear and Arrhenius plots of ln k_f that are curved. Since proteins have large changes in heat capacity on denaturation and $\Delta C_p = [\partial \Delta H_U(T)/\partial T]_p$, the enthalpy change of unfolding, ΔH_U, is temperature dependent. The logarithm of the equilibrium constant for unfolding, ln K_u, is related to the change in enthalpy of unfolding through the van't Hoff relationship (eq 2) so that the logarithm of the equilibrium constant has a nonlinear temperature dependence. Since ln K_u = ln (k_f/k_u), either or both of ln k_f or ln k_u should have a nonlinear temperature dependence. If Arrhenius plots of ln k_u are linear, one would expect ln k_f plots to be nonlinear. This is observed also for the Eyring plots [ln (k/T) vs $1/T$] for unfolding and refolding of CI2. Following the analysis of Chen et al. (1989), the data can be fitted to

$$\ln (k_f/T) = A + B(T_o/T) + C \ln (T_o/T) \quad (4)$$

where

$$A = [-\Delta C_{pf}^{\ddagger} + \Delta S_f^{\ddagger}(T_o)]/R - \ln (h/k_B)$$

$$B = [\Delta C_{pf}^{\ddagger} - \Delta S_f^{\ddagger}(T_o)]/R - \Delta G_f^{\ddagger}(T_o)/RT_o$$

$$C = -\Delta C_{pf}^{\ddagger}/R$$

where $\Delta C_{pf}^{\ddagger}$ is the heat capacity change between denatured state and transition state, $\Delta S_f^{\ddagger}$ is the activation entropy of folding, $\Delta H_f^{\ddagger}$ is the activation enthalpy of folding, and $\Delta G_f^{\ddagger}$ is the activation energy of folding. Following this analysis, the nonlinear regression fitting routine ENZFITTER was used to calculate the activation parameters for refolding of CI2 at T_o = 25 °C. The results are summarized in Table II.

The temperature dependence of the unfolding, k_u, and refolding, k_f, rate constants can also be used to calculate the free energy change of unfolding, $\Delta G_u(H_2O)$, as a function of temperature. It was shown that, at 25 °C, CI2 follows a two-state model of folding in which only the native, N, and unfolded, U, states are populated (Jackson & Fersht, 1991).

$$U \underset{k_u}{\overset{k_f}{\rightleftharpoons}} N$$

Table III: Comparison of $\Delta G_u(H_2O)$, the Free Energy of Unfolding in the Absence of Denaturant, Calculated from Equilibrium and Kinetic Experiments as a Function of Temperature

temp (°C)	$\Delta G_u(H_2O)$ (kcal/mol) equilibrium expts[a]	$\Delta G_u(H_2O)$ (kcal/mol) kinetic expts[b]
17	−7.47 ± 0.52	−7.94 ± 0.40
21	−7.28 ± 0.44	−7.54 ± 0.38
25	−7.04 ± 0.37	−7.45 ± 0.37
29	−6.76 ± 0.31	−7.11 ± 0.36
32	−6.52 ± 0.27	−6.97 ± 0.35

[a] Calculated from thermal denaturation studies (Jackson & Fersht, 1991) by extrapolating $\Delta H(T_m)$ and $\Delta S(T_m)$ at various [GdnHCl] to 17, 21, 25, 29, and 32 °C. $\Delta G_u(H_2O)$ was then calculated by extrapolation of the linear plot of ΔG_u versus [GdnHCl] to 0 M denaturant. [b] Calculated from the ratio of the refolding and unfolding rate constants, taking into account the equilibrium due to proline isomerization.

Table IV: $\Delta G^*_{H_2O}$, the Activation Energy, and m^*, the Dependence of $\Delta G^*_{H_2O}$ on [GdnHCl], for Unfolding and for the Fast Refolding Phase of CI2 in the Absence of Denaturant

	$\Delta G^*_{H_2O}$ (kcal mol^{-1})	m^* (kcal mol^{-1} M^{-1})
unfolding[a]	+22.3 ± 0.1	−0.69 ± 0.01
refolding[b]	+15.4 ± 0.1	+1.04 ± 0.01

[a] Calculated from the linear plot of ΔG^* for unfolding versus [GdnHCl] (Figure 5A) according to eq 5. [b] Calculated from the linear plot of ΔG^* for refolding versus [GdnHCl] (Figure 5B) according to eq 5, under refolding conditions: 0 M GdnHCl, 25 °C, pH 6.3.

It follows that $K_U = k_f/k_u$. The values for $\Delta G_u(H_2O)$ calculated from the kinetic experiments are shown in Table III. Values for $\Delta G_u(H_2O)$ as a function of temperature can also be calculated from equilibrium experiments using thermal denaturation (Table III). The values calculated from kinetic and equilibrium experiments are similar at all the temperatures studied and do not differ by more than the experimental error, i.e., there is not a significant deviation from the two-state model between 17 and 32 °C.

[GdnHCl] Dependence of the Activation Energy for Unfolding and Refolding. The temperature dependence of the rate of refolding, as well as the temperature dependence of the rate of unfolding, was determined at various GdnHCl concentrations. The plot of the free energy of activation against [GdnHCl] is linear for both unfolding and refolding (Figure 5) and can be represented by the general equation

$$\Delta G^* = \Delta G^*_{H_2O} + m^*[\text{GdnHCl}] \quad (5)$$

where ΔG^* is the activation energy at a particular GdnHCl concentration, $\Delta G^*_{H_2O}$ is the activation energy in water, and m^* is the gradient. The results are summarized in Table IV.

The free energies of activation of unfolding, $\Delta G_u^*(H_2O)$, and refolding, $\Delta G_f^*(H_2O)$, in water, calculated by extrapolation of the linear plots of ΔG_u^* and ΔG_f^* versus [GdnHCl] (Figure 5) to 0 M GdnHCl are +22.30 and +15.37 kcal mol^{-1}, respectively. From these values, an equilibrium change in free energy, ΔG_{H_2O}, can be calculated by using

$$\Delta G_{H_2O} = \Delta G_u^*(H_2O) - \Delta G_f^*(H_2O) \quad (6)$$

and an equilibrium m value can be calculated from

$$m = m_u^* - m_f^* \quad (7)$$

where m_u^* and m_f^* are the gradients of the plots for unfolding and refolding, respectively. The values calculated for ΔG_{H_2O} and m are +6.93 kcal mol^{-1} and 1.73 kcal mol^{-1} M^{-1}, respectively. These agree with the values measured by equilibrium experiments using both GdnHCl and thermal denaturation (Jackson & Fersht, 1991).

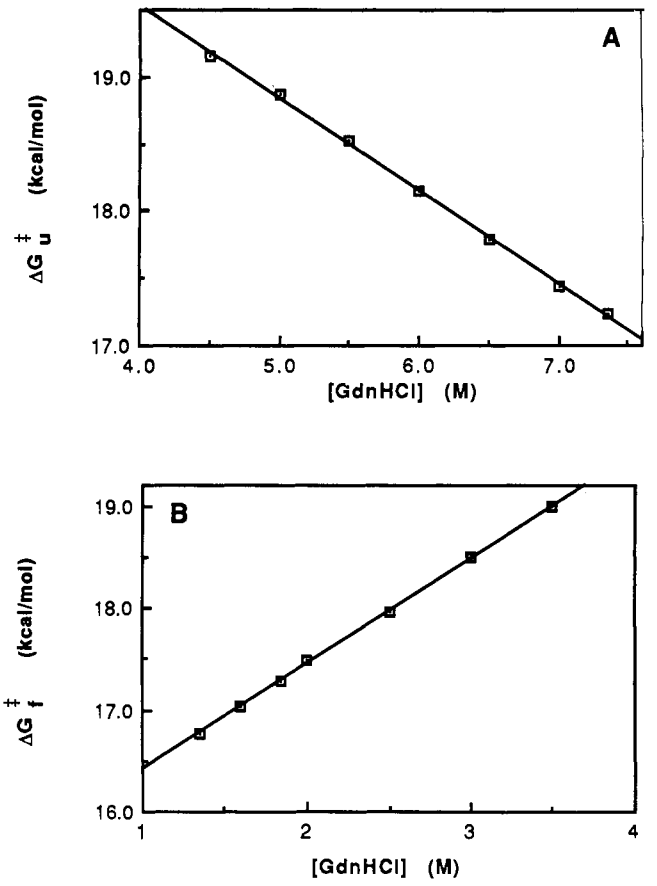

FIGURE 5: (A) Free energy change of activation for unfolding, ΔG_u^*, as a function of [GdnHCl]. (B) Free energy change of activation for refolding, ΔG_f^*, as a function of [GdnHCl]. Extrapolation of the linear plots to 0 M GdnHCl yields values for $\Delta G_u^*(H_2O)$ and $\Delta G_f^*(H_2O)$.

Discussion

Evidence That the Intermediate and Slow Phases in the Refolding of CI2 Are Due to Proline Isomerization. Proline isomerization can be a rate-limiting step in protein folding (Brandts et al., 1975; Kim & Baldwin, 1982). CI2 has five proline residues, four of which are in a trans configuration in the native structure. In the denatured state, however, both cis and trans isomers exist in equilibrium. Isomerization of proline residues that are in the wrong conformation, i.e., in a cis conformation, in the denatured state may be expected to give rise to slow folding species in the folding of CI2. The fifth proline is in a region that is not defined in either the crystal structure or in the NMR solution structure and is not thought to affect the folding process.

The strongest evidence that the intermediate and slow phases observed in the refolding of CI2 are due to rate-limiting proline isomerizations comes from experiments with peptidyl–prolyl isomerase. PPI is an enzyme that has been shown to catalyze the slow isomerization of peptide–proline bonds both in model oligopeptides (Fischer et al., 1984) and in the refolding of proteins where proline isomerization has been shown to be a rate-limiting step (Lang et al., 1987, Lin et al., 1988). PPI catalyzes both the intermediate and slow refolding phases of CI2 but does not affect the rate of folding of the fast phase. This suggests that the intermediate and slow refolding phases are due to slow rate-limiting proline isomerizations, the result of the fraction of protein with a proline in a nonnative cis conformation in the denatured state. It also suggests that the fast phase does not involve proline isomerization and corresponds to the fraction of protein that has all its prolines in a

native trans conformation in the denatured state. PPI does not affect the relative amplitudes of the three phases, suggesting that there is no change in the pathway of folding and that it acts only as a catalyst.

The ratio of cis to trans isomers of proline bonds in short oligopeptides varies between 0.1 and 1.0 (Grathwohl & Wüthrich, 1976a), and the half-lives for interconversion at 25 °C are in the range 10–100 s in aqueous solution (Grathwohl & Wüthrich, 1981). The cis/trans ratio for the intermediate and slow refolding species of CI2 are 0.09 and 0.19, respectively, and the half-lives, in the absence of denaturant, are 1.6 and 30 s, respectively, at 25 °C. Although the half-life for the slow phase is within the range found for model peptides, the half-life for the intermediate phase is smaller than expected from these studies. Possible reasons for this are discussed later.

The activation enthalpy for proline isomerization is characteristically large. Model compounds have activation enthalpies in the order of 19 ± 3 kcal mol^{-1} (Cheng & Bovey, 1977), and studies on proteins where isomerization is known to be a rate-limiting step find values in the range 16–20 kcal mol^{-1} (Brandts et al., 1975). The activation enthalpies for the intermediate and slow refolding phases of CI2 are 8 and 16 kcal mol^{-1}, respectively. Whereas the activation enthalpy for the slow phase is high and characteristic of a process involving proline isomerization, the activation enthalpy for the intermediate phase is low and suggests that this phase does not correspond to a simple proline isomerization.

The enthalpy change associated with the isomerization of proline residues is characteristically small. Values from model peptide studies are in the range 0–1 kcal mol^{-1} (Cheng & Bovey, 1977; Steinberg et al., 1960). The enthalpy change associated with the intermediate and slow refolding phases of CI2 is also very small, less than 1 kcal mol^{-1}, consistent with the hypothesis that these species are formed by proline isomerization.

The rate of proline isomerization in unfolded RNase A has been measured, under unfolding conditions, and found to be independent of [GdnHCl] (Schmid & Baldwin, 1979). In addition, the rate of proline isomerization in oligopeptides is also found to be independent of denaturant concentration (Nall et al., 1978). This suggests that the rate of isomerization in the denatured state should be independent of denaturant concentration. The slow refolding phase of CI2 is found to be almost independent of [GdnHCl], between 0 and 1.25 M, consistent with this model. Studies on carbonic anhydrase B have also found that the rate constant for the slow refolding phase, corresponding to proline isomerization, is independent of denaturant concentration (Semisotnov et al., 1990). The intermediate refolding phase of CI2, however, has a strong dependence on [GdnHCl]. This suggests that isomerization does not occur in the denatured state and that another process exists that both affects the rate of isomerization of this proline and that is dependent upon the denaturant concentration.

Evidence That the Intermediate Phase Is Due to Proline Isomerization in a Partially Structured State. Unlike the slow refolding phase of CI2, the intermediate phase shows behavior that is not consistent with a proline isomerization step occurring in the denatured state. The data are consistent, however, with a phase resulting from proline isomerization within a partially structured state. This may be an intermediate state on the folding pathway, or it may be a partially structured state not on the folding pathway but in rapid equilibrium with the denatured state. It is not possible to distinguish between these two mechanisms. It has been proposed that proline isomerization occurs in a partially structured state that is an intermediate on the folding pathway of RNase A (Nall et al., 1978).

The rates of isomerization of prolines in strained cyclic peptides are considerably higher than those observed for prolines in linear peptides (Grathwohl & Wüthrich, 1976b). Conformational strain in the cyclic peptide results in a lowering of the activation enthalpy of isomerization. The intermediate refolding phase of CI2 has a faster rate than normally observed for linear peptides, and correspondingly there are changes in both the enthalpy and entropy of activation. The enthalpy of activation is much less than the values found for linear peptides. This is consistent with the hypothesis that the isomerization step takes place in a partially structured state analogous to a strained peptide.

The rate of refolding increases with decreasing guanidinium chloride concentration, and so does any proline isomerization that occurs in a partially folded state and is thus coupled with folding. The rate of refolding of the intermediate phase is observed to increase with decreasing [GdnHCl]. The rate of refolding of the slow phase, corresponding to an isomerization already taking place in the denatured state, however, is virtually unaffected by the addition of denaturant.

In conclusion, it has been shown that the intermediate and slow phase observed in the refolding of CI2 result from rate-limiting proline isomerizations. The slow phase results from a proline isomerization within the denatured state, whereas the intermediate phase results from proline isomerization within a partially structured state.

Characterization of the Transition State of Folding. CI2 represents the simplest system for studying the thermodynamics of protein folding. Both equilibrium and kinetic experiments show that CI2 behaves as a two-state system where only the native and denatured states are significantly populated (Jackson & Fersht, 1991). The principle of microscopic reversibility requires that, under the same conditions, the transition state for unfolding is the same as the transition state for refolding. Studies on the temperature dependence of unfolding and on the temperature dependence of the fast phase of refolding, as well as the [GdnHCl] dependence of these processes, gives thermodynamic data on the nature of this transition state and data on its relative exposure to solvent.

Curvature in Eyring plots can be observed when there is a significant change in heat capacity between the initial state and the transition state. For CI2, the Eyring plot for unfolding is linear over the temperature range investigated, 17–40 °C, suggesting that the heat capacity change between the native state and the transition state is small. The Eyring plot for refolding, however, shows significant curvature over a similar temperature range, indicating that the heat capacity change from the denatured state to the transition state is large. Such behavior has also been observed for unfolding and refolding of hen egg white lysozyme (Segawa & Sugihara, 1984), chymotrypsin (Pohl, 1968a), and trypsin (Pohl, 1968b).

Curvature in the Eyring plot for refolding of CI2 may result not only from changes in heat capacity. CI2 is denatured under acidic conditions, and it may be possible, under such conditions, that another process affects the rate of refolding and results in curvature. For example, many proteins have residual structure at low pH and can adopt molten-globule-like equilibrium states (Goto et al., 1990). If such an equilibrium between denatured and partially folded states exists at low pH, increasing the temperature would destabilize the partially folded state with respect to the denatured state and force folding to occur from the denatured state rather than from the partially folded state. If folding from the denatured state

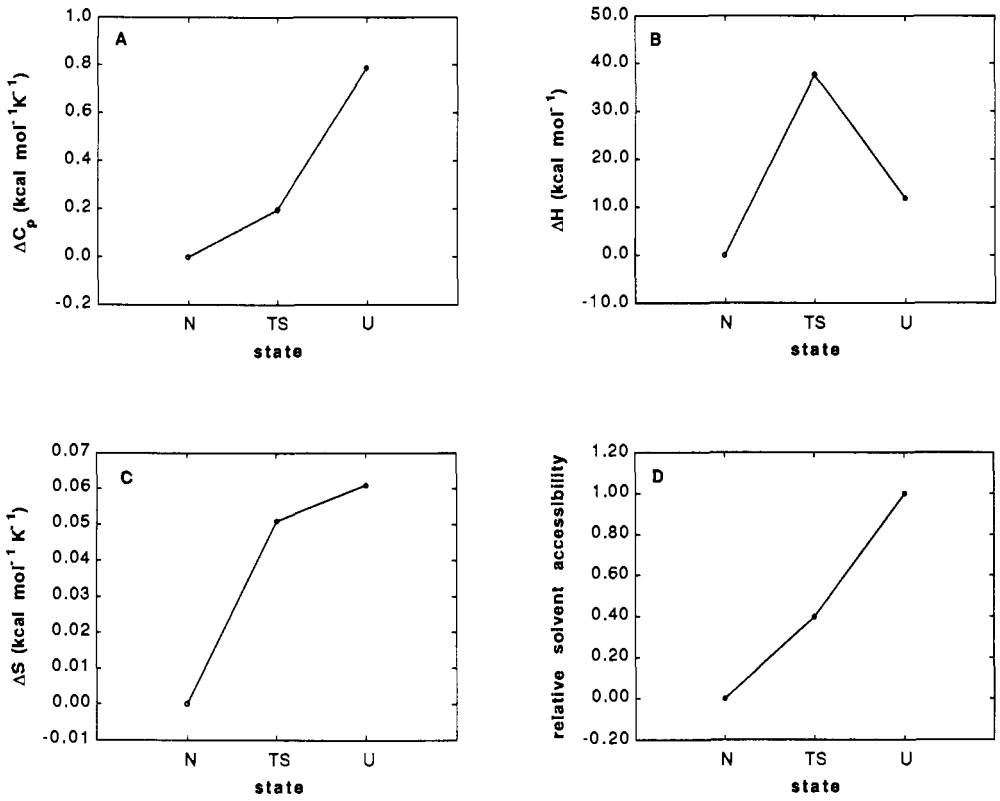

FIGURE 6: Reaction profiles for CI2 under native conditions, 0 M GdnHCl, at pH 6.3. The reaction proceeds from the native state "N", through the transition state "TS", to the denatured state "D". The native state is arbitrarily taken as the reference point. (A) Changes in heat capacity between the three states. (B) Changes in enthalpy between the three states. (C) Change in entropy between the three states. (D) Changes in solvent accessibility. In this case, the solvent accessibility of the native state is taken as 0, and the solvent accessibility of the denatured state is taken as 1.

was slower than from a partially folded state, this would result in curvature of the kind observed. To show that this is not the case for CI2, the experiment was repeated in the presence of 1.5 M GdnHCl in both denaturing and renaturing buffer to destabilize any partially folded species and ensure that there is no residual structure in the denatured sample. Although GdnHCl affects the absolute rates of refolding (Jackson & Fersht, 1991), significant curvature is still observed (Figure 4B). In addition, irreversible modification of the protein under acidic conditions is also excluded as the rates of refolding, measured first increasing in temperature and then decreasing in temperature, coincide, i.e., there is no hysteresis in the Eyring plot (Figure 4B). In the analysis of the refolding data, it is therefore assumed that the curvature is due entirely to changes in heat capacity between the denatured state and the transition state.

Using the analysis of Chen et al. (1989), we calculate the value for the change in heat capacity between the denatured and the transition state for CI2 to be −0.591 kcal mol^{-1} K^{-1}. This is 75% of the total heat capacity change between denatured and native states measured from scanning microcalorimetry experiments (Jackson & Fersht, 1991). Since $\Delta C_p = \Delta C_{pu}^{\ddagger} + \Delta C_{pf}^{\ddagger}$, this should result in a heat capacity change between native state and transition state of 0.197 kcal mol^{-1} K^{-1}. Over the temperature range studied, such a small change in heat capacity would not result in detectable curvature of the Eyring plot. The Eyring plot for unfolding does appear to be linear, and, therefore, it is possible only to say that the value of $\Delta C_{pu}^{\ddagger}$ is small and an accurate value cannot be measured directly from the Eyring plot.

Changes in heat capacity reflect changes in the degree of exposure of hydrophobic residues (Privalov et al., 1989, Privalov & Gill, 1989). The changes in heat capacity calculated from the analysis of the Eyring plots for unfolding and refolding of CI2 can, therefore, be used as a measure of the change in exposure of hydrophobic residues between the denatured, native, and transition state. Figure 6A shows that the heat capacity increases progressively from the native state through the transition state to the denatured state. The largest change occurs between the denatured state and the transition state, indicating a significant burying of hydrophobic residues. There is relatively little change in the exposure of hydrophobic residues between the native state and the transition state.

$\Delta H^{\ddagger}$, the activation enthalpy, and $\Delta S^{\ddagger}$, the activation entropy, can also be calculated from the analysis of the Eyring plots for unfolding and refolding. $\Delta H_u^{\ddagger}$ and $\Delta S_u^{\ddagger}$ for unfolding are 37.7 kcal mol^{-1} and 50.6 cal mol^{-1} K^{-1}, respectively. The large positive $\Delta H_u^{\ddagger}$ reflects the loss of interactions in the transition state that are present in the native state. Breaking these interactions seems to result in a less ordered state and an increase in the entropy of the system. For refolding, $\Delta H_f^{\ddagger}$ and $\Delta S_f^{\ddagger}$ are 11.92 kcal mol^{-1} and −10.4 cal mol^{-1} K^{-1}, respectively. The negative entropy change shows that the transition state is more ordered than the denatured state. There is a balance between the decrease in the conformational entropy of the chain on forming a "compact" transition state and the entropy gained by the release of water molecules associated with hydrophobic residues becoming buried in the transition state. At 25 °C, the loss in the conformational entropy of the chain outweighs the increase in entropy due to the hydrophobic effect. The large positive change in enthalpy on refolding is indicative of the formation of hydrophobic interactions. Figure 6B,C shows reaction profiles for the change in entropy and enthalpy, respectively, under native conditions.

The change in free energy of activation of unfolding and

refolding (the change in free energy between native and transition state or denatured and transition state), as well as the equilibrium change in free energy of unfolding, are linearly dependent on GdnHCl concentration (Figure 5). The slope of such plots is a measure of the relative interaction of the initial state and the transition state with denaturant. A negative sign indicates that the interaction is stronger in the transition state than in the initial state and a positive sign that the interaction is weaker in the transition state than in the initial state. The magnitude of the slope is a measure of the relative strength of the interactions.

The plot of the free energy of activation of unfolding versus [GdnHCl] (Figure 5A) has a negative gradient, indicating that the transition state interacts more strongly with GdnHCl than the native state. The transition state is partially opened, and there is an average increase in the exposure of residues. The value of m_u^* is approximately one-third of that for the total transition between native and denatured states. Conversely, the plot of free energy of activation of folding against [GdnHCl] (Figure 5B) has a positive gradient. The denatured state interacts more strongly with GdnHCl than the transition state. Residues that interact with solvent in the denatured state become buried in the transition state. The magnitude of m_f^* is about two-thirds of that for the transition between native and denatured states. The average degree of exposure of residues decreases progressively from the denatured state through the transition state to the native state (Figure 6D). The largest change occurs between the denatured state and the transition state.

In summary, the kinetic data from unfolding and refolding experiments have been used to characterize the transition state of folding for CI2. The transition state is compact with many hydrophobic residues buried. This is shown by the large negative change in heat capacity between denatured state and transition state and by the large positive enthalpy of activation of folding. In addition, the average degree of exposure of residues to solvent has greatly decreased from denatured state to transition state. The magnitudes of both the change in heat capacity and the change in degree of exposure of residues between the three states suggest that the transition state is native-like.

The transition state is possibly a high-energy form of the native state in which stabilizing interactions, present in the native structure, have been lost. This is shown by the large positive enthalpy of activation of unfolding. Loss of such interactions seems to be accompanied by a partial opening of the hydrophobic core. This is shown by the slight increase in solvent accessibility of the transition state compared to the native state and, in addition, the small increase in the exposure of hydrophobic residues.

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References

Brandts, J. F., Halvorson, H. R., & Brennan, M. (1975) *Biochemistry 14*, 4953–4963.

Chen, B., Baase, W. A., & Schellman, J. A. (1989) *Biochemistry 28*, 691–699.

Cheng, H. N., & Bovey, F. A. (1977) *Biopolymers 16*, 1465–1472.

Clore, G. M., Gronenborn, A. M., Kjaer, M., & Poulsen, F. M. (1987a) *Protein Eng. 1*, 305–311.

Clore, G. M., Gronenborn, A. M., James, M. N. G., Kjaer, M., McPhalen, C. A., & Poulsen, F. M. (1987b) *Protein Eng. 1*, 313–318.

Fischer, G., & Schmid, F. X. (1990) *Biochemistry 29*, 2205–2212.

Fischer, G., Bang, H., & Mech, C. (1984) *Biomed. Biochim. Acta 43*, 1101–1111.

Goto, Y., Calciano, L. J., & Fink, A. L. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 573–577.

Grathwohl, C., & Wüthrich, K. (1976a) *Biopolymers 15*, 2025–2041.

Grathwohl, C., & Wüthrich, K. (1976b) *Biopolymers 15*, 2043–2057.

Grathwohl, C., & Wüthrich, K. (1981) *Biopolymers 20*, 2623–2633.

Jackson, S. E., & Fersht, A. R. (1991) *Biochemistry* (preceding paper in this issue).

Kiefhaber, T., Quaas, R., Hahn, U., & Schmid, F. X. (1990a) *Biochemistry 29*, 3053–3061.

Kiefhaber, T., Quaas, R., Hahn, U., & Schmid, F. X. (1990b) *Biochemistry 29*, 3061–3070.

Kiefhaber, T., Grunert, H. P., Hahn, U., & Schmid, F. X. (1990c) *Biochemistry 29*, 6475–6480.

Kim, P. S., & Baldwin, R. L. (1982) *Annu. Rev. Biochem. 51*, 459–489.

Kim, P. S., & Baldwin, R. L. (1990) *Annu. Rev. Biochem. 59*, 631–660.

Laidler, K. J. (1950) *Chemical Kinetics*, Chapter 3, McGraw-Hill, New York.

Lang, K., Schmid, F. X., & Fischer, G. (1987) *Nature 329*, 268–270.

Lin, L.-N., Hasumi, H., & Brandts, J. F. (1988) *Biochim. Biophys. Acta 956*, 256–266.

Liu, J., Albers, M. W., Chen, C.-M., Schreiber, S. L., & Walsh, C. T. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 2304–2308.

Longstaff, C., Campbell, A., & Fersht, A. R. (1990) *Biochemistry 29*, 7339–7347.

McPhalen, C. A., & James, M. N. G. (1987) *Biochemistry 26*, 261–269.

Nall, B. T., Garel, J. R., & Baldwin, R. L. (1978) *J. Mol. Biol. 118*, 317–330.

Pohl, F. M. (1968a) *Eur. J. Biochem. 4*, 373–377.

Privalov, P. L. (1979) *Adv. Protein Chem. 33*, 167–241.

Privalov, P. L., & Gill, S. J. (1989) *Adv. Protein Chem. 39*, 191–234.

Schellmann, J. A. (1978) *Biopolymers 17*, 1305–1322.

Schmid, F. X., & Baldwin, R. L. (1979) *J. Mol. Biol. 133*, 285–287.

Segawa, S.-I., & Sugihara, M. (1984) *Biopolymers 23*, 2473–2488.

Semisotnov, G. V., Uversky, V. N., Sokolovsky, I. V., Gutin, A. M., Razgulyaev, O. I., & Rodionova, N. A. (1990) *J. Mol. Biol. 213*, 561–568.

Steinberg, I. Z., Harrington, W. F., Berger, A., Sela, M., & Katchalski, E. (1960) *J. Am. Chem. Soc. 81*, 62–67.