Effect of an Engineered Disulfide Bond on the Folding of T4 Lysozyme at Low Temperatures†

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ABSTRACT: Equilibrium and kinetic effects on the folding of T4 lysozyme were investigated by fluorescence emission spectroscopy in cryosolvent. To study the role of disulfide cross-links in stability and folding, a comparison was made with a mutant containing an engineered disulfide bond between Cys-3 (Ile-3 in the wild type) and Cys-97, which links the C-terminal domain to the N terminus of the protein [Perry & Wetzel (1984) *Science 226*, 555]. In our experimental system, stability toward thermal and denaturant unfolding was increased slightly as a result of the cross-link. The corresponding reduced protein was significantly less stable than the wild type. Unfolding and refolding kinetics were carried out in 35% methanol, pH 6.8 at −15 °C, with guanidine hydrochloride as the denaturant. Unfolding/refolding of the wild-type and reduced enzyme showed biphasic kinetics both within and outside the denaturant-induced transition region and were consistent with the presence of a populated intermediate in folding. Double-jump refolding experiments eliminated proline isomerization as a possible cause for the biphasicity. The disulfide mutant protein, however, showed monophasic kinetics in all guanidine concentrations studied.

Although disulfide bonds are a common feature of extracellular proteins, their role in protein structure and function is not clear, and recent experiments on disulfide bonds engineered into proteins have, if anything, complicated the picture (Wetzel, 1988). There are two central questions regarding the anticipated stabilizing effect of these cross-links. (1) In the conversion of two spatially proximal residues into disulfide-cross-linked cysteines, how do revised nonbonded interactions, disulfide geometry, chain entropy, and other effects interact to determine the contribution of the disulfide to the free energy of stabilization? (2) Is there a straightforward relationship between this free energy effect (i.e., stability toward reversible denaturation) and the disulfide's contribution to irreversible denaturation/inactivation processes such as thermal aggregation and autolysis, or does the disulfide possess more subtle attributes that must be invoked to account for such stability effects? For example, in the subtilisin system, cross-links that are expected to provide free energy of stabilization do not produce molecules of enhanced stability to thermal inactivation (Wells & Powers, 1986; Mitchinson & Wells, 1989). Experiments with T4 lysozyme present a different dichotomy: although an engineered disulfide does contribute somewhat to the free energy of stabilization in reversible unfolding experiments, the stability it contributes to irreversible thermal denaturation is clearly not related to its effect on unfolding thermodynamics (Wetzel et al., 1988).

This paper describes equilibrium and kinetic studies of denaturant-induced unfolding and refolding of wild-type T4 lysozyme at subzero temperature in cryosolvent, as monitored by tryptophan fluorescence, and compares its behavior to that of the cross-linked and non-cross-linked forms of a T4 lysozyme mutant. Although these experiments are considerably different from thermal unfolding experiments (Wetzel et al., 1988), we observe a similar stabilizing effect of the disulfide on the reversible transition. In addition, while non-cross-linked T4 lysozymes show biphasic kinetics in folding, consistent with involvement of a folding intermediate, the 3–97 cross-linked molecule shows more rapid monophasic folding kinetics. These results may bear on the question of the role of the disulfide in stabilizing T4 lysozyme against thermal denaturation. Subzero temperatures and aqueous methanol cryosolvent were used because of their potential to stabilize partially folded intermediate states (Biringer & Fink, 1982a,b, 1988a,b; Fink, 1986; Biringer et al., 1988).

Materials and Methods

Materials

Wild-type and mutant (I3C/C54V or I3C/C54T) T4 lysozyme were prepared as described previously (Mulkerrin et al., 1985; Perry & Wetzel, 1987). The oxidized forms of I3C/C54T or I3C/C54V, in which there is a disulfide bond between Cys-3 and Cys-97, will be referred to as the disulfide or cross-linked mutant. The reduced forms, in which Cys-3 and Cys-97 are in the SH form, will be referred to as the reduced mutant. The storage buffer, 0.2 M phosphate and 1 mM EDTA, pH 6.5, was changed by dialyzing against 50 mM cacodylate and 0.2 M NaCl, pH 6.0. For the wild-type and the reduced mutant the solution was made 1 mM in β-mercaptoethanol. The concentration of the stock enzyme solution was 4 mg/mL as measured by the absorbance at 280 nm, using $\epsilon_{280}^{0.1\%}$ of 1.22 (Tsugita, 1971). Ultrapure Gdn·HCl (Research Plus Laboratories) dithiothreitol (Sigma), mercaptoethanol (J. T. Baker Chemical Co.), and HPLC grade methanol (Fisher Scientific) were used; all other chemicals were of reagent grade.

Two cryosolvent solutions were prepared; both were sodium cacodylate (0.05 M) buffers of pH 6.8 containing 0.2 M NaCl, 1 mM β-mercaptoethanol, and 35% methanol. One of the solutions was 5 M in Gdn·HCl. These solutions were made by adding a 35% methanol solution to the solids in a volumetric

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flask. The 35% methanol solution was prepared on a volume/volume basis. Refolding and unfolding solutions were made by adding varying amounts of both buffers to make a 5-mL solution; e.g., if a 4 M Gdn·HCl solution was desired, 4 mL of the 5 M Gdn·HCl buffer and 1 mL of the standard buffer were mixed together. All solutions were filtered through a 0.45-μm Teflon filter prior to use.

Formation of the Reduced Mutants. Initial attempts at reducing the disulfide bond were performed in various concentrations of methanol and in aqueous medium, at pH 8, under native conditions. Aggregation resulted in all these experiments. However, the reduction could be satisfactorily carried out under denaturing conditions, namely, 4 M Gdn·HCl, 35% methanol, 50 mM cacodylate, 0.2 M NaCl, 1 mM EDTA, and 10 mM DTT, pH 8.0. A 10-mL reducing buffer was prepared prior to each reduction experiment by adding the solids, excluding the DTT, to a 10-mL volumetric flask and then adding a 35% methanol (v/v) solution to the 10-mL mark. The solution was then degassed by bubbling nitrogen. After the solution was degassed for at least 30 min, the DTT was added and mixed. Reduction was carried out in 1-mL volumes by adding the appropriate aliquot of cross-linked I3C/C54T enzyme to yield an enzyme concentration of 0.15 mg/mL. After reduction, the solution was stored at 4 °C. The Gdn·HCl was removed to obtain native enzyme by dialyzing 1–2 mL of reduced enzyme against 250 mL of degassed 35% methanol, 50 mM cacodylate, 0.2 M NaCl, and 1 mM EDTA, pH 6.8, at 4 °C three times over 24 h. The dialyzing solution was degassed by subjecting it to a vacuum while magnetically stirring for 30 min and then bubbling nitrogen gas through.

Methods

Spectroscopy. Fluorescence spectroscopy with a Perkin-Elmer MPF-4 spectrophotometer was used to follow the thermal and Gdn·HCl-induced transitions and the kinetics of unfolding and refolding. The thermally induced unfolding transition and the kinetics of unfolding and refolding were monitored by the change in fluorescence intensity at 358 nm. Denaturant-induced transitions were measured by scanning the fluorescence emission spectra of protein samples in known Gdn·HCl concentrations and noting the change in λ_{max}. In the above experiments an excitation wavelength of 280 nm was employed and the excitation and emission slit widths were 3 and 8 nm, respectively. An insulated brass-block cell holder attached to a low-temperature circulation bath maintained the temperature of the cell contents at -15 ± 0.2 °C. The cell compartment was constantly purged with dry nitrogen.

Guanidine Hydrochloride Induced Unfolding Transitions. Solutions of 5 mL of cross-linked mutants and wild-type enzymes were prepared with varied concentrations of Gdn·HCl. This was accomplished by first adding 1 mL of native enzyme in standard buffer (0.05 mg/mL) to the 5-mL volumetric flask. Depending on the desired Gdn·HCl concentration, aliquots of the 5 M Gdn·HCl buffer solution were then added, and the remainder was filled with the standard buffer. The prepared solutions were allowed to stand overnight at −20 °C. Emission scans were then performed after 1 mL of the enzyme solution was equilibrated at −15 °C for 20 min and stirred at the low temperature.

Kinetics of Unfolding and Refolding. Stock solutions of T4 lysozyme for unfolding and refolding were prepared in an analogous manner to the above. The final enzyme concentration was 0.32 mg/mL in both cases. The enzyme solutions were stored at −20 °C prior to each experiment. For the kinetic experiments 50 μL of the stock solution was added to 1.0 mL of precooled cryosolvent which ranged in final Gdn·HCl concentration from 2.4 to 4 M for unfolding and from 0.6 to 2.4 M for refolding. The resulting enzyme concentration in the cell was 0.80 μM. Immediately after injection of the protein, the solution was stirred with a vibrating mixer. Mixing and thermal artifacts lasted less than 30 s on the basis of control experiments. The conditions employed for following the kinetics of unfolding and refolding were 35% methanol, −15 °C, and pH* 6.8.

Double-Jump Folding Assays for Proline Isomerization. The wild-type protein sample was unfolded in 5.6 M Gdn·HCl, pH 2, at 0.5 °C. The final T4 lysozyme concentration was 0.1 mg/mL. Aliquots were removed after various time periods and injected into solutions of cacodylate buffer, pH 7, containing various concentrations of Gdn·HCl (in the region of the C_m). The final pH was 6.4. The kinetics of unfolding and refolding in aqueous solutions were monitored by using the ellipticity at 222 nm.

Data Analysis. Data from all experiments were analyzed by a curve-fitting program based on the algorithm of Marquardt (1963) as described by Bevington (1969) using a microcomputer (Koerber & Fink, 1987). The expected amplitudes in the kinetics experiments were calculated by scanning the emission spectrum of the cell contents after each unfolding or refolding experiment and noting the difference between spectra of the native or denatured state, respectively. The amplitudes obtained from the microcomputer analysis for both phases were then summed and compared to this value.

Thermal Unfolding Transitions. The thermal unfolding transitions of the wild-type lysozyme and of the reduced and cross-linked mutants were performed under identical conditions, namely, 35% methanol, 0.2 M NaCl, and 50 mM cacodylate, pH 6.8. The enzyme concentration for each experiment was 0.015 mg/mL. In each case the appropriate amount of enzyme was added to the precooled cryosolvent, about 0 °C, in the cell. For the reduced enzyme, the cryosolvent buffer was degassed by bubbling nitrogen through prior to the experiment. The temperature was raised by 0.5 °C/min with a linear temperature programmer (Neslab). The temperature of the cell contents was monitored by a thermocouple taped to the outside of the cell. The cell contents were constantly stirred by a motor-driven paddle (SpectroCell) which also acted as a stopper for the cell. The temperature was increased to 55 °C for each run, while the data were collected on a microprocessor. The temperature was then decreased to the original starting temperature to check for reversibility.

RESULTS

In preliminary experiments (data not shown) we determined that the kinetics of unfolding and refolding of the I3C/C54T and I3C/C54V mutants, in the oxidized or reduced forms, were very similar. That is, the nature of the residue at position 54 did not affect the folding kinetics.

Fluorescence Properties of T4 Lysozyme. The fluorescence emission spectra of wild-type, the disulfide mutant I3C-C97/C54V, and the reduced mutant (I3C/C54V) T4 lysozyme in the native and unfolded states are shown in Figure 1. The protein contains six tyrosines and three tryptophans; the tryptophans are responsible for most of the fluorescence emission. The largest difference in intensity between the native and the unfolded state occurred in the 350–360-nm region. Therefore, the change in fluorescence emission at 358 nm was used to follow the conformational changes associated with folding.

Denaturant-Induced Unfolding Transitions. The unfolding transition was monitored by fluorescence emission as a function

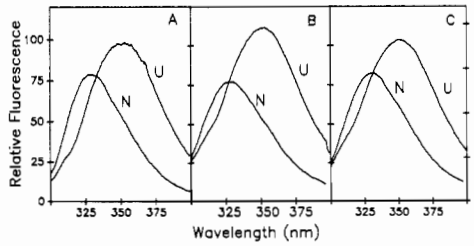

FIGURE 1: Fluorescence emission spectra of the native and unfolded states of T4 lysozymes in 35% methanol, pH* 6.8, at −15 °C. The unfolded proteins are in 4 M Gdn·HCl. Excitation was at 280 nm. (A) Wild-type enzyme; (B) disulfide mutant I3C-C97/C54T; (C) reduced mutant I3C/C54T.

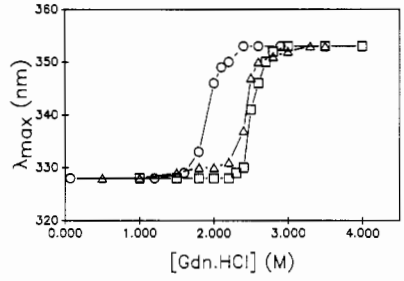

FIGURE 2: Unfolding transitions of T4 lysozymes as a function of Gdn·HCl concentration. The reaction was monitored by fluorescence emission with excitation at 280 nm. The figure shows the shift in λ_{max} as a function of Gdn·HCl concentration at −15 °C, pH* 6.8, in 35% methanol. The wild-type protein is denoted △, the disulfide mutant I3C-C97/C54V □, and the reduced mutant I3C/C54V ○.

Table I: Comparison of Relative Stabilities of T4 Lysozymes[a]

protein	C_m (M)	T_m (°C)
wild type	2.45	38.8
disulfide (I3C/C54V)	2.55	41.0
reduced (I3C/C54V)	1.9	35.5

[a] Conditions were 35% methanol, pH* 6.8. For Gdn·HCl denaturation the temperature was −15 °C. The transitions were monitored by fluorescence emission.

of Gdn·HCl (Figure 2). The conditions were 35% methanol, pH* 6.8, and −15 °C. The wild-type enzyme and the disulfide mutant have similar C_m values around 2.5 M, whereas the reduced mutant is substantially less stable than either the disulfide mutant or the wild-type enzyme. The data for each protein are summarized in Table I.

Thermal Unfolding Transitions. The thermally induced unfolding transition of the three proteins was monitored by fluorescence emission (Figure 3). The cross-linked mutant, I3C-C97/C54V, had a T_m slightly higher than that found for the wild-type protein and significantly higher than that for the corresponding reduced mutant (Table I).

In each of the thermal denaturations the cell contents were heated to about 55 °C. The temperature was then decreased to the starting temperature. Only the disulfide mutant showed complete renaturation, and the T_m of the reverse transition was nearly identical with that for unfolding. The disulfide mutant also showed an identical emission spectrum after return to the starting temperature, whereas the others did not. The relative stabilities, and reversibility in unfolding, in cryosolvent are properties identical with those observed in aqueous buffer at pH 6.5 when unfolding was monitored by circular dichroism (Wetzel et al., 1988).

Kinetics of Folding of Wild-Type T4 Lysozyme. The kinetics of *unfolding* were monitored at various final concentrations of Gdn·HCl in 35% methanol at −15 °C and pH* 6.8. In all Gdn·HCl concentrations studied, in and above the transition region, the kinetics were biphasic. A typical kinetic

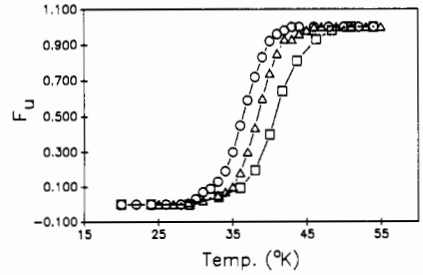

FIGURE 3: Thermal unfolding transitions of T4 lysozymes. The conditions employed were 35% methanol, pH* 6.8. The change in fluorescence emission intensity at 358 nm (excitation at 280 nm) was used to follow the transition. The raw data were converted to fraction of unfolded protein by linear extrapolation of the regions outside the transition. The wild-type protein is denoted △, the disulfide mutant I3C-C97/C54V □, and the reduced mutant I3C/C54V ○.

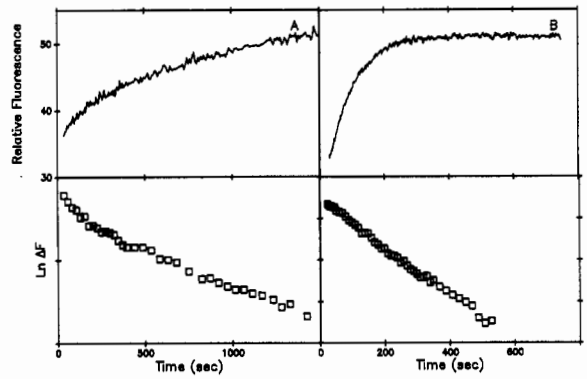

FIGURE 4: Kinetics of unfolding of T4 lysozyme. Conditions were 35% methanol, pH* 6.8, and −15 °C, measured by fluorescence emission at 358 nm. The protein concentration was 0.015 mg/mL. (A) Kinetic trace for unfolding the wild-type protein in 3.0 M Gdn·HCl. The lower panel shows the semilog plot of the data for unfolding in 3.0 M Gdn·HCl illustrating the biphasic nature of the reaction. (B) Kinetics of unfolding of the disulfide mutant I3C-C97/C54V in 3.0 M Gdn·HCl. The lower panel shows a semilog plot of the data for unfolding in 2.6 M Gdn·HCl illustrating the monophasic kinetics.

trace, for unfolding in 3.0 M Gdn·HCl, is shown in Figure 4A. The semilog plot of the data is also shown to illustrate the biphasic nature of the kinetics. The sum of the amplitudes of each phase accounted for the total expected difference based on equilibrium data. This indicates that there is no additional faster phase occurring prior to the first observed transient. The amplitudes of the fast and slow phases relative to the total change will be denoted α_2 and α_1, respectively, where $\alpha_1 + \alpha_2 = 1$.

Figure 5 shows the dependence of the rate constants relative amplitude for unfolding on the final Gdn·HCl concentration. The relative amplitudes of the fast and slow phases in unfolding were relatively independent of the final Gdn·HCl concentration.

Refolding kinetics of wild-type T4 lysozyme were studied by using enzyme denatured in 4.0 M Gdn·HCl as a function of Gdn·HCl, in 35% methanol at −15 °C and pH* 6.8. Kinetic traces of the refolding process showed biphasic data above 1.6 M Gdn·HCl. Typical data are shown in Figure 6A. The biphasic nature of the data is readily apparent in the semilog plot in Figure 6A. As in unfolding, the sum of the amplitudes of the fast and slow phases corresponded to the expected change, within experimental error, based on equilibrium data.

The observed kinetics of refolding into Gdn·HCl concentrations below 1.0 M appeared monophasic. However, comparison of the associated amplitude with the equilibrium measurements suggested the presence of a faster phase that was complete within the dead-time of mixing. The amplitude

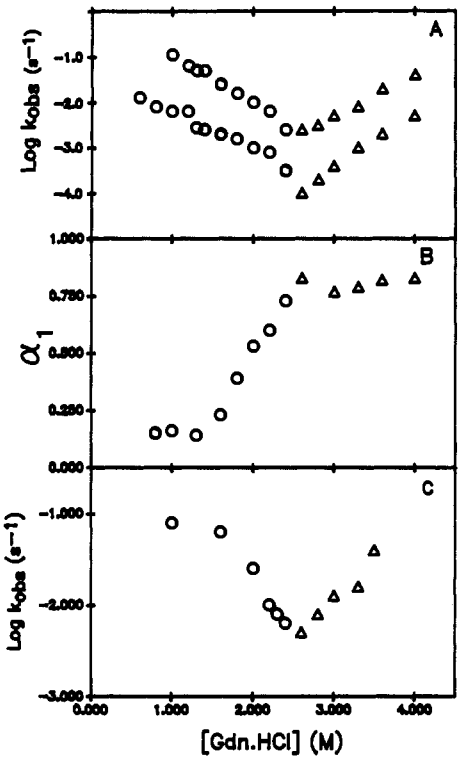

FIGURE 5: (A) Kinetic dependence of the fast and slow rates of folding of wild-type T4 lysozyme as a function of Gdn·HCl concentration. Refolding experiments are shown by O and unfolding experiments by Δ. For the refolding experiments the protein was unfolded in 4 M Gdn·HCl in cryosolvent. (B) Relative amplitude of the slow phase, α_1, as a function of Gdn·HCl concentration for refolding (O) and unfolding (Δ) of the wild-type protein. (C) Kinetic data for the disulfide mutant 13C-C97/C54V; unfolding results are shown as Δ and refolding data as O.

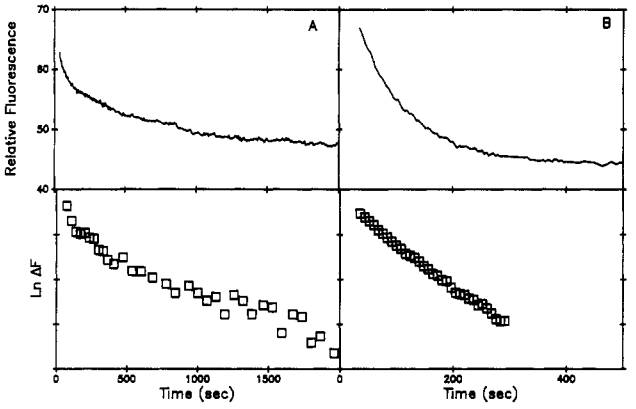

FIGURE 6: Kinetics of refolding of T4 lysozyme. The experimental conditions were the same as those used in the unfolding experiments given in the legend to Figure 4. The protein was unfolded in 4 M Gdn·HCl. (A) Refolding of the wild-type protein in 2.0 M Gdn·HCl. The lower panel shows the corresponding semilog plot demonstrating the biphasic kinetics. (B) Kinetics of refolding of the disulfide mutant 13C-C97/C54V in 2.2 M Gdn·HCl. The lower panel shows the corresponding semilog plot illustrating the monophasic kinetics.

of the fast phase was obtained by subtracting the amplitude of the slow phase from the expected intensity change.

The amplitude of the slow phase is fairly constant below the transition region, about 15–20% of the total change and then increases to a constant value of about 80–85% of the total change above the transition region (Figure 5B). The midpoint of the amplitude transition occurs at a Gdn·HCl concentration slightly lower than the C_m.

Several points argue in favor of the slow phase, shown in Figure 6, being a real process and not the result of aggregation or photooxidation of tryptophan. In experiments with higher enzyme concentration, aggregation was noticed and resulted in an apparent increase in fluorescence intensity due to light scattering, in contrast to the *decrease* in fluorescence emission in refolding. Photolysis processes actually did occur in several of the refolding experiments under the settings employed after long time periods. These resulted in the presence of an even slower phase following the slow phase. This very slow phase was stripped out by extrapolation prior to analysis of the biphasic refolding kinetics. The very slow phase was attributed to Trp photooxidation on the basis of experiments which were run for extended periods of time. After the expected completion of the folding reaction, an emission scan showed the λ_{max} of the native state. After prolonged periods of monitoring only the intensity of the spectrum decreased, there was no change in λ_{max}. Refolding kinetics experiments performed at 315 nm, where the folding corresponded to an increase in fluorescence intensity, gave kinetics results, for both phases, very similar to those obtained at 358 nm and did not show the presence of the very slow phase.

Kinetics of Folding of the Disulfide Mutant. In all Gdn·HCl concentrations studied the unfolding kinetics could be described with a *single* exponential function. Results from such an experiment are presented in Figure 4B. The semilog plot is linear, consistent with the monophasic nature of the reaction.

Figure 5C shows the effect of Gdn·HCl on the observed rate constant of the single phase of unfolding. The rate of this monophasic process is similar to that of the fast phase in unfolding of the wild-type protein, in terms of both the magnitude of the rate constant and the dependence on Gdn·HCl concentration.

Refolding was initiated by adding protein, unfolded in 4 mM Gdn·HCl, into various Gdn·HCl concentrations. A typical refolding kinetic trace is depicted in Figure 6B, along with a corresponding semilog plot. Monophasic refolding kinetics were obtained in all Gdn·HCl concentrations studied. The amplitude of the observed phase, when compared to the expected amplitude from equilibrium measurements, accounted for the entire change and ruled out the possibility of the presence of a faster phase occurring within the dead-time of the system. The observed rate constants and the Gdn·HCl dependence are simlar to those found for the fast phase of refolding of wild-type T4 lysozyme. A minimum in the refolding rate occurs near the C_m. In several refolding experiments evidence for photooxidation was noted at long time periods.

Kinetics of Folding of the Reduced Mutant. The unfolding kinetics of the reduced mutant were studied in a manner similar to those of the wild-type protein. The unfolding kinetics were biphasic at all Gdn·HCl concentrations examined (Figure 7). Comparing this plot to that found for the wild-type enzyme reveals two major differences. The minimums in the rates for the fast and slow phases are shifted to lower Gdn·HCl concentrations and appear to be accelerated, relative to that of the wild-type protein. Figure 7 also shows the relative amplitude of the slow phase, α_1, in unfolding as a function of Gdn·HCl. It is fairly constant for all final Gdn-HCl concentrations studied and is of a similar value as that found for the wild-type protein.

The kinetics of refolding for the reduced mutant (Figure 7) were biphasic at higher guanidine concentrations but became apparently monophasic at lower denaturant concentrations. Examination of the observed amplitude for the monophasic reactions indicated a fast phase complete within the

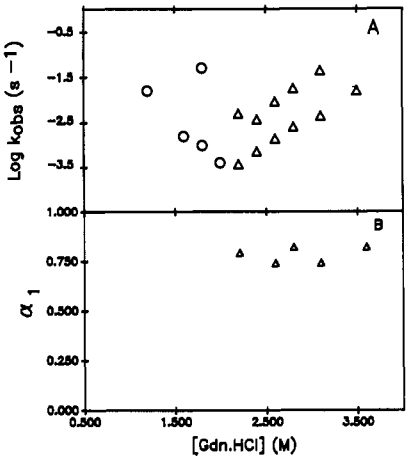

FIGURE 7: Effect of Gdn·HCl on the kinetics of unfolding and refolding of the reduced mutant I3C/C54T. Data from refolding experiments are shown by circles, and unfolding data are shown by triangles.

dead-time of the manual mixing.

Temperature Dependence of the Kinetics of Unfolding and Refolding. The kinetics of refolding of the wild-type protein as a function of temperature were also measured. Figure 8A shows the temperature dependence for refolding in 1.8 M Gdn·HCl. This concentration was chosen since the amplitude of the slow phase has an appreciable value. The activation energy for the fast phase is 10 kcal/mol. The rate of the slow phase, however, shows very little temperature dependence. The amplitude of the slow phase increases as the temperature is lowered. This most likely results from the different activation energies for the rates of the fast and slow phases. The relative amplitude of the slow phase as a function of temperature is also shown in Figure 8B.

Figure 8C shows the temperature dependence of the kinetics for the disulfide mutant in refolding experiments with 2.0 M Gdn·HCl. The temperature dependence of the refolding rates gave an activation energy of 19 kcal/mol. The temperature dependence of unfolding in 3.3 M Gdn·HCl shows an activation energy of 12 kcal/mol.

Assays for Proline Isomerization. A common source of multiple phases in the refolding kinetics of proteins is the presence of proline isomerization. This can be monitored by double-jump folding experiments (Schmid, 1986). A simple method involves unfolding the protein in denaturant at 0 °C (to minimize proline isomerization, which has a large energy of activation) and then refolding in the vicinity of the C_m. An initial rapid formation of native material is observed, followed by a slower decrease in concentration of the native state as the equilibrium between denatured states with different proline conformations is established (T. Nakano and A. L. Fink, unpublished results). In the case of T4 lysozyme only the initial refolding to native state was observed, the rates being comparable to those observed in normal refolding kinetics experiments. No evidence of a slower phase corresponding to formation of a species with nonnative proline conformation was observed.

Discussion

The results of the equilibrium studies show that the presence of a disulfide bond between residues 3 and 97 of T4 lysozyme results in only a small increase in stability toward reversible thermal and Gdn·HCl denaturation when compared to the enhancements resulting from chemical cross-links in ribonuclease and lysozyme (Johnson et al., 1975; Lin et al., 1984). Reduction of the disulfide bond leads to a significant decrease in stability, especially toward denaturant. Interestingly, the

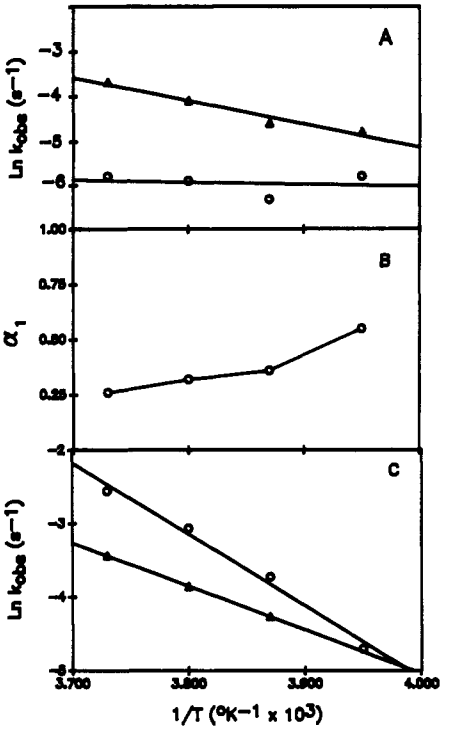

FIGURE 8: Temperature effects on the rates of folding of T4 lysozymes. (A) Temperature dependence on refolding of the wild-type protein in 1.8 M Gdn·HCl for both phases in refolding. The data for the fast phase are shown by triangles, those for the slow phase by circles. (B) Corresponding temperature dependence of the fraction of the total amplitude for the slow phase. (C) Temperature dependence on the single phase in refolding (open circles) and unfolding (solid triangles) for the disulfide mutant I3C-97/C54V.

C_m with Gdn·HCl for wild-type protein at −15 °C, 35% methanol, pH* 6.8, 2.45 M, is quite similar to that for aqueous solution at 15.5 °C and pH 7.4, namely, 2.25 M (Desmadril & Yon, 1984).

The net stability of a protein depends on the difference in free energy of the unfolded and folded state, ΔG_u. The enhanced stability of cross-linked enzymes has been attributed to an increase in conformational free energy of the unfolded state, due to the reduced conformational flexibility of the unfolded state, which results in decreased entropy. Chicken lysozyme and ribonuclease showed large increases in ΔG_u, as evidenced by a 20–30 °C rise in the T_m on formation of chemical cross-links. In the present study the disulfide mutant of T4 lysozyme showed very small increases in the T_m and C_m, relative to those of the wild type. The relatively small stabilization of the 3–97 cross-link has also been observed in thermal unfolding studies in aqueous solution (Wetzel et al., 1988). It appears that the stabilization derived from the chain entropy effect is partially compensated for by other factors, such as interactions associated with side-chain replacement and dihedral strain in the disulfide (Wetzel, 1987a,b).

Folding Model for Wild-Type T4 Lysozyme. A two-state model is ruled out by the biphasic kinetics. A three-state model involving the interconversion between two unfolded species differing by proline isomerization (eq 1) can be ruled out on

$$U_1 \Leftrightarrow U_2 \Leftrightarrow N \qquad (1)$$

the basis of the results of the assays for proline isomerization and the fact that proline isomerization is characterized by a high activation energy, about 20 kcal/mol, in contrast to the low value observed for the slow phase (Figure 8).

The results are most reasonably interpreted in terms of either a three-state model in which an intermediate state exists between the native and unfolded state or an independent do-

main folding model (Ikai & Tanford, 1973), which predict biphasic kinetics with constant amplitudes for the fast and slow phases above and below the transition region (eq 2). Figure

$$U \Leftrightarrow I \Leftrightarrow N \quad (2)$$

5 shows that the amplitude of the slow phase is constant over a wide range of Gdn·HCl concentrations below and above the transition and that the change in amplitude below and above the transition obeys the required reciprocal relationship.

The independent folding domain model is a reasonable interpretation of the data in light of the results of Desmadril and Yon (1984), in aqueous solution, which showed noncoincidence of the transitions when monitored by fluorescence or CD and were consistent with the loss of secondary structure (corresponding to the N-terminal domain) prior to the loss of the fluorescence signal from the three Trp residues located in the C-terminal domain. This suggests that the C-terminal domain is more stable and that the observed changes in fluorescence amplitude in unfolding/refolding correspond predominantly to changes in the structure of the C-terminal domain.

Folding of the Disulfide-Bridged Protein. In contrast to the wild-type and reduced proteins, the disulfide mutant followed two-state kinetics under all concentrations of denaturant. Our results are in general agreement with urea-gradient gel analysis of the unfolding of T4 lysozyme, which shows that while non-cross-linked T4 lysozyme unfolds slowly on the time scale of the gel experiment, a 3–97 cross-linked molecule undergoes relatively rapid unfolding (Wetzel, 1989). Comparison of the results for the disulfide cross-linked and reduced mutant suggests that the disulfide bond, rather than amino acid subtitution, accounts for the monophasic kinetic behavior of the disulfide mutant.

The simplest explanation for the difference between the protein with and without the disulfide is that the presence of the cross-link between the domains strongly couples the domain folding process, such that the independent domain folding is abolished. Alternatively, it is possible that the presence of the disulfide decreases the stability of the folding intermediate or affects the fluorescence properties of the intermediate so that it becomes indistinguishable from either U or N.

Stability against Unfolding-Dependent Aggregation (Irreversible Denaturation). Aggregation of folding or unfolding intermediates has been implicated in the irreversible denaturation of a number of proteins, particularly multidomain and multisubunit molecules (Ghélis & Yon, 1982). T4 lysozyme is a two-domain protein, and these and other (Desmadril & Yon, 1984) studies have implicated a metastable intermediate in the reversible folding/unfolding of this molecule. At the same time there is no direct evidence for the involvement of an intermediate in denaturation-dependent aggregation of T4 lysozyme. Furthermore, the thermal unfolding pathway may be different from the pathway studied here. Nonetheless, our finding that the 3–97 disulfide bond increases the decay rate of a folding intermediate highly populated with non-cross-linked lysozymes suggests that this may be the mechanism by which it produces its stabilizing effect in the thermal irreversible denaturation of this protein.

Especially in the context of a possible effect by altering the unfolding pathway, the location of the 3–97 cross-link, spanning an N-terminal residue to a residue of the C-terminal domain, suggests the positioning may be an important aspect of the stabilizing ability of these bonds. However, we have constructed a mutant containing a disulfide between residues 83 and 112 of the C-terminal domain, and this molecule exhibits the same dramatic stability against thermal denaturation as the 3–97 bridged molecule (Wetzel et al., 1988). The role of this cross-link in unfolding kinetics has not been assessed.

Effects of Mutations on Transition-State and Ground-State Free Energy. The difference between the transition-state free energy and the free energy of the unfolded state will be reduced if the latter is raised by the cross-link. Theoretically, the rate of refolding should be accelerated with little or no change in the unfolding rate, depending on the change in the transition-state free energy. A cross-link introduced into ribonuclease is believed to cause an increase of the transition-state free energy, in addition to the conformational free energy of the unfolded state, because the rate of unfolding was reduced (Lin et al., 1984). To account for the increase in the unfolding rate with the T4 lysozyme disulfide mutant, the free energy of the transition state would have to be lowered or the free energy of the native state raised.

Beastey et al. (1986) have postulated four hypothetical classes of folding mutants and described their effects in terms of reaction coordinate diagrams. The mutants of T4 lysozyme examined here show both equilibrium and kinetic effects, reflecting effects on the free energies of both ground states and transition states.

The results reported herein indicate that aqueous methanol cryosolvents are well suited for studying the folding of T4 lysozyme. The folding system appeared very well behaved, and the use of low temperatures facilitated the measurement of fast rates. At the same time, the results of equilibrium experiments in the cryosolvent were similar to those reported in aqueous buffers, suggesting that the kinetic data might also be relevant in aqueous systems.

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A Spectroscopic Study of the Binding of N-7-Substituted Cap Analogues to Human Protein Synthesis Initiation Factor 4E†

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ABSTRACT: The binding of N-7-substituted cap analogues to eIF-4E from human erythrocytes is described. Data presented here indicate that there is a correlation between the tightness of binding of these cap analogues to eIF-4E and their potency as inhibitors of protein synthesis. This result indicates that the inhibitory activity of the cap analogues is strictly a function of the affinity of the analogue for eIF-4E under equilibrium conditions. The pH dependence of binding of the cap analogues to eIF-4E indicates that the enolate form of the cap is preferred, as originally postulated by Rhoads et al. [(1983) *Biochemistry 22*, 6084-6088]. Data indicate that there are differences in the mode of binding of alkyl-substituted and aryl-substituted cap analogues to eIF-4E arising from favorable interactions of the phenyl ring with the guanosine moiety. These differences may explain the enhanced recognition of the aryl-substituted cap analogues by eIF-4E.

All eukaryotic cellular mRNA contains a 5′-cap structure which has been shown to facilitate ribosome binding to mRNA during initiation (Rhoads, 1985; Shatkin, 1985; Sonenberg, 1988) and impart stability to mRNA (Furuichi et al., 1977). Important structural features of the mRNA cap include an N-7-substituted, positively charged guanosine (Adams et al., 1978; Furuichi et al., 1979), the C-2 amino and C-6 keto substituents of the capping guanosine (Adams et al., 1978), and a hydrogen-bond acceptor α-phosphate (Canaani et al., 1976; Hickey et al., 1976; Darzynkiewicz et al., 1981, 1987). Increasing the number of phosphates was shown to enhance the inhibitory effects of m^7G[1] cap analogues by maintaining

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[1] Abbreviations: m^7G, 7-methylguanosine; m^7GMP, 7-methylguanosine 5′-monophosphate; m^7GTP, 7-methylguanosine 5′-triphosphate; e^7GMP, 7-ethylguanosine 5′-monophosphate; bn^7GMP, 7-benzylguanosine 5′-monophosphate; (2-phet)^{7}GMP, 7-(2-phenylethyl)-guanosine 5′-monophosphate; $m^{2,7}GMP$, 2,7-dimethylguanosine 5′-monophosphate; $m^{2,2,7}GMP$, 2,2,7-trimethylguanosine 5′-monophosphate; CD, circular dichroism; eIF, eukaryotic initiation factor; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetate disodium salt.

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