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Correlation between Mutational Destabilization of Phage T4 Lysozyme and Increased Unfolding Rates†

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ABSTRACT: The thermodynamics and kinetics of unfolding of 28 bacteriophage T4 lysozyme variants were compared by using urea gradient gel electrophoresis. The mutations studied cause a variety of sequence changes at different residues throughout the polypeptide chain and result in a wide range of thermodynamic stabilities. A striking relationship was observed between the thermodynamic and kinetic effects of the amino acid replacements: All the substitutions that destabilized the native protein by 2 kcal/mol or more also increased the rate of unfolding. The observed increases in unfolding rate corresponded to a decrease in the activation energy of unfolding ($\Delta G_u^{\ddagger}$) at least 35% as large as the decrease in thermodynamic stability (ΔG_u). Thus, the destabilizing lesions bring the free energy of the native state closer to that of both the unfolded state and the transition state for folding and unfolding. Since a large fraction of the mutational destabilization is expressed between the transition state and the native conformation, the changes in folding energetics cannot be accounted for by effects on the unfolded state alone. The results also suggest that interactions throughout much of the folded structure are altered in the formation of the transition state during unfolding.

Structural and thermodynamic comparisons of genetically altered proteins have resulted in rapid progress in understanding the factors that contribute to conformational stability (Alber, 1989; Goldenberg, 1988; Matthews, B. W., 1987; Shortle, 1989). Although the effects of amino acid replacements on protein stability are often attributed to alterations of interactions in the native structure, it is generally not possible to determine whether a change in thermodynamic stability (i.e., the free energy change for unfolding) is due primarily to a change in the native state or the unfolded state. This has generated considerable controversy about how the two states are affected by mutations.

One line of evidence indicating that destabilizing amino acid replacements have significant effects on the native state comes from the observation that most destabilizing replacements alter residues that are rigid or buried in the folded structure. This correlation between destabilization and features of the native structure—which has been observed for several proteins including the repressor (Hecht et al., 1984) and cro (Pakula et al., 1986) proteins of bacteriophage λ and bacteriophage T4 lysozyme (Alber et al., 1987a)—would be unlikely if many of the mutations acted primarily through effects on the unfolded state (Alber et al., 1987a).

On the other hand, it has been suggested that the effects of mutations on the stability of staphylococcal nuclease may be attributable to changes in the unfolded protein (Shortle, 1989). Amino acid replacements have been shown to change the steepness of the denaturant-induced unfolding transition (Shortle & Meeker, 1986) and to alter the hydrodynamic volumes of incompletely folded fragments of this protein (Shortle & Meeker, 1989). These observations have been interpreted as indicating that the mutations change the distribution of conformations in the unfolded state and the interactions of the unfolded chain with solvent. However, it is not yet known what effects these changes in the unfolded state have on the free energy change for unfolding.

Additional insights about the effects of substitutions on the different species making up a folding transition can be gained from kinetic studies, since effects on the free energy change for different steps in folding can be compared (Beasty et al.,

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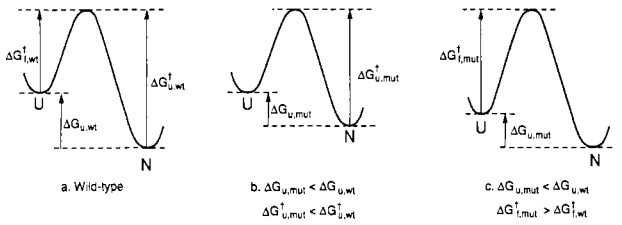


FIGURE 1: Hypothetical reaction coordinates for folding transitions of a wild-type protein (a) and thermodynamically destabilized mutants (b, c). The energies of the transition states are aligned in the figure to emphasize the effects of mutations on the folding and unfolding rates. For the variant depicted in (b), the reduction in thermodynamic stability (ΔG_u) is expressed kinetically as an increase in the rate of unfolding, reflecting a decrease in the free energy of activation for unfolding ($\Delta G^{\ddagger}_u$). In (c), the destabilization is expressed kinetically as a decrease in folding rate, reflecting an increase in $\Delta G^{\ddagger}_f$. The profiles illustrated in (b) and (c) represent extreme possibilities since both the folding and unfolding rates may be altered by an amino acid substitution.

1986; Perry et al., 1987; Goldenberg et al., 1989; Matouschek et al., 1989). In the simplest case, where kinetic intermediates are not detected, the effects of a mutation on the free energies of the native and unfolded states can be determined relative to the transition state by measuring the rates of folding and unfolding (Figure 1). If a decrease in thermodynamic stability is expressed kinetically as an increased unfolding rate, then the native protein is destabilized relative to the transition state, as well as the unfolded state (Figure 1b). Such an observation would indicate that the environment of the altered amino acid is different in the native structure and the transition state. On the other hand, if the destabilizing substitution causes a decrease in the rate of folding (Figure 1c), then the change of environment of the altered residue occurs during the formation of the transition state from the unfolded state.

Like thermodynamic measurements, kinetic studies cannot establish how the absolute free energies of the different species are changed by an amino acid replacement. A comparison of thermodynamic and kinetic effects, however, can indicate what steps of folding are most altered. For example, the extent to which the thermodynamic destabilization is expressed between the transition state and native conformation can be expressed by the ratio of the change in activation free energy for unfolding ($\Delta\Delta G^{\ddagger}_u$) to the change in thermodynamic stability ($\Delta\Delta G_u$) (Goldenberg et al., 1989; Matouschek et al., 1989). For the extreme cases represented in Figure 1, $\Delta\Delta G^{\ddagger}_u/\Delta\Delta G_u$ is 1 when only the unfolding rate is increased (Figure 1b), or 0 when only the folding rate is decreased (Figure 1c). Generally, both rates may be altered by a substitution. If the thermodynamic destabilization is associated with both an increase in unfolding rate and a decrease in folding rate, the ratio will be between 0 and 1. If both rates are either increased or decreased, the ratio will be greater than 1 or less than 0, respectively. This occurs when the transition state is selectively stabilized or destabilized by an amino acid replacement (Beasty et al., 1986). In general, a larger value of the ratio $\Delta\Delta G^{\ddagger}_u/\Delta\Delta G_u$ indicates that more of the thermodynamic destabilization is expressed between the transition state and native state.

In the present study, we have used urea gradient gel electrophoresis to examine the thermodynamics and kinetics of unfolding for 28 variants of bacteriophage T4 lysozyme. Wild-type T4 lysozyme and a large number of mutant forms have been extensively studied by structural and thermodynamic methods [e.g., see Alber et al (1987a,b, 1988), Alber and Matthews (1987), Hawkes et al. (1984), Hudson et al. (1986), Kitamura and Sturtevant (1989), Matsumura et al. (1988), McIntosh et al. (1987), Nicholson et al. (1988), and Weaver and Matthews (1987)], but there have been relatively few kinetic studies of these proteins (Chen et al., 1989). The mutations examined here alter a variety of different residue types at sites throughout the three-dimensional structure (Figure 2) and result in a wide range of thermodynamic stabilities. All of the variants destabilized by more than 2 kcal/mol were found to display markedly enhanced unfolding rates, and in each case, the increase in unfolding rate represented a significant fraction of the thermodynamic destabilization. These observations show that a large number of amino acid replacements destabilize the native protein with respect to the transition state as well as the unfolded state and suggest that contacts throughout the native lysozyme structure are altered in the transition state.

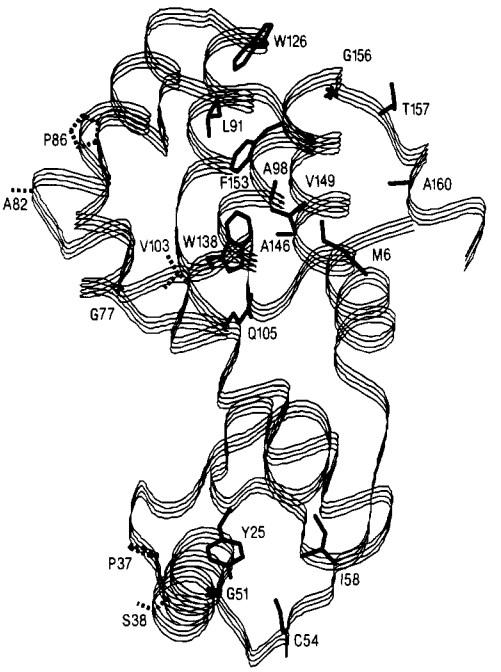


FIGURE 2: Ribbon representation of wild-type bacteriophage T4 lysozyme, drawn from the crystal structure coordinates (Weaver & Matthews, 1987; Protein Data Bank coordinate set 2LZM). Side chains are shown for the residues altered in the lysozyme variants used for this study. The side chains drawn with solid lines are the sites of amino acid replacements that lead to reduced thermodynamic stability and enhanced rates of unfolding, while replacements at the residues drawn with dashed lines do not greatly increase the rate of unfolding.

EXPERIMENTAL PROCEDURES

Purification of Lysozyme Variants. Mutations in the T4 lysozyme gene were generated previously either by oligonucleotide-directed mutagenesis or by screening for temperature-sensitive lysozyme activity following chemical mutagenesis (Alber et al., 1987a,b, 1988; Alber & Matthews, 1987; Nicholson et al., 1989). The substitutions used for this study were M6I,[1] Y25G, P37A, S38D, G51D, C54Y, I58Y, A82P, P86G, L91P, A98T, A98V, A98V/T152S, V103A, Q105G, W126R, W138Y, A146I, A146T, A146V, V149A, F143C, T155A/T157I, G156D, T157D, T157F, and A160T. Wild-type and mutant lysozymes were produced in *Escherichia coli* RR1 using plasmids derived from pHSe5 (Muchmore et al.,

[1] Abbreviations: MOPS, 3-(*N*-morpholino)propanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; BPTI, bovine pancreatic trypsin inhibitor; amino acid substitutions are identified by the wild-type residue type (using the one-letter code for the amino acids), followed by the residue number and the mutant residue type.

1989) and were purified by ion-exchange chromatography using CM-Sepharose and SP-Sephadex media (Muchmore et al., 1989). Protein samples were stored at 4 °C. After storage for several months, some samples were found to display heterogeneity in the form of multiple bands on urea gradient gels. Fresh samples of the same mutant proteins appeared homogeneous, and only samples displaying a single band on urea gradient gels were considered in the comparisons described here.

Urea Gradient Gel Electrophoresis. Gels were prepared with a linear 0–8 M urea gradient and a compensating 15–11% (w/v) gradient of acrylamide as described previously (Creighton, 1979; Goldenberg, 1989). The gels and electrode solutions were buffered with 0.05 M imidazole/0.05 M MOPS (pH 7.0). Before samples were applied, the gels were preelectrophoresed for 15 min.

For each lysozyme variant, parallel samples of native and urea-unfolded protein were prepared. In order to reduce any oxidized cysteine or methionine residues, the proteins were first incubated (at a protein concentration of approximately 15 mg/mL) in 10 mM dithiothreitol, 10 mM EDTA, and 5 mM Tris-HCl, pH 8, for 20 min at room temperature. The sample of native protein was prepared by diluting the preincubated protein to a final concentration of 0.5 mg/mL in 0.01 M imidazole, 0.01 M MOPS, 10% glycerol, and 0.04% methyl green. The sample of unfolded protein was prepared similarly, except that solid urea was added to a final concentration of 8 M and the sample was incubated at 22 °C for 30 min before addition of the glycerol and methyl green.

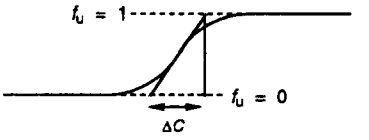
The native and unfolded samples (100 μL) of each variant were applied to identical gels prepared at the same time and electrophoresed in parallel in the same apparatus. Temperature was maintained at 22 °C by circulating water through glass tubing immersed in the anode buffer solution. The gels were stained with Coomassie blue R-250 (Goldenberg, 1989).

The urea concentration at the midpoint of the unfolding transition (C_m) was estimated by measuring the position on the gel where the electrophoretic mobility of the protein was the simple average of the mobilities of the folded and unfolded forms. For variants that displayed blurred or discontinuous transitions, the C_m values were estimated by determining the midpoint of a line joining the inner limits of the smooth sections of the folded and unfolded bands. For each protein, the value of C_m observed was the same (within the accuracy of the measurements, about 0.2 M) whether native or unfolded protein was applied to the gel, and each value reported represents the average of measurements from a minimum of two gels of unfolded protein and two of folded protein.

To compare the stabilities of different lysozyme variants at the same urea concentration, the free energy change for unfolding (ΔG_u) was assumed to depend linearly on urea concentration (C) (Schellman, 1978). For proteins displaying a two-state unfolding transition and a smooth continuous gel pattern, the electrophoretic pattern can be interpreted as a plot of the fraction of protein unfolded (f_u) versus C. Hollecker and Creighton (1982) have shown that the derivative of f_u with respect to C at the midpoint (where $f_u = 0.5$ and $C = C_m$) is proportional to the slope (m) of ΔG_u versus C according to the relationship:

$$\frac{df_u(C_m)}{dC} = -\frac{m}{4RT} \quad (1)$$

where R is the gas constant and T the absolute temperature. To estimate the derivative of f_u with respect to C, a line was drawn tangent to the gel band at the midpoint, and the change in urea concentration (ΔC) corresponding to a change of f_u from 0 to 1 was determined, as illustrated below:



(2)

The value of m was then calculated according to

$$m = -4RT/\Delta C \quad (3)$$

and used to estimate ΔG_u at other urea concentrations (C) by using the relationship

$$\Delta G_u = m(C - C_m) \quad (4)$$

Rate constants for unfolding at the transition midpoints were estimated from the continuity and sharpness of the gel bands, which depend upon the rates for both folding and unfolding and the dependence of the rates on urea concentration (Creighton, 1979). The observed distributions of stained protein at the midpoint were compared with the patterns predicted by Creighton (1979) for different values of the rate constants (relative to the time of electrophoresis). For a two-state transition, the rate constants for folding and unfolding are equal at the midpoint. At higher urea concentrations, the rate constant for folding is expected to decrease, and the rate constant for unfolding is expected to increase (Tanford, 1968).

Results

To characterize simultaneously the thermodynamics and kinetics of the unfolding transitions of the T4 lysozyme variants, we used urea gradient gel electrophoresis (Creighton, 1979, 1980). This method utilizes polyacrylamide slab gels prepared with a transverse gradient of urea concentration. A protein sample is applied across the top of the gel and electrophoresed in a direction perpendicular to the urea gradient. Urea-induced unfolding is detected by the reduced mobility of the unfolded form, and the resulting electrophoretic pattern provides semiquantitative information about the stability of the native protein and the rate of the unfolding transition.

If unfolding and refolding are rapid on the time scale of the electrophoresis, a continuous band of protein is generated. Provided there are no stable intermediates present, the mobility at any urea concentration reflects the fraction of molecules in the native and unfolded states. At the midpoint of the transition, the concentrations of native and unfolded forms are equal, and the free energy change for unfolding (ΔG_u) is zero. The free energy change for unfolding at other urea concentrations can be estimated by assuming a linear dependence of ΔG_u on urea concentration (Hollecker & Creighton, 1982; Schellman, 1978).

If folding and unfolding are slow on the electrophoresis time scale, a discontinuity between the positions of the native and unfolded forms will be generated. A smeared or blurred band of protein will arise if the half-times for folding and unfolding are comparable to the time of electrophoresis. The rates of folding and unfolding within the transition zone can be estimated by comparing the observed electrophoretic patterns with those predicted for different values of the rate constants (Creighton, 1979; Goldenberg & Creighton, 1984; Mitchell, 1976).

When native wild-type lysozyme was applied to a urea gradient gel and electrophoresed for 20 min at 22 °C, pH 7.0, a discontinuous transition between the native and unfolded forms was observed at about 6.3 M urea (Figure 3a). A

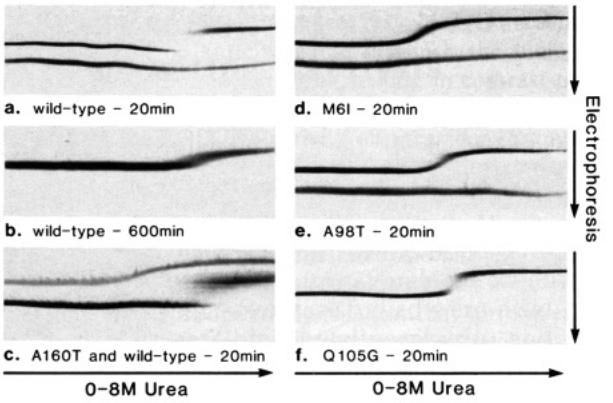


FIGURE 3: Urea gradient gel electrophoresis of wild-type and mutant forms of T4 lysozyme. The gels contained a continuous 0–8 M urea gradient with the urea concentration increasing from left to right. Protein samples were applied across the tops of the gels and electrophoresed at 250 V (except in b) toward the the cathode at 22 °C, pH 7. (a) Native wild-type lysozyme electrophoresed for 20 min at 250 V. The lower band is native BPTI, which does not unfold under these conditions. (b) Native wild-type T4 lysozyme electrophoresed for 600 min at 10 V. (c) Wild-type and A160T lysozyme applied to the gel after unfolding in 8 M urea. The wild-type protein was applied to the gel first and electrophoresed for 10 min. The mutant protein was then applied, and electrophoresis was continued for another 20 min. (d) Native M6I lysozyme and BPTI applied in a single sample and electrophoresed for 20 min. (e) Native A98T lysozyme and BPTI electrophoresed for 20 min. (f) Native Q105G lysozyme electrophoresed for 20 min.

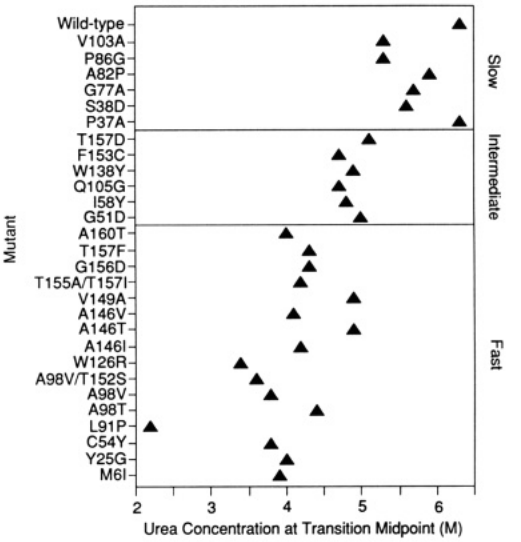


FIGURE 4: Urea concentrations at the midpoints of the unfolding transitions (C_m) grouped according to apparent transition rates. The transition rate for each lysozyme variant was classified according to the appearance of the urea gradient gel band in the transition region after electrophoresis for 20 min. "Fast" transitions were characterized by sharp, continuous bands (e.g., M6I and A98T in Figure 3d,e); those classified as "intermediate" displayed continuous, but blurred, bands (e.g., Q105G, Figure 3f) while those classified as "slow" displayed a clear discontinuity between the native and unfolded protein (e.g., the wild-type protein in Figure 3a). Within each kinetic class, mutants are ordered according to the sequence numbers of the altered residue(s).

similar pattern was generated when unfolded protein was applied (Figure 3c). A more continuous band was generated, however, when wild-type lysozyme was electrophoresed for a longer time (600 min) at lower voltage (Figure 3b), indicating that the transition was reversible under these conditions and that the discontinuous pattern observed after 20 min of electrophoresis was caused by slow folding and unfolding in the transition region. Comparison of the patterns observed after electrophoresis for 20 or 600 min with those predicted by simulations indicated that the rate constants for folding and unfolding of wild-type T4 lysozyme were about 0.01 min^{-1} at the transition midpoint (~6.3 M urea).

Twenty-eight variants of T4 lysozyme with amino acid substitutions throughout the sequence were purified and characterized on urea gradient gels. The different proteins displayed unfolding transitions with a wide range of midpoint urea concentrations (2.3–6.3 M) and rates. The transition rates varied by at least 100-fold, as indicated by the observation of smooth, blurred, and discontinuous gel patterns (Creighton, 1979). For each protein, a similar pattern was generated when the native or unfolded protein was applied to the gel. There was no evidence for the accumulation of intermediates in folding or unfolding or for multiple unfolded forms of any of the proteins.

The proteins that displayed a discontinuous band after 20 min of electrophoresis were estimated to have rate constants for folding and unfolding less than about 0.05 min^{-1} at the midpoint. A blurred band (e.g., for Q105G; Figure 3f) indicates folding and unfolding rates of 0.1–0.5 min^{-1} at the midpoint, while a smooth continuous band (e.g., M6I, A98T, and A160T; Figure 3c–e) reflects rate constants greater than 0.5 min^{-1}.

A striking correlation between the transition midpoints and rates was observed (Figure 4). Lysozyme variants that unfolded at the lowest urea concentrations showed rapid interconversion between the native and unfolded states, while proteins that unfolded at higher urea concentrations displayed slower transitions. No variants with transition midpoints below ~5 M urea unfolded as slowly as wild-type lysozyme did at its midpoint. Since the unfolding rate is expected to increase with urea concentration (Matthews, C. R., 1987; Tanford, 1968), the unfolding rates of the destabilized variants at their own transition midpoints represent minimum estimates of their unfolding rates at the higher C_m of the wild-type protein. Thus, all of the substitutions that lead to a C_m below 5 M urea also significantly increase the unfolding rate constant.

The gel patterns also indicated that the folding rates of the more extensively destabilized variants at their transition midpoints are greater than that of the wild-type protein at its midpoint. However, since the folding rates are expected to decrease at higher urea concentration (Matthews, C. R., 1987; Tanford, 1968), the gel patterns cannot be used to compare the folding rates at a common urea concentration.

To compare the thermodynamic stabilities of the T4 lysozyme variants under identical conditions, the free energy changes for unfolding were extrapolated to 6.3 M urea (where $\Delta G_u = 0$ for the wild-type protein). The urea dependence of ΔG_u was estimated from the gels to be about −2 kcal mol^{-1} M^{-1} for the variants that undergo rapid unfolding transitions. The C_m values for these variants (except one extremely destabilized variant, L91P) were between 3.4 and 4.8 M urea, yielding values of −6 to −3 kcal/mol for ΔG_u extrapolated to 6.3 M urea. If the variants with intermediate rates of unfolding have similar urea dependences, their C_m values of 4.7–5.2 M urea correspond to ΔG_u values of −3 to −2 kcal/mol at 6.3 M urea.

The observed increases in unfolding rate correspond to a large fraction of the thermodynamic destabilization from the amino acid replacements. The variants that generate smooth gel bands unfold at least 100-fold faster than wild-type lysozyme. This is a conservative estimate, since it does not consider the expected increase in unfolding rate at the higher urea concentration, and corresponds to a decrease in the activation free energy of unfolding ($\Delta G_u^{\ddagger}$) of about 2.3 kcal/mol.

Therefore, the minimum values of $\Delta\Delta G_u^{\ddagger}/\Delta\Delta G_u$ range from 0.35 to 0.75, indicating that a large fraction of the thermodynamic destabilization (3–6 kcal/mol) arises from an increase in the unfolding rate. For the variants showing blurred gel bands, $\Delta G_u^{\ddagger}$ is decreased by at least 1–2 kcal/mol, again representing a significant fraction of the decrease in stability (2–3 kcal/mol).

Five of the variants (V103A, P86G, A82P, G77A, and S38D) displayed reduced C_m values (5–5.7 M urea, versus 6.3 M for the wild-type protein) and also gave discontinuous electrophoresis patterns (Figure 4). Thus, these mutants have unfolding rate constants less than about 0.05 min^{-1} at their transition midpoints, compared to 0.01 min^{-1} for the wild-type protein (at 6.3 M urea). For some of these mutants, it is possible that the thermodynamic destabilization may be expressed primarily through decreased folding rates, corresponding to stabilization of the unfolded protein relative to the transition state. The P86G replacement, for example, has been proposed to increase the change in conformational entropy during folding (Matthews et al., 1987), and this entropic effect may be expressed between the unfolded state and the transition state. The unfolding rate constants estimated from the urea gradient gels, however, are not suficiently accurate to rule out the possibility that the V103A, P86G, A82P, G77A, and S38D mutants do, in fact, unfold faster than wild-type lysozyme. In addition, the unfolding rates of the mutants are expected to be greater at the C_m of the wild-type protein. Because these substitutions destabilize the protein by only 1–2 kcal/mol, an increase in the unfolding rate constant of only 5-fold would correspond to values of $\Delta\Delta G_u^{\ddagger}/\Delta\Delta G_u$ of 0.45–0.9. Consequently, for these variants, detailed kinetic studies using spectroscopic probes will be required to assess the mutational effects.

Discussion

By surveying a large collection of mutants, we have found that destabilizing substitutions in phage T4 lysozyme generally increase the rate of protein unfolding. Thus, amino acid substitutions that destabilize the native conformation with respect to the unfolded state also destabilize the native protein with respect to the transition state for folding and unfolding. These results do not establish the absolute effects of the substitutions on the free energies of the native and unfolded forms. Nonetheless, it is apparent that much of the destabilization is expressed between the transition state and the native state. This indicates that the altered residues are in diffeent environments in the native and transition states, consistent with the hypothesis that the amino acid replacements weaken interactions in the native structure that are disrupted or weakened when the transition state is formed during unfolding of the wild-type protein. Such changes in interactions may well be identifiable through high-resolution studies of the native structures of mutant proteins. Alternatively, the substitutions may stabilize both the unfolded state and the transition state, perhaps by making interactions with solvent more favorable. In either case, the correlation between thermodynamic destabilization and increased rates of unfolding cannot be accounted for by the effects on the unfolded state alone.

It is perhaps to be expected that destabilizing mutations increase the rate of unfolding, since many other perturbations that decrease thermodynamic stability, such as the addition of denaturants, also enhance unfolding rates. However, the urea gradient gels demonstrate that the majority of the destabilizing substitutions have a greater effect on the unfolding rate of T4 lysozyme than does adding urea to destabilize the protein by the same amount. This can be seen by considering the wild-type protein at a urea concentration corresponding to the transitin midpoint of one of the destabilized mutants, for instance A160T in Figure 3c. At 4 M urea, the native state of the wild-type protein predominates and ΔG_u is positive. If the wild-type protein is destabilized by increasing the urea concentration to 6.3 M, so that ΔG_u equals zero, the rate constant for unfolding is approximately 0.01 min^{-1}. On the other hand, the thermodynamic stability (at 4 M urea) is reduced by the same amount by the change of Ala-160 to Thr, but the rate constant is at least 0.5 min^{-1}.

Chaotropic denaturants such as urea are thought to act by favoring solvation of regions of the polypeptide chain that become exposed when the protein unfolds (Creighton, 1979; Schellman, 1978; Tanford, 1968). The relative degree of solvent exposure of the transition state can be estimated from the dependence of folding and unfolding rates on denaturant concentration (Chen et al., 1989; Kuwajima et al., 1989; Matouschek et al., 1989; Tanford, 1970): If a change in denaturant concentration primarily alters the folding rate, for example, the major change in solvent exposure occurs between the unfolded and transition states. Conversely, if the effect on the unfolding rate is greater, then most of the difference in solvent exposure is between the native protein and the transition state. Measurements of the effects of denaturants on rates have revealed that the degree of solvent exposure in the transition state varies somewhat for different proteins (Chen et al., 1989; Kuwajima et al., 1989; Matouschek et al., 1989; Tanford, 1970). Generally at least 20% of the increase in exposure due to complete unfolding occurs between the native and transition states. The observation that the destabilizing substitutions cause a larger increase in unfolding rate than does increasing the urea concentration (to yield the same degree of thermodynamic destabilization) indicates that the transition state for T4 lysozyme is less "native-like" with respect to sensitivity to each of the amino acid replacements than it is with respect to overall solvent exposure.

It is particularly striking that 23 of 28 destabilized variants displayed this pattern. These substitutions are located at 15 different sites in the polypeptide chain and alter a variety of different kinds of residues and interactions (Figure 2). That substitutions at many sites throughout the three-dimensional structure increase the unfolding rate argues against a mechanism of unfolding in which the rate is determined by disruption of a small region of the native protein, followed by rapid complete unfolding. Although unfolding may take place via a preferred sequence of rapid steps that precede the major transition state, the mutational effects observed here suggest that the folded protein has undergone perturbations throughout much of its structure by the time this transition state is reached.

Similar enhancements of unfolding rates have been reported for the majority of mutations that destabilize bovine pancreatic trypsin inhibitor (Goldenberg et al., 1989), dihydrofolate reductase (Perry et al., 1987; Garvey & Matthews, 1989), and the α-subunit of tryptophan synthase (Beasty et al., 1986). Somewhat different results have been seen for barnase, where some destabilizing substitutions primarily affect the folding rate, indicating that some sites are quite nativelike in the transition state (Matouschek et al., 1989). Even for barnase, however, there are substitutions at sites in different regions of the three-dimensional structure for which most of the thermodynamic destabilization is expressed between the transition state and the native conformation. Thus, it may be quite general that the passage of proteins between the folded structure and the transition state involves extensively distrib-

uted energetic and conformational changes.

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CORRECTION

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