Effects of Guanidine Hydrochloride on the Refolding Kinetics of Denatured Thioredoxin†

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ABSTRACT: The effect of guanidine hydrochloride concentration on the kinetics of the conformational change of *Escherichia coli* thioredoxin was examined by using fluorescence, absorbance, circular dichroic, and viscosity measurements. Native thioredoxin unfolds in a single kinetic phase whose time constant decreases markedly with increasing denaturant concentration in the denaturation base-line zone. This dependency merges with the time constant of the slowest refolding kinetic phase at the midpoint of the equilibrium transition in 2.5 M denaturant. The time constant of the slowest refolding phase becomes denaturant independent below 1 M denaturant in the native base-line region. The denaturant-independent slowest refolding phase has an activation energy of 16 kcal/mol and is generated in the denatured base-line zone in a denaturant-independent reaction having a time constant of 19 s at 25 °C. The fractional amplitude of the slowest refolding phase diminishes in the native base-line zone to a minimum value of 0.25. This decrease is accompanied by an increase in the fractional amplitudes of two faster refolding kinetic phases, an increase describing a sigmoidal transition centered at about 1.6 M denaturant. Manual multimixing measurements indicate that only the slowest refolding kinetic phase generates a product having the stability of the native protein. We suggest that the two faster refolding phases reflect the transient accumulation of folding intermediates which can contain a nonnative isomer of proline peptide 76.

Escherichia coli thioredoxin has several features which make it attractive for studies of protein folding. The protein is relatively small, having 108 residues on a single polypeptide chain of known sequence (Holmgren, 1968). This relatively short chain is folded into two well-defined domains in the native protein (Holmgren et al., 1975). The larger N-terminal domain contains a $\beta\alpha\beta\alpha\beta$ structure, an architectural feature characteristic of many larger more complex proteins. The smaller C-terminal domain contains a $\beta\beta\alpha$ structure whose strands join those of the N-terminal domain to form a central twisted sheet with flanking helices. The oxidized form of the protein contains a single disulfide bond which bridges the first and fourth residues of a reverse turn. This disulfide is reversibly reduced during hydrogen transfer, which is the biofunction of thioredoxin.

The native structure of oxidized thioredoxin is unfolded at neutral pH and 25 °C in solvents containing more than 2 M guanidine hydrochloride (Gdn·HCl)[1] (Holmgren, 1972). Native thioredoxin unfolds in 4 M Gdn·HCl in a single kinetic phase while denatured thioredoxn refolds in 2 M Gdn·HCl in three kinetic phases of which the slowest is dominant (Kelley & Stellwagen, 1984). In this paper, we examine the effect of Gdn·HCl on the kinetic profiles of both the unfolding and refolding of thioredoxin. The principal conformational probe is the fluorescence emission of the two tryptophan residues located at positions 28 and 31 in the N-terminal domain.

Experimental Procedures

Materials. Oxidized thioredoxin was purified from an *E. coli* strain (Lunn et al., 1984) containing multiple copies of a plasmid with the gene for *E. coli* thioredoxin. The protein was purified by a modification of the procedure of Holmgren & Reichard (1967) described previously (Kelley & Stellwagen, 1984). All measurements were done with oxidized thioredoxin, that is, thioredoxin with the disulfide bond linking cysteine residues 32 and 35 intact. All solutions of thioredoxin contained 50 mM phosphate buffer, pH 7.0, and were maintained at 25 °C unless noted otherwise.

Methods. All equilibrium and kinetic measurements of reduced viscosity, fluorescence emission, and far-ultraviolet ellipticity were performed and analyzed as described previously (Kelley & Stellwagen, 1984). Some of the fluorescence measurements were made for using an SLM Model 4800 fluorometer. Absorbance measurements were made by using a Gilford Model 2600 spectrophotometer.

Results

Equilibrium Measurements. The dependence of the fluorescence emission intensity of the two tryptophan residues in thioredoxin on Gdn·HCl concentration is shown in Figure 1. This dependence may be divided into three zones: the native base-line zone between zero and 2 M Gdn·HCl, the transition zone between 2 and 3 M Gdn·HCl. The intensity dependence observed in the denatured base-line zone is equivalent to that observed for an equimolar concentration of a model tryptophan residue (Kelley & Stellwagen, (1984). The diminished fluorescence intensity dependence observed in the native base-line zone presumably reflects that of the partially quenched tryptophan residues in the native conformation. The midpoint of the transition zone occurs at 2.5 M Gdn·HCl as reported previously (Kelley & Stellwagen, 1984).

Single Mixing Kinetic Measurements. The kinetic profiles observed for the unfolding of thioredoxin in 3 M Gdn·HCl and the refolding of denatured thioredoxin in 2 M Gdn·HCl were selected as representative of each conformational change.

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[1] Abbreviation: Gdn·HCl (Gu in figures), guanidine hydrochloride.

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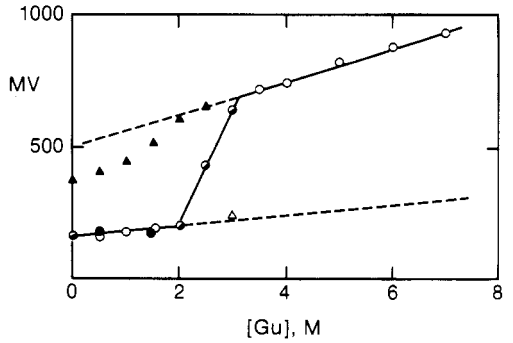

FIGURE 1: Equilibrium and kinetic measurements of the tryptophan fluorescence emission intensity of thioredoxin in Gdn·HCl. All solutions contained 20 μM thioredoxin, 50 mM phosphate buffer, pH 7.0, and the indicated concentrations of Gdn·HCl. Samples were excited at 276 nm, and emission intensity was measured at 350 nm in millivolts. The open circles indicate values obtained at equilibrium starting with native thioredoxin. The open triangle indicates the fluorescence emission intensity value predicted at time zero from analysis of the kinetic profile of a sample of native thioredoxin introduced into 3 M Gdn·HCl by manual mixing. The closed circles indicate equilibrium values obtained starting with denatured thioredoxin in 6 M Gdn·HCl. The closed triangles indicate intensity values predicted at time zero from analyses of the kinetic profiles of samples of denatured thioredoxin diluted to the indicated Gdn·HCl concentrations by manual mixing.

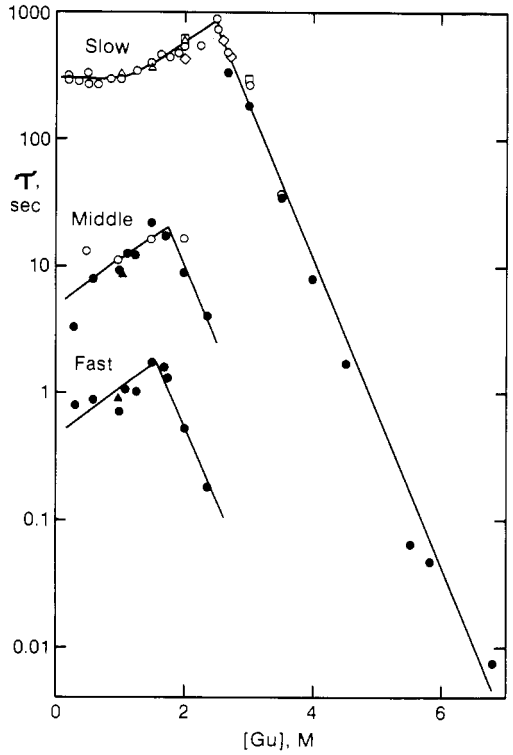

FIGURE 2: Dependence of the observed time constants on the concentration of Gdn·HCl. Solutions of either denatured or native thioredoxin were mixed with either 50 mM phosphate buffer, pH 7.0, or concentrated Gdn·HCl in buffer, respectively, to give thioredoxin solutions containing the indicated Gdn·HCl concentrations. The open symbols indicate measurements obtained following manual mixing, and the closed symbols indicate measurements obtained following stopped-flow mixing. The circles indicate fluorescence emission measurements at 350 nm, the triangles indicate absorbance measurements at 287 nm, the squares indicate circular dichroic measurements at 219 nm, and the diamonds indicate reduced viscosity measurements.

Samples of native thioredoxin were equilibrated with various concentrations of Gdn·HCl in the native base-line zone or in the transition zone prior to observation of the unfolding kinetics in 3 M Gdn·HCl following manual mixing. All such samples

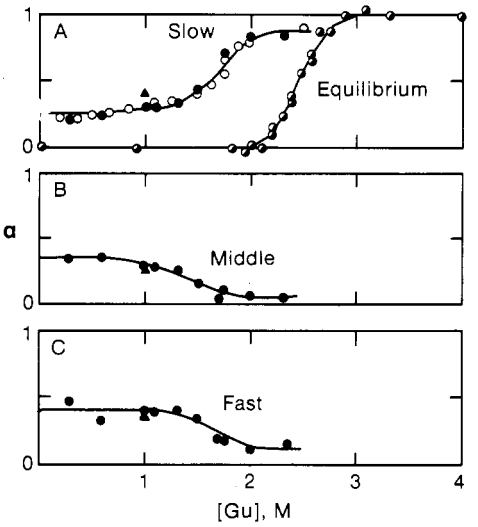

FIGURE 3: Dependence of the fractional change associated with each refolding kinetic phase on the concentration of Gdn·HCl. The open symbols indicate measurements obtained following manual mixing, and the closed symbols indicate measurements obtained following stopped-flow mixing. The circles indicate measurements of fluorescence emission at 350 nm, and the triangles indicate measurements of absorbance at 287 nm. Panel A illustrates values for the slow refolding phase, panel B the middle refolding phase, and panel C the fast refolding phase. Equilibrium measurements observed by fluorescence emission are also illustrated in panel A by the half-filled circles.

unfolded in a single kinetic phase having a time constant of 275 ± 34 s. Samples of denatured thioredoxin were equilibrated with various concentrations of Gdn·HCl in the denatured base-line zone and in the transition zone prior to refolding in 2 M Gdn·HCl following manual or stopped-flow mixing. All the refolding kinetic profiles contained a phase having a time constant of 503 ± 65 s accounting for 77% ± 4% of the total change. A second phase having a time constant of 12 ± 6 s accounting for 12% ± 2% of the total change and a third phase having a time constant of 0.65 ± 0.20 s accounting for 12% ± 4% of the total change were also observed in all the refolding kinetic profiles. These results indicate that the kinetic patterns observed for both unfolding and refolding are independent of the initial concentration of Gdn·HCl.

By contrast, the kinetic profiles for both unfolding and refolding of thioredoxin are dependent upon the final concentration of Gdn·HCl. As shown in Figure 2, the time constant for the unfolding of thioredoxin decreases markedly as the final concentration of Gdn·HCl increases in the denaturation base-line zone. No evidence could be obtained for the presence of more than one kinetic phase during unfolding at any Gdn·HCl concentration. The kinetic profiles for the refolding of denatured thioredoxin in all Gdn·HCl concentrations below 2.5 M Gdn·HCl evidence three kinetic phases, termed the slow, middle, and fast refolding phases. The time constant and fractional amplitude of each refolding phase are dependent upon Gdn·HCl concentration in the native base-line zone as shown in Figures 2 and 3. The kinetic features of the slowest refolding kinetic phase are more precisely determined following manual mixing as opposed to stopped-flow mixing, owing to the long-term stability of the equilibrium fluorometer. As shown in Figure 2, the time constant of the slow refolding phase decreases from about 800 s to a constant value of about 300 s in Gdn·HCl concentrations of 1 M or less. Similarly, the fractional amplitude of the slow refolding phase diminishes from 1.0 to a constant value of 0.25 in a single transition having a midpoint at 1.7 M as shown in Figure 3A. The middle refolding phase having a time constant of about

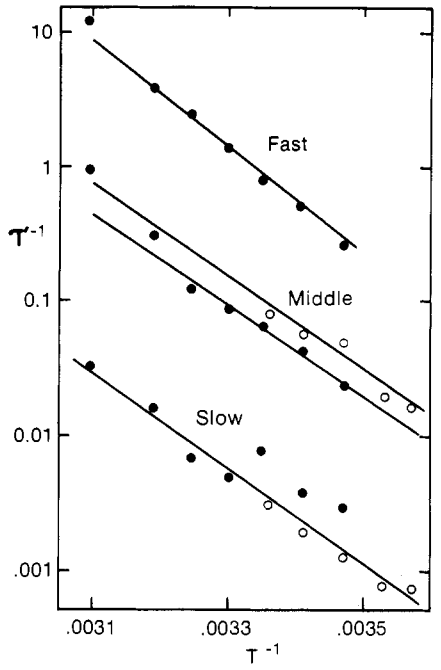

FIGURE 4: Dependence of time constants for refolding on temperature. All values were obtained from fluorescence emission measurements. The open symbols indicate values obtained in 0.5 M Gdn·HCl following manual mixing, and the closed symbols indicate values obtained in 1.4 M Gdn·HCl following stopped-flow mixing.

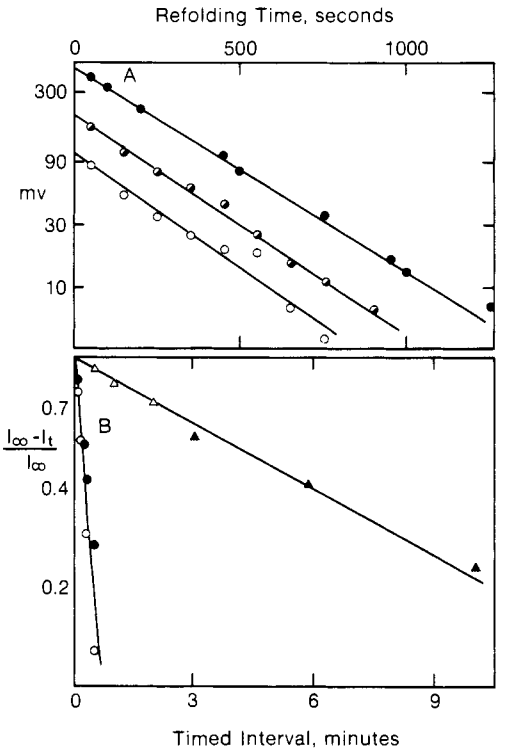

FIGURE 5: Fluorescence emission measurements following manual multimixing. (A) Refolding kinetics following manual multimixing. Concentrated Gdn·HCl was mixed at zero time with a solution of native thioredoxin to generate a protein solution 5 M in Gdn·HCl. After a measured time interval, sufficient phosphate buffer was added to generate a 20 μM protein solution in 0.5 M Gdn·HCl. The fluorescence emission intensity of this solution was recorded until a constant value was obtained. The emission intensity values were used to construct a first-order plot for each multimixing experiment. Results are shown for refolding after exposure of the native protein to 5 M Gdn·HCl for 5 (○), 10 (◒), and 600 s (●). The observed first-order plots have a common slope corresponding to a time constant of 271 ± 9 s but differing intensities at zero folding time. The intensity predicted at zero refolding time following exposure to 5 M Gdn·HCl for a limited time, I_t, is compared with the intensity at zero folding time for a sample exposed to 5 M Gdn·HCl for 600 s, I_∞. These values are used to construct a secondary first-order plot shown in panel B. The open circles indicate values obtained after a timed exposure of the native protein to 5 M Gdn·HCl prior to refolding in 0.5 M Gdn·HCl. The closed circles indicae values obtained after a timed exposure of the native protein to 6.8 M Gdn·HCl prior to refolding in 0.5 M Gdn·HCl. The triangles indicate results obtained after execution of a reverse protocol in which equilibrium-denatured thioredoxin was exposed to 0.5 M Gdn·HCl for a timed interval prior to observation of the kinetics of unfolding. The open triangles indicate values obtained by observing unfolding in 3 M Gdn·HCl in which the observed unfolding time constant had a value of 290 ± 63 s. The closed triangles indicate values obtained by observing unfolding in 3.53 M Gdn·HCl in which the observed unfolding time constant had a value of 35 ± 7 s.

10 s is just barely detectable when our manual mixing protocol is used. The summed amplitudes for the slow and middle refolding phases observed below 2 M Gdn·HCl predict the presence of a faster kinetic phase as shown in Figure 1. Accordingly, refolding was observed following stopped-flow mixing in order to improve the precision of measurement of the middle refolding phase and to directly observe the fast refolding phase. The kinetic profiles obtained following stopped-flow mixing were analyzed by holding the time constant of the slow phase constant to the value appropriate to the Gdn·HCl concentration shown in Figure 2. The time constants for both the middle and fast refolding phases exhibit a triangular dependence on Gdn·HCl concentration in the native base-line zone as shown in Figure 2. The fractional amplitudes of both the middle and fast refolding phases increase as the Gdn·HCl refolding concentration is dimished below 2 M as shown in Figure 3. The fractional amplitude of the middle phase is increased to a maximal value of 0.35 in a single transition centered at about 1.5 M Gdn·HCl. The fracitonal amplitude of the fast phase is increased to a maximal value of 0.40 in a single transition centered at 1.7 M Gdn·HCl. It should be noted that the fractional refolding amplitude which is not detected following manual mixing is observed as a fast phase amplitude following stopped-flow mixing. These results suggest that the refolding of thioredoxin can be described by three kinetic phases throughout the native base-line zone.

The time constant for each of the three refolding kinetic phases is markedly dependent on temperature as shown in Figure 4. Refolding measurements were made both within and oustide the kinetic transition region by using manual and stopped-flow mixing. The reciprocal of the time constant of each kinetic refolding phase exhibits a linear dependence on the reciprocal of the temperature of measurement as shown in Figure 4. The slopes of the relationships shown in Figure 4 for the fast, middle, and slow refolding phases correspond to activation energies of 18, 16, and 16 kcal/mol, respectively.

Manual Multimixing Measurements. The origin of the protein exhibiting slow phase refolding was examined by using two successive manual mixes. A solution of concentrated Gdn·HCl was rapidly added at time zero to a solution of native thioredoxin in a fluorescence cuvette to initiate unfolding of the protein in 5 M Gdn·HCl. After a timed interval of unfolding in 5 M Gdn·HCl, sufficient buffer solution was added to the protein solution to initiate refolding in 0.5 M Gdn·HCl. The fluorescence emission intensity of the refolding solution was recorded until equilibrium was obtained. The decrease in the fluorescence intensity observed during refolding was analyzed by a first-order plot to determine the amplitude of the slow refolding phase. As shown in Figure 5A, the amplitude of the slow refolding phase increases with increasing

denaturation time prior to refolding. This relationship was analyzed by using a second first-order plot shown in Figure 5B. Such analysis indicates that the slow refolding phase is generated during denaturation in 5 M Gdn·HCl with a time constant of 19 s. A similar value was obtained when unfolding was done in 6.8 M Gdn·HCl instead of 5.0 M Gdn·HCl, as shown in Figure 5B. By contrast, a higher value of 42 s was reported when unfolding was done at 4.0 M Gdn·HCl (Kelley & Stellwagen, 1984). It is likely that this higher value reflects a perturbation contributed by the slower unfolding in 4.0 M Gdn·HCl, whose time constant (Figure 2) is comparable to that for the generation of the slow refolding phase. Finally, it should be noted that the kinetics of the refolding of thioredoxin initially denatured after the briefest exposure to either 5.0 or 6.8 M Gdn·HCl was completed within the dead time of the manual mixing protocol employed. This observation indicates that the denatured protein from which the slow refolding protein is generated rapidly refolds.

The identity of the kinetic phase(s) which generate(s) the native protein was investigated by using an opposite manual multimixing protocol. Equilibrium-denatured thioredoxin was allowed to refold for a limited time in 0.5 M Gdn·HCl prior to addition of Gdn·HCl to increase its concentration to 3 M or greater to initiate unfolding. The increase in tryptophan fluorescence amplitude accompanying the unfolding of thioredoxin was recorded as a function of time until equilibrium was obtained. The increase in fluorescence intensity accompanying unfolding was analyzed by using a first-order plot. In all such measurements, only a single phase was detected having a time constant of 290 ± 63 s in 3.0 M Gdn·HCl and 35 ± 7 s in 3.5 M Gdn·HCl. These values are characteristic for unfolding of native thioredoxin as shown in Figure 2. The fractional amplitude of the unfolding observed in 3.0 or 3.5 M Gdn·HCl increased with increasing refolding time in 0.5 M Gdn·HCl prior to unfolding. This dependency generates a linear first-order plot having a time constant of 410 s as shown in Figure 5B. The magnitude of this value suggests that the native protein is only produced by the slow refolding phase.

Other Measurements. The near-ultraviolet absorbance spectrum of native thioredoxin was compared with that of the denatured protein in 6 M Gdn·HCl using the latter solution as a reference. The resultant difference spectrum has three difference maxima located at 280, 287, and 298 nm, characteristic for a conformational change involving both tyrosine and tryptophan residues (Donovan, 1969). The dependence of the absorbance of thioredoxin at 287 nm, the principal difference maximum, on the concentration of Gdn·HCl is shown in Figure 6. This dependence, like that of the fluorescence emission, can be divided into a native base-line zone, a transition zone, and a denatured base-line zone. The transition zone described by absorbance measurements has the same midpoint and span as that described by the fluorescence measurements shown in Figure 1. Kinetic profiles of the increase in absorbance at 287 nm were obtained following dilution of the denatured protein by either manual or stopped-flow mixing. The time constants for the single refolding phase detected by absorbance measurements following manual mixing are characteristic for slow phase refolding detected by fluorescence measurements as shown in Figure 2. The amplitude of the slow refolding phase detected by absorbance measurements decreases markedly with decreasing Gdn·HCl concentration as shown in Figure 6. This decrease describes a single transition comparable to that observed for the slow refolding phase observed by fluorescence emission shown in Figure 3. The kinetic profile of the absorbance change accompanying refolding in 1 M Gdn·HCl observed following stopped-flow mixing can be resolved into three kinetic phases whose time constants and fractional amplitudes are comparable to those obtained by fluorescence measurements as shown in Figures 2 and 3.

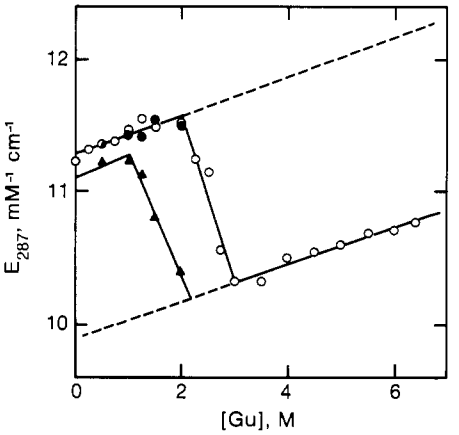

FIGURE 6: Dependence of the absorbance of thioredoxin on Gdn·HCl concentration. All solutions contained between 62 and 68 μM thioredoxin, 50 mM phosphate buffer, pH 6.5 ± 0.2, and the indicated concentrations of Gdn·HCl. The open circles indicate values obtained at equilibrium starting with native thioredoxin. The closed circles indicate equilibrium values obtained starting with the protein denatured in 6 M Gdn·HCl. The closed triangles indicate the values predicted for the slow refolding phase at zero time.

The reduced viscosity of native globular thioredoxin, 3.6 mL/g, is increased to 13.0 mL/g upon denaturation in 4 M Gdn·HCl (Kelley & Stellwagen, 1984). Since both the unfolding and refolding of thioredoxin are very slow between 2 and 3 M Gdn·HCl, it was possible to observe the kinetics of these processes by viscometric measurements. As shown in Figure 2, the time constants for the changes in viscosity accompanying the unfolding and refolding of thioredoxin are within experimental variation of the values obtained by using fluorescence measurements.

It has been shown previously that the far-ultraviolet circular dichroic spectrum changes from that characteristic of a globular protein to a random coil between 2 and 3 M Gdn·HCl (Kelley & Stellwagen, 1984). The kinetic profiles of the changes in circular dichroism at 219 nm accompanying the unfolding of thioredoxin in 3 M Gdn·HCl and the refolding of denatured thioredoxin in 2 M Gdn·HCl were obtained following manual mixing. These profiles were each resolved into a single kinetic phase whose time constants are comparable to those observed by fluorescence measurements as shown in Figure 2.

Discussion

The time constants for the unfolding and for the slow phase refolding of thioredoxin describe a continuous inverted triangular dependence on Gdn·HCl concentration above 1.5 M, as shown in Figure 2. Such a dependence is characteristic for a protein conformational change in which both the native and denatured conformations weakly bind denaturant (Tanford, 1968). The common time constants for unfolding and refolding in this Gdn·HCl concentration range detected by fluorescence, absorbance, circular dichroism, and reduced viscosity measurements indicate that the observed conformational change is global and not restricted to the environment about the two tryptophan residues.

However, the time constant of the slow refolding phase becomes independent of Gdn·HCl concentration at concen-

trations of 1 M or less as shown in Figure 2. This suggests that a reaction not involving a conformational change becomes rate limiting in this Gdn·HCl concentration range. Several observations are consistent with the Gdn·HCl-independent reaction involving a *cis*/*trans*-proline peptide configurational isomerization (Brandts et al., 1975). As shown in Figure 4, the Gdn·HCl-independent slow phase has an activation energy of 16 kcal/mol, characteristic for peptide isomerization. Second, the Gdn·HCl-independent slow phase is generated in the denatured state with a time constant within the range observed for proline peptide isomerizations in model peptides and polypeptides (Grathwohl & Wüthrich, 1981). Third, the time constant for generation of the Gdn·HCl-independent slow refolding phase is itself Gdn·HCl independent in the denatured base-line region as is the time constant for proline peptide isomerization in model peptides (Nall et al., 1978).

While *E. coli* thioredoxin has five proline residues, the Gdn·HCl-independent slow refolding phase is generated by a single relaxation in the denatured state. Since the time constant for proline peptide isomerization in model peptides and in denatured proteins is somewhat sequence dependent (Grathwhol & Wüthrich, 1981; Lin & Brandts, 1983), observation of a single-generation time constant suggests that the isomerization of only one of the five proline peptide bonds is germain to the Gdn·HCl-independent slow refolding phase. Assuming this to be correct, the time constants for the Gdn·HCl-independent slow refolding phase and for the generation of this phase can be combined to predict that 95% of the proline peptide bond has a nonnative configuration in the denatured state. This prediction strongly suggests that the proline peptide being considered is cis in the native conformation since proline peptides are normally predominantly trans in the denatured state. Of the five proline peptides in thioredoxin, only proline-76 is cis in the native conformation (Brändén et al., 1983). Accordingly, we tentatively assign the Gdn·HCl-independent slow phase refolding to the peptide isomerization of the nonnative trans configuration of proline-76 in the denatured state. The remaining 5% of the protein which is predicted to have the cis peptide of proline-76 in the denatured state would be expected to have Gdn·HCl-dependent refolding time constant smaller than that of the middle refolding phase.

The fractional amplitude of each of the three observed refolding phases changes markedly in the native base-line zone, as shown in Figure 3. Such an amplitude dependence suggests a significant change in the population of transiently stable refolding intermediate forms. The inverted triangular dependence of the time constant of the middle and fast refolding phases on Gdn·HCl concentration, shown in Figure 2, indicates that these phases involve a conformational rather than a configurational isomerization. The apex of such a triangular dependence should correspond to the equilibrium midpoint of the conformational change considered. Accordingly, the sigmoidal-shaped dependence of the fractional amplitude of the middle and fast refolding phases on Gdn·HCl concentration, shown in Figure 3, may be interpreted to represent the stability curves of two folding intermediates.

The multimixing measurement described in Figure 5B indicates that only the slow refolding phase generates a product having an unfolding time constant characteristic for the native protein. Presumably then, the folded products of the middle and fast refolding phases unfold more rapidly than can be detected by the multimixing protocol employed in these measurements. Such a presumption is consistent with the time constant dependency of the middle and fast phases on Gdn·HCl concentration shown in Figure 2.

One way to accommodate the observations described and interpreted above is to consider that the refolding of denatured thioredoxin involves two transiently stable intermediates generated by the fast and middle phases and located either on a sequential pathway or on alternate pathways. The changes in the fractional amplitude of these phases would reflect the stability of these intermediates. The slow refolding phase would then represent the conversion of the intermeidate(s) to the native conformation. At low Gdn·HCl concentration, the slow refolding phase is limited by proline isomerization, suggesting that isomerization preceded the formation of the native protein but not the folding intermediates. While this scenario likely represents only one of many reaction schemes that can accommodate the experimental observations, many of the intrepretations and predictions of the proposed scheme can be tested by using a range of technologies. This report does establish that the refolding pathway of thioredoxin is perturbed in a major way by Gdn·HCl, that intermediate forms can be detected, and that proline peptide isomerization may occur within the intermediate forms.

Registry No. Gdn·HCl, 50-01-1.

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