Refolding of Denatured Thioredoxin Observed by Size-Exclusion Chromatography†

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ABSTRACT: Molecular sieve chromatography can resolve interactive systems into populations having different effective hydrodynamic volumes. In this report, the advantages of such resolution to protein folding are illustrated by using moderate pressure to decrease analysis time and lowered temperature to slow down the kinetics of conformational change. A 300-mm Bio-Sil TSK-125 size-exclusion column was equilibrated with a series of different concentrations of guanidine hydrochloride at 2 °C in 50 mM phosphate buffer, pH 7.0. Samples of native *Escherichia coli* thioredoxin, denatured thioredoxin, or thioredoxin equilibrated with the column solvent were injected, and the effluent was monitored at 220 nm. Injection of equilibrated protein samples defined three denaturant concentration zones identical with those observed by spectral measurements: the native base-line zone where only compact protein is observed in the effluent profile; the transition zone in which both compact and denatured forms are observed in slow exchange; and the denatured base-line zone in which only denatured protein is observed. Unfolding was observed by injection of native protein into columns having isocratic denaturant concentrations in the transition and denatured base-line zones. Effluent profiles indicated a dynamic conversion of compact to denatured protein with a time constant which appeared to decrease markedly with increasing denaturant concentration. Refolding was observed by injection of denatured protein into columns having isocratic concentrations in the transition and native base-line zones. As the denaturant concentration was decreased, the effluent profiles evidenced a persistent slow conversion of denatured to compact protein which was suddenly accelerated about midway in the native base-line zone. Separate multimixing measurements indicated that the slow refolding protein was generated in the denatured state and that the compact products of the accelerated refolding in the native base-line zone were less stable than the native protein. The observed effluent profiles were simulated by using a computer program based on the equations of Endo et al. [Endo, S., Saito, Y., & Wada, A. (1983) *Anal. Biochem. 131*, 108–120]. Such simulations indicate that population of partially folded conformations having an elution time intermediate between that of the native and denatured thioredoxin is not obligatory. All the observed profiles can be simulated by assuming that the denatured protein is dominated by molecules containing one or more nonnative configurational isomers. At relatively high denaturant concentrations in the native base-line zone, only the denatured molecules containing the native configurational isomers appear to be able to fold. However, at lower denaturant concentrations, the denatured molecules containing nonnative configurational isomers appear to be able to fold into stable compact conformations in which configurational isomerization to the native protein can subsequently occur.

Most laboratory studies of the kinetics of protein folding involve measurement of the changes in absorbance or fluorescence which accompany the folding of a denatured protein into its biofunctional conformation. Such spectrophotometric measurements are popular because they utilize an intrinsic chromophore such as a tryptophan residue and because they are amenable to rapid data collection. However, the spectral properties of an intrinsic chromophore can be as responsive to subtle changes in a biofunctional conformation as to the collapse of a denatured protein into a more compact conformation.

Hydrodynamic measurements, by contrast, should focus on events early in protein folding which are involved in the collapse of the denatured protein into a more compact conformation. Unfortunately, traditional hydrodynamic measurements are usually too slow to be very useful to kinetic measurements of protein folding. However, the introduction of matrices for size-exclusion chromatography stable to moderate pressures makes possible hydrodynamic measurements in the time scale of protein folding at temperature below ambient. In this report, we illustrate how such measurements can be useful to the studies of protein folding using the small single-chained protein thioredoxin.

Experimental Procedures

Materials. Thioredoxin was purified from an *Escherichia coli* strain (Lunn et al., 1984) containing multiple copies of a plasmid with the structural gene for *E. coli* thioredoxin. The protein was purified by a modification of the procedure of Holmgren and Reichard (1967) described previously (Kelley & Stellwagen, 1984). All measurements were made by using oxidized thioredoxin, that is, thioredoxin containing a single disulfide bridge which links cysteine residues 32 and 35.

Methods. Fluorescence measurements were obtained by using an SLM Model 4800 fluorometer. Solutions were prepared by manual mixing, excited at 280 nm, and monitored at 350 nm. Resultant kinetic profiles were fit to simultaneous first-order reactions using a Dec Model 11/780 VAX computer, as described previously (Kelley & Stellwagen, 1984).

Size-exclusion chromatography was done by using a 300-mm Bio-Sil TSK-125 gel filtration column, an IBM Model LC9533 liquid chromatograph, and an Isco Model V4 varia-

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ble-wavelength absorbance detector, unless noted otherwise. The column was jacketed and maintained at a fixed temperature by using a circulating water bath. Twenty-microliter protein samples were injected into the column and subjected to isocratic chromatography at a flow rate of 1 mL/min and a pressure between 25 and 30 bar. The absorbance of the column effluent was monitored at 220 nm, encoded on disk, and transmitted to a Dec 11/780 VAX computer for further analysis. The absorbance of the protein was found to be independent of denaturant concentration at 220 nm by using an Aviv/Cary spectrophotometer. The column effluent detector gave a linear response to protein concentration over the range employed, 0–500 μg/mL. The chromatographic system had an excluded volume of 5.5 mL and an included volume of 10.8 mL determined by using blue dextran and either tyrosine or potassium dichromate, respectively, as calibration reagents.

Chromatographic elution profiles were simulated by using a Dec 11/780 computer and a menu-driven program based on eq 13 and 14 of Endo et al. (1983). The program can accommodate any desired mechanism involving coupled conformational and/or configurational isomerization reactions. Required information for a simulation includes the following: the volume, concentration, and nature of the protein sample injected, whether native, denatured, or equilibrated with the isocratic solvent; the flow rate and denaturant concentration of the isocratic solvent; the elution time and relative absorbance for each component in the reaction mechanism; the width, midpoint, and time constant for each conformational isomerization; the equilibrium distribution and time constant for each configurational isomerization; and the number of theoretical plates on the column. The real and simulated chromatographic profiles were displayed simultaneously on a graphics terminal. Real elution profiles containing a single component in either base-line region were simulated exactly by using a minimum of 300 theoretical plates. All conformational transitions fit best with a transition width such that 80% of the observed transition occurred over a denaturant concentration change of 1.0 M.

Each observed real chromatographic profile was superimposed by a simulated profile using the reaction mechanism and constants described here unless noted otherwise. The sensitivity of the simulated elution profiles was modeled for a protein which exhibits a denaturant-dependent two-state conformational transition having the peak shape and elution times for native and denatured thioredoxin. All elution profiles in the transition zone were visually responsive to changes in a midpoint denaturant concentration of ±0.05 M. Elution profiles in all zones are visually responsive to time constants ranging from about 5 to 5000 s. The sensitivity of a profile varied ±2% to the changes in the time constant in the middle of this range and to ±20% at the extremes.

Results

The denaturant used throughout this study was guanidine hydrochloride which is abbreviated Gdn-HCl in the figures. All the chromatographic solvents were isocratic in denaturant concentration, contained 50 mM phosphate buffer, pH 7.0, and were maintained at 2 °C, unless noted otherwise. Three different kinds of samples were injected into the chromatographic solvents: native protein; equilibrated protein, that is, protein equilibrated with the chromatographic solvent; and denatured protein, that is, protein equilibrated with an excess of denaturant. Protein elution profiles observed after injection of these samples are referred to as unfolding, equilibrium, and refolding profiles, respectively. In some measurements, a protein sample was preincubated for a controlled period of time in a given denaturant concentration prior to injection into a chromatographic solvent containing a different denaturant concentration. This procedure is termed multimixing. All illustrated profiles have 220-nm absorbance as the oridinate and elution times between 6.0 and 10.5 min as the abscissa, unless noted otherwise. The maximum ordinate in each elution profile was automatically made full scale by the encoding program.

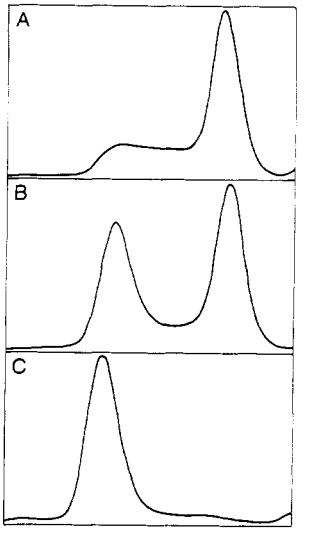


FIGURE 1: Chromatographic profiles in the transition zone. All chromatographic measurements were made in 2.5 ± 0.05 M denaturant. (A) Unfolding profile obtained by injection of native protein. (B) Equilibrium profile. (C) Refolding profile obtained by injection of protein denatured in 5.8 M denaturant for 20 min. In each panel, the ordinate is absorbance at 220 nm, and the abscissa is chromatography time ranging from 6.0 to 10.5 min.

Equilibrium Profiles. Equilibrium profiles of thioredoxin exhibit a single symmetrical peak having an elution time of about 10 min in denaturant concentrations less that 2 M. By contrast, two peaks are observed in equilibrium profiles obtained in denaturant concentrations between 2 and 3 M, as illustrated in Figure 1B. The additional peak elutes about 1.8 min earlier and is well resolved, indicating two components in slow exchange. In denaturant concentrations above 3 M, only a single symmetrical peak is again observed, but now having an elution time characteristic for the slower component, about 8 min.

These results are coincident with the conformational transition of thioredoxin observed in guanidine hydrochloride at neutral pH and 2 °C using far-ultraviolet circular dichroic measurements at 219 nm (Kelley et al., 1987). In these measurements, the native conformation predominates in the native base-line zone below 2 M denaturant; the protein undergoes a conformational change in the transition zone between 2 and 3 M having a midpoint at 2.4 M, and the denaturated protein predominates in the denatured base-line zone above 3 M. The 1.8-min difference observed here in the elution time of thioredoxin in the native and denatured base-line zones presumably results from the increase in the effective volume of the protein accompanying denaturation. The bimodal shape of the elution profiles observed in the transition zone suggests that conformations having an effective volume intermediate between those of the native and denatured proteins are not populated significantly at equilibrium.

Unfolding Profiles. As illustrated in Figure 1A, unfolding profiles observed in the transition region at or below the midpoint concentration are dominated by a component having the elution time of the native protein. This suggests that the

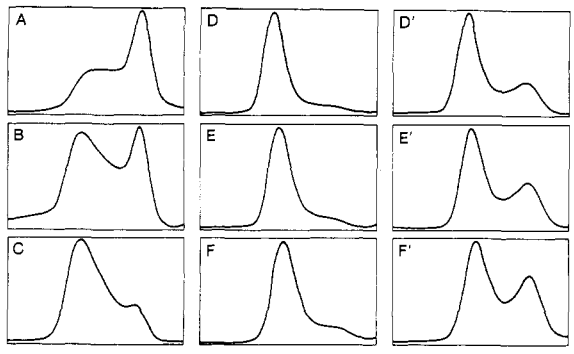


FIGURE 2: Chromatographic profiles symmetrical with the transition midpoint. In each panel, the ordinate is absorbance at 220 nm, and the abscissa is chromatography time ranging from 6.0 to 10.5 min. Panels A–C are unfolding profiles observed following injection of native thioredoxin into a column containing the following denaturant concentrations: (A) 2.75 M; (B) 2.85 M; (C) 3.00 M. Panels D–F are refolding profiles observed following injection of thioredoxin incubated in 5.8 M denaturant for 20 min. Panels D′–F′ are refolding profiles observed following injection of thioredoxin incubated in 5.8 M denaturant for 15 s. The denaturant concentration in the column was 1.83 M in panels D and D′, 1.68 M in panels E and E′, and 1.59 M in panels F and F′.

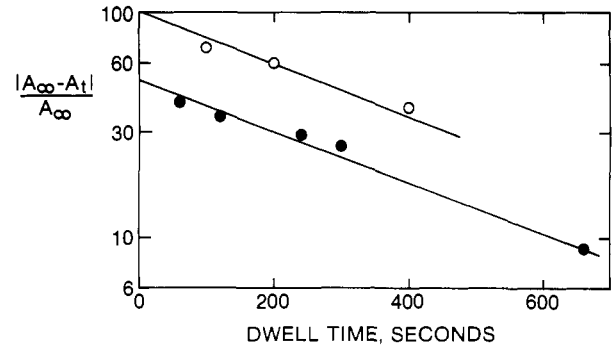


FIGURE 3: Multimixing measurements observed in the native base-line zone. Samples of native thioredoxin were exposed to 5.8 M denaturant for the indicated times and then immediately injected into a column containing 1.45 M denaturant. The closed circles indicate the relative area of the component having an elution time characteristic for the denatured protein. The open circles indicate the fractional amplitude associated with the slow refolding component. A_∞ indicates the values observed after exposure to 5.8 M denaturant for several hours. A_t indicates the area of amplitude observed after exposure to 5.8 M denaturant for the indicated dwell times in seconds.

time constant for unfolding is also slow in this denaturant concentration range. In denaturant concentrations above 2.6 M, a significant amount of protein is distributed between two components in the unfolding profiles as illustrated in Figure 2, panels A–C. The two principal components in each profile have elution times characteristic for native and denatured thioredoxin. However, the fractional area associated with the native protein and the height of the valley connecting the native and denatured components in the unfolding profiles are significantly greater than these features in equilibrium profiles at the same denaturant concentrations. Such differences indicate that unfolding has not been completed during chromatography and that the net conversion of native to denaturated protein is being observed. By contrast, unfolding profiles observed in denaturant concentrations of 4 M or greater indicate the presence of a single component having an elution time characteristic of the denatured protein in equilibrium profiles at the same denaturant concentrations. This equivalence indicates that the rate of unfolding is accelerated in high denaturant concentrations and is quickly completed during chromatography.

Refolding Profiles in 1.45–2.50 M Denaturant. Refolding profiles in the transition zone are dominated by a component having an elution time characteristic for denaturated thioredoxin as illustrated in Figure 1C. As the concentration of denaturant on the column is decreased into the native base-line zone, a small amount of well-resolved component having the elution time of native thioredoxin is observed as illustrated in Figure 2, panels D–F. However, the extent of refolding is significantly less than the extent of unfolding observed at denaturant concentrations approximately symmetrical with the equilibrium transition midpoint, as illustrated in Figure 2. The unfolding profiles, panels A–C, illustrate a marked increase in the concentration of denatured protein as the denaturant concentration is increased. By contrast, the refolding profiles, panels D–F, remain relatively constant in shape with the denatured protein dominant as the denaturant concentration is decreased. Thus, it would appear either that refolding is very slow or that the bulk of the denatured protein is being withheld from refolding by a coupled reaction in the denatured state.

The occurrence of a coupled reaction was investigated by using a multimixing protocol. Samples of native thioredoxin were incubated in 5.8 M denaturant either for 15 s or for 20 min at 2 °C prior to injection in a column equilibrated with a denaturant concentration in the native base-line zone. As shown in Figure 2, elution profiles observed after 15-s denaturation, panels D′–F′, evidence much more material eluting at the position of native protein than do corresponding profiles observed after 20-min denaturation, panels D–F. The enhanced concentration of native protein observed after brief denaturation does not result from incomplete denaturation prior to the initiation of refolding. This was established by injection of protein exposed to 5.8 M denaturant for only 15 s into a column equilibrated with 2.5 M denaturant. The observed refolding profile was identical with that illustrated in Figure 1C. Since protein both unfolds and refolds slowly in 2.5 M denaturant, the absence of any detectable native protein in the refolding profile indicates that all the protein exposed to 5.8 M denaturant for only 15 s was denatured. Accordingly, the denatured protein must isomerize to a more slowly refolding component upon continued exposure to denaturant.

The kinetics of the formation of the more slowly refolding component were examined by varying the dwell time of native protein in 5.8 M denaturant prior to injection into a column equilibrated with 1.45 M denaturant, a refolding concentration in the native base-line zone. The relative area under the native component decreases with increasing dwell time in 5.8 M denaturant as shown in Figure 3. This decrease describes a first-order relationship having a time constant of about 400 s. Execution of the same multimixing protocol using tryptophan fluorescence measurements gives a similar time constant as shown in Figure 3. The lower initial fraction of protein detected by the chromatographic measurements illustrated in Figure 3 results from interconversions which occur during residence of the protein on the chromatographic column prior to elution and is quantitatively predicted by the cube mechanism, considered under Discussion.

Refolding Profiles below 1.45 M Denaturant. The relatively constant refolding profiles illustrated in Figure 2, panels D–F, do not persist at lower denaturant concentrations in the native base-line zone. As illustrated in Figure 4, the elution time of the major component, which corresponds to the denatured protein in high denaturant concentrations in the native base-line zone, systematically increases as the denaturant concentration is described below 1.45 M. Such a systematic increase in elution time is characteristic for a two-component system

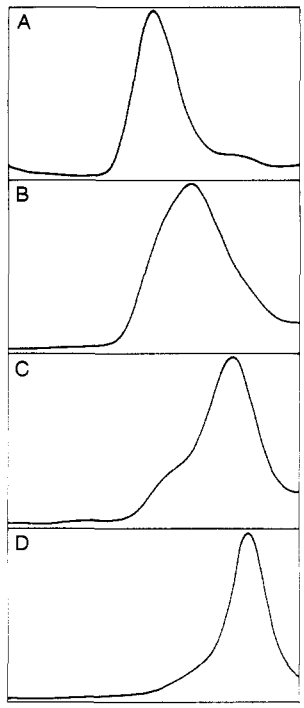


FIGURE 4: Refolding profiles observed below 1.5 M denaturant. Thioredoxin denatured in 5.8 M denaturant for at least 20 min was injected in a column containing the following denaturant concentrations: (A) 1.45 M; (B) 1.22 M; (C) 1.02 M; (D) 0.82 M. The ordinate in each panel is absorbance at 220 nm, and the abscissa is chromatography time ranging from 6.0 to 10.5 min.

in rapid exchange. It is curious to observe the abrupt appearance of this rapid exchange system since all refolding profiles observed in denaturant concentrations greater than 1.45 M are characteristic of slow exchange. As the concentration of denaturant in the column is decreased below 1.25 M, an additional refolding component is observed as a shoulder on the trailing edge of the major refolding component. The major refolding component, whose elution time systematically increases in the native base-line zone, is termed the walking peak, and the minor refolding component appearing as a shoulder on the walking peak is termed the lag peak. The small amount of protein which has an elution time characteristic for native thioredoxin in refolding denaturant concentrations above 1 M is termed the native refolding component.

All the observed refolding elution profiles were fit to the sum of three Gaussian curves representative of the native, walking, and lag refolding components. Such fittings partition 16% ± 1% of the total area of the elution profile to the lag component, 74% ± 1% to the walking component, and 10% ± 1% to the native refolding component. The dependence of the fitted elution time for each component on denaturant concentration is shown in Figure 5. The elution times for the lag and walking components each describe a sigmoidal dependence which spans the range between the elution times observed or extrapolated for native and denatured thioredoxin, respectively, obtained from equilibrium profiles. These observations suggest that three different forms of denatured thioredoxin can be observed to fold into a compact conformation equilivalent in elution position to the native protein.

The refolding profiles observed in 1.1 M denaturant for native thioredoxin exposed to 5.8 M denaturant for 15 s and for 20 min at 2 °C are shown in Figure 6. The fractional area contributed by the walking and lag components increases markedly with increasing dwell time in concentrated denaturant. This indicates that both the walking and lag components

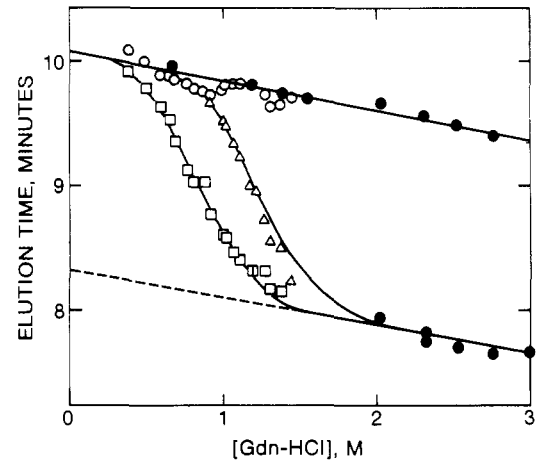


FIGURE 5: Dependence of chromatographic elution times on denaturant concentration. The open circles, triangles, and squares represent the elution times for the native, walking, and lag refolding components, respectively. These values were obtained from analysis of single and multimixing refolding profiles. The closed circles represent the elution times for the native and denatured components observed in equilibrium profiles.

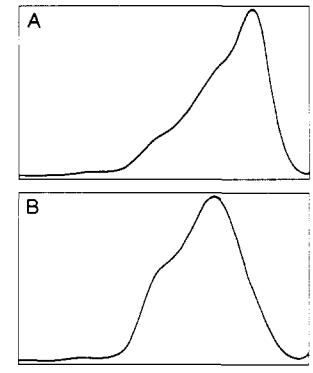


FIGURE 6: Refolding profiles in 1.1 M denaturant. Panel A illustrates the refolding profiles observed following injection of a sample of native thioredoxin exposed to 5.8 M denaturant for 15 s. Panel B illustrates the refolding profile observed following injection of a sample of native thioredoxin exposed to 6.8 M denaturant for 20 min. The ordinate in each panel is absorbance at 220 nm, and the abscissa is chromatography time ranging from 6.0 to 10.5 min.

are generated in the denatured state. The ratio of the area of the walking component to the area of the lag component is independent of the dwell time in concentrated denaturant. This indicates that both components are generated with the same time constant.

Multimixing Measurements in the Native Base-Line Zone. The relative stabilities of the compact conformations produced by refolding denatured thioredoxin were compared by using a multimixing protocol. These experiments were conducted at 25 °C using a 75-mm chromatographic column. A supply of denatured protein was prepared by exposing native protein to 5.8 M denaturant for several hours, ensuring that the slow folding form was dominant in the denaturated protein. Samples of this denatured protein were diluted to a given concentration in the native base-line zone to facilitate refolding. After refolding for a controlled period of time, termed the dwell time, an aliquot of the refolded protein was injected into a chromatographic column equilibrated with 2.5 M denaturant. It was anticipated that any compact protein having a stability significantly less than native thioredoxin, such as the walking or lag component, would be denatured following injection into this column. Thus, the native refolding component could be anticipated to be the only compact form which should appear in the elution profiles using this multimixing protocol. Analysis

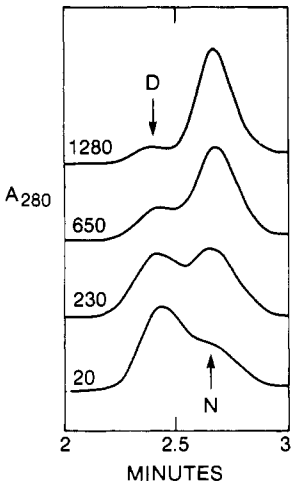


FIGURE 7: Unfolding profiles observed at 2.5 M denaturant following multimixing. Samples of denatured thioredoxin were exposed to 0.17 M denaturant for various dwell times prior to injection into a column containing 2.5 M denaturant. The unfolding profiles were obtained after the indicated dwell times in seconds. The arrows indicate the elution positions of native thioredoxin, N, and denatured thioredoxin, D, observed by using a sample of thioredoxin equilibrated with 2.5 M denaturant prior to injection. In contrast to other measurements, all these manipulations were done at 25 °C the chromatography was done with a 75-mm column, and the absorbance was measured at 280 nm.

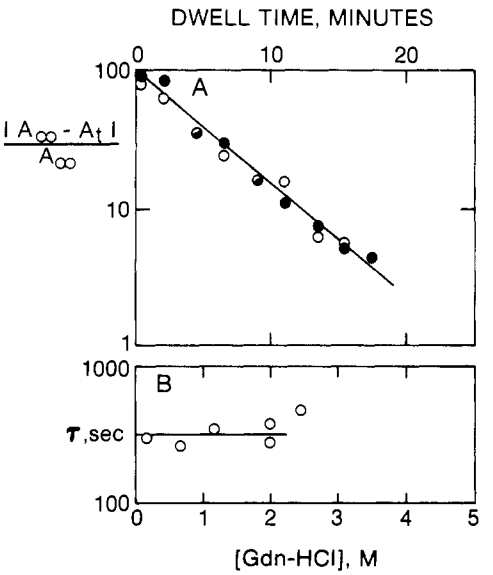


FIGURE 8: Analysis of multimixing measurements in the transition zone. Panel A illustrates analysis of the multimixing measurement described and illustrated in Figure 7. A_t is the area after a dwell time of t seconds, and A_∞ is the area after a dwell time of several hours. The open circles indicate changes in the area associated with the compact protein, and the closed circles indicate changes in the area associated with the denatured protein. Panel B indicates the dependence of the time constant obtained by these measurements on the denaturant concentration in which refolding took place. All measurements illustrated in this figure were obtained at 25 °C.

of the area of the compact component as a function of dwell time should generate the time constant(s) for the conversion of the compact refolding products(s) into native thioredoxin.

Representative elution profiles for a refolding multimixing protocol conducted at 0.17 M denaturant are shown in Figure 7. As the dwell time for refolding is increased, there is a net conversion of material from an elution time characteristic for denaturated thioredoxin, D, to that characteristic for native thioredoxin, N. The kinetics for this conversion generate a first-order plot having a time constant of 350 s as illustrated in Figure 8A. Similar results were obtained by varying the refolding dwell times throughout the native base-line zone as shown in Figure 8B. Taken together, these results suggest that the compact products generated by both the lag and walking components are converted to the native protein in a reaction having a time constant of about 350 s. The time constant and denaturant independence of this reaction are equivalent to the slow refolding phase observed by tryptophan fluorescence measurements of refolding (Kelley, et al., 1986). The measured activation energy of the slow-phase refolding (Kelley et al., 1986) predicts that the observed reaction would have a time constant of about 3900 s at 2 °C.

Discussion

Two kinds of protein isomerization reactions are considered under Discussion. An isomerization involving a very local region of the polypeptide chain, e.g., the cis/trans isomerization of a peptide bond, is termed a configurational isomerization. The isomeric form present in the native protein is termed the native isomer, and the isomeric form(s) not present in the native protein is (are) termed the nonnative isomer(s). The kinetic time constant for a configurational isomerization is expected to be independent of denaturant concentration. In all reactions considered, the time constant is the reciprocal sum of the rate constants for the forward and reverse reactions. An isomerization involving a much larger portion of the polypeptide chain, e.g., protein unfolding and refolding, is termed a conformational isomerization. The time constant for a conformational isomerization reaction is expected to be denaturant dependent, since the denaturant perturbs the equilibrium between the two conformational isomers. The denaturant dependence takes the form of an inverted triangle when the logarithm of the observed time constant is plotted as a function of denaturant concentration. The apex of the inverted triangle, i.e., the largest observed time constant, should occur at the midpoint of the denaturant-dependent conformational transition. This is because the rate of unfolding increases as denaturant concentration increases while the rate of refolding increases as denaturant concentration decreases. The reciprocal sum of these two rates, the time constant, is maximal at the midpoint where the two rates are equal.

Fitting to a Mechanism. Analysis of the elution profiles was begun by assuming the simplest reaction mechanism, a two-state conformational transition, N = DN, between native thioredoxin, N, and denatured thioredoxin, DN. All the equilibrium profiles can be fit by this mechanism with a transition midpoint of 2.35 ± 0.19 M and the time constants indicated by the open squares in Figure 9A. The logarithm of these time constants exhibits an inverted triangular dependence on denaturant concentration characteristic for a conformational transition. All the unfolding profiles can also be fit by this two-state mechanism with a transition midpoint of 2.24 ± 0.05 M and the time constants indicated by the open triangles in Figure 9A. The logarithm of the unfolding time constants exhibits a linear dependence on denaturant concentration coincident with one leg of the dependence obtained by fitting the equilibrium profiles. However, this simple two-state mechanism cannot accommodate the generation of slow-refolding components in the denatured state, three distinct refolding components, or the transient accumulation of compact forms having a stability less than the native protein. Clearly, the reaction mechanism will have to be expanded in order to incorporate these features. Nevertheless, the midpoint and time constants obtained from fitting with a two-state transition can serve as useful starting parameters for subsequent fitting procedures.

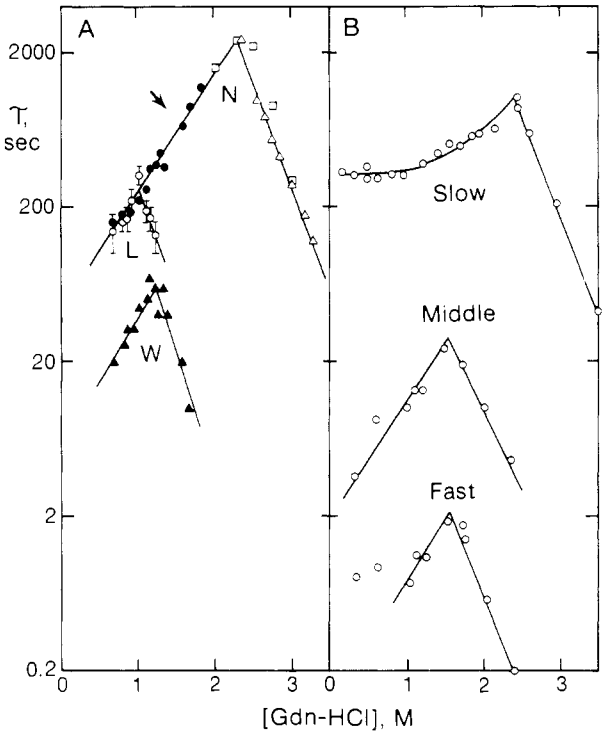
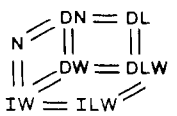


FIGURE 9: Dependence of fitted time constants, τ, on denaturant concentration. Panel A illustrates values obtained from chromatographic measurements at 2 °C. The open squares were obtained from analysis of equilibrium profiles using a two-state mechanism, N = DN. The open triangles were obtained from analysis of unfolding profiles using the same two-state mechanism or the cube mechanism. The open and closed circles and the closed triangles were obtained from analysis of refolding profiles using the cube mechanism. The closed circles indicate values for the native refolding component whose inverted triangle is labeled N. The open circles indicate values for the lag component whose inverted triangle is labeled L. The closed triangles indicate values for the walking component whose inverted triangle is labeled W. Panel B illustrates values obtained from stopped-flow tryptophan fluorescence emission measurements obtained at 25 °C (Kelley et al., 1986). The slow, middle, and fast kinetic refolding phases are labeled.

It is likely that a number of reaction mechanisms can be fitted with the refolding profiles observed following both the single and multimixing protocols described above. However, we wish to propose for further consideration one particular mechanism, termed the cube mechanism, since it appears to be relatively simple, employs reactions characteristic for the refolding of other small globular proteins, and is consistent with previous refolding measurements obtained by using tryptophan fluorescence. The denatured protein in the cube mechanism is represented by four configurational isomers: DN, which is the native isomeric form; DW, which contains one nonnative isomeric form; DL, which contains a different nonnative isomeric form; and DLW, which contains both nonnative isomeric forms. While the refolding profiles can be fit equally well by using only DN, DW, and DLW, DL is included for reasons of symmetry. The compact protein is considered to be populated by at least three components: N, the native protein; IW, a compact intermediate containing one nonnative configurational isomer; and ILW, a compact intermediate containing two nonnative configurational isomers. These components are coupled as diagrammed in the cube mechanism:



Both the single and multimixing refolding profiles were fit with the cube mechanism by using the following conditions. The time constant for each of the configurational isomerizations in the denatured state was held at 400 s, the value obtained from multimixing measurements. The concentration ratios [DN]/[DL] and [DW]/[DLW] were initially set but not held at 62/38, and the concentration ratios [DN]/[DW] and [DL]/[DLW] were initially set but not held at 12/88. These values are compatible with the Gaussian fits of refolding profiles in the native base-line zone. The midpoint of the conformational isomerization N = DN was initially set but not held at 2.35 M, the average value obtained from two-state fitting. The midpoint of the conformational change IW = DW was initially set but not held at 1.2 M, the midpoint of the denaturant dependence of the elution time of the walking component shown in Figure 5. Similarly, the midpoint of the conformational change ILW = DLW was initially set but not held at 0.8 M, the midpoint of the denaturant dependence of the elution time of the lag component. All of the compact intermediates were assumed to convert to the native protein since all equilibrium profiles observed using spectral or hydrodynamic probes indicate a single transition. The time constant for these conversions was held at 3900 s, the value obtained from multimixing measurements.

These set values were refined until all the unfolding and refolding profiles, obtained by using both single and multimixing protocols, were simulated. The refined time constants generated a regular logarithmic dependence on denaturant concentration characteristic for three conformational transitions as shown in Figure 9A. The highest inverted triangle, labeled N for the native refolding component, represents the N = DN transition having a refined midpoint of 2.35 M. The middle inverted triangle, labeled L for the lag component, represents the ILW = DLW transition having a refined midpoint of 0.9 M. The lowest inverted triangle, labeled W for the walking component, represents the IW = DW transition having a refined midpoint of 1.2 M. Most time constants ranged over the values covered by the symbols except for those of the lag component which ranged as indicated by the bars. The time constants shown in Figure 9A were obtained by using ratios for the denatured configurational isomers pairs [DN]/[DL] and [DW]/[DLW] refined to 60/40 and for the pairs [DN]/[DW] and [DL]/[DLW] refined to 15/85. Essentially the same time constants are observed upon increasing the latter ratio to a maximum of 25/75, but the midpoint of the ILW = DLW is raised to 1.0 M.

Comparison with Other Measurements. The denaturant dependence of the time constants obtained from simulations of the chromatographic measurements compares favorably with those obtained previously from analysis of stopped-flow fluorescence measurements (Kelley et al., 1986). Both data sets exhibit the same general featurs, namely, three inverted triangles separated by about an order of magnitude as shown in Figure 9. The inverted triangles in the individual data sets may be paired as follows: the walking component W and the fast kinetic phase; the lag component and the middle kinetic phase; the native refolding component and the slow refolding phase. The middle and fast refolding phases dominate the amplitude of the fluorescence change in the native base-line zone. Similarly, the lag and walking components are dominant in the refolding chromatographic profiles in the native base-line zone. The apex of the inverted triangles for the middle and fast refolding phases occurs at similar denaturant concentrations as do the apexes for the lag and walking components. The apex of the inverted triangle for the slow refolding phase

occurs at a denaturant concentration about 1.0 M greater than the apexes for the inverted triangles of the middle and fast refolding phases. Similarly, the apex of the inverted triangle for the native refolding component occurs at a denaturant concentration about 1.3 M greater than the apexes for the inverted triangles of the lag and walking components. The time constants for the slow, middle, and fast refolding phases can be predicted at 2 °C by using the time constants observed at 25 °C and the measured activation energy for each phase (Kelley, 1984; Kelley et al., 1986). The time constants predicted at the apexes of the triangles for the slow, middle, and fast phases at 2 °C are within a factor of 3 of the time constants observed at 2 °C at the apexes of the triangles for the native, lag, and walking refolding components, respectively. Finally, the time constant for the proposed configurational isomerization in the denatured state obtained from chromatographic measurements, 400 s, is within a factor of 2 of that calculated from the time constant, 19 s, and activation energy, 22 kcal/mol, obtained from fluorescence measurements at 25 °C (Kelley et al., 1986).

Features unique to the chromatographic measurements include the detection of time constants for the refolding leg of the N = DN transition and the absence of the denaturant-independent portion of the slow refolding phase. The refolding leg indicated by the arrow in Figure 9A was made visible by performing multimixing refolding chromatographic measurements in which the relative concentration of DN was enhanced. The concentration of DN in a single mixing protocol is normally too low to observe its refolding as a distinctive kinetic phase in fluorescence measurements. The slow denaturant-independent refolding phase was not directly observed in the chromatographic measurements presumably because it occurs subsequent to formation of a compact product. However, a reaction having the features of the slow denaturant-independent refolding phase was observed by using a multimixing chromatographic protocol. This reaction presumably represents the configurational isomerizations within the compact forms, IW = N and ILW = N, which are observed by tryptophan fluorescence measurements.

Configurational Isomerizations. We believe that the configurational isomerizations considered in the cube mechanism involve proline peptide isomerization reactions. Thioredoxin has five proline residues; four of these proline residues participate in trans peptide bonds in the native protein, and one, proline-76, is cis (Brändén et al., 1983). The trans isomer of each proline peptide bond likely predominates in the denatured state. This would be the native isomer of four of the proline peptide bonds but the nonnative isomer of the fifth proline at position 76. Accordingly, it is likely that DN = DW and DL = DLW involve proline-76 since the nonnative isomer predominates with fitted ratios ranging from 15/85 to 25/75. The DN = DL and DW = DLW configurational isomerizations then must involve one of the other four prolines. The isomerization of the remaining three proline peptide bonds is not detected chromatographically and may not perturb refolding.

Preliminary chromatographic measurements using thioredoxin Ala-76, a site-directed mutagenic product provided by Dr. Robert Kelley, indicate that the N = DN transition is missing and that the major transition has the features of the walking peak. These are precisely the changes that would be predicted were the DN = DW isomerization to involve proline-76.

Equilibrium Profiles. While the cube mechanism can accommodate all the intricate features of the refolding chromatographic profiles as well as the unfolding chromatographic profiles, it does not simulate the equilibrium profiles such as that shown in Figure 1B. This failure likely results from the coupled configurational isomerization reactions in the denatured state which are not offset by populated coupled reactions in the compact state. (Neither IW nor ILW is populated owing to equilibria which strongly favor N.) For example, the midpoint of the conformational change N = DN would need to be at 2.9 M denaturant in order for the cube mechanism to have an equilibrium midpoint at 2.4 M. Conversely, if the midpoint of the conformational change were 2.4 M, then the equilibrium midpoint would need to be 1.9 M. Clearly, neither appears to be the case.

One resolution to this dilemma, for which we have no direct evidence, is to replace N in the cube mechanism with N = IN, with IN being a compact intermediate containing only native configurational isomers. The combination of midpoint values and time constants for the coupled reactions N = IN = DN which simulate both the equilibrium and unfolding profiles are very limited. Reaction N = IN needs a very fast time constant, and IN = DN needs a modest midpoint to facilitate adequate unfolding. Midpoint values for N = IN of 3.0 M and for IN = DN of 2.35 M and a time constant for N = IN of no greater than 0.01 s will simulate the unfolding and equilibrium profiles rather well. Such an expansion of the mechanism will not affect the refolding profiles since the added reaction occurs after the generation of the compact folding products.

General Conclusions. The chromatographic measurements and their simulation have revealed several unique features of the conformational transition of thioredoxin which may be of broad scope. First, all the kinetic profiles can be simulated without involving a transient population of conformations having an effective volume intermediate between those of the native and denatured protein. This is not to say that the simulations rule out such intermediates; they just are not obligatory. Second, the transiently populated intermediates which are detected have an effective volume characteristic for the native protein. Thus, the detected intermediates are nativelike as opposed to denatured-like. Third, hydrodynamic measurements do not appear to detect any kinetic phases not also observed by spectral measurements. Nonetheless, hydrodynamic measurements do have the unique ability to directly observe and quantitate denatured and compact populations. Finally, chromatographic measurements can resolve equilibrium distributions in an effort to discern silent components.

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