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Equilibrium and Kinetic Measurements of the Conformational Transition of Thioredoxin in Urea†

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ABSTRACT: Addition of urea to solutions of *Escherichia coli* thioredoxin results in a cooperative unfolding of the protein centered at 6.7 M urea at 25 °C and 5.1 M urea at 2 °C and neutral pH as judged by changes in tryptophan fluorescence emission, far-ultraviolet circular dichroism, and exclusion chromatography. Kinetic profiles of changes in tryptophan fluorescence emission intensity were analyzed following either manual or stopped-flow mixing to initiate unfolding or refolding. Unfolding of the native protein occurs in a single kinetic phase whose time constant is markedly dependent on urea concentration. Refolding of the urea-denatured protein occurs in a multiplicity of kinetic phases whose time constants and fractional amplitudes are also dependent upon urea concentration. Urea gradient gel electrophoretic and exclusion chromatographic measurements suggest the transient accumulation of at least one and likely two compact nativelike intermediate conformations during refolding. Simulations of both electrophoretic and chromatographic results suggest that the intermediate conformations are generated by the concerted action of the middle and fast refolding phases.

The protein thioredoxin obtained from *Escherichia coli* contains a single polypeptide chain of 108 residues of known sequence (Holmgren, 1968). In the native protein, the chain is folded into two domains, a large N-terminal domain having a $\beta\alpha\beta\alpha\beta$ structure and a small C-terminal domain having a $\beta\beta\alpha$ structure (Holmgren et al., 1975). The structure of the native protein unfolds cooperatively and reversibly at neutral pH and 25 °C in solvents containing guanidine hydrochloride in excess of 2 M (Holmgren, 1972; Kelley & Stellwagen, 1984). Unfolding occurs in a single kinetic phase while refolding occurs in three kinetic phases as detected by changes

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in tryptophan fluorescence emission (Kelley et al., 1986a). The dependence of the time constant and fractional amplitude of each of the refolding phases on the concentration of guanidine hydrochloride suggests the transient accumulation of intermediates in the refolding pathway. In this report, we describe the results in equilibrium and kinetic measurements of the conformational changes of thioredoxin using urea as the denaturant. We find that the hydrodynamic changes of thioredoxin observed in urea using gel electrophoresis and using exclusion chromatography suggest the presence of one and likely two nativelike intermediates in the conformational transition. A preliminary account of some of these results has been reported previously (Kelley et al., 1986b,c).

Experimental Procedures

Materials. Thioredoxin was purified from an *E. coli* strain (Lunn et al., 1984) containing multiple copies of a plasmid with a 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 obtained in 50 mM phosphate buffer, pH 7.0, using thioredoxin having a disulfide bond linking cysteine residues 32 and 35.

Methods. Equilibrium fluorescence measurements as well as those kinetic fluorescence measurements initiated by manual mixing were obtained by using an SLM Model 4800 fluorometer. Kinetic fluorescence measurements initiated by stopped-flow mixing were obtained by using a Durrum Model D-110 spectrometer as described previously (Kelley & Stellwagen, 1984). Kinetic profiles observed following either manual mixing or stopped-flow mixing were fit to simultaneous first-order reactions using a DEC Model 11/780 VAX computer as described previously (Kelley & Stellwagen, 1984). All fitted kinetic profiles are described in terms of the time constant and fractional amplitude of the component reactions which are called kinetic phases. Only kinetic phases having fractional amplitudes of 0.05 or greater are considered. When choices were possible, the minimum number of kinetic phases needed to generate an equivalent goodness of fit was chosen.

Urea gradient electrophoresis was done as described by Creighton (1979). Gels were cast in 50 mM phosphate buffer, pH 7.0, containing a 0–8 M linear urea gradient. Zone applications of either native or denatured thioredoxin were subjected to electrophoresis for 120 min using an LKB flat-bed electrofocusing apparatus and a potential difference of 300 V. Water maintained at 2 °C was pumped through the thermostable plate on which the gel was placed during electrophoresis. Selected gels were stained with either Coomassie blue or silver salt after electrophoresis and photographed.

Exclusion chromatography was done by using a 7.5 × 300 mm Bio-Sil TSK-125 gel filtration column, an IBM Model LC9533 liquid chromatograph, and either an IBM fixed-wavelength or an Iso V4 variable-wavelength absorbance detector. Water maintained at 2 °C was circulated through the column jacket. Twenty-microliter samples containing 1 mg/mL protein were injected into the column at zero time and subjected to isocratic chromatography at a flow rate of 1 mL/min.

Results obtained by using urea gradient electrophoresis and exclusion chromatography were simulated with the dynamic exclusion chromatographic equations of Endo et al. (1983). Input values for simulation of the chromatographic profiles include the elution times for material totally excluded by the column (5.5 min using blue dextran) and totally included by the column (10.8 min using potassium dichromate), the elution times for folded and unfolded protein at the isocratic urea

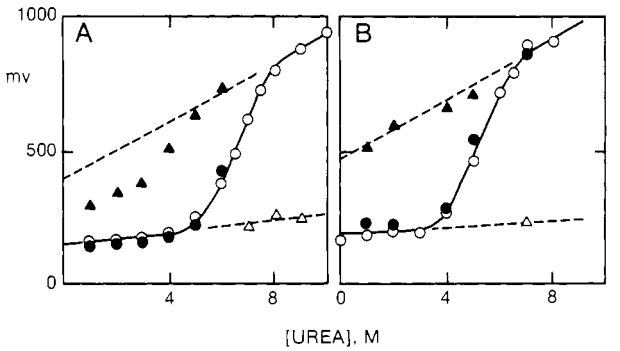

FIGURE 1: Dependence of the fluorescence emission intensity of thioredoxin on the concentration of urea. All solutions contained 20 μM thioredoxin, 50 mM phosphate buffer, pH 7.0, and the indicated concentration of urea. Panel A illustrates the fluorescence emission intensity measured at 350 nm in millivolts (mV) at 25 °C using 276-nm excitation. Panel B illustrates the same measurements performed at 2 °C. The open circles indicate intensity values observed at equilibrium starting with native thioredoxin, and the closed circles indicate equilibrium values starting with thioredoxin denatured in excess urea. The open triangles indicate intensity valules predicted at time zero from analysis of the kinetic profiles of the unfolding of native thioredoxin following manual mixing. The closed triangles indicate the same values predicted from analysis of the refolding of denatured thioredoxin following manual mixing.

concentration (Figure 8A), the midpoint of the folding transition, the width of 80% of the conformational transition (1.8 M urea), the time constant for the conformational transition, the isocratic urea concentrations, the nature of the protein sample (whether native or denatured or equilibrated with the isocratic solvent), the volume of protein sample, the diffusional broadening of the protein sample (10%), and the number of theoretical plates. Preliminary calculations indicated that the simulations would not be changed significantly by increasing the number of theoretical plates beyond 500. The equations and input quantities were modified slightly to simulate electrophoresis as opposed to chromatographic results. All simulations were performed by using a DEC model 11/780 computer.

Results

Fluorescence Measurements at 25 °C. The near-ultraviolet fluorescence excitation spectra of solutions of native and of urea-denatured thioredoxin were observed with an emission wavelength of 350 nm. Both forms of the protein exhibit an excitation spectrum having a broad maximum centered at about 276 nm and a shoulder centered at about 290 nm which is similar in profile to the near-ultraviolet absorbance spectrum of the protein. Fluorescence emission spectra of the native and urea-denatured thioredoxin were observed following excitation at 276 nm. The emission spectrum of native thioredoxin exhibits a broad maximum centered at 344 nm characteristic for a tryptophan residue. The intensity of the entire fluorescence emission profile of thioredoxin is markedly enhanced in the presence of excess urea, and its emission maximum is shifted from 344 to 351 nm. These changes are characteristic for the denaturation of thioredoxin and are assumed to reflect in large measure the steric separation of tryptophan residue 28 from the quenching disulfide bond linking cysteine residues 32 and 35 (Reutimann et al., 1981; Kelley & Stellwagen, 1984).

The dependence of the fluorescence emission intensity of thioredoxin observed at 350 nm on the concentration of urea is shown in Figure 1A. This dependence can be considered to comprise three zones: the native base-line zone between 0 and 4 M urea, the transition zone between 4 and 8 M urea, and the denatured base-line zone above 8 M urea. Extrapo-

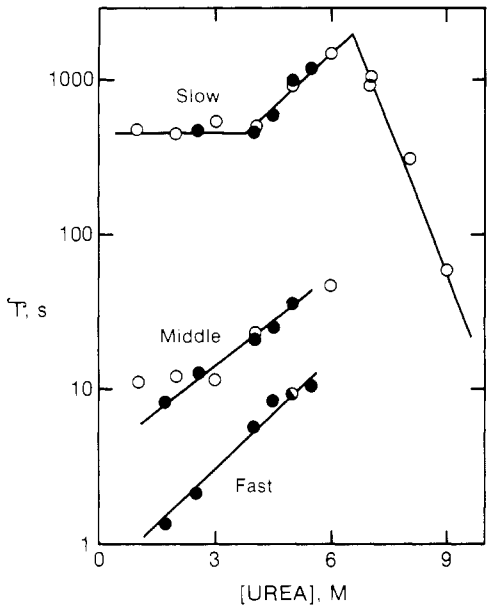

FIGURE 2: Dependence of the kinetic time constants measured at 25 °C on the concentration of urea. Solutions of either urea-denatured or native thioredoxin were mixed with either 50 mM phosphate buffer, pH 7.0, or concentrated urea in buffer, respectively, to generate solutions containing 20 μM protein and the indicated concentration of urea. The open circles indicate analysis of fluorescence emission measurements obtained following manual mixing, and the closed circles indicate analysis of fluorescence emission measurements obtained following stopped-flow mixing.

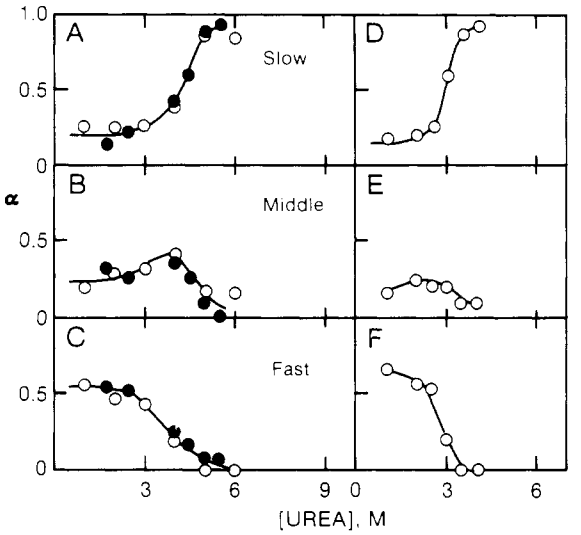

FIGURE 3: Dependence of the fractional amplitude of the kinetic phases on the concentration of urea. The fractional amplitude has the symbol α. Panels A–C illustrate results obtained at 25 °C, and panels D–F illustrate results obtained at 2 °C. The top, middle, and bottom panels illustrate the fractional amplitude of the slow, middle, and fast kinetic phases, respectively. The open circles indicate analysis of measurements obtained following manual mixing, and the closed circles indicate analysis of measurements obtained following stopped-flow mixing.

lation of the denatured base-line zone to 0 M urea indicates that the fluorescence emission intensity of thioredoxin is increased by a factor of 2.6 in the presence of excess urea. A similar increase in the fluorescence emission intensity of thioredoxin at 350 mn has been observed following cooperative denaturation of the protein in guanidine hydrochloride at neutral pH and 25 °C (Reutimann et al., 1981; Kelley & Stellwagen, 1984). The transition zone has a midpoint at about 6.5 M urea, and the observed changes are completely reversible. A similar transition in urea has been partially characterized (Holmgren, 1972).

A solution of native thioredoxin was manually mixed with a solution of concentrated urea to generate a series of thioredoxin solutions whose final urea concentration was either in the transition zone or in the denatured base-line zone. The increase in fluorescence emission intensity was recorded following mixing until a constant value was obtained. Each kinetic profile of the unfolding of thioredoxin so obtained fit very well with a single first-order unfolding reaction. The time constant for the unfolding reaction is markedly dependent on the concentration of urea present in the unfolding solvent as shown in Figure 2. Such a dependence is characteristic for a protein conformational change and presumably results from the differential binding of the denaturant urea to the native and denatured conformations. The total change in fluorescence emission intensity associated with the single unfolding reaction at each urea concentration is equivalent to that predicted from equilibrium measurements as shown in Figure 1A. These results are consistent with the view that thioredoxin unfolds in a single kinetic phase observable by fluorescence measurements.

A solution of denatured thioredoxin equilibrated with a concentration of urea in the denatured base-line zone was manually mixed with buffer to generate a series of thioredoxin solutions whose final urea concentrations were either in the native base-line zone or in the transition zone. The decrease in fluorescence emission intensity following mixing was recorded until a constant value was obtained. Each refolding reaction profile so obtained fit much better with two simultaneous first-order reactions, termed the slow and middle refolding phases, than with a single first-order reaction, as was the case for unfolding. The time constants for the slow and middle refolding phases each exhibit an inverse dependence on the concentration of urea in the transition zone as shown in Figure 2. However, in the native base-line zone, the time constant for the slow refolding phase, but not that for the middle refolding phase, becomes independent of urea concentration. The sum of the change in the fluorescence emission intensity associated with the slow and middle refolding phases obtained from the fitting procedure does not account for the change in intensity predicted from equilibrium measurements in the native base-line zone as shown in Figure 1A. This discrepancy suggests that the refolding of thioredoxin involves a third kinetic phase too rapid to be detected by manual mixing protocols at 25 °C.

Accordingly, the changes in fluorescence emission intensity accompanying the refolding of denatured thioredoxin in urea solutions were observed following stopped-flow mixing. The reaction profiles so obtained clearly fit better with three simultaneous first-order reactions, termed the slow, middle, and fast refolding phases, than with only two such reactions. The time constants for the slow and middle refolding phases obtained by analysis of refolding reactions initiated by manual mixing and by stopped-flow mixing are in good agreement, as shown in Figure 2. As predicted, the third reaction uniquely observed following stopped-flow mixing is a fast refolding phase. The time constant for this refolding phase exhibits an inverse dependence on urea concentration both within and outside the transition zone, as shown in Figure 2. The fractional fluorescence emission intensities observed or predicted for each of the three refolding phases following manual mixing and following stopped-flow mixing also are in good agreement, as shown in Figure 3A. It should be noted that the fractional fluorescence intensity associated with each of the three refolding phases is quite dependent on denaturant concentration between 2 and 5 M urea.

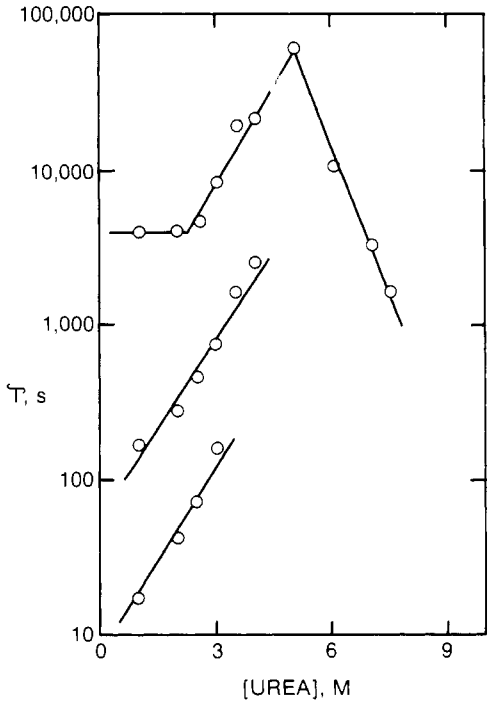

FIGURE 4: Dependence of the kinetic time constants measured at 2 °C on the concentration of urea. Solutions of either urea-denatured or native thioredoxin were mixed either with 50 mM phosphate buffer, pH 7.0, or with a concentrated urea solution in buffer, respectively, to generate solutions containing 20 μM thioredoxin and the indicated concentrations of urea. Changes in fluorescence emission intensity were observed following manual mixing.

Fluorescence Measurements at 2 °C. The near-ultraviolet fluorescence excitation and emission spectra of thioredoxin observed in the absence and presence of excess urea at 2 °C are virtually identical with those obtained at 25 °C as described above. The dependence of the fluorescence emission intensity of thioredoxin at 350 nm on urea concentration is shown in Figure 1B. This dependence can also be resolved into a native base-line zone, a transition zone, and a denatured base-line zone. While the cooperativity of the transition appears comparable at 2 and 25 °C, the midpoint of the transition is shifted from 6.5 M urea at 25 °C to 5.1 M urea at 2 °C. Although the denatured base-line zone is abbreviated due to the solubility of urea at 2 °C, extrapolation suggests that the fluorescence intensity at 350 nm is increased by a factor of about 2.5 with denaturation, essentially the same increment observed at 25 °C.

The kinetics of both the unfolding and refolding of thioredoxin at 2 °C were observed by recording changes in fluorescence emission intensity initiated by manual mixing. As was observed at 25 °C, the kinetic profiles for the unfolding of thioredoxin in urea at 2 °C fit well with a single reaction. The time constant for unfolding is inversely dependent on urea concentration below the equilibrium midpoint value as shown in Figure 4. Most of the kinetic profiles observed for refolding of the denatured protein required more than one reaction to obtain a good fit and to account for the change in fluorescence emission amplitude predicted by equilibrium measurements. The refolding reaction profiles observed in urea concentrations greater than 3 M fit well using two kinetic phases while the reaction profiles observed in urea concentrations of 3 M or less required three kinetic phases for a fit of comparable quality. The dependence of the time constant for each refolding phase on the concentration of urea present during refolding is shown in Figure 4. In contrast to the kinetic refolding measurements obtained following manual mixing at

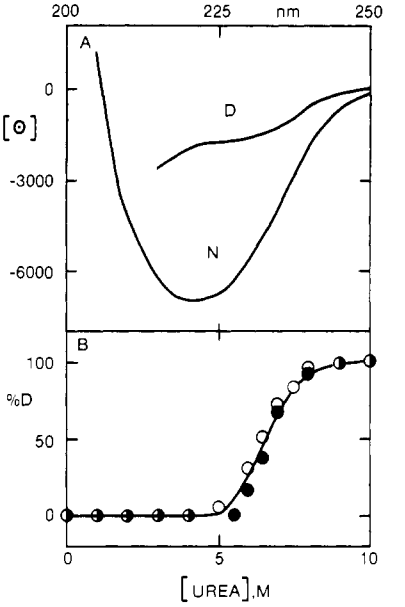

FIGURE 5: Far-ultraviolet circular dichroic measurements. The upper spectrum in panel A illustrates the spectrum for the denatured protein, D, in 8.8 M urea and the lower spectrum for the native protein, N. The molar ellipticity, [θ], has the units degrees decimole of amino acid per residue centimeter squared. These spectra were obtained by using thioredoxin solutions containing 0.663 mg of protein/mL and a cell having an optical path of 1.5 mm. The fractional increase in the ellipticity observed at 230 nm as a function of the concentration of urea present is indicated by the closed circles in panel B. The fractional increase in the fluorescence emission intensity with increasing urea concentration, obtained from the measurements shown in Figure 1A, is indicated by the open circles. All measurements were performed at 25 °C in 50 mM phosphate buffer, pH 7.0.

25 °C, all the change in fluorescence intensity predicted by equilibrium measurements was accounted for following manual mixing at 2 °C, as shown in Figure 1B. This results from the order of magnitude increase in the time constant of each of the kinetic phases on decreasing the refolding temperature from 25 to 2 °C. It should be noted that the dependence of the time constant and fractional amplitude of each of the refolding kinetic phases on urea concentration is qualitatively similar at 2 °C and at 25 °C.

Circular Dichroic Measurements at 25 °C. The far-ultraviolet circular dichroic spectrum of thioredoxin at neutral pH and 25 °C, shown in Figure 5A, is characteristic for the native protein. In the presence of 9 M urea, the circular dichroic spectrum of thioredoxin is markedly changed and very comparable with the spectrum of the protein denatured in excess guanidine hydrochloride (Reutimann et al., 1981; Kelley & Stellwagen, 1984). The fractional change in ellipticity with increasing urea concentration is shown in Figure 5B. It should be noted that this transition is coincident with the fractional change in fluorescence emission intensity observed using the same solvent conditions.

Urea Gradient Gel Electrophoresis Measurements at 2 °C. Polyacrylamide slab gels containing linear concentration gradients of urea were obtained by using the procedure described by Creighton (1979). A sample of either native or urea-denatured thioredoxin was applied as a zone across the top of a gel in parallel with the urea gradient. The applied protein was subjected to electrophoresis normal to the urea gradient and then stained for protein. The stained protein profiles observed following application of native and denatured thioredoxin are quite dissimilar as shown in Figure 6. The upper gel to which native thioredoxin had been applied shows a discontinuous staining pattern with the discontinuity centered

FIGURE 6: Urea gradient gel electrophoresis. The gels are oriented such that 0 M urea is on the left edge and 8 M urea on the right edge, the protein sample having been applied across the top edge. Thus, the urea concentration increases linearly from left to right, and the protein migrates from top to bottom. The gels were cast in 50 mM phosphate buffer, pH 7.0. Native protein was applied to the upper gel which is referred to as the unfolding gel. Urea-denatured protein was applied to the lower gel which is referred to as the refolding gel. Electrophoresis was done for 120 min at 2 °C. The upper gel was stained with Coomassie blue and the lower gel with silver stain.

at about 7 M urea. By contrast, the lower gel to which the denatured protein had been applied shows a continuous sigmoidal-shaped staining band having a midpoint at about 3.5 M urea.

These profiles can be interpreted by using the model reactions considered by Creighton (1980). The observed profiles are not characteristic for a fast conformational transition between native and denatured protein coupled with a slower configurational isomerization in the denatured protein, N = DF = DS. Such a mechanism would generate a transition zone common to both gels, N = DF, plus a denatured band extending across the transition zone in the refolding gel, DS = DF → N. The observed profiles are also not characteristic for two coupled two-state transitions, DS = N = DF, in which one transition has a slow time constant and the other a fast time constant. Such a mechanism would generate a transition zone common to both gels and a spur extending from the native band in the unfolding gel and from the denatured band in the refolding gel. The observed profiles are characteristic for a three-component conformational transition having a nativelike intermediate conformation which is in slow exchange with the native protein and in rapid exchange with the denatured protein (Creighton, 1980). The midpoint of the sigmoidal-shaped staining band, 3.5 M, would indicate the transition midpoint of the nativelike intermediate.

Exclusion Chromatography at 2 °C. Samples of thioredoxin equilibrated with various concentrations of urea were injected into an exclusion column equilibrated with the same solvent and subjected to isocratic chromatography at 32–35 atm. Typical elution profiles are illustrated in Figure 7. No more than two protein components were evident in any of the profiles obtained. The component having an elution time of about 9 min is assumed to represent the more compact native protein, and the component having an elution time of about 7 min is assumed to represent the less compact denatured protein. The shape of these profiles indicates that the native and denatured forms of thioredoxin are in slow exchange under the chromatographic conditions employed. The dependence of the elution time and fractional urea of the native and denatured protein on urea concentration is shown in Figure 8.

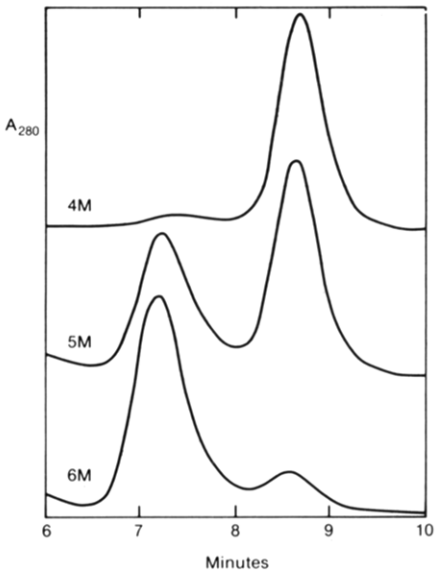

FIGURE 7: Equilibrium exclusion chromatographic profiles. The exclusion column was equilibrated in turn with 4, 5, and 6 M urea solutions at 2 °C. Samples of thioredoxin equilibrated in the same solvent were injected at zero time, and the absorbance of the column effluent was monitored at 280 nm.

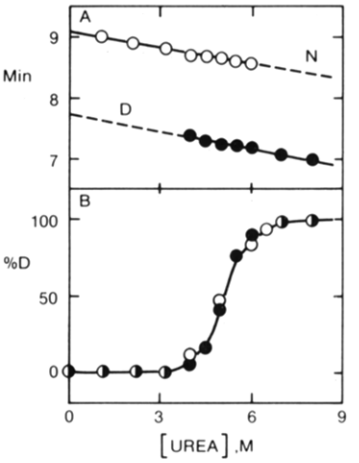

FIGURE 8: Dependence of exclusion chromatography of thioredoxin on urea concentration at equilibrium. Experimental values were obtained as described in the legend to Figure 7. Panel A illustrates the dependence of elution times for native, N, and denatured, D, thioredoxin in the elution profiles obtained at equilibrium. The dependence of the relative area of the denatured component in the elution profiles on urea concentration is indicated by the closed circles in panel B. The fractional increase in fluorescence emission intensity with increasing urea concentration, obtained from the measurements shown in Figure 1B, is indicated by the open circles.

It should be noted that the fractional change in the area of the denatured protein is coincident with the fractional change in fluorescence emission intensity measured using the same solvent conditions.

Samples of denatured thioredoxin were also injected into the exclusion chromatographic column equilibrated with various concentrations of urea. The elution profiles obtained at three representative urea concentrations in the native base-line zone are shown in Figure 9. The shape of the elution profile observed in 4 M urea indicates that the majority of the denatured protein applied to the column remained denatured during its residence on the column. This suggests that the time constant for the hydrodynamic change in 4 M urea is long relative to the residence time on the column. By contrast, the elution profiles observed in urea concentrations less than 4 M indicate the presence of significant amounts of a compact form

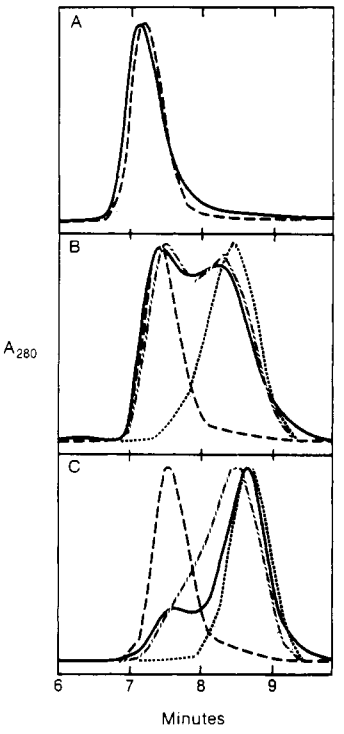

FIGURE 9: Nonequilibrium exclusion chromatographic profiles. A sample of thioredoxin equilibrated with an excess of urea was injected into the exclusion column equilibrated at 2 °C in turn with 4.00 M urea (panel A), 2.75 M urea (panel B), and 2.00 M urea (panel C). The solid lines indicate the elution profiles observed following injection of denatured protein at time zero. The dashed lines indicate elution profiles simulated by assuming that the slow refolding phase solely generates the compact conformation in a transition centered at 5.1 M. The alternately dashed and dotted lines indicate the elution profiles simulated by assuming that the middle refolding phase solely generates the compact conformation in a transition centered at 3.75 M. The dotted lines indicate the elution profiles simulated by assuming that the fast refolding phase generates the compact conformation in a transition centered at 3.5 M. In simulating the elution profiles, it was assumed that each transition had the width observed for the native protein, 1.8 M, that the product of each kinetic phase had an elution time equivalent to that of the native protein, and that the refolding phases had the time constants shown in Figure 4.

of the protein. The shape of the elution profiles observed at 2.75 and 2 M urea, shown in Figure 9, suggests time constants for the hydrodynamic change comparable to the residence time on the column.

Discussion

Addition of urea to solutions of thioredoxin causes an extensive reversible change in the conformation of the protein. The extent of the conformational change is comparable to that observed using guanidine hydrochloride as a denaturant (Reutimann et al., 1981; Kelley & Stellwagen, 1984), indicating that the native conformation is largely, if not completely, unfolded. The observed changes in hydrodynamic volume, secondary structure, and fluorescence quenching describe a common dependence on the concentration of urea, suggesting that the conformational features of the native protein are lost in a highly cooperative single transition. As commonly observed, urea is a less effective denaturant than is guanidine hydrochloride in that the midpoint for the conformational transition of thioredoxin occurs in 6.5 M urea but only 2.5 M guanidine hydrochloride using the same solvent conditions.

The kinetic profiles of the change in tryptophan fluorescence emission accompanying the conformational change of thioredoxin in urea are similar to but not identical with the kinetic profiles observed with guanidine hydrochloride as the denaturant (Kelley et al., 1985). In both denaturants, unfolding occurs in a single kinetic phase whose time constant is sharply dependent on denaturant concentration while refolding occurs in up to three kinetic phases whose time constants and fractional amplitudes are rather intricately dependent on denaturant concentration. The denaturant concentration dependence of the time constant for unfolding joins with the denaturant concentration dependence of the time constant for the slow refolding phase to form an inverted triangle, characteristic of a conformational change. The apex of the triangle has a time constant of about 1000 s in both denaturants. The time constant of the slow refolding phase becomes independent of denaturant concentration in the native base-line zone with a limiting time constant of about 400 s in each solvent. Recent evidence (Kelley et al., 1986a; R. F. Kelley and F. M. Richards, unpublished results) suggests that the guanidine hydrochloride independent portion of the slow refolding phase reflects the configurational isomerization of the peptide bond of proline-76 which is cis in the native protein and predominantly trans in the denatured protein. The time constants for the middle of fast refolding phases observed in urea remain inversely dependent on denaturant concentration throughout their range of detection and separated by about an order of magnitude. The time constants for these phases in guanidine hydrochloride are also separated by an order of magnitude but exhibit an inverted triangular dependence on denaturant concentration. It should be noted, however, that the very small amplitudes of the middle and fast kinetic phases in high denaturant concentration render a precise description of their denaturant dependence rather difficult. The fractional amplitude of the slow refolding phase diminishes to a limiting value of about 0.2 in the native base-line zone in both denaturants. In urea, the fractional amplitude of the fast unfolding phase becomes dominate in this zone while in guanidine hydrochloride the fractional amplitudes of the middle and fast phases increase in parallel and codominate in this zone. The differences in fractional amplitude profiles of the refolding phases in the two denaturants do not necessarily reflect differences in the relative population of conformational forms but may result from the combination of time constants of similar magnitude (Hagerman & Baldwin, 1976; Hagerman, 1977). Taken together, the general features of the conformation transitions of thioredoxin in urea and in guanidine hydrochloride are very similar.

Although equilibrium measurements of the reversible unfolding of thioredoxin in guanidine hydrochloride and in urea can be fit with a two-component transition involving the native and denatured forms of the protein, detection of the three kinetic refolding phases in both solvents indicates the involvement of at least two more components. Hydrodynamic measurements uniquely performed in urea indicate that at least one and likely both additional components are nativelike intermediate conformations. Urea gradient gel electrophoresis staining profiles simulated by using the time constants of the kinetic phases observed by fluorescence measurements are shown in Figure 10. The time constants for the single unfolding phase emulate the discontinuous staining profile observed in the upper (unfolding) gel illustrated in Figure 6. However, the time constants for the linked slow refolding phase do not emulate the sigmoidal staining profile observed in the lower refolding gel illustrated in Figure 6. The staining profile in the refolding gel can be emulated by using the time constants for either the middle or the fast refolding phase assuming that each phase generates a compact conformation whose stability to urea denaturation is less than that of the

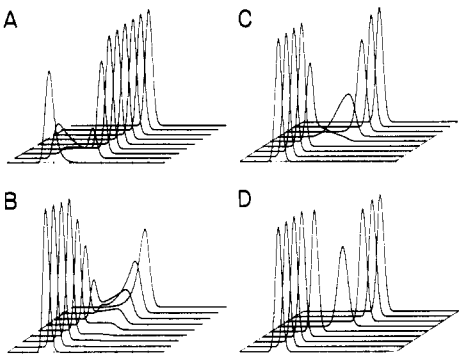

FIGURE 10: Simulations of urea gradient gel electrophoresis profiles. The stained protein band on a gel is mimicked by a series of protein profiles simulated at 1 M urea intervals using the time constants obtained from fluorescence measurements and shown in Figure 4. The ordinate represents increasing protein concentration and the abscissa the distance in the gel from the point of application of the protein, the left edge of each profile. Electrophoretic migration proceeds from left to right. In all simulations, the denatured protein was assumed to migrate 2.5 distance units and the native protein 5.0 distance units, in keeping with the relative mobilities observed on the real gels. All transitions were assumed to have an 80% width of 1.8 M urea. The offset lines in each display represent the profiles simulated at 1 M urea concentration intervals where the simulated protein profile in 8 M urea is always nearest the viewer and the profile in 0 M urea furthest from the viewer. The display in panel A simulates a urea gradient gel to which native thioredoxin was applied. The individual profiles in this panel were obtained by using the unfolding time constants and a transition centered at 5.1 M urea. The remaining panels display simulated urea gradient gels to which denatured thioredoxin was applied. The display in panel B was simualted by assuming that the compact protein was generated solely by the slow refolding phase in a transition centered at 5.1 M urea. It was assumed that the urea dependence of the time constant for the refolding phase continued below 2 M. The display in panel C was simulated by assuming that the compact protein was generated solely by the middle refolding phase having a transition centered at 3.75 M urea. The display in panel D was simulated by assuming that the compact protein was generated solely by the fast refolding phase having a transition centered at 3.5 M urea.

native protein. Of the two emulations, that generated by using the time constants for the fast refolding phase is superior. By contrast, the emulations of exclusion chromatographic profiles are superior using the time constants for the middle refolding phase. As shown in Figure 9A, the elution profile observed for refolding in 4 M urea, a concentration in which the middle and slow refolding phases exhibit little if any amplitude, can be reasonably emulated by using the time constant for the slow refolding phase. However, the time constant in 2.75 M clearly does not emulate the refolding elution profiles observed in 2.75 and 2 M urea shown in Figure 9B,C. The profile simulated by using the time constant for the middle unfolding phase nicely approximates the observed profile in 2.75 M urea but appears to need a contribution from the fast refolding phase in 2 M urea.

The exclusion chromatographic and urea gradient electrophoretic results clearly indicate that the conformational transition of thioredoxin is a two-state process in the sense that no conformational form having an effective volume intermediate between that of the native and denatured conformation accumulates. In the transition zone, the compact conformation appears to be the native conformation, requiring the isomerization of any nonnative peptide isomers to precede folding. In the native base-line zone, the compact conformation initially formed and highly populated is likely an intermediate conformation containing at least one nonnative peptide isomer, the trans isomer of proline peptide 76 being a likely candidate. While the compact intermediate conformation is rapidly formed, it likely contains more structure than that associated with a molten globule (Dolgikh et al., 1981; Ohgushi & Wada, 1983) since the time constants for the rapid and middle refolding phases detected by fluorescence quenching measurements are compatible with the time constant of the formation of the compact intermediate conformation at the present state of analysis.

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