Girg, R., Jaenicke, R., & Rudolph, R. (1983a) *Biochem. Int. 7*, 433–441.
Girg, R., Rudolph, R., & Jaenicke, R. (1983b) *FEBS Lett. 163*, 132–135.
Goldberg, M. E. (1969) *J. Mol. Biol. 46*, 441–446.
Goldberg, M., & Zetina, C. (1980) in *Protein Folding* (Jaenicke, R., Ed.) pp 525–546, Elsevier/North-Holland, Amsterdam.
Grandits, W. (1979) Thesis, Regensburg University.
Gray, W. R. (1972a) *Methods Enzymol. 25*, 121–138.
Gray, W. R. (1972b) *Methods Enzymol. 25*, 333–344.
Hartley, B. S. (1970) *Biochem. J. 119*, 805–822.
Hayashi, R. (1978) *Methods Enzymol. 47*, 84–93.
Hermann, R., Jaenicke, R., & Rudolph, R. (1981) *Biochemistry 20*, 5195–5201.
Hermann, R., Jaenicke, R., & Kretsch, G. (1983) *Naturwissenschaften 70*, 517–518.
Holbrook, J. J., Liljas, A., Steindel, S. J., & Rossmann, M. G. (1975) *Enzymes (3rd Ed.) 11*, 191–292.
Hunkapiller, M. W., & Hood, L. E. (1983) *Methods Enzymol. 91*, 486–493.
Jaenicke, R. (1983) in *Mobility and Recognition in Cell Biology* (Sund, H., & Veeger, C., Eds.) pp 67–81, de Gruyter, Berlin and New York.
Jaenicke, R. (1984) *Angew. Chem., Int. Ed. Engl. 23*, 395–413.
Jaenicke, R., & Knof, S. (1968) *Eur. J. Biochem. 4*, 157–163.
Jaenicke, R., & Rudolph, R. (1983) in *Biological Oxidations* (Sund, H., & Ullrich, V., Eds.) 34th Colloquium Mosbach, pp 62–90, Springer-Verlag, Berlin, Heidelberg, New York, and Tokyo.
Jaenicke, R., Vogel, W., & Rudolph, R. (1981) *Eur. J. Biochem. 114*, 525–531.
Jeckel, D., Anders, R., & Pfleiderer, G. (1973) *Hoppe-Seyler's Z. Physiol. Chem. 354*, 737–748.
Kirschner, K., & Szadkowski, H. (1980) *J. Mol. Biol. 143*, 395–409.
Lang, K., & Schmid, F. X. (1986) *Eur. J. Biochem. 159*, 275–281.
Matsubara, H. (1970) *Methods Enzymol. 19*, 642–651.
Robson, B., & Pain, R. H. (1976a) *Biochem. J. 155*, 325–330.
Robson, B., & Pain, R. H. (1976b) *Biochem. J. 155*, 331–344.
Rossmann, M. G., Liljas, A., Brändén, C.-I., & Banaszak, L. J. (1975) *Enzymes (3rd Ed.) 11*, 61–102.
Rudolph, R. (1985) *Biochem. Soc. Trans. 13*, 308–311.
Rupley, J. A. (1967) *Methods Enzymol. 11*, 905–917.
Schmid, F. X., & Blaschek, H. (1984) *Biochemistry 23*, 2118–2133.
Taylor, R. P., & Silver, A. (1976) *J. Am. Chem. Soc. 98*, 4650–4651.
Teale, J. M., & Benjamin, D. C. (1977) *J. Biol. Chem. 252*, 4521–4526.
Teschner, W., Rudolph, R., & Garel, J.-R. (1987) *Biochemistry* (in press).
Zettlmeissl, G., Rudolph, R., & Jaenicke, R. (1979) *Biochemistry 18*, 5567–5571.
Zettlmeissl, G., Rudolph, R., & Jaenicke, R. (1981) *Eur. J. Biochem. 121*, 169–175.
Zettlmeissl, G., Rudolph, R., & Jaenicke, R. (1982) *Biochemistry 21*, 3946–3950.
Zettlmeissl, G., Rudolph, R., & Jaenicke, R. (1983) *Arch. Biochem. Biophys. 224*, 161–168.

Equilibrium and Kinetic Measurements of the Conformational Transition of Reduced Thioredoxin†

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ABSTRACT: The single disulfide bond in *Escherichia coli* thioredoxin was reduced by reaction with a 20-fold excess of reduced dithiothreitol at neutral pH and 25 °C. For some measurements, reduced thioredoxin was further reacted with iodoacetamide to alkylate the cysteinyl residues. The denaturation transitions of oxidized, reduced, and reduced alkylated thioredoxin were observed by using far-ultraviolet circular dichroic and exclusion chromatographic measurements. Cleavage of the disulfide bond lowers the stability of the native thioredoxin to denaturation by about 2.4 kcal/mol, and subsequent alkylation lowers the stability by a further 1.6 kcal/mol. The kinetics of the conformational change of reduced thioredoxin in guanidine hydrochloride were observed by using exclusion chromatography at moderate pressure and 2 °C. Analyses of single and multimixing protocols are consistent with a predominant nonnative configuration in the denatured state and the transient accumulation of a compact nativelike intermediate during refolding. The intermediate can incorporate the nonnative configuration and can accommodate its isomerization. No compelling chromatographic evidence was found for a conformation having an elution time different from that characteristic for either the native or the denatured protein.

The crystallographic model of *Escherichia coli* oxidized thioredoxin (Holmgren et al., 1975) indicates that the single disulfide bond in the protein bridges the first and fourth residues of a type III reverse turn involving residues 32–35. Such a reverse turn likely persists in the denatured protein and could in principal direct the folding of the polypeptide into its native conformation. In this report, we describe equilibrium and kinetic measurements of the conformational transition of *E. coli* thioredoxin having its single disulfide bond reduced. We find that the conformational features of soluble native

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thioredoxin are largely preserved upon reduction of the disulfide, that the stability of the native conformation to denaturants is decreased by reduction, and that the folding pathway deduced for the denatured oxidized protein (Kelley et al., 1986a–c; Wilson et al., 1986) appears to be executed by the denatured reduced protein as well.

Experimental Procedures

Materials. Thioredoxin was purified from an *E. coli* strain (Lunn et al., 1984) containing multiple copies of a plasmid with the structure 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). Oxidized thioredoxin refers to the product of the purification procedure which has an intact disulfide bond linking cysteine residues 32 and 35. Reduced thioredoxin refers to the protein obtained following reaction of oxidized thioredoxin with a 20-fold molar excess of reduced dithiothreitol for at least 15 min in 50 mM phosphate buffer, pH 7.0, at room temperature. The reduced protein was maintained in this solvent. Reduced alkylated thioredoxin refers to the reduced protein in which the sulfhydryl groups of cysteine residues 32 and 35 have been alkylated by reaction with an excess of iodoacetamide at pH 8.0 for 2 h at room temperature. Amino acid analysis indicated that the two cysteine residues were quantitatively alkylated by this procedure and that no other residues were detectably altered.

Methods. Fluorescence measurements were obtained by using an SLM Model 4800 fluorometer and an optical cell having a light path of 10 mm in both directions. Circular dichroism measurements were obtained with an Aviv-Cary circular dichroism apparatus and an optical cell having a light path of 1 mm. Thermal denaturation was observed by using a Microcal Model MC-1 differential scanning calorimeter. Absorbance measurements were obtained by using a Gilford Response spectrophotometer.

Exclusion chromatography was done with a 7.5 × 300 mm Bio-Sil TSK-125 gel filtration column, an IBM Model LC9533 liquid chromatograph, and an Isco Model V4 variable-wavelength absorbance detector. Twenty-microliter samples were injected into the column and subjected to isocratic chromatography at a flow rate of 1 mL/min. Elution profiles were observed by using either 280- or 220-nm absorbance. The chromatographic system had an excluded volume of 5.5 mL using blue dextran and an included volume of 10.8 mL using either free tyrosine or potassium dichromate. Chromatographic results were simulated by using the dynamic equations of Endo et al. (1983) programmed on a DEC Model 11/780 computer. The menu-driven program facilitates selection of a desired mechanism, the width, midpoint, and time constant for each conformational isomerization, the equilibrium distribution and time constant for each configurational isomerization, the elution times for the compact and denatured protein, the flow rate and concentration of denaturant in the column solvent, the volume and nature of the protein sample injected, whether native, denatured, or equilibrated with the column solvent, the number of theoretical plates, and the diffusional broadening occurring. All protein components were assumed to have 80% of their conformational transitions distributed over 0.7 M denaturant, to experience 10% diffusional broadening during chromatography, and to be equilibrated with 500 theoretical plates.

Results

Fluorescence Measurements. The quenching of the tryptophan fluorescence of native thioredoxin (Stryer et al., 1967) and a model thioredoxin peptide (Kishore et al., 1983) is relieved by reduction of the single disulfide linking residues 32 and 35. As shown in Figure 1, addition of reduced dithiothreitol to a solution of native oxidized thioredoxin increases the tryptophan fluorescence emission intensity of the protein at a rate dependent upon the concentration of reducing agent. The increase in fluorescence emission intensity observed following reduction of the disulfide is equivalent to that observed following denaturation of the oxidized protein as observed previously (Holmgren, 1972). The increased fluorescence emission intensity observed for the native protein in the presence of a 20-fold molar excess of dithiothreitol is maintained for many hours at room temperature following addition of the reducing agent. On the basis of this experience, the reduced thioredoxin characterized in this study was generated and maintained by reaction of the oxidized protein with a 20-fold molar excess of reduced dithiothreitol for at least 15 min at room temperature and pH 7.0.

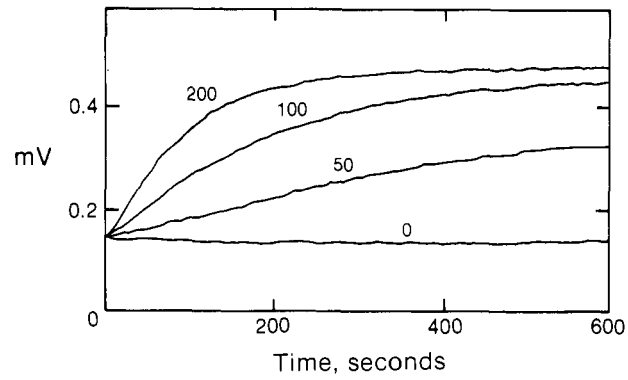

FIGURE 1: Kinetic profiles of the formation of reduced thioredoxin at 25 °C. Each sample contained 20 μM oxidized thioredoxin, 50 mM phosphate buffer, pH 7.0, and the indicated concentration of reduced dithiothreitol in micromolar. The fluorescence emission intensity of the protein solution was recorded in millivolts (mV) at 350 nm using 276-nm excitation. The reaction was initiated by addition of reduced dithiothreitol.

Circular Dichroic Measurements. The far-ultraviolet circular dichroic spectrum of native oxidized thioredoxin exhibits a broad minimum at about 219 nm appropriate to the secondary structural content of the protein determined by crystallographic measurements (Holmgren et al., 1975). As shown in Figure 2, the dichroic spectrum of the native oxidized protein is not perturbed significantly by reduction of the disulfide or by alkylation subsequent to reduction. Upon addition of excess urea or guanidine hydrochloride, the broad minimum at 220 nm disappears, and the dichroic spectrum becomes characteristic for a denatured protein. Again, the dichroic spectra of the oxidized, reduced, and reduced alkylated protein in excess denaturant at neutral pH and 25 °C are equivalent within experimental variation. Similar results are observed at 2 °C for all forms of thioredoxin.

Since the tryptophan emission intensity of native reduced thioredoxin is unchanged by denaturation, the conformational transitions of oxidized, reduced, and reduced alkylated thioredoxin were observed and compared by using ellipticity measurements at 219 nm. Typical results are illustrated in Figure 3. Each transition can be described by three denaturant concentration ranges: a native base-line zone in which the ellipticity remains characteristic of the native protein; a transition zone in which the ellipticity is markedly dependent on denaturant concentration; and a denatured base-line zone in which the ellipticity remains characteristic of the denatured protein. As shown in Figure 3A, reduction of the disulfide and reduction followed by alkylation each lower the midpoint

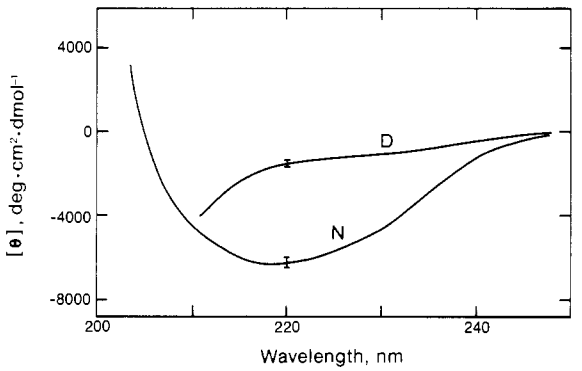

FIGURE 2: Far-ultraviolet circular dichroic spectra of thioredoxin. All spectra were obtained by using a protein solution containing between 0.66 and 1.19 mg/mL thioredoxin in 50 mM phosphate buffer, pH 7.0, maintained at either 25 °C or 2 °C. Solutions of reduced thioredoxin contained, in addition, a 20-fold molar excess of reduced dithiothreitol. The lower spectrum, labeled N, represents the average spectrum of native oxidized, native reduced, or native reduced alkylated thioredoxin. The upper spectrum, labeled D, represents the average spectrum of each of these proteins in either 8.5 M urea or 4 M guanidine hydrochloride. The bars indicate the range of values observed among the samples.

Table I: Stability Comparisons of Thioredoxins[a]

protein	denaturant	transition midpoint	$-\Delta G°$ (kcal/mol)
oxidized	heat	82 °C	
	Gdn-HCl	2.6 M	8.6
	urea	6.7 M	8.7
reduced	Gdn-HCl	1.8 M	6.2
reduced alkylated	heat	64 °C	
	GDN-HCl	1.4 M	4.6
	urea	3.3 M	4.3

[a] The abbreviation Gdn-HCl represents guanidine hydrochloride. Measurements in urea and Gdn-HCl were obtained at 25 °C, and all measurements were obtained in 50 mM phosphate buffer, pH 7.0.

of the transition zone of thioredoxin without changing the shape of the transition. The midpoint values observed in guanidine hydrochloride and in urea at neutral pH and 25 °C are compared in Table I. As shown in Figure 3B, the midpoint values for oxidized and for reduced thioredoxin are each lowered by 0.2 M guanidine hydrochloride upon changing the temperature of the measurements from 25 to 2 °C.

Calorimetric Measurements. The thermal stability of oxidized and of reduced alkylated thioredoxin was measured by differential scanning calorimetry. The change in heat capacity observed upon heating solutions of each protein maintained at neutral pH is illustrated in Figure 4. The melting temperature, defined as the maximum ordinate, is substantially higher for the oxidized protein as shown in Table I. The change in heat capacity of each protein with temperature is reversible if the temperature of the protein solution is only raised to the melting temperature. However, if the temperature is increased into the denatured base-line zone, a significant amount of irreversible thermal denaturation of both proteins occurs.

Absorbance Measurements. The near-ultraviolet absorbance spectrum of reduced thioredoxin is only modestly perturbed by denaturation in excess guanidine hydrochloride at neutral pH and 25 °C. The difference spectrum between native and denatured reduced thioredoxin exhibits a difference maximum of no greater than 1700 cm^{-1} M^{-1} between 250 and 300 nm. This small change was judged not to be of sufficient sensitivity for observation and resolution of the multiphasic refolding kinetics anticipated for the denatured reduced protein.

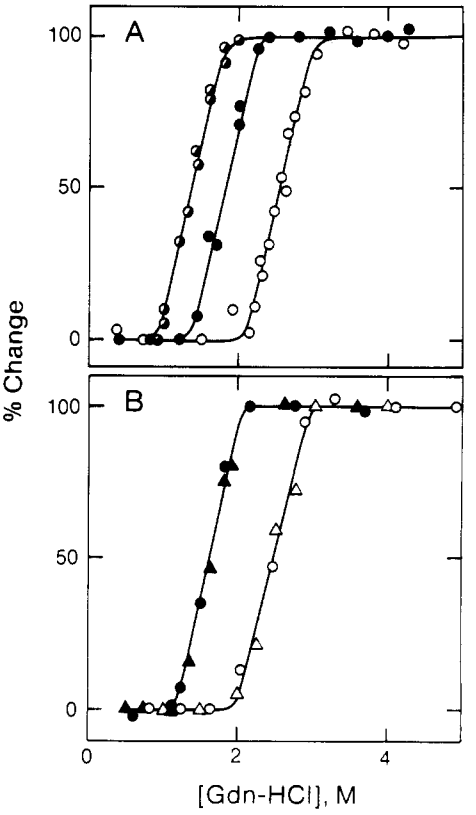

FIGURE 3: Equilibrium measurements of the conformational transition of thioredoxins. The circles indicate the increase in ellipticity observed at 219 nm, and the triangles indicate the increase in the area of the component which elutes between 7 and 8 min after injection of a sample into the exclusion chromatographic column. Values represented by open symbols were obtained with oxidized thioredoxin, by closed symbols with reduced thioredoxin, and by half-filled symbols with reduced alkylated thioredoxin. The values illustrated in panel A were obtained at 25 °C and those in panel B at 2 °C. All measurements were done in solvents containing 50 mM phosphate buffer, pH 7.0, and for solutions of reduced thioredoxin, a 20-fold molar excess of reduced dithiothreitol.

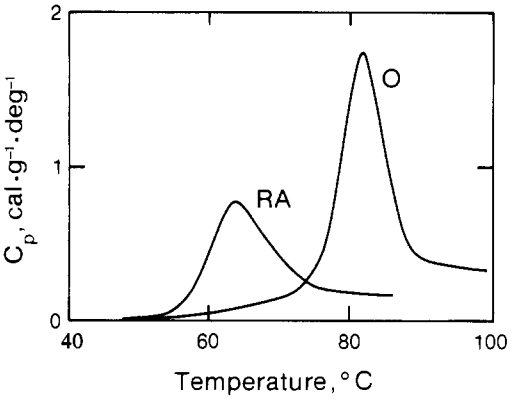

FIGURE 4: Thermal denaturation of thioredoxin. The curves illustrate the change in heat capacity, C_p, observed during the initial heating of a solution of oxidized thioredoxin, O, and a solution of reduced alkylated thioredoxin, RA. The oxidized protein solution contained 4.0 mg/mL thioredoxin in 50 mM phosphate buffer, pH 7.0, and the reduced alkylated protein contained 2.1 mg/mL thioredoxin in the same solvent. Heat was applied to raise the temperature 1 °C/min.

Exclusion Chromatographic Measurements. Reduced thioredoxin was equilibrated with a series of guanidine hydrochloride concentrations at neutral pH and 2 °C. Each sample was injected into a gel exclusion column equilibrated with the same denaturant concentration at moderate pressure. In the native base-line zone and in the denatured base-line zone, the protein eluted as a single symmetrical component

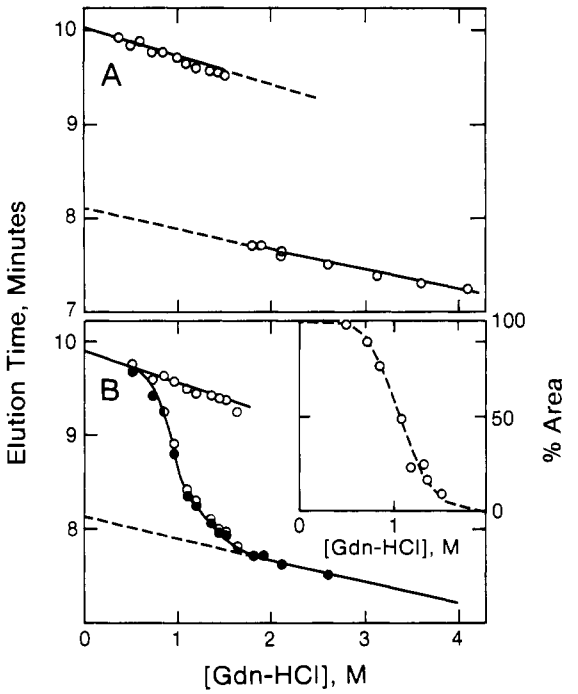

FIGURE 5: Exclusion chromatographic measurements. Panel A illustrates the dependence of the elution time of reduced thioredoxin on the concentration of guanidine hydrochloride, Gdn-HCl, outside the transition zone. Each sample of reduced thioredoxin was equilibrated with the isocratic column solvent prior to injection. Panel B illustrates the dependence of the elution time of reduced thioredoxin on denaturant concentration following injection of reduced denatured thioredoxin. The open circles indicate that reduced native thioredoxin was exposed to 5.8 M guanidine hydrochloride for 15 s at 25 °C prior to injection. The closed circles indicate that the reduced native protein was exposed to 5.8 M guanidine hydrochloride for 20 min at 25 °C prior to injection. The inset indicates the fractional area associated with the component eluting at approximately 9.5 min following injection of the sample exposed to denaturant for 15 s prior to injection. All thioredoxin solutions contained 1 mg/mL protein, a 20-fold molar excess of reduced dithiothreitol, and 50 mM phosphate buffer, pH 7.0. All column solvents contained the same concentrations of reduced dithiothreitol and phosphate buffer and the indicated concentration of guanidine hydrochloride. Chromatography was done in jacketed columns maintained at 2 °C.

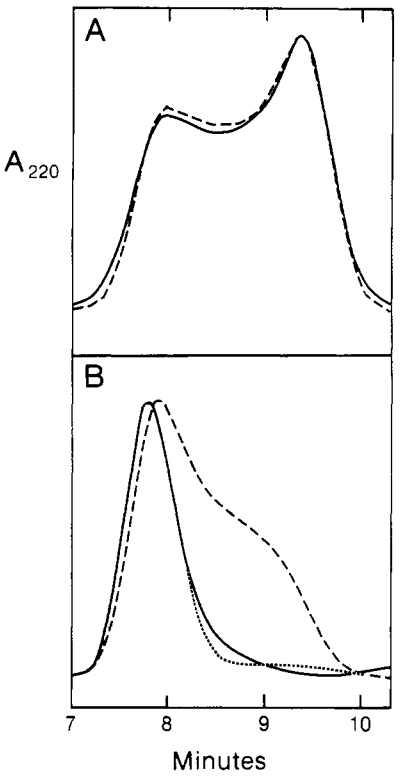

FIGURE 6: Elution profiles observed in the transition zone. Each profile was obtained by using the exclusion column equilibrated with 1.63 M guanidine hydrochloride, 1.5 mM reduced dithiothreitol, and 50 mM phosphate buffer, pH 7.0, at 2 °C. The elution profile shown by the solid line in panel A was obtained following injection of a sample equilibrated with the column solvent. The elution profile shown by the solid line in panel B was obtained by using a sample of reduced thioredoxin equilibrated with 5.8 M guanidine hydrochloride prior to injection. The longer dashed lines indicate profiles simulated for each sample using a two-state transition having a midpoint of 1.62 M denaturant and an exchange time constant of 250 s. The dotted line in panel B indicates the profile simulated for this sample and a three-component mechanism, $N \rightleftharpoons DF \rightleftharpoons DS$, in which the conformational transition has a midpoint of 1.93 M denaturant and a time constant of 550 s and the configurational isomerization is 90% DS at equilibrium in the denatured state and has a time constant of 375 s.

having a constant width at half-height. The dependence of the elution time of the protein on denaturation concentration in these two zones is shown in Figure 5A. Extrapolation of the observed dependencies indicates that denaturation of the protein decreases the elution time by about 2 min, presumably due to expansion of the hydrodynamic volume of the protein. A similar difference in the elution times of native and denatured oxidized protein has been observed (Wilson et al., 1986). By contrast, the elution profiles of the reduced protein in the transition zone are characteristic for two components in moderate dynamic exchange. A typical elution profile observed in the transition zone is illustrated at the top of Figure 6. All elution profiles observed in the transition zone can be simulated by using a two-state transition having a midpoint at 1.62 M guanidine hydrochloride, a time constant which varies between 100 and 250 s, and the extrapolated elution times for native and denatured protein. A typical simulation is illustrated at the top of Figure 6. The change in the fractional area associated with the denatured protein correlates very well with the fractional change in ellipticity in the transition zone, as shown in Figure 3B. Similar analyses of the elution profiles of the oxidized protein, previously described (Kelley et al., 1986), also correlate very well with the ellipticity measurements of the oxidized protein in the transition zone as shown in Figure 3B.

The chromatographic profile observed at a given denaturant concentration can be very dependent upon the nature of the protein sample injected, particularly in the transition zone. As shown in Figure 6, the profile observed after injection of a sample of denatured reduced protein is intermediate between the profile of pure denatured protein and the profile observed for a sample equilibrated with the column solvent prior to injection. We denote such profiles as refolding and equilibrium profiles dependent upon whether a sample of denatured or equilibrated protein, respectively, was injected. The refolding profile clearly evidences an ongoing conversion of denatured to compact protein which is not completed during the residence time of the protein on the column.

The observed refolding profile is dependent upon the concentration of denaturant in the native base-line zone. A typical refolding profile observed in this zone at 1.08 M guanidine hydrochloride is shown in Figure 7A. All such profiles reveal two components, a well-defined major component which elutes first followed by a less well-defined minor component which elutes as a broad relative short trailing peak. The elution time for the major component exhibits a sigmodial dependence on the column denatured concentration, centered at 0.95 M guanidine hydrochloride, as shown by the closed circles in Figure 5B. The elution time for the minor component is in

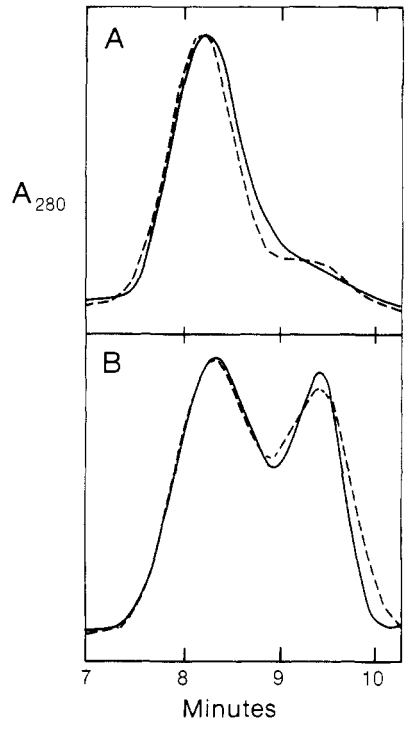

FIGURE 7: Elution profiles observed in the native base-line zone. The column was equilibrated with 1.08 M guanidine hydrochloride, 1.5 mM reduced dithiothreitol, and 50 mM phosphate buffer, pH 7.0 at 2 °C. The solid profile in panel A was observed following injection of a sample of reduced native thioredoxin denatured for 20 min in 5.8 M guanidine hydrochloride, 1.5 mM reduced dithiothreitol, and 50 mM phosphate buffer, pH 7.0, at 25 °C. The solid profile observed in panel B was observed by using the same protocol except that native reduced thioredoxin was denatured for only 15 s prior to injection. The dashed profile was simulated by using mechanism 2 in which the conformational transition N ⇌ DF has a transition midpoint at 1.93 M denaturant and a time constant of 160 s; the conformational transition IS ⇌ DS has a transition midpoint at 0.90 M denaturant and a time constant of 20 s; the configurational transition IS ⇌ N is 99.9% N at equilibrium in the native base-line zone and has a time constant of 3500 s; the configurational isomerization DF ⇌ DS is 90% DS at equilibrium in the denatured base-line zone and has a time constant of 375 s. The time constants for denaturation and configurational isomerization at 25 °C indicate that the protein injected into the column to generate the profiles shown in panel B was more than 99% denatured and that 53% of the denatured protein was in the DS configuration. Time constants for the configurational transitions at 2 and 25 °C and for denaturation at 25 °C were assumed to be the same as measured for the oxidized protein under similar conditions (Kelley et al., 1986a).

the range characteristic for the native protein, but its position cannot be reliably determined owing to the breadth and small area of this component.

The observed refolding profile also depends upon the length of time the native protein is incubated with excess denaturant prior to injection. If the native protein is incubated in excess denaturant only briefly prior to injection, more of the protein elutes as the second component as seen by comparing panels A and B in Figure 7. The elution position of the first component again exhibits a sigmoidal dependence on column denaturant concentration as shown by the open circles in Figure 5B. Since more material elutes as the second component, its elution position can be more reliably determined. The elution time for the second component exhibits a monotonic dependence on denaturant concentration, characteristic for the native protein, as shown by the open circles in Figure 5B. The relative area of the second component observed in these refolding profiles is inversely dependent upon the column denaturant concentration, as illustrated in the inset to Figure 5B. This dependence indicates that the presence of the second component, which has an elution time characteristic for the native protein, does not result from incomplete denaturation prior to injection, since all samples used to generate the measurements in the inset to Figure 5B were denatured for the same length of time.

Discussion

The gross structural features of native oxidized thioredoxin are maintained following reduction of the disulfide bond linking residues 32 and 35. The effective volume of the protein as measured by the elution time from an exclusion chromatographic column is unchanged. The complement of secondary structural elements as measured by the far-ultraviolet circular dichroic spectrum is also unchanged within the precision of measurement. However, proton NMR measurements (Holmgren & Roberts, 1976) suggest that the microenvironments about tryptophan residues 28 and 31 are perturbed by reduction of the adjacent disulfide bond. These authors suggest that the active-site protrusion formed by residues 29–37 undergoes a localized conformation change during reduction. Such a perturbation together with the loss of the quenching disulfide may contribute to the observed enhancement in tryptophan fluorescence emission following reduction.

As shown in Table I, reduction of the single disulfide bond in thioredoxin with or without alkylation increases the susceptibility of the protein to denaturation by heat, urea, and guanidine hydrochloride at neutral pH. The reversible transitions observed in urea and in guanidine hydrochloride were analyzed by assuming a two-state model with no preferential binding as described by Schellman (1978). Such analysis indicates that reduction of the disulfide destabilizes the native conformation of the protein by about 2.4 kcal/mol. This value is within a factor of 2 of the decrease in free energy predicted to result from the change in chain entropy associated with cleavage of a disulfide bridge, 1.6 kcal/mol at 25 °C, using the equation of Flory (1956). Alkylation of the sulfhydryl groups in reduced thioredoxin was observed to destabilize the native conformation by an additional 1.6 kcal/mol. This destabilization likely results from a further perturbation of the conformation of the active-site protrusion following changes in the size and polarity of the two cysteinyl residues by alkylation. The thermal transitions were not analyzed in detail owing to their irreversibility.

While equilibrium circular dichroic and exclusion chromatographic measurements can be fit with a two-state transition between native and denatured reduced thioredoxin, the observed refolding chromatographic profiles indicate the participation of additional components in the conformational transition. The equilibrium elution profile observed in the transition zone and shown in Figure 6A can be fit nicely with a two-state transition having a transition midpoint at 1.62 M denaturant and a time constant of 250 s. However, these values poorly simulate the refolding elution profile observed on the same column as shown in Figure 6B. The difference between the simulated and observed refolding profiles suggests that the refolding of denatured protein is being delayed. One way to accomplish such a delay would be to couple a slow configurational isomerization in the denatured state with folding, as illustrated in mechanism 1. In this mechanism,

$$N \rightleftharpoons DF \rightleftharpoons DS \quad (1)$$

N is the native protein, DF is a fast-folding denatured protein, and DS is a slow-folding denatured protein containing a nonnative isomer which must isomerize prior to folding. This mechanism can be used to fit the refolding profile in Figure

6B assuming 90% of the denatured protein applied to the column is DS, a time constant for the configurational isomerization of 375 s, a midpoint for the conformational transition of 1.93 M denaturant, and a time constant for the conformational transition of 550 s. The fractional concentration of DS and the time constant for its isomerization are the measured values obtained for oxidized thioredoxin (Kelley et al., 1986a) which are assumed to pertain to the reduced protein as well. The midpoint for the conformational transition was calculated from the overall midpoint, 1.62 M denaturant (Figure 3B), assuming 90% of the denatured protein is in the DS form at equilibrium.

The formation of a slow-folding form, DS, in the denatured base-line zone is consistent with the refolding elution profiles observed in the native base-line zone, as illustrated in Figure 7. Mechanism 1 predicts that the amount of native protein observed in the refolding profiles will be inversely related to the length of time the native protein is incubated with excess denaturant. As shown in Figure 7, the relative amount of protein having a elution time characteristic for the native protein is markedly diminished as the incubation time in excess denaturant is increased from 15 to 1200 s.

The simulation of the refolding profile shown in Figure 7B using equilibrium-denatured protein indicates that the first component to elute originated as DS and the second component as DF. If mechanism 1 were correct, then the elution time for the first component should exhibit the denaturant concentration dependence characteristic for the denatured protein, since the refolding of DS is limited by the slow denaturant-independent isomerization of DS to DF, having a one-way time constant of 3750 s. However, the elution time for the first component exhibits a sigmoidal dependence on denaturant concentration, spanning the range from that characteristic for the denatured and native proteins, as shown in Figure 5B. One way to accommodate this dependence is to assume that DS can refold to a compact conformation containing a nonnative configurational isomer, IS, in mechanism 2. This mechanism can simulate the observed refolding profiles, as shown in Figure 7.

$$\begin{array}{ccc} N & \rightleftharpoons & DF \\ \uparrow\downarrow & & \uparrow\downarrow \\ IS & \rightleftharpoons & DS \end{array} \qquad (2)$$

Several features of this mechanism merit comment. First, the chromatographic results can be simulated by using only compact and denatured components. No partially folded conformations such as proposed by Aune et al. (1967) are necessarily required. Second, the denatured form having a nonnative isomer, DS, folds more quickly into a compact form than does the all-native isomer, DF, at a given denaturant concentration in the native base-line zone. Under these conditions, the standard DF/DS nomenclature is misleading. Third, the stability of IS to denaturation is less than that of N, as expected for a folding intermediate with a nonnative configurational isomer. Fourth, the mechanism requires that the dominant configurational isomer at equilibrium is DS in the denatured protein and N in the compact protein. Such an inversion can be accomplished by coupling the configurational isomerization with the conformational isomerization. A plot of the time course for the concentration of each component in mechanism 2 during native base-line refolding beginning with equilibrium-denatured protein predicts IS to be the dominant component for a considerable time during refolding. This persistence should facilitate characterization of the conformation of IS and measurement of its configurational isomeric distribution, assuming the latter to represent proline peptide isomerization. Finally, the mechanism is analogous to that proposed (Goto & Hamaguchi, 1982) for the conformational transition of the constant domain of immunoglobulin IgG.

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REFERENCES

Aune, K. C., Salahuddin, A., Zarlengo, M. H., & Tanford, C. (1967) *J. Biol. Chem. 242*, 4486–4489.

Endo, S., Saito, Y., & Wada, A. (1983) *Anal. Biochem. 131*, 108–120.

Flory, P. J. (1956) *J. Am. Chem. Soc. 78*, 5222–5235.

Goto, Y., & Hamguchi, K. (1982) *J. Mol. Biol. 156*, 891–910.

Holmgren, A. (1972) *J. Biol. Chem. 247*, 1992–1998.

Holmgren, A., & Reichard, P. (1967) *Eur. J. Biochem. 2*, 187–196.

Holmgren, A., & Roberts, G. (1976) *FEBS Lett. 71*, 261–265.

Holmgren, A., Söderberg, B.-O., Eklund, H., & Brändén, C.-I. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 2305–2309.

Kelley, R. F., & Stellwagen, E. (1984) *Biochemistry 23*, 5095–5102.

Kelley, R. F., Wilson, J., Bryant, C., & Stellwagen, E. (1986a) *Biochemistry 25*, 728–732.

Kelley, R., Wilson, J., Bryant, C., Shalongo, W., Ledger, R., Lowery, D., & Stellwagen, E. (1986b) in *Thioredoxin and Glutaredoxin Systems: Structure and Function* (Holmgren, A., Brändén, C.-I., Jörnvall, H., & Sjöberg, B.-M., Eds.) pp 77–85, Raven Press, New York.

Kelley, R., Ledger, R., Shalongo, W., & Stellwagen, E. (1986c) *UCLA Symp. Mol. Cell. Biol., New Ser. 39*, 269–281.

Kishore, R., Mathew, M. K., & Balaram, P. (1983) *FEBS Lett. 159*, 221–224.

Lunn, C. A., Kathju, S., Wallace, B. J., Kushner, S. R., & Pigiet, V. (1984) *J. Biol. Chem. 259*, 10469–10474.

Schellman, J. A. (1978) *Biopolymers 17*, 1305–1322.

Stryer, L., Holmgren, A., & Reichard, P. (1967) *Biochemistry 6*, 1016–1020.

Wilson, J., Kelley, R. F., Shalongo, W., Lowery, D., & Stellwagen, E. (1986) *Biochemistry 25*, 7560–7566.