Replacement of Proline-76 with Alanine Eliminates the Slowest Kinetic Phase in Thioredoxin Folding†

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ABSTRACT: The conformational transition observed upon addition of guanidine hydrochloride (Gdn-HCl) to solutions of oxidized *Escherichia coli* thioredoxin is dominated by a slow kinetic phase (time constant τ_1 = 300–800 s) that has features appropriate to a proline peptide isomerization. This observation has been interpreted as reflecting a folding pathway involving an obligatory isomerization of the imide peptide bond between isoleucine-75 and proline-76 [Kelley, R. F., & Stellwagen, E. (1984) *Biochemistry 23*, 5095–5102]; this peptide bond is known to have the cis configuration in the folded state [Eklund, H., Cambillan, C., Sjöberg, B.-M., Holmgren, A., Jörnvall, H., Höög, J.-O., & Brändén, C.-I. (1984) *EMBO J. 3*, 1443–1449]. We have tested this hypothesis by examining the conformational transitions of two thioredoxin mutants, *trxA76* having an alanine substituted for proline-76 and *trxA2* [Russel, M., & Model, P. (1983) *J. Bacteriol. 154*, 1064–1070] having proline-34 replaced with serine. Both mutant proteins display far-ultraviolet circular dichroic spectra similar to that of native wild-type thioredoxin. The tryptophan fluorescence emission of native *trxA2* is equivalent to that of wild-type thioredoxin, while the emission intensity of native *trxA76* at 350 nm is 2-fold greater. Tryptophan fluorescence and peptide ellipticity measurements indicate that the mutant proteins undergo two-state and reversible equilibrium unfolding transitions upon addition of guanidine hydrochloride (Gdn-HCl). These transitions are centered at 2.4 and 1.5 M Gdn-HCl for *trxA2* and *trxA76*, respectively, as compared to a midpoint of 2.5 M denaturant for wild-type thioredoxin. As observed for wild-type thioredoxin, fluorescence measurements reveal monophasic unfolding kinetics for *trxA2* at a variety of final denaturant concentrations. The τ for unfolding varies monotonically from 210 s in 2.4 M Gdn-HCl to 7 s for a final Gdn-HCl concentration of 3.5 M. Refolding of denatured *trxA2* in 1.5 M Gdn-HCl detected by fluorescence measurements is described by three kinetic phases with time constants and fractional amplitudes (α) similar to those of wild-type thioredoxins. The fractional amplitude (α_1) of the slowest of these phases, τ_1 = 430 ± 38 s in 2.0 M Gdn-HCl, decreases with final Gdn-HCl concentration. Multimixing experiments suggest that this phase results from an equilibration between denatured forms and has a τ of 34 s in 4 M denaturant, features previously observed for the wild-type protein. In contrast, both unfolding and refolding of *trxA76* (monitored by fluorescence measurements) are described by two kinetic phases having time constants of 24 s (τ_1) and 1 s (τ_2) for a final Gdn-HCl concentration of 1.5 M. The faster and slower phases have activation enthalpies of 25 and 17 kcal/mol, respectively, for refolding in 0.2 M Gdn-HCl. In refolding experiments, α_1 and α_2 are equivalent and independent of both initial and final denaturant concentration, while τ_1 and τ_2 show small decreases with final denaturant concentration. For unfolding of *trxA76* initially equilibrated in 1.0 M Gdn-HCl, α_2 has a value of 0.8 and is independent of final denaturant concentration. The time constant of the faster phase in unfolding (τ_2) decreases from 1 s in 1.5 M Gdn-HCl to 0.04 s in a final denaturant concentration of 3.0 M while τ_1 decreases about 3-fold over this range of final Gdn-HCl concentration. In unfolding experiments starting with protein equilibrated in 1.5 M Gdn-HCl and ending in 2.5 M Gdn-HCl, only the faster phase is observed, and α_2 has a value of 1. These results confirm the assignment of the slowest phase in thioredoxin folding to the isomerization of proline-76. Also, the dependence of α_2 for unfolding on initial denaturant concentration suggests that folding of *trxA76* is described by a three-state mechanism involving a folded intermediate.

The thioredoxin produced by *Escherichia coli* is a 108 amino acid polypeptide having 2 cysteine, 5 proline, and 2 tryptophan residues (Holmgren, 1968). Thioredoxin is usually isolated in the oxidized form, which contains an intramolecular disulfide bond between the two cysteines located at positions 32 and 35 in the primary sequence. This disulfide can be reduced chemically or enzymatically with thioredoxin reductase and NADPH. The reduced form of thioredoxin can function, at least in vitro, as a donor of reducing equivalents in a variety of reactions (Holmgren, 1985). Thioredoxin is required in vivo for replication of T7 (Chamberlin, 1974) and assembly of f1 bacteriophage (Russel & Model, 1985).

Thioredoxin is a good model protein for detailed studies of polypeptide chain folding. Crystallographic measurements of oxidized thioredoxin have indicated that the protein is composed of an N-terminal $\beta/\alpha/\beta/\alpha/\beta$ structural unit joined to a C-terminal $\beta/\beta/\alpha$ unit by an α-helix (Holmgren et al., 1975; Eklund et al., 1984). As shown in Figure 7, the β-strands form a central core twisted sheet that is flanked by two α-helices on each side. The short disulfide loop is located at the N-terminal end of the second α-helix and forms a protrusion from the molecule with the sulfurs partially exposed to solvent. The tryptophans, positions 28 and 31, are in close proximity to the disulfide, consistent with solution measurements demonstrating

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a strong quenching of tryptophan fluorescence in the native protein that is relieved upon reduction of the disulfide or denaturation of the protein (Holmgren, 1972; Kelley & Stellwagen, 1984). Tryptophan fluorescence measurements provide a convenient and sensitive probe for monitoring thioredoxin conformational changes. Since thioredoxin is a normal constituent of *E. coli* and the structural gene has been cloned and sequenced (Wallace & Kushner, 1984), attempts at studying folding of thioredoxin by directed mutagenesis should be straightforward.

Refolding of Gdn-HCl[1]-denatured thioredoxin occurs in three kinetic phases (Kelley & Stellwagen, 1984; Kelley et al., 1986). The slowest kinetic phase (τ_1 = 300–800 s) has a fractional amplitude (α_1) of 0.8 for refolding in 2 M Gdn-HCl and has features characteristic of a peptide isomerization (Kelley & Stellwagen, 1984). Kelley and Stellwagen (1984) have suggested that this phase results solely from configurational isomerization of proline-76 since a refinement of the model of thioredoxin (Eklund et al., 1984) indicates that this proline has the cis configuration while the other four proline residues have the trans configuration. Unfolded molecules having proline-76 in the trans configuration are proposed to fold slowly as expected from the proline isomerization hypothesis (Brandts et al., 1975). However, the observation that α_1 for refolding is dependent on the final, but not initial, denaturant concentration (Kelley et al., 1986) is not easily accomodated by a folding pathway involving only native and denatured states. One mechanism that is compatible with this result is shown in Figure 1.

The rate and magnitude of the slowest kinetic phase observed for thioredoxin folding could result from isomerization of all five proline residues (Creighton, 1978). Also, a quantitative analysis of the mechanism of Figure 1 is very difficult since analytical solutions for four-state mechanisms with two denatured forms are not available. We have attempted to confirm the assignment of the slowest phase to Pro-76 isomerism, and hopefully simplify the kinetic behavior, by studying the folding of single-site thioredoxin mutants. Since an amide peptide bond should have a different equilibrium and rate of isomerization than an imide peptide bond, replacement of a proline whose isomerization must precede acquisition of the native state should manifest an effect on the folding kinetics. This paper describes the folding properties of *trxA76* (Pro-76 → Ala), constructed by us using oligonucleotide-directed mutagenesis, and *trxA2* (Pro-34 → Ser), a thioredoxin variant originally identified as a ts mutant for f1 phage production (Russel & Model, 1983). Our results are consistent with assignment of the slowest phase to Pro-76 isomerism since this kinetic phase is not detected for folding of *trxA76* thioredoxin.

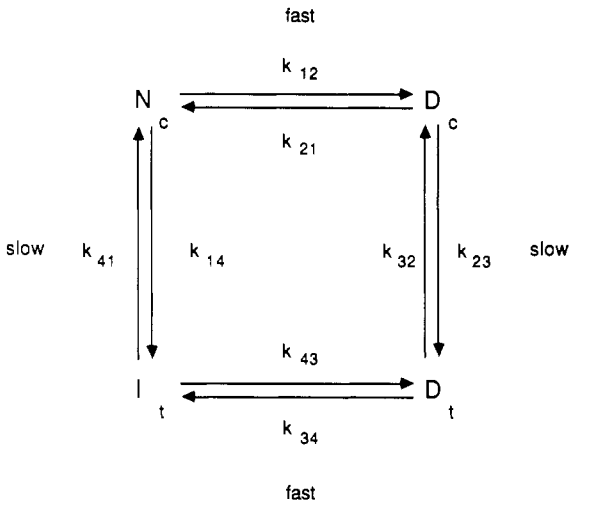

FIGURE 1: Folding mechanism proposed for wild-type thioredoxin (Kelley, 1984). In this mechanism, N is the native state, D represents Gdn-HCl-denatured protein, and I is a transient, globular intermediate state populated for refolding below the equilibrium transition zone. This intermediate is presumed to have far-UV circular dichroism similar to that of native protein but tryptophan fluorescence emission intensity intermediate between those of native and denatured states. The subscript refers to the configuration of the imide peptide bond between Ile-75 and Pro-76. The time constant for isomerization of this peptide bond in the unfolded state $\tau_{ct} = (k_{23} + k_{32})^{-1}$ has a value of 30 s on the basis of measurements of the increase in α_1 for refolding with time in denaturant (Kelley & Stellwagen, 1984; Kelley et al., 1986a). The equilibrium for this step, $K_{23} = [D_c]/[D_t]$, is estimated to have a value of 0.1 on the basis of the value of τ_{ct} and the values measured for τ_1 and α_1 for refolding in 2 M denaturant of protein initially equilibrated in 4 M Gdn-HCl. The equilibria of *horizontal* steps in the mechamism are presumed to be denaturant dependent since α_1 for refolding decreases with final [Gdn-HCl] (Kelley et al., 1986a). The equilibria of the *vertical* interconversions should be relatively denaturant independent since τ_{ct}, determined from multimixing experiments, is independent of [Gdn-HCl]. A similar mechanism has been proposed for folding of the constant fragment of the immunoglobulin light chain (Goto & Hamaguchi, 1982).

Materials and Methods

Guanidine hydrochloride (Gdn-HCl) was purchased from Sigma Chemical Co., St. Louis, MO, and was recrystallized from methanol. Concentrations of stock solutions of Gdn-HCl were determined by refractive index measurements. Ultrogel AcA54 was purchased from LKB, DE-52 cellulose from Whatman, Ltd., and DTNB from Aldrich Chemical Co.

Bacterial Strains and Plasmids. Bacterial strains M5219 (M72*lac* am *trp* am Sm^R λ*bio*252 *cI857ΔH1*), CJ109 (λcI^+ lysogen of S165), and RZ1032 (*dut*$^-$ *ung*$^-$) (Kunkel, 1985) were obtained from C. Joyce (Yale University). A317 (K12 HfrC *trxA*::kan) and K91.pMR14 (a thioredoxin reductase overproducing strain) were obtained from M. Russel (The Rockefeller University). AR120 (N99 *cI*$^+$ *-gal nadA*::tn10) was supplied by J. Mott (Yale University). Strains RK52 and RKAR120 were constructed by p1 transduction of M5219 and AR120, respectively, with phage grown on A317. The plasmids pKC30 (Rosenberg et al., 1983) and pDL-41, having a 477-bp *Hin*cII fragment carrying the *trxA* gene inserted in the *Hpa*I site of pKC30, were provided by D. LeMaster (Yale University). A pBR322 derivative carrying the *trxA2* gene was the kind gift of M. Russel.

Construction of trxA76 Mutant. A 634-bp *Hpa*II fragment isolated from digestion of pDL-41 was ligated with *Acc*I-cleaved M13mp9 and competent JM101 transformed as described by Messing (1983). Recombinant phage having the coding strand of the *trxA* gene in the single-stranded form of the phage were identified by dideoxynucleotide sequencing (Sanger et al., 1977). An oligonucleotide, $^{5'}$GCAGAGTCGC*GATACCA$^{3'}$ (C* is the mismatched base), designed to introduce a C to G mutation in the first nucleotide of the proline codon, as well as a unique *Nru*I site, was synthesized according to a modified phosphotriester method (Efimov et al., 1982). The oligonucleotide was made on a solid support with the automated synthesizer in the laboratory of N. Grindley (Yale University). Single-stranded template DNA was prepared for mutagenesis from phage replicated in RZ1032 as described by Kunkel (1985). Phosphorylation of the oligonucleotide, annealing of template and primer, in vitro polymerization, and transformation of

[1] Abbreviations: Gdn-HCl, guanidine hydrochloride; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); *trxA76*, Pro-76 → Ala thioredoxin; *trxA2*, Pro-34 → Ser thioredoxin.

competent JM101 were performed according to the protocols of Smith and Gillam (1981). Transformants having the desired mutation were identified by isolating single-stranded template DNA and performing the C* reaction of the dideoxynucleotide sequencing protocol. Mutants identified in this manner were plaque purified. About 15% of the phage screened had the mutant genotype while the remainder were wild type. A mutant phage isolate having no other alterations in the thioredoxin gene was identified by performing a complete dideoxynucleotide sequencing protocol. This recombinant (M13mp9*trxA76*) was used as the source of *trxA76* DNA in further manipulations. Replicative-form DNA and plasmid DNA were isolated according to the procedure of Birnboim and Doly (1979).

Expression and Purification of Mutant Proteins. Replicative-form DNA isolated from infection of JM101 with M13mp9*trxA76* was digested with *Hin*cII and *Hae*III, a 473-bp fragment encoding the entire *trxA76* gene and some flanking sequences was isolated, ligated with *Hpa*I-cleaved pKC30, and the reaction mixture was used to transform CJ109 to ampicillin resistance. A recombinant plasmid having *trxA76* inserted in the correct orientation for transcription from the λ pL promoter on pKC30 was identified by digestion with *Nru*I. A 477-bp *Hin*cII fragment carrying the *trxA2* gene was cloned in pKC30 by D. LeMaster (Yale University). For protein expression under control of the λ pL promoter, these pKC30 recombinants were introduced into M5219, which has a temperature-sensitive *cI* repressor (*cI857*). Cells were grown at 37 °C until the optical density at 550 nm was 1.0, the temperature was rapidly raised to 42 °C to inactivate the *cI857* repressor, and cells were harvested 5 h after the temperature shift. Expression of *trxA2* under this protocol was similar to that of the wild-type protein in this system (20–50 mg/L), while *trxA76* was expressed at approximately a 50-fold lower level. The lower level of *trxA76* expression necessitated production of this protein in bacterial strains in which the chromosomal thioredoxin gene had been inactivated by insertion of a kanamycin-resistance marker. Expression of *trxA76* in RKAR120 by induction of the λ pL promoter with 40 μg/mL nalidixic acid (Mott et al., 1985) at 30 °C was about 2-fold higher than induction by the temperature shift described above.

For purification of the mutant proteins, 10-L cultures were grown at 37 °C with vigorous agitation and aeration in a fermentor. Transcription from the λ pL promoter was induced as described above by use of heat induction for *trxA2* and chemical induction for *trxA76*. Upon chemical induction the temperature was lowered to 30 °C, and cells were harvested by centrifugation 8 h after addition of nalidixic acid. Both mutant proteins were purified according to a procedure described for the wild-type protein (LeMaster & Richards, 1985). A brief outline of the protocol is (i) cell breakage by sonication, (ii) concentration of the cell supernatant by lyophilization, (iii) gel filtration chromatography on a column of Ultrogel AcA54, and (iv) chromatography on a column of DE-52 cellulose. A DTNB-coupled assay (Moore et al., 1964) was used to monitor elution of thioredoxin in the two column chromatography steps. Thioredoxin reductase used in the assay was purified from a homogenate of K91.pMR14 according to the procedure of Pigiet and Conley (1977). No significant differences in chromatographic behavior were observed for either mutant protein relative to wild-type thioredoxin. However, since *trxA76* was expressed at a low level, the gel filtration chromatography step was repeated in order to obtain a level of purity approaching that obtained with the wild-type protein. A typical yield of *trxA2* was about 500 mg and for *trxA76* 25 mg. Each protein comigrated with wild-type thioredoxin upon electrophoresis on a sodium dodecyl sulfate–15% polyacrylamide gel and appeared to be >95% pure. The purified proteins had ultraviolet absorbance spectra similar to that of wild-type thioredoxin and had an A_{260}/A_{280} ratio of 0.6. Both mutant thioredoxins were isolated with the disulfide bond intact as judged by the lack of reaction with DTNB in the presence of concentrated Gdn-HCl. The concentration of solutions of the purified proteins was determined by absorbance measurements at 280 nm with the extinction coefficient for wild-type thioredoxin being 13.7 mM^{-1} cm^{-1} (Holmgren & Reichard, 1967).

Equilibrium Measurements. All measurements were made on solutions that contained 50 mM potassium phosphate, pH 7. Unless noted otherwise, experiments were performed on thioredoxin samples that had the disulfide intact. Fluorescence spectra were recorded on a Hitachi Model MPF-2A spectrofluorometer as described previously (Kelley & Stellwagen, 1984). Square quartz cuvettes of 1-cm path length were used. Circular dichroic spectra were recorded on a Jasco Model J-500A spectropolarimeter in cylindrical cells of 0.1-cm path length, with a time constant of 8 s and a sensitivity of 0.5 mdeg/cm. The protein concentration for fluorescence and CD measurements was 10 μM. For both measurements the sample temperature was maintained by circulating thermoregulated water around the cuvette, and the actual temperature of the sample solution was measured with a thermistor.

Kinetic Measurements. As for the equilibrium experiments, all measurements were made on protein samples having the disulfide bond intact in solutions containing 50 mM potassium phosphate, pH 7. Fluorescence measurements of the kinetics of folding were performed on the Hitachi spectrofluorometer. An excitation wavelength of 295 nm, emission wavelength of 350 nm, and sensitivity of 3 were employed. Excitation and emission slits were 10 or less. Kinetic experiments were initiated by pipetting 0.2 mL of protein solution into 1.8 mL of solution having a varied Gdn-HCl concentration contained in a 1-cm path length quartz cuvette. Both solutions had been equilibrated at the specified temperature. Mixing was accomplished by inverting the cuvette several times. The protein concentration after mixing was 10 μM. The dead time between addition of protein to the buffer solution and the first measurement was 10 s except for the determination of the temperature dependence of refolding in which the dead time was 5 s. In all experiments, the observed change in tryptophan fluorescence with reaction time $f(t)$ could be described as the sum of one or more exponentials of the form

$$f(t) = A + \sum f_i e^{-t/\tau_i} \quad (1)$$

for measurements of refolding and

$$f(t) = A + \sum f_i (1 - e^{-t/\tau_i}) \quad (2)$$

for unfolding experiments. The time constant τ and fluorescence amplitude f were extracted by a first-order analysis of the change in tryptophan fluorescence with a linear form of these equations:

$$\ln |f(t) - f_\infty| = \ln f - t/\tau \quad (3)$$

The infinite time value (f_∞) used in the analysis was the fluorescence measured 2 h after mixing. The kinetic parameters extracted in this manner were used to calculate an exponential and compared to the observed fluorescence profile. When more than one kinetic phase was apparent, as evidenced by significant deviations between the observed and calculated exponentials, the kinetic parameters were determined by a

"peel-back" procedure. A first-order analysis of the slowest kinetic phase was performed with data collected after the faster kinetic phase(s) appeared completed. The slowest kinetic phase is designated with the subscript 1. The calculated exponential was extrapolated to zero reaction time and then subtracted from the observed fluorescence profile, and the residual was fit to a first-order process. This procedure was repeated until the observed and calculated fluorescence curves appeared identical. Faster kinetic phases are designated with the subscripts 2 and 3 in order of decreasing time constant.

For experiments in which only a single kinetic phase could be directly observed, the functional amplitude α was calculated from eq 4, where f_0 is the initial fluorescence value. This initial

$$\alpha = f/(f_0 - f_\infty) \quad (4)$$

value is that obtained by extrapolation of the linear dependence of the equilibrium fluorescence of either native protein (unfolding experiment) or denatured protein (refolding experiment) on denaturant concentration, observed outside the equilibrium transition zone, to the final denaturant concentration used in the experiment. An initial value f_0' was also determined by extrapolation of the calculated exponential(s) to zero reaction time. A significant difference (>10%) between f_0 and f_0' would suggest that a faster kinetic phase has escaped detection. For experiments where more that one kinetic phase was directly observed and the difference between f_0 and f_0' was within the experimental error in the measurement, the fractional amplitude was calculated from eq 5 (if $f_0 = f_0'$, then

$$\alpha_i = f_i/\sum f_i \quad (5)$$

$\sum f_i = |f_0 - f_\infty|$ and $\sum \alpha_i = 1$) without correction for the effect of solvent on native and denatured base-line fluorescence. Since the dependence of native and denatured base-line fluorescence on denaturant concentration is shallow, neglect of this effect introduces only small errors (<10%) in the calculation of α. It was necessary to neglect this effect for analysis of data from stopped-flow mixing experiments since it was not possible to reliably extract these base-line dependencies.

Fluorescence measurements of folding kinetics were also performed with a Durrum Model D-110 stopped-flow mixing spectrometer as described previously (Kelley & Stellwagen, 1984). Folding kinetics were initiated by mixing equal volumes of protein and buffer solution, and the mixing dead time was 10 ms. The fluorescence profile was displayed on an oscilloscope, photographed, and analyzed as described above except that f_∞ was determined from a Guggenheim analysis of the slowest kinetic phase.

RESULTS

Characterization of Native Proteins. The capacity of the mutant and wild-type proteins to function as substrate for thioredoxin reductase at 25 °C was compared with a DTNB-coupled assay (Holmgren & Reichard, 1967). As previously reported (Russel & Model, 1986), *trxA2* has a specific activity in this assay about 1.2 times that of the wild-type protein. In contrast, the specific activity of the *trxA76* variant as a substrate for thioredoxin reductase is 5-fold less than that measured for wild-type thioredoxin.

As shown in Figure 2A, all three thioredoxins display similar far-ultraviolet circular dichroic spectra. The dichroic spectra are characterized by a broad negative peak at 220 nm, a crossover to positive ellipticity values around 206 nm, and a positive maximum at 202 nm, features typical of an α/β protein (Hennessey & Johnson, 1981). These data suggest that the gross structural features of the native wild-type protein

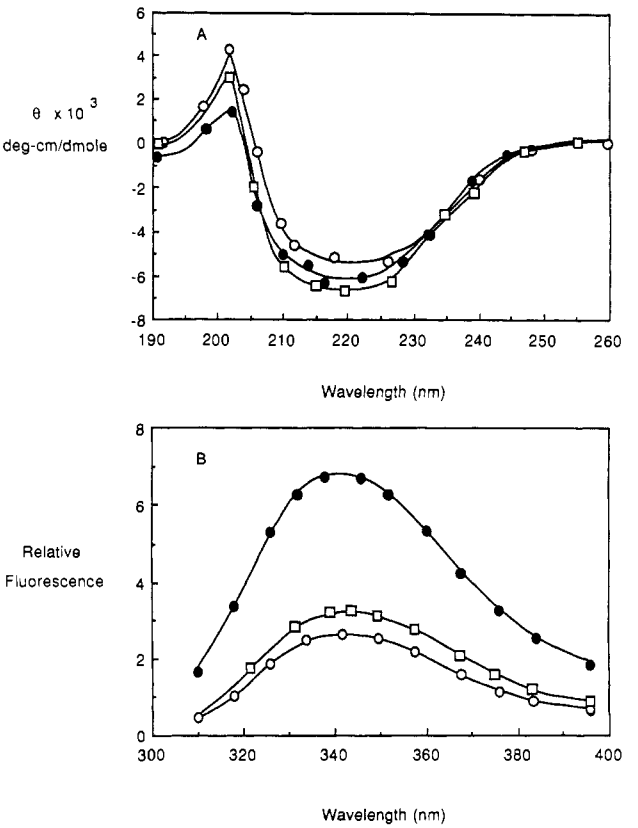

FIGURE 2: Spectroscopic characteristics of native thioredoxins. Selected data points from measurements of 10 μM wild-type (○), *trxA76* (●), and *trxA2* (□) thioredoxins in 50 mM potassium phosphate, pH 7, are shown. *Solid* lines represent the best curve drawn through all the data for each protein without weighting. Measurements were made at 25 °C. (A) Far-ultraviolet circular dichroic spectra collected with a 0.1-cm path length cylindrical cell. (B) Tryptophan fluorescence emission spectra obtained with an excitation wavelength of 295 nm and 1-cm path length cuvettes.

are maintained in the mutant proteins. In addition, in solutions containing 3.5 M or higher concentrations of Gdn-HCl all three proteins display spectra expected for a denatured protein (data not shown).

The tryptophan fluorescence emission spectra observed for native thioredoxins upon excitation with 295-nm wavelength light are shown in Figure 2B. Each protein displays an emission maximum of 342 nm. Under identical instrumental and solution conditions, the model fluorophore *N*-acetyltryptophan ethyl ester has an emission maximum at 355 nm. As previously reported for the wild-type protein (Kelley & Stellwagen, 1984), the emission intensity of native *trxA2* is quenched, having about 30% of the fluorescence yield at 350 nm as an equimolar solution of *N*-acetyltryptophan ethyl ester. However, we have noticed that if *trxA2* is stored as a frozen, concentrated solution and then thawed and diluted for measurement, then the emission intensity observed is 2–3 times greater. The excess fluorescence decays slowly over several days and is not observed if the protein is incubated in 6 M Gdn-HCl for a few hours and then renatured by dilution. Although concentrated solutions of *trxA2* do not appear turbid, the excess fluorescence may indicate that the native protein aggregates when stored frozen. A thermal melting curve determined for a freshly thawed and diluted solution of *trxA2* has features suggesting intermolecular cooperativity (J. Sturtevant and D. LeMaster, personal communication). In order to avoid this artifact, all experiments described in this paper we performed on a stock solution of *trxA2* that was stored at 4 °C.

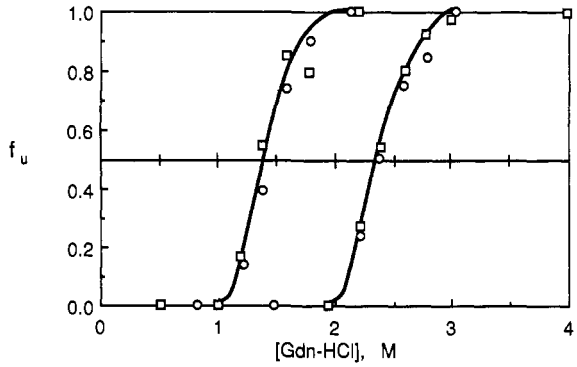

FIGURE 3: Equilibrium denaturation of thioredoxin mutants. Measurements were made at 25 °C on solutions containing either 10 μM *trxA76* (left curve) or 10 μM *trxA2* (right curve), 50 mM potassium phosphate, pH 7, and the indicated concentration of Gdn-HCl. The magnitude of the fluorescence emission intensity at 350 nm with 295-nm excitation (○) or of the mean residue ellipticity at 230 nm (□) was used to calculate the fraction unfolded (f_u) with the equation $f_u = (y - y_n)/(y - y_u)$. In this equation, y is the measured value of the observable, and y_n and y_u are the values expected for native and denatured protein in the same solution. These values were obtained by extrapolation of the shallow, linear dependence of the observable on denaturant concentration below and above the transition zone, respectively. Each value is the average of measurements made by addition of denaturant to native protein or by refolding of protein denatured in 6 M Gdn-HCl. Solutions were allowed to equilibrate overnight prior to measurement. The $\Delta G°$ associated with folding given in the text was obtained by extrapolation of the linear dependence of ln K_{app} on [Gdn-HCl] in the transition zone to zero denaturant concentration [$K_{app} = f_u/(1 - f_u)$].

The emission intensity of native *trxA76* is approximately 2-fold larger than that measured for the two other thioredoxins. Addition of Gdn-HCl to 6 M results in a shift in the emission maximum for all three thioredoxins to 350 nm. The emission intensity measured at this wavelength for *trxA76* is 78 ± 5% and for *trxA2* is 83 ± 4% of the intensity measured for an equimolar solution of *N*-acetyltryptophan ethyl ester in 6 M denaturant. Previous measurements of wild-type thioredoxin in 6 M Gdn-HCl have shown that the emission intensity at 350 nm is also less than 100% (87 ± 3%) of the value determined for the model fluorophore although viscosity measurements suggest a random-coil conformation with one disulide cross-link for this protein in 6 M denaturant (Kelley & Stellwagen, 1984). This comparison suggests that the two thioredoxin variants are unfolded in solutions containing concentrated Gdn-HCl at neutral pH and ambient temperature.

Equilibrium Denaturation. Addition of Gdn-HCl to solutions of native *trxA76* or *trxA2* results in a cooperative unfolding transition as evidenced by an increase in tryptophan fluorescence emission at 350 nm and a dimunition of the negative ellipticity at 230 nm. Outside the transition region, the values of the observables for both native and denatured protein show a small but linear increase with denaturant concentration. The data in the transition zone were normalized by extrapolation of these base-line dependencies and are shown as the fraction of unfolded protein as a function of denaturant concentration in Figure 3. Equilibrium unfolding of both proteins appears to be a two-state process since the transitions are cooperative and the two spectroscopic probes yield coincident transitions. The transitions for *trxA76* and TrxA2 are centered at 1.5 and 2.4 M Gdn-HCl, respectively, whereas the midpoint for denaturation of wild-type thioredoxin is 2.5 M. Unfolding of either mutant in Gdn-HCl is reversible as similar values of the observables are obtained if protein is first denatured in 6 M Gdn-HCl and then refolded by dilution. Analysis of these transitions in terms of a two-state model with extrapolation of the equilibrium constant for unfolding to zero denaturant concentration provides $\Delta G°$ values for folding of 8.8 and 5.0 kcal/mol for *trxA2* and *trxA76*, respectively. A $\Delta G°$ value for folding of 8.9 kcal/mol has been previously determined for wild-type thioredoxin from similar equilibrium unfolding data (Kelley & Stellwagen, 1984). This result suggests that the native conformations of wild-type thioredoxin and *trxA2* have equivalent stability while the native form of *trxA76* is significantly destabilized relative to unfolded protein.

The stability of the native conformation of *trxA76* was further examined by performing thermal melting experiments using circular dichroism at 230 nm as the observable. In a solution containing 50 mM potassium phosphate, pH 7, a midpoint of 65 °C is observed for thermal denaturation of native *trxA76*. A midpoint of 82 °C was previously determined for thermal denaturation of native wild-type thioredoxin from differential scanning calorimetry experiments (Kelley et al., 1987). Thermal melting experiments were also performed with *trxA76* that had been reduced by incubation with a 20-fold excess of dithiothreitol. Reduction of the disulfide does not cause any significant changes in the far-UV CD spectrum of native *trxA76*, but the midpoint for thermal denaturation decreases to 50 °C. By comparison, calorimetric experiments indicate that a sample of wild-type thioredoxin that has been reduced and the resultant cysteines alkylated with iodoacetamide (Kelley et al., 1987) has a midpoint for thermal denaturation of 65 °C. Incomplete reversibility of thermal denaturation for both reduced and oxidized thioredoxins precludes a quantitative comparison of these transitions; nonetheless, it is apparent that *trxA76* is significantly less stable than wild-type thioredoxin but retains the differential stability between the reduced and oxidized proteins.

Folding Kinetics. The principal goal of this investigation was to determine whether the slow kinetic phase characteristic of refolding and unfolding of wild-type thioredoxin would be observed for either mutant protein. Folding kinetics were observed by tryptophan fluorescence measurements since fluorescence detection is sensitive and can be used on conjunction with rapid-mixing devices. Changes in tryptophan fluorescence reflect a global conformational transition as indicated by the equilibrium denaturation experiments described above. In initial experiments, a manual mixing protocol was used to either cause protein unfolding by denaturant addition or to initiate refolding of denatured protein by dilution. Typical kinetic profiles observed with this mixing protocol are shown in Figure 4, and the results of a series of kinetic experiments are summarized in Table I. Manual mixing was preferred over stopped-flow mixing since it allows greater control of experimental variables, i.e., temperature and mixing ratio, requires less protein, and is sufficient for measurement of the slowest phase in wild-type thioredoxin folding over a wide range of experimental conditions (Kelley et al., 1986). However, when a significant fraction of the total fluorescence change was completed within the dead time of manual mixing, kinetic experiments were repeated with stopped-flow mixing. The standard error in determination of kinetic parameters from manual mixing experiments was usually 10% for *trxA2* thioredoxin but was substantially greater for *trxA76*, about 20%, owing to the smaller fluorescence change associated with denaturation of this variant. While kinetic experiments with *trxA2* were usually repeated 5 or more times with the mean values reported in Table I, the small supply of *trxA76* limited the number of repetitions to 2 or 3.

Kinetics of trxA2 Folding. The change in tryptophan fluorescence accompanying either unfolding or refolding of

Table I: Thioredoxin Folding Kinetics[a]

sample	method[b]	[Gdn-HCl] (M) initial	[Gdn-HCl] (M) final	τ_1	τ_2	τ_3	α_1	α_2
wild type	m	1.0	2.5	800			1	
	m	1.0	3.0	275 ± 32			1	
	sf	1.0	3.0	167			1	
	m	1.0	3.5	35			1	
	m	4.0	2.5	800			1	
	m	4.0	2.0	560 ± 60			0.80 ± 0.06	
	sf	3.0	1.5	340	14	1	0.5	0.3
	m	4.0	0.2	300			0.2	
trxA2	m	1.0	2.4	210	1		1	
	m	1.0	3.0	34 ± 5			1	
	sf	1.0	3.0	34			1	
	m	1.0	3.5	7			1	
	m	4.0	2.4	260			0.7	
	m	4.0	2.0	430 ± 38			0.83 ± 0.07	
	m	4.0	1.0	850			0.4	
	m	4.0	0.2	600			0.2	
	sf	3.0	1.5	420	23	1.8	0.5	0.2
trxA76	m	0	1.2	70			0.8	
	m	0	1.4	22 ± 5			0.50 ± 0.05	
	sf	1.0	1.5	18	1		0.2	0.8
	m	0	2.0	28			0.4	
	sf	1.0	2.0	24 ± 10	0.6 ± 0.3		0.20 ± 0.05	0.80 ± 0.12
	m	1.0	2.0	32			0.3	
	m	0	2.4	20			0.3	
	sf	1.0	2.5	13	0.1		0.2	0.8
	m	1.5	2.5		fast		0	1
	sf	1.5	2.5		0.1		0	1
	sf	1.0	3.0	5	0.04		0.2	0.8
	sf	2.0	1.5	29	1		0.4	0.6
	m	2.5	1.4	23 ± 9			0.4 ± 0.1	
	m	1.5	1.0	23			0.5	
	sf	1.5	1.0	16	0.8		0.5	0.5
	sf	2.0	1.0	22	1		0.4	0.6
	m	2.0	1.0	14			0.5	
	m	4.0	1.0	28			0.4	
	m	2.0	0.2	13			0.5	

[a] Values shown with standard deviation are the mean of five or more determinations. [b] Mixing method used: m = manual mixing; sf = stopped-flow mixing.

trxA2 in 2.4 M Gdn-HCl initiated by manual mixing is shown in Figure 4A. Within the experimental error in this determination (10%), all of the increase in fluorescence intensity associated with unfolding of *trxA2* can be accounted for by a single kinetic phase having the time constant (τ_1) given in Table I. Both the time constant and fractional amplitude (α_1) of this phase, determined for unfolding in 3 M Gdn-HCl, are independent of the initial denaturant concentration (data not shown). As shown in Table I, τ_1 decreases with an increase in the denaturant concentration used to unfold the protein, but α_1 has a constant value of 1. In the range of 2.5–4.0 M final Gdn-HCl concentration, the change in τ_1 with an increase in denaturant concentration parallels that observed for the wild-type protein (Kelley et al., 1986), but the absolute rate at a given Gdn-HCl concentration is faster for *trxA2*. As indicated in Table I, no additional kinetic phases are observed when stopped-flow mixing is employed in a measurement of unfolding in 3 M Gdn-HCl. This result supports the conclusion that all of the equilibrium fluorescence change associated with unfolding in 2.4 M denaturant is described by the single kinetic phase detected with manual mixing.

Fluorescence measurements of refolding of denatured *trxA2* in 2.4 M Gdn-HCl reveal a kinetic phase having a time constant (τ_1) of similar magnitude to that measured for unfolding. The observation of similar τ_1 values for unfolding and refolding under the same final conditions provides further evidence of the reversibility of denaturation. The values determined for τ_1 and α_1 from refolding experiments are not altered by a change in the initial denaturant concentration in the range 2.5–5.0 M (data not shown). This phase accounts for the majority of the decrease in fluorescence intensity accompanying refolding in 2.4 M Gdn-HCl. As shown in Table I, α_1 decreases when the final denaturant concentration for refolding is decreased. The loss in fractional amplitude is accounted for by a concomitant increase in the fraction of fluorescence change occurring in the manual mixing dead time. This rapid decrease in tryptophan fluorescence can be monitored with stopped-flow mixing. Two faster kinetic phases having the time constants (τ_2 and τ_3) and fractional amplitudes given in Table I must be included in the analysis to precisely describe the complete change in fluorescence upon refolding in 1.5 M Gdn-HCl. Thus, all of the equilibrium fluorescence decrease associated with refolding is contained in three kinetic phases that are similar in rate and magnitude to those observed for wild-type thioredoxin refolding (Kelley & Stellwagen, 1984; Kelley et al., 1986).

Kinetics of trxA76 Folding. A typical tryptophan fluorescence profile observed upon unfolding or refolding *trxA76* in 1.4 M Gdn-HCl obtained with manual mixing is shown in Figure 4B. A single kinetic phase having the time constant (τ_1) given in Table I precisely describes the increase in fluorescence observed for unfolding. No slower kinetic events can be resolved, but a significant fraction of the equilibrium increase in fluorescence intensity is completed within the manual mixing dead time. As indicated in Table I, the kinetic constants for unfolding of protein initially equilibrated in the absence of denaturant depend on the final denaturant concentration attained. The time constant (τ_1)

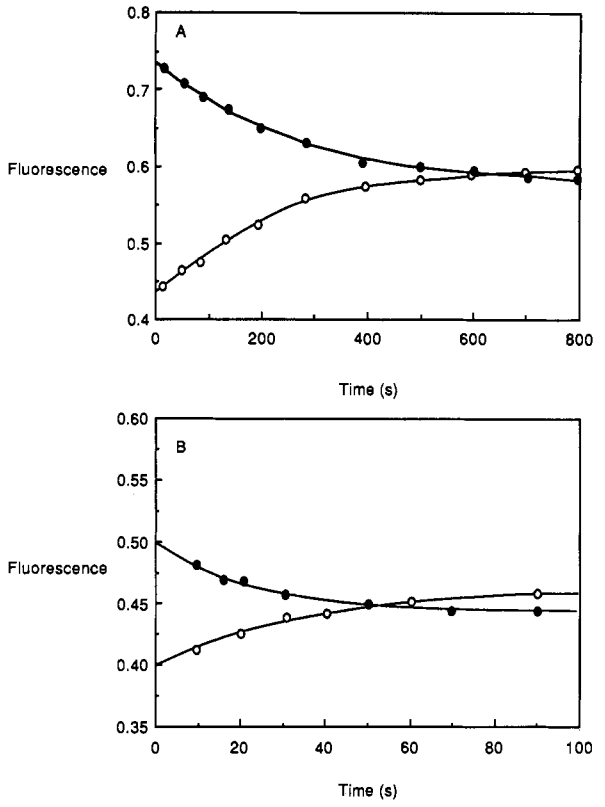

FIGURE 4: Fluorescence measurements of thioredoxin folding kinetics. Folding kinetics were initiated by manual mixing (dead time = 10 s). Emission intensity at 350 nm with 295-nm excitation is represented by (○) for unfolding experiments and by (●) for refolding experiments. The temperature was 25 °C, and all solutions contained 50 mM potassium phosphate, pH 7. The small differences in final values between unfolding and refolding are assumed to result from volume fluctuations in mixing. The solid lines are single exponentials calculated from a first-order analysis of the data with the fluorescence measured 2 h after mixing as f_∞. The lower and upper boundaries of the ordinate correspond approximately to the values expected for native and denatured protein, respectively, in the final conditions. (A) Folding kinetics of *trxA2* in a final denaturant concentration of 2.4 M. For unfolding, protein equilibrated in 1 M Gdn-HCl was mixed with concentrated Gdn-HCl and for refolding protein was denatured overnight in 4 M Gdn-HCl and renatured by dilution. (B) Folding kinetics of *trxA76* in a final Gdn-HCl concentration of 1.4 M. Protein was denatured in 2.5 M Gdn-HCl overnight prior to measurement of refolding kinetics while unfolding experiments were performed by adding denaturant to native protein.

decreases 3-fold, and the fractional amplitude (α_1) approaches a limiting value of 0.3, as the final denaturant concentration is varied from 1.2 to 2.4 M. The effect of initial denaturant concentration on the kinetics of unfolding was examined with a final denaturant concentration of 2 M. As illustrated in Table I, τ_1 for unfolding is dependent only on the final conditions. However, a larger fraction of the fluorescence increase accompanying unfolding in 2 M Gdn-HCl is completed within the manual mixing dead time as the initial denaturant concentration is increased. Indeed, for unfolding of protein initially equilibrated in 1.5 M denaturant, all of the fluorescence increase occurs too rapidly to follow with manual mixing.

The rapid increase in fluorescence intensity was further examined by employing stopped-flow mixing to cause unfolding in 1.5 M Gdn-HCl of protein initially equilibrated in 1.0 M denaturant. Analysis of the observed fluorescence profile indicates that the rapid change in fluorescence is associated with a kinetic phase having a time constant (τ_2) of 1 s. None of the change in fluorescence intensity occurs in the 10-ms dead time of stopped-flow mixing. Thus, in contrast to the wild-type protein, two phases are required to describe the kinetics of *trxA76* unfolding. Comparable values for α_1 and τ_1 are obtained from either mixing method. As shown in Table I, τ_2 is quite dependent on final denaturant concentration. Despite this increase in rate of the faster phase, α_2 remains constant with an increase in final denaturant concentration. Results of the manual mixing unfolding experiments suggested that α_1 decreased to a limiting value as the final denaturant concentration was increased. This inconsistency probably reflects the different initial denaturant concentrations used for the two mixing methods. As observed with manual mixing, all of the change in fluorescence intensity resulting when the denaturant concentration is rapidly increased from 1.5 to 2.5 M is associated with the faster kinetic phase.

A first-order analysis of the refolding data shown in Figure 4B reveals a kinetic phase having a time constant (τ_1) of 23 s and fractional amplitude (α_1) of 0.4. A slower kinetic phase is not detected, and the remainder of the fluorescence decrease is too rapid to monitor with manual mixing. As summarized in Table I, only small variations in τ_1 and α_1 are observed when either the initial or final denaturant concentration in a refolding experiment is varied. In contrast to measurements of the kinetics of unfolding, the slower phase is detected for refolding of protein previously equilibrated in 1.5 M Gdn-HCl. As shown in Table I, analysis of refolding experiments performed with stopped-flow mixing indicate that all of the rapid decrease in fluorescence intensity is associated with a faster kinetic phase similar in time constant (τ_2) to the one detected in unfolding experiments. In contrast to wild-type thioredoxin, two phases are sufficient to describe the kinetics of *trxA76* refolding. For experiments in which either unfolding or refolding in 1.5 M Gdn-HCl is measured, similar time constants for the two phases are determined as expected for a reversible conformational transition.

Manual Multimixing Experiments. A multimixing protocol was used to determine whether the slow kinetic phase observed for refolding *trxA2* results from an equilibration between fast and slow folding denatured states. Native *trxA2* was unfolded for a brief but varied time period in 4 M Gdn-HCl and then refolded in 2 M Gdn-HCl and the fractional amplitude of the slow phase (τ_1 = 430 s) determined by a first-order analysis of the fluorescence change accompanying refolding. In 4 M Gdn-HCl, the fluorescence changes associated with unfolding are completed within 1 s. The complete mixing protocol was repeated with a series of timed intervals between the start of unfolding and the initiation of refolding. While τ_1 for refolding has no dependence on the incubation period in the unfolding solvent, α_1 for refolding increases with unfolding time as shown in Figure 5. The increase in α_1 with time in 4 M denaturant is described by a single exponential having a time constant of 34 s. This value compares favorably with the time constant of 20–40 s determined for the wild-tye protein in identical multimixing experiments (Kelley & Stellwagen, 1984; Kelley et al., 1986).

Since the second phase in refolding of wild-type thioredoxin also seems to originate from an equilibration between unfolded forms (R. F. Kelley, unpublished results), we attempted multimixing experiments with *trxA76* to examine the origin of the slower of the two kinetic phases. However, these experiments were inconclusive because of two factors. First, both the total fluorescence change for denaturation and the fraction associated with the slowest phase in refolding of *trxA76* are smaller than for the other thioredoxins such that the precision in amplitude measurements is decreased. Second, the rate of the slower phase for unfolding in 3 M Gdn-HCl (τ_1 = 5 s) is not much greater than the rate of this phase for refolding

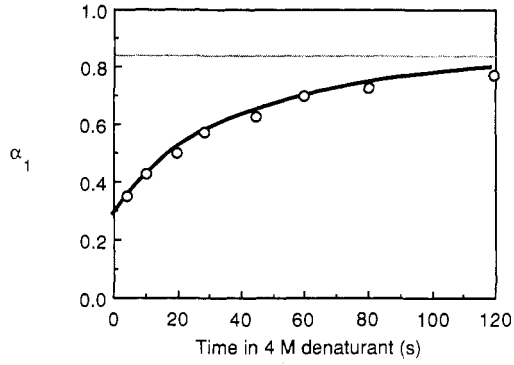

FIGURE 5: Examination of *trxA2* slow refolding using a multimixing protocol. The fractional amplitude of the slow kinetic phase in refolding of *trxA2* ($\tau \simeq 500$ s) as a function of incubation period in concentrated denaturant is shown. Experimental values are given as (O), and the *solid* line is a single exponential having a time constant of 34 s calculated from a first-order analysis of these data. The *dotted* line represents the value of α_1 measured for refolding of protein denatured in 4 M Gdn-HCl for 2 h. This value was used as f_∞ in the analysis of the increase in α_1 with time in denaturant. In these experiments, aliquots of native *trxA2* were manually mixed with an unfolding solution to obtain a mixture containing 4 M Gdn-HCl, 50 mM potassium phosphate, pH 7, and 100 μM protein. This mixture was incubated at 25 °C for the indicated time period, and then refolding was initiated by manual dilution to give a solution containing 2 M Gdn-HCl, 50 mM potassium phosphate, pH 7, and 10 μM protein. Refolding was monitored by the change in tryptophan fluorescence, and the kinetic data were analyzed as described in the text.

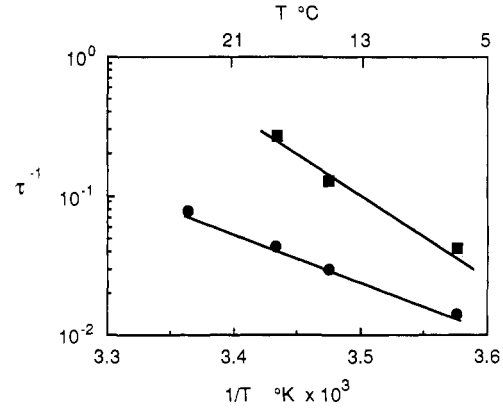

FIGURE 6: Temperature dependence of *trxA76* refolding kinetics. Rate constants of the fast (●) and slow (■) phases determined for refolding *trxA76* in 0.2 M Gdn-HCl–50 mM potassium phosphate, pH 7, at several temperatures are shown in an Arrhenius plot. Protein was denatured overnight at 25 °C in 2 M Gdn-HCl and equilibrated at the appropriate temperature, and then refolding was initiated by manual 10-fold dilution with 50 mM potassium phosphate, pH 7. Refolding was followed by tryptophan fluorescence measurements, and the rate constants were determined as described in the text. The temperature is that measured inside the fluorescence cuvette at the completion of the kinetic profile. Each value shown is the average of three or more refolding determinations. The *solid* lines are the result of least-squares regression, providing activation enthalpies of 25 and 17 kcal/mol for the fast and slow phases, respectively.

in 1 M denaturant (τ_1 = 20 s). Thus, it is difficult to kinetically separate the conformational changes in unfolding that manifest a spectroscopic change from any chain equilibrations that occur after unfolding.

Temperature Dependence of trxA76 Refolding Kinetics. The effect of temperature on the kinetics of *trxA76* refolding was examined with manual mixing. Protein denatured in 2 M Gdn-HCl was refolded by 10-fold dilution with 50 mM potassium phosphate, pH 7, and refolding was monitored by tryptophan fluorescence measurements. At temperatures below 20 °C, the faster phase can be detected by manual mixing so that data were analyzed by peeling off exponentials. The time constant of both phases shows a strong dependence on temperature. As shown in Figure 6 an Arrhenius plot of this dependence is linear, providing activation enthalpies of 25 and 17 kcal/mol for the fast and slow phases, respectively. Extrapolation of the temperature dependence of the faster phase time constant to 25 °C predicts a τ_2 value of 1.4 s for refolding in 0.2 M Gdn-HCl. This result suggests that the rate of the faster phase is denaturant independent since the time constant for refolding in 1.5 M Gdn-HCl measured directly with stopped-flow mixing at 25 °C is 1 s. The time constant (τ_3) of the fastest phase in refolding of wild-type thioredoxin decreases with final denaturant concentration (Kelley et al., 1986). The fractional amplitudes determined for the two phases do not vary with temperature except for measurements made at the lowest temperature, 6 °C. At this temperature the ratio of the time constants is small such that is it is difficult to resolve the two kinetic phases, and a larger fractional amplitude is assigned to the slower phase.

DISCUSSION

Examination of a space-filling model of the short disulfide loop of thioredoxin, ^{32}C-G-P-C^{35}, suggests that isomerization of the G–P peptide bond is severely restricted by steric hindrance. Therefore, it is expected that the native configuration of this peptide bond is maintained in the denatured protein if the disulfide bond is intact. Consistent with this expectation is our observation that replacement of Pro-34 by serine has only small effects on the equilibrium and kinetics of oxidized thioredoxin folding. The principal features of the slowest phase in refolding, most importantly the observation that this phase results from an equilibration between denatured states, are comparable for wild-type and *trxA2* thioredoxins. The fractional amplitudes and time constants for the three kinetic phases in refolding have similar values for wild-type and *trxA2* thioredoxin. One difference noted is the dependence of τ_1 for refolding on denaturant concentration (see Table I). This discrepancy may result from the presumed tendency of native *trxA2* to aggregate. We conclude that isomerization of proline-34 does not limit the rate of any step in thioredoxin folding that involves a change in conformation.

Replacement of proline-76 by alanine results in decreases in biological activity and equilibrium stability as well as a loss of a kinetic phase for refolding of Gdn-HCl-denatured thioredoxin. A sequence comparison of three thioredoxins (Eklund et al., 1984) indicates that proline-76 is a conserved residue that is part of a surface postulated as the site of interaction with thioredoxin and ribonucleotide reductases. The thioredoxin encoded by T4 bacteriophage, which is active with *E. coli* thioredoxin reductase, retains the cis configuration for this proline. The decrease in stability may reflect a localized change in conformation since there are no changes in peptide ellipticity, but the proximity or orientation of the tryptophans relative to the disulfide is perturbed as indicated by the increased fluorescence yield. As illustrated in Figure 7, this result is consistent with the crystallographic structure of thioredoxin, which shows that the side chain of Pro-76 is in close contact with the disulfide (Eklund et al., 1984). The decreased equilibrium stability may account for the low expression of *trxA76* in *E. coli*. The majority of *trxA76* is probably in the reduced form intracellularly as observed for wild-type thioredoxin (Fagerstedt & Holmgren, 1982). Given the low melting temperature of reduced *trxA76*, it is possible that a significant fraction of this protein is unfolded in vivo. Pulse–chase labeling experiments indicate that wild-type thioredoxin is stable in vivo at 37 °C while *trxA76* is rapidly

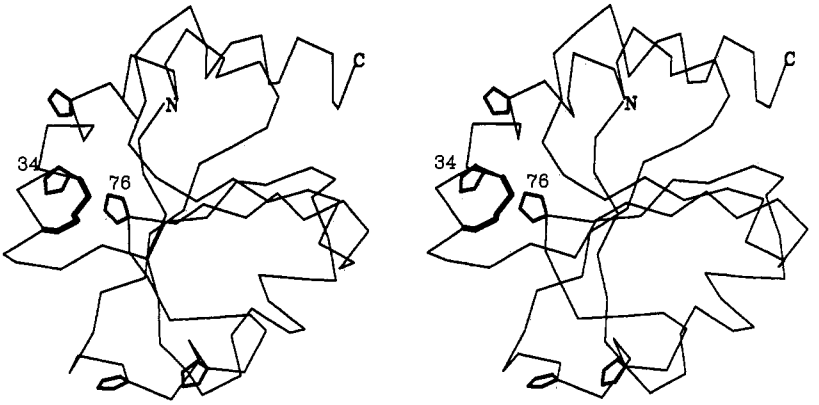

FIGURE 7: Stereoscopic view of the α-carbon chain of *E. coli* thioredoxin with the N- and C-termini labeled. A disulfide bond between the redox pair of cysteine residues 32 and 35 is shown as a thick line. The five proline residues with numbers 34 and 76 labeled are the only side chains shown. This figure was prepared from a coordinate list kindly supplied by Drs. H. Ecklund and C.-I. Bränden.

degraded (M. Russel, personal communication).

Although the change in equilibrium stability complicates a comparison of folding kinetics under identical conditions, it is clear that the slowest phase observed for *trxA76* refolding is at least an order of magnitude faster than the slowest phase detected for refolding of the wild-type protein. This suggests that the dominant slowest phase in thioredoxin folding is lost, but the two other phases are retained, when an alanine is substituted for proline-76. These results confirm the assignment (Kelley & Stellwagen, 1984) of the slowest kinetic phase to the isomerization of proline-76.

Our results can be constrasted with the results of a study on Pro-71 mutants of iso-1-cytochrome *c* (Ramdas & Nall, 1986). Replacement of Pro-71 with Val resulted in a change in the rate of the fastest phase in folding of iso-1-cytochrome *c*, but the α and τ of the slowest phase remained unchanged. The results of our study and that of Ramdas and Nall support the notion that not all proline residues in a protein contribute to slow phase folding through peptide bond isomerization as envisaged by Brandts et al. (1975). Isomerization of a proline residue may not lead to slow folding either because the native configuration is retained in the unfolded state, as might be the case for proline-34 in thioredoxin, or folding can proceed without isomerization of this residue (Levitt, 1981). As shown in Figure 7, proline-76 precedes an interior strand in the β-sheet. Stabilization of the β-sheet may require a cis peptide at position 76 such that complete folding must await isomerization of this residue.

The 3–4 kcal/mol decrease in stability observed for *trxA76* seems too large for a proline to alanine substitution. An alanine substitution is one of the most frequent allowed changes for a proline residue in related proteins from homologous organisms (Schulz & Schirmer, 1979). These two residues also have similar hydrophobicity (Bull & Breese, 1974; Eisenberg & McLachlan, 1986). Although our results do not directly address the question of the configuration of Ala-76 and *cis*-amide peptide bonds are found in natural proteins (Quiocho & Lipscomb, 1971), the decreased stability seems likely to result from incorporation of a trans peptide at position 76 in the native state. Obviously, a crystallographic determination of the structure of *trxA76* is needed to resolve this question; nonetheless, a reasonable argument for a *trans*-Ala-76 can be offered. First, if the native state tolerates only a cis peptide bond at position 76 and conformational events are faster than isomerization, then refolding of the alanine variant should be dominated by a single kinetic phase since almost 100% of denatured molecules would have this peptide in the trans configuration (Brandts et al., 1975). Second, several features proposed for the thioredoxin folding intermediate I_t (Kelley, 1984) are similar to those reported here for *trxA76*. I_t is proposed to have peptide ellipticity similar to that of the native state, tryptophan fluorescence intensity between those of native and denatured material, and little activity as a substrate for thioredoxin reductase. I_t appears to be populated transiently in the same range of denaturant concentration that native *trxA67* has equilibrium stability (Kelley et al., 1986a). Both species appear to have faster kinetics for unfolding and refolding than native wild-type thioredoxin. Finally, energy minimization calculations suggest that incorporating a nonnative proline isomer in BPTI results in small, localized structural changes (Levitt, 1981). Levitt's analysis indicates that at some positions in the polypeptide chain the wrong isomer may destabilize the native state, but not enough to prevent incorporation in a nativelike intermediate (a type II proline residue). Thus, it seems plausible that *trxA76* has the properties expected for a folded thioredoxin incorporating a trans peptide at position 76. This hypothesis predicts that the lower stability results from a restricted conformational space, a native state with Ala-76 cis may not be kinetically or thermodynamically accessible, and is relatively independent of the type of residue substituted for the proline.

Two kinetic phases are observed for both unfolding and refolding of *trxA76*, implying that a three-state mechanism describes the folding of this protein. Although a rigorous discrimination between kinetic mechanisms is not possible on the basis of the limited data presented here, at least one folding pathway can be readily eliminted. The simple three-state mechanism $U_1 \leftrightarrow U_2 \leftrightarrow N$, where the two unfolded states differ in the configuration of one or more of the proline residues 40, 64, and 68, is incompatible with the kinetic data. Although the rate and activation enthalpy of the slower kinetic phase in refolding are consistent with a peptide isomerization, this mechanism cannot account for the observed dependence of α_2 for unfolding on denaturant concentration (see Table I). For this mechanism, α_2 for unfolding must increase as the ratio τ_2/τ_1 decreases (Hagerman, 1977). The lack of dependence of α_2 on final Gdn-HCl can only be accounted for by this mechanism if the $[U_1]/[U_2]$ equilibrium is strongly denaturant dependent. This feature seems unlikely for a proline peptide isomerization in an unfolded protein (Nall et al., 1978). This mechanism also predicts that α_2 should be the same for unfolding jumps starting from within or below the equilibrium transition zone. The disappearance of the slower phase for unfolding starting from 1.5 M Gdn-HCl suggests that folding involves a denaturant-dependent step in addition to the [N]/[U] equilibrium. A folded intermediate is required to

explain this result. However, it is unclear whether this state is on the folding pathway for denatured material, U ↔ I ↔ N, or is an abortive species, I ↔ U ↔ N. Two folded states may be populated below the transition zone, but only one within the transition zone since monophasic unfolding kinetics are observed for an initial Gdn-HCl concn. of 1.5 M.

Replacement of Pro-76 by Ala has simplified the folding kinetics of thioredoxin. A direct solution of the folding mechanism for *trxA76* should be possible especially since analytical solutions for three-state mechanisms are available (Hagerman, 1977; Ikai & Tanford, 1973). Since both kinetic phases are observed when the folding equilibrium is perturbed in either direction, a comparison of the unfolding and refolding kinetics at the same final conditions throughout the equilibrium transition zone should provide sufficient information to solve the mechanism.

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Registry No. Gdn-HCl, 50-01-1; Pro, 147-85-3.

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