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Amino Acid Residues Important for Folding of Thioredoxin Are Revealed Only by Study of the Physiologically Relevant Reduced Form of the Protein†

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ABSTRACT: Thioredoxin-1 from *Escherichia coli* has frequently been used as a model substrate in protein folding studies. However, for reasons of convenience, these studies have focused largely on oxidized thioredoxin and not on reduced thioredoxin, the more physiologically relevant species. Here we describe the first extensive characterization of the refolding kinetics and conformational thermodynamics of reduced thioredoxin. We have previously described a genetic screen that yielded mutant thioredoxin proteins that fold more slowly in both the oxidized and reduced forms. In this study, we apply our more detailed analysis of reduced thioredoxin folding to a larger number of folding mutants that includes those obtained from continuation of the genetic screen. We have identified mutant proteins that display folding defects specifically in the reduced state but not the oxidized state. Some of these substitutions represent unusual folding mutants in that they result in semiconservative substitutions at solvent-exposed positions in the folded conformation and do not appear to affect the conformational stability of the protein. Further, the genetic selection yields mutants at only a limited number of sites, pointing to perhaps the most critical amino acids in the folding pathway and underscoring, in particular, the role of the carboxy-terminal amino acids in the folding of thioredoxin. Our results demonstrate the importance of studying the physiologically relevant folding species.

Thioredoxin-1 (TrxA) of *Escherichia coli* is a cytoplasmic enzyme that maintains the cysteines of substrate proteins in the reduced form (*3*). It does this by utilizing one of its two redox-active cysteines (cysteine 32) to attack a substrate's disulfide bonds, thus initiating the reductive process that leads to a reduced substrate and an oxidized thioredoxin (*4*). The enzyme thioredoxin reductase then transfers electrons from NADPH to thioredoxin, returning its cysteines to the reduced state. Because of its abundance and relative ease of purification, thioredoxin has been the subject of numerous in vitro protein folding studies (e.g., refs (*5*−*10*), etc). However, most studies of thioredoxin folding have been conducted on the oxidized form of thioredoxin because it is rapidly air oxidized in the absence of a reducing agent, because oxidized thioredoxin is more stable than the reduced form, and because oxidized thioredoxin folds with simplified kinetics.

We previously described our use of a genetic screen to isolate new classes of mutants of thioredoxin that were defective for folding (*11*). When we examined their conformational stability and refolding kinetics in vitro, all but one of these folding mutants also showed significant defects in the refolding of the oxidized proteins. The exceptional mutant contained a relatively conservative substitution (aspartate to asparagine) at position 15, which is completely solvent-exposed in structural models of thioredoxin and had not been previously implicated in thioredoxin folding. Furthermore, the D15N mutant protein, which we presumed to cause a defect in thioredoxin folding in vivo, did not exhibit such a defect in preliminary in vitro studies on a reduced form of this mutant protein. Surprisingly, the D15N substitution had no effect on the rate of refolding of oxidized thioredoxin and even increased its conformational stability.

In this study, we revisited our genetic screen to isolate more thioredoxin folding mutants. We submitted several of the purified mutant proteins to a more rigorous analysis of their conformational dynamics and refolding kinetics. In addition, we conducted the first extensive folding analysis of the reduced form of thioredoxin and applied it to some of the mutant proteins. Among the mutations that we isolated were some that caused novel substitutions at D15 as well as substitutions at D13, which is also completely solvent exposed in the thioredoxin structure. These substitutions cause defects in the refolding kinetics of the reduced, but not the oxidized, forms of these proteins. We also find that a high proportion of the folding mutations either affect the penultimate amino acid, L107, or alter the chain-terminating codon causing extension of the carboxy-terminal amino acid sequence. These findings, along with a L107A mutant constructed here, strengthen previous evidence of a key role of L107 in folding and suggest that altering the

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carboxy terminus of the protein in other ways may also interfere with folding (*11*). Our results demonstrate the importance of studying the physiologically relevant form of a protein in vitro and suggest that genetic studies of this sort may allow a significant narrowing of the list of candidates for key residues in folding.

EXPERIMENTAL PROCEDURES

Strain and Plasmid Construction and Growth Conditions. Strains and plasmids were constructed using standard genetic and molecular techniques (*12*, *13*). *E. coli* K-12 strains DRH119 [*araD139* Δ(*ara, leu*)*7697 galU galK* Δ*lacX74 rpsL thi* Δ*malF3* Δ(*phoA*[*PvuII*]) *phoR* Δ*dsbA*::Kan^R Δ*dsbC dsbD*::mini-Tn*10*Cam^R/F′ *lac-pro lacI*^{q}], DRH245 [F$^-$ Δ*lacX74 galE galK thi rpsL* Δ*phoA*(*PvuII*) *degP41*(Δ*Pst*I)::ΩKan^R Δ*ompT ptr-32*::ΩCam^R Δ*tsp3*::ΩKan^R *eda51*::Tn*10* Δ*trxA*], and JF521 [Δ(*lac, pro*) *thi supE metE46 srl300*::Tn*10 trxA2*(*7004*) *recA*/F′ *traD36 proAB lacI*^{Q} Δ*lacZ*(M15)] as well as plasmids pLMD82 (bearing the *phoAss-trxA* gene under control of the *phoA* promoter), pCFS122 [a modified pTrc99a plasmid (Promega) bearing the sequence encoding the *phoA* signal sequence], and pTK10trxA were described previously (*11*). All restriction enzymes were obtained from New England Biolabs. The mutant *trxA* genes were placed under *lac* control by PCR[1] amplification of a DNA fragment corresponding to the *trxA* gene from the mutant plasmids, into which a *Bsp*HI site was introduced at the 5′ end of the gene. The PCR fragment was subsequently digested with *Xba*I and *Bsp*HI and ligated with pCFS122 cut with *Nco*I and *Xba*I. Point mutations in the *trxA* gene were introduced into plasmid pTK10trxA by QuickChange mutagenesis (Invitrogen). Cells were generally grown at 37 °C in NZ medium (*14*). When necessary, ampicillin was added at a concentration of 200 μg/mL, chloramphenicol at 10 μg/mL, kanamycin at 40 μg/mL, and tetracycline at 15 μg/mL. Induction of *lac* promoter constructs was accomplished by addition of IPTG to a final concentration of 10 μM.

Mutagenesis. Mutagenesis of the *trxA* gene was conducted by mutagenic PCR using two general methods: (1) skewing the ratio of purine to pyrimidine dNTPs in the PCR and (2) conducting traditional PCR in the presence of mutagenic dNTP analogues. The first method was modified from that of Chen et al. (*15*) and is described in detail in the Supporting Information. Alternatively, four independent PCRs each were conducted in the presence of either 50 μM 8-oxo-dGTP (8-oxo-2′-deoxyguanosine 5′-triphosphate) or 50 μM dPTP (6*H*,8*H*,4-dihydropyrimido[4,5-*c*][1,2]-oxazin-7-one-8-β-D-2′-deoxyribofuranoside 5′-triphophate) (Jena Bioscience, Jena, Germany) using Invitrogen Platinum PCR Supermix (20 cycles of PCR) (*16*). To maintain the independence of any mutations that were isolated, each PCR mixture was digested and cloned separately. Each PCR mixture was individually cut with *Age*I and *Xba*I and ligated into pLMD82 cut with the same enzymes.

Genetic Screen for Thioredoxin Folding Mutants. The genetic screen for thioredoxin folding mutants was conducted as described previously (*11*). See the Supporting Information for a more detailed description of the genetic screen.

Subcellular Fractionation. Fractionations were performed as described previously (*1*).

Expression and Purification of Thioredoxin Mutant Proteins. Thioredoxin was purified as described previously (*11*). See the Supporting Information for details.

Thermal Denaturation Curves and Equilibrium Transition Curves Using Chemical Denaturation of Reduced Thioredoxin. Thermal denaturation curves and equilibrium denaturation and renaturation curves in the presence of guanidinium chloride (GdmCl) of reduced thioredoxin were determined by circular dichroism spectroscopy and fitted using standard procedures derived from a two-state model. Thioredoxin was kept reduced by the inclusion of 1 mM DTT in all solutions. For a more detailed description of the experimental procedures and data fitting, see the Supporting Information.

Thioredoxin Refolding Kinetics. The refolding kinetics of thioredoxin were determined as previously described (*11*). The refolding kinetics of reduced thioredoxin were determined in the presence of 1 mM DTT to maintain thioredoxin in the reduced state. A detailed description of the experimental procedures and data modeling can be found in the Supporting Information.

RESULTS

When the posttranslational signal sequence of the periplasmic protein alkaline phosphatase (PhoAss) is attached to thioredoxin-1, very little of the protein appears in the periplasm because it rapidly folds in the cytoplasm before it can be exported. However, amino acid substitutions that interfere with the folding of the PhoAss−thioredoxin fusion protein allow significant amounts of the protein to be exported to the periplasm (*11*). Although thioredoxin is normally involved in the reduction of disulfide bonds in the cytoplasm, when the mutant thioredoxins are localized to the periplasm, they can partially replace the activity of DsbA in promoting the formation of disulfide bonds (*2*). We previously detected such mutants by selecting for restoration of motility to an *E. coli* Δ*dsbA* strain carrying a *phoAss-trxA* gene that was mutagenized using a *mutD* mutator strain (*11*). Motility is dependent on a component of the flagellum, the FlgI protein, which contains a disulfide bond essential for its activity (*17*).

To obtain new classes of mutations, we mutagenized the thioredoxin-encoding portion of the *phoAss-trxA* gene using mutagenic PCR. Compared to mutagenesis of the plasmid in a *mutD* mutator strain, PCR has the potential to generate a greater variety of types of mutations (e.g., increased frequency of transversion mutations) by altering the ratio of dNTPs present in the PCR or by doping the PCR with mutagenic base analogues, such as 8-oxo-dGTP (see Experimental Procedures). After ligating the mutagenized PCR product into plasmid pLMD82 cut with *Age*I and *Xba*I, we plated transformations of the mutagenized ligants into the *dsbA* screening strain (DRH119) onto the center of LB plates containing 0.35% agar and selected for increased motility by picking from the edge of the motility swarm. To ensure that the mutants were the result of independent mutational events, we isolated only one mutant per mutagenic PCR. We purified and sequenced plasmids from isolates that (1) exhibited increased motility after restreaking to colonies and (2) retained the increased motility phenotype upon retransformation. A more detailed description of the screen can be found in the Supporting Information and in ref *11*.

We obtained a total of 20 independently isolated *trxA* mutants (9 single mutants, 10 double mutants, and 1 triple mutant) in this

[1]Abbreviations: WT, wild-type; PhoA, alkaline phosphatase; PhoAss, alkaline phosphatase signal sequence; IPTG, isopropyl β-D-thiogalactopyranoside; 8-oxo-dGTP, 8-oxo-2′-deoxyguanosine 5′-triphosphate; dPTP, 6*H*,8*H*,4-dihydropyrimido[4,5-*c*][1,2]oxazin-7-one-8-β-D-2′-deoxyribofuranoside 5′-triphophate; DTT, dithiothreitol; GdmCl, guanidinium chloride; PCR, polymerase chain reaction.

selection, which resulted in the following amino acid substitutions: D13A, D15V, P34R, L107P, L107R, Stop109Q, Stop109Y, D9G/Stop109K, D13N/A29V, D15Y/Stop109K, L17P/Stop109K, G33C/Stop109L, P34S/Stop109Q, A46P/Stop109Q, A46T/A87V, T54P/Stop109S, I75T/Stop109K, D9N/A19V/Stop109Q (Table 1). In summary, we obtained mutations that resulted in substitutions at a total of 13 amino acid positions (including mutations at the UAA stop codon, which changed it to a sense codon and appended the sequence SRVDRCP to the C-terminus). Seven of these substitutions resulted in unique amino acid substitutions that were not identified in our previous screen. However, mutations at only three of these positions (changes to positions D13, I75, and Stop109) were isolated as single mutants. Mutations at the five remaining unique positions were all isolated as double or triple mutants, and only one double mutant (A46T/A87V) resulted in substitutions in which both amino acid substitutions were not otherwise isolated as single mutants. However, in this case, when separated from one another, neither single mutant caused increased motility in the *dsbA* screening strain. It appears that these separated mutations may not strongly affect folding unless they are combined with another similarly very weak mutation.

Table 1: Summary of Mutations Obtained in the Genetic Screen

amino acid substitution[a]	mutation	no. of independent isolations[b]
D9N[c,d]	GAC → AAC	1 (2)
D9G[c]	GAC → GGC	1
D13A	GAC → GCC	1
D13N[c]	GAC → AAC	1
D15V	GAT → GTT	1 (3)
D15N	GAT → AAT	0 (1)
D15Y[c]	GAT → TAT	1
A29V[c,d]	GCA → GTA	1 (8)
P34S[c,d]	CCG → CTG	1 (2)
P34R	CCG → CGG	1
I41T	ATT → ACT	0 (1)
I75T[c,d]	ATC → ACC	1 (3)
S95P	TCT → CCT	0 (2)
L107P[c,d]	CTG → CCG	1 (2)
L107R	CTG → CGG	1
Stop109Q	TAA → CAA	4
Stop109Y	TAA → TAT	1
Stop109S[c]	TAA → TCA	1
Stop109L[c]	TAA → TTA	1
Stop109K[c]	TAA → AAA	4

[a]Substitutions that were only isolated as double mutants in combination with a substitution at a position already known to cause an increased level of export of the PhoAss−thioredoxin fusion protein are not included. [b]The number in parentheses is the sum of the independent isolations in this work and ref 11. [c]Isolated only in combination with a second mutation in this study. [d]Isolated as a single mutation in ref 11.

trxA Mutants Result in an Increased Level of Export of Thioredoxin into the Periplasm. The increased motility promoted by our *trxA* mutants could result from either an increased level of export to the periplasm or increased activity of the small amount of thioredoxin that is translocated across the cytoplasmic membrane. If the amino acid substitutions affected protein folding, more thioredoxin should be translocated to the periplasm. We therefore placed a representative set of single mutants under the control of the inducible lactose promoter in plasmid pCFS122 and assayed for translocation by subcellular fractionation. According to a densitometric analysis of the periplasmic fractions in Western blots against thioredoxin, the mutants that we tested caused a 7−82% increase in the steady-state amount of thioredoxin in the periplasm (Figure 1).

The L107A Substitution Causes an Increased Level of Export of Thioredoxin to the Periplasm. On the basis of the folding defects in an L107P mutant of thioredoxin obtained in the previous study, we suggested that the penultimate amino acid of thioredoxin, L107, plays an important role in thioredoxin folding (*11*).

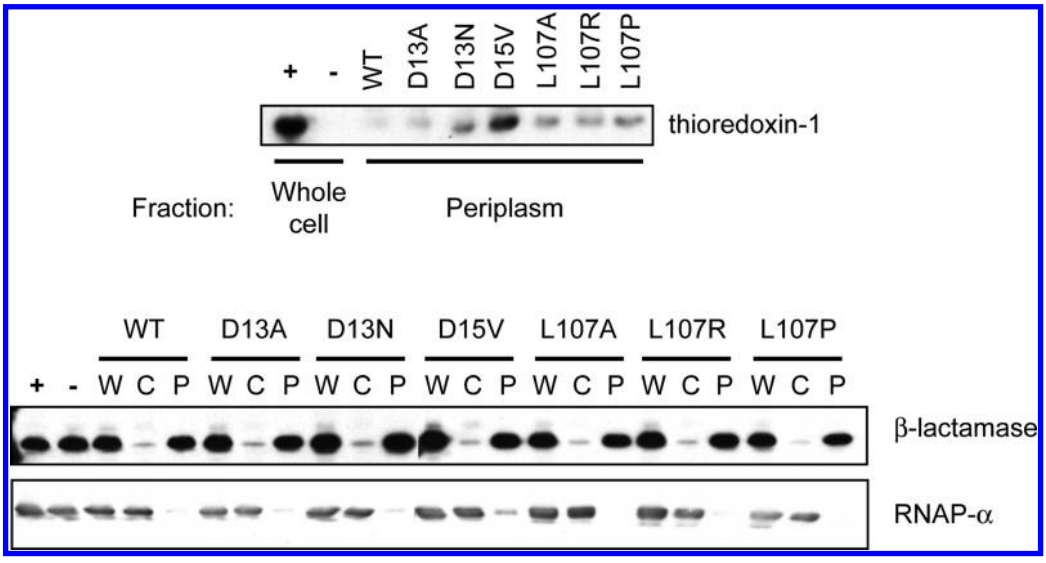

FIGURE 1: Mutations in thioredoxin that cause increased motility also result in an increased level of translocation to the periplasm. DRH245 (Δ*trxA*) cells, which are deficient for multiple periplasmic proteases, were transformed with plasmid pCFS122 (PhoAss), pCFS126 (PhoAss-thioredoxin), pDRH503 [PhoAss-thioredoxin(D13A)], pDRH504 [PhoAss-thioredoxin(D15V)], pDRH506 [PhoAss-thioredoxin-(L107R)], pMER209 [PhoAss-thioredoxin(L107A)], or pMER210 [PhoAss-thioredoxin(D13N)] and separated into whole cell, cytoplasm, and periplasm fractions by subcellular fractionation. (Top) Equal amounts of the whole cell or periplasmic fractions, as indicated, were analyzed by SDS−PAGE and Western blotting against thioredoxin-1. (Bottom) To ensure that we separated the periplasmic and cytoplasmic fractions and that the fractions contained similar amounts of protein, equal amounts of the whole cell (W), cytoplasmic (C), and periplasmic (P) fractions were resolved by SDS−PAGE and analyzed by Western blotting against β-lactamase as a periplasmic control or the α subunit of RNA polymerase (RNAP-α) as a cytoplasmic control.

The isolation of the L107R alteration reported here is consistent with this proposal. However, it may not be that L107 is essential for folding because both of these amino acid substitutions could have drastic effects via replacement of a hydrophobic amino acid with either a strongly charged amino acid or an amino acid that can significantly alter structure. To determine whether a less dramatic change would have an effect on thioredoxin folding, we generated a mutant protein that contained an alanine substitution at L107, which reduces the length of the aliphatic side chain at position 107. The L107A substitution caused increased motility when expressed in our *dsbA* screening strain, and we observed an increased amount of the L107A variant in the periplasm (Figure 1). We therefore included L107A, along with several other mutants, in the following studies of folding and stability.

Conformational Stability of Reduced Mutant Thioredoxins. We proceeded to measure and compare the conformational stability of several thioredoxin folding mutant proteins obtained both here and in the previous study. To this end, we introduced mutations into the *trxA* gene on plasmid pTK100, resulting in L107P, L107A, D15N, D15V, and D13N substitutions, by site-directed mutagenesis and purified these variants in addition to wild-type thioredoxin. Our interest in studying the substitutions at D13 and D15 comes from the surprising finding in our previous study that the D15N variant of thioredoxin, which restores motility to the same extent as many of the other mutants, had an increased overall conformational stability in its oxidized state compared to that of wild-type thioredoxin and had no measurable effect on the kinetics of folding (*11*). The isolation of a mutation resulting in a D15V change reported here enhanced our interest in the role of this residue. Further, amino acids D15 and D13 are part of a local cluster of surface-exposed aspartate residues near the N-terminus of thioredoxin, which drew our attention to the D13N substitution (*6*). D9, which we identified in our previous study as being important for thioredoxin folding, is part of the same cluster but is partially buried in the tertiary structure.

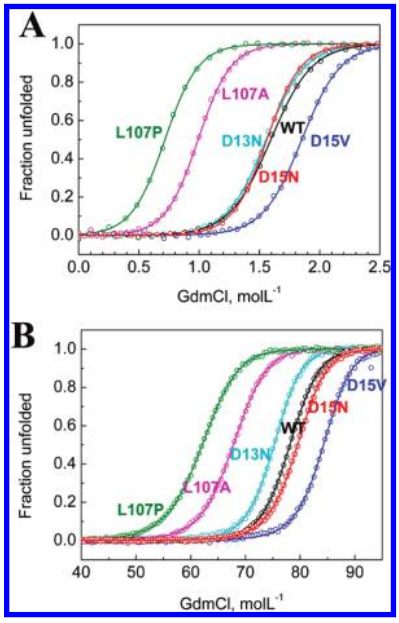

FIGURE 2: Chemical and thermal denaturation melting curves of reduced thioredoxin variants as determined by CD spectroscopy. The ellipticity at 222 nm (a measure of α-helical content) of wild-type thioredoxin and the indicated thioredoxin variants was measured (A) in the presence of chemical denaturant or (B) as a function of temperature for calculation of the thermodynamic parameters for the folding of these proteins: black for wild-type thioredoxin, cyan for D13N thioredoxin, red for D15N thioredoxin, blue for D15V thioredoxin, and pink for L107A thioredoxin.

Because we hadn't seen any effect on folding of D15N using the oxidized form of thioredoxin (*11*), we conducted most of the following experiments on the mutant thioredoxin proteins under reducing conditions (1 mM DTT). Reduced thioredoxin is the form of the protein that appears from the ribosome and is almost certainly the species of the protein that folds into its final conformation.

We first probed the conformational stability of the reduced mutant thioredoxins by monitoring circular dichroism at 222 nm in the presence of a chemical denaturant (Figure 2A) or heat (Figure 2B). Although Kelley et al. (*18*) reported that thermal denaturation of reduced or alkylated thioredioxin is not reversible, Ladbury et al. (*19*) found a minimal 90% reversibility (at a minimal protein concentration of 1.9 mg/mL) when heated to a temparature not more than 10 °C above the T_M. We therefore deduced the thermodynamic parameters from the temperature-dependent ellipticity of the wild type and thioredoxin variants at 0.1 mg/mL within the high-temperature limit of reversible unfolding (i.e., 10 °C above the T_M value). GdmCl denaturation was reversible because the folding and unfolding curves, which were acquired independently, were superimposable within the experimental accuracy. No significant difference in the ΔG_{H_2O} and half unfolding GdmCl concentration was found when the unfolding−refolding curve was established from mixing at equilibrium native and totally unfolded thioredoxin at the same concentration in the presence of GdmCl at various concentrations.

As expected, both the L107P and L107A substitutions result in a substantial destabilization in the conformational stability of reduced thioredoxin (Table 2). The L107P substitution causes a 16 °C decrease in the T_M, a 24 kcal/mol decrease in the ΔH_{T_M} according to the thermal denaturation curves, and a >2.5 kcal/mol increase in the ΔG_{H_2O} according to the chemical denaturation and renaturation curves. The destabilization caused by the L107A substitution was less severe: a 10 °C decrease in T_M, a 15 kcal/mol decrease in ΔH_{T_M}, and a 1.5 kcal/mol decrease in ΔG_{H_2O}.

Table 2: Thermodynamic Parameters of the Reduced Forms of the Thioredoxin Variants Determined by CD Spectroscopy

	$\Delta G_{H_2O}{}^{a,b}$ (kcal/mol)	$m^{a,b}$ (kcal mol^{-1} M^{-1})	$[GdmCl]_{1/2}{}^{a}$ (M)	$\Delta H_{T_M}{}^{c,b}$ (kcal/mol)	$\Delta C_p{}^{c,b}$ (kcal mol^{-1} K^{-1})	$T_M{}^{c,b}$ [°C (K)]
wild type	5.41 ± 0.19	3.39 ± 0.12	1.60	91.00 ± 0.67	1.52 ± 0.12	78 (351) ± 0.04
D13N	5.59 ± 0.08	3.60 ± 0.05	1.55	95.07 ± 0.95	2.67 ± 021	75 (348) ± 0.04
D15N	6.38 ± 0.08	4.09 ± 0.05	1.56	91.28 ± 0.66	2.73 ± 0.04	80 (353) ± 0.03
D15V	6.69 ± 0.14	3.61 ± 0.08	1.85	95.81 ± 2.14	2.40 ± 0.14	84 (357) ± 0.14
L107P	3.00 ± 0.13	4.20 ± 0.16	0.71	66.62 ± 0.44	1.43 ± 0.07	62 (335) ± 0.03
L107A	4.23 ± 0.11	4.35 ± 0.10	0.97	75.65 ± 0.59	1.55 ± 0.11	68 (341) ± 0.04

[a]Determined by chemical denaturation. [b]Confidence intervals are given as the standard error. [c]Determined by thermal denaturation.

In contrast to the L107 substitutions, the D13N, D15N, and D15V substitutions either had a minimal effect on the conformational stability or even slightly increased the stability of thioredoxin. The D13N substitution caused a small decrease (3 °C) in the T_M but did not have a significant effect on the ΔH_{T_M} or ΔG, and the D15N substitution resulted in a moderate stabilization of reduced thioredoxin (2 °C increase in T_M and 0.5 kcal/mol increase in ΔG), which is consistent with the parameters reported in our previous study. We observed a larger stabilization by the D15V substitution, which caused a 6 °C increase in T_M and an almost 1 kcal/mol increase in ΔG, as evidenced by a shift in the transition melting curves for both chemical and thermal denaturation.

Folding Kinetics of the Oxidized Mutant Thioredoxins. To evaluate the impact of the D13N, D15N, D15V, L107P, and L107A substitutions on the kinetics of thioredoxin folding, we recorded kinetics after rapid dilution of unfolded protein to native conditions using a stopped-flow device under both oxidizing and reducing conditions. According to the GdmCl-induced equilibrium unfolding and refolding curves, wild-type thioredoxin and all the mutant proteins were totally unfolded at 3.5 M GdmCl and recovered an ellipticity and intrinsic fluorescence indicative of the native state at GdmCl concentrations of < 0.1 M. We therefore observed the refolding by monitoring tryptophan fluorescence as a function of time after a rapid 40-fold dilution into a buffer of oxidized or reduced thioredoxin denatured in 3.5 M GdmHCl. Under these standard conditions, any change in the kinetic characteristics (number of phases, rate constants, or amplitudes) of variants relative to wild-type thioredoxin can be interpreted as an alteration in folding without having to consider the mechanism.

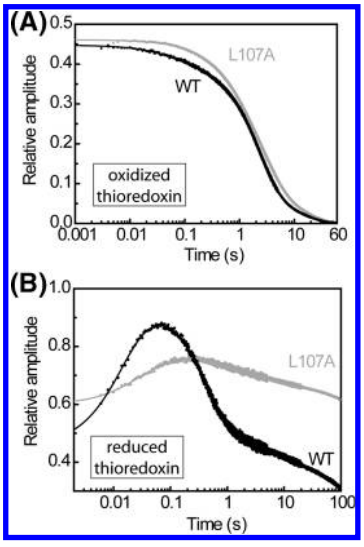

FIGURE 3: Example kinetic traces of tryptophan fluorescence. Wild-type (black) and L107A (gray) thioredoxin, denatured in 3.5 M GdmCl, were rapidly diluted 40-fold to refolding conditions by stopped-flow mixing. The refolding kinetics were recorded on three adjacent regions of time with the three following sampling periods: 0.001 s from 0 to 5 s, 0.01 s from 5 to 20 s, and 0.2 s from 20 to 600 s. The refolding was monitored by the intrinsic fluorescence of tryptophans 28 and 31, and the kinetics were normalized to the total amplitude between the native and unfolded states. (A) Refolding under oxidizing conditions. (B) Refolding in the presence of 1 mM DTT. The solid lines correspond to the best fits from nonlinear least-squares analysis using a multiexponential equation.

Oxidized WT thioredoxin folds with at least five phases: a rapid burst phase followed by four measurable folding phases (*5*). The initial burst phase generates a change in fluorescence with a time constant smaller than the observation dead time (2.5 ms). This phase is thought to correspond to the initial hydrophobic collapse of thioredoxin upon its return to renaturing conditions. The recorded fluorescence trace following the burst phase was best described by a curve containing three kinetic constants corresponding to three folding phases. We could not measure the slowest folding phase with stopped-flow mixing because of drift in the fluorescence signal over long periods of time. This folding phase corresponds to the slow trans to cis isomerization of P76 (*5*). However, none of the substitutions we identified are located immediately proximal to P76 and are thus not likely to influence the intrinsic rate of cis−trans proline isomerization at this position.

The rate constants that we derived for the first four folding phases of oxidized wild-type thioredoxin were consistent with the previously obtained kinetic constants (Table S1 of the Supporting Information) (*5*, *11*). As reported in our previous study, the L107P substitution caused a substantial defect in the first and second folding phases of oxidized thioredoxin, while the D15N substitution appears to have little or no effect on the folding kinetics of oxidized thioredoxin. Of the substitutions discussed in this study, the L107A substitution causes a defect in the rate of thioredoxin folding that is less severe than the defect caused by the L107P substitution. In addition, fitting of the L107A folding curve required the inclusion of a fourth kinetic component between the fastest and second refolding phase ($k = 1.27\ s^{-1}$). In contrast, the two mutants D13N and D15V did not cause any apparent defects in the folding kinetics of oxidized thioredoxin and, in the case of the D13N substitution, even appeared to accelerate the first folding step following the burst phase.

Folding Kinetics of the Reduced Mutant Thioredoxins. In an effort to resolve the perplexing results with the three mutant proteins, D13N, D15N, and D15V, which had no apparent folding (or stability) defects, we determined the refolding kinetics of the reduced form of these proteins, the biologically more relevant species (Figure 3B). Again, we included L107P and L107A in these studies. Under a standard set of conditions in the presence of 1 mM DTT, we found that reduced WT thioredoxin folds with at least six kinetic phases, including a burst phase and

Table 3: Folding Rate Constants of the Reduced Thioredoxin Variants Determined via Tryptophan Fluorescence

	burst (%)[a]	k_1 (s^{-1})[b]	k_2 (s^{-1})[b]	k_3 (s^{-1})[b]	k_4 (s^{-1})[b]	k_5 (s^{-1})[b]
WT	57	75.6 ± 7.5[b]	20.4 ± 2.7	2.52 ± 0.05	0.248 ± 0.037	(8.7 ± 2.9) × 10^{-4}
D13N	43	104 ± 21	45.6 ± 17.5	2.19 ± 0.23	0.090 ± 0.020	(9.0 ± 1.8) × 10^{-4}
D15N	54	100 ± 28	14.3 ± 1.3	2.19 ± 0.11	0.109 ± 0.013	(0.00110 ± 2.6) × 10^{-4}
D15V	43	34.0 ± 7.9	4.61 ± 0.27	0.734 ± 0.017	0.087 ± 0.026	(7.4 ± 2.9) × 10^{-4}
L107P	57	46.3 ± 10.7	6.76 ± 1.48	0.933 ± 0.172	0.070 ± 0.013	(9.5 ± 1.4) × 10^{-4}
L107A	40	75.1 ± 8.8	20.6 ± 4.5	0.751 ± 0.099	0.082 ± 0.029	(6.5 ± 1.0) × 10^{-4}

[a]The amplitude of the burst phase is given as the percentage of the total fluorescence change. [b]The confidence intervals (standard deviation) were calculated by fitting at least five individual kinetics.

five observable folding phases represented by rate constants k_1-k_5. Four of these phases, the burst phase and the three slowest observable phases (k_3-k_5), resulted in quenching of the tryptophan fluorescence, whereas the two remaining phases (k_1 and k_2) resulted in a gain in tryptophan fluorescence.

In contrast to our studies with the oxidized form of thioredoxin mutant proteins, we found that all of the reduced mutant thioredoxins examined were defective in at least one of the five observable folding phases (Table 3). As expected from our previous study (*11*), the L107P substitution was the most severe and resulted in defects in four of the five observable folding phases (k_1-k_4). The L107A substitution appeared to cause less severe, but significant, defects in two of the folding phases (k_3 and k_4). This is consistent with our in vivo results that suggest that the folding of L107A thioredoxin is intermediate between those of WT and L107P thioredoxin in the cell.

The D15V mutant protein also had severe effects, causing defects in the first four folding phases of reduced thioredoxin. The defects caused by the D13N and D15N substitutions were more subtle. Both of these substitutions caused a moderate apparent increase (~25%) in k_1, and the D13N substitution caused a substantial increase (~2-fold) in k_2. However, both substitutions caused a >2-fold defect in the fourth rate constant (k_4). Because k_4 is nearly 2 orders of magnitude slower than k_1 or k_2, this defect could make up for the moderate increase in the rate of folding caused by the D13N and D15N substitutions. The defects observed for the reduced form of each of these three proteins are in marked contrast to the lack of any effects when the oxidized form of the proteins was studied.

DISCUSSION

We describe here the isolation of 20 catalytically active mutants of thioredoxin, which when fused to a posttranslational signal sequence, display more activity in the periplasm than the wild-type protein, presumably because they are better exported to the periplasm. In combination with those obtained in a previous study (*11*), we have now identified a total of 39 independently isolated mutants that result in increased activity of thioredoxin in the periplasm. In our previous publication, we mention several other attributes of thioredoxin that these mutations could affect (*11*). However, the combined observations that (i) all of the mutants that we examined display an increased level of export of thioredoxin to the periplasm in vivo and (ii) all of the mutant proteins that we examined in vitro are affected for folding suggest that the majority of the mutations that we identified cause folding defects. In some cases, the effect of these substitutions on thioredoxin folding seemed small. However, these small differences may be sufficient to allow more productive interaction with a component of posttranslational translocation machinery, such as the dedicated secretion chaperone SecB.

Although we used different mutagenic techniques with different mutational biases, many of the amino acid positions in the protein sequence were identified multiple times, sometimes with mutations that resulted in substitutions of different amino acids (often with different properties) in the same position. Some of these sites could represent mutational hot spots, although different mutagenic techniques usually give different hot spots (*20*−*23*). However, it seems likely that further screening using this method would not result in the identification of novel amino acid positions that are important for folding, which may be due to the limitations discussed in our initial paper (*11*).

The isolation of an L107R substitution, along with the previously obtained L107P substitution (*11*), suggests that position L107 is critical for stable folding of thioredoxin in vivo, and our finding that an L107A substitution also altered protein folding appears to support this notion. Structural modeling studies suggest that the shortening of the side chain at position 107 as in the L107A and L107P variants could reduce the number of van der Waals contacts with L53, L80, and L102 and could open the hydrophobic cluster formed by these residues to access by the solvent. In addition, these studies suggest that the arginine residue in the L107R variant does not fit in the hydrophobic pocket and would protrude into the solvent, which would open the pocket to access by the solvent. Moreover, loss of the L107 hydrophobic anchor might decrease the zipping rate of the amphiphilic terminal helix. Santos et al. (*7*) have recently reported studies on the folding of a subdomain of thioredoxin that also suggests L107 plays an important role. Our results with L107A thioredoxin suggest that alanine scanning in combination with our genetic screen could be a useful approach for identifying additional thioredoxin folding mutants.

The isolation of multiple, independently isolated mutations that alter residues near the C-terminus of thioredoxin (e.g., mutations that cause substitutions at S95, L107, and the termination codon) seems to suggest the general importance of the C-terminal region of thioredoxin for its folding. The mechanism via which the C-terminal extension, which resulted from a mutations at the termination codon, could cause increased motility in vivo is unknown. However, this C-terminal extension could also slow protein folding, perhaps by a mechanism similar to that of signal sequences (*24*−*26*). Regardless of the mechanism, the isolation of these substitutions could provide insight into the mechanism of protein folding in vivo. For example, the sequestration of residues critical for thioredoxin folding (e.g., L107) in the polypeptide exit channel of the ribosome ensures that thioredoxin cannot stably fold until it is fully synthesized. If this phenomenon is generalizable, other proteins, or domains of proteins, could contain residues critical to their folding near their C-termini. Alternatively, extending the open reading frame by eight amino acids in the termination codon mutants could allow more time for the cotranslational association of the translocation machinery with the nascent chain (*1*). Other studies of the export of PhoAss-thioredoxin have reported that extending the reading frame at the N-terminus between the signal sequence- and thioredoxin-encoding portions of the *phoA-trxA* gene results in a similar increase in the level of export (*2*).

Because the D13N, D15N, and D15V substitutions did not cause altered folding of oxidized thioredoxin, our in vitro characterization of these variants led us to a more extensive analysis of folding of the reduced form of thioredoxin. This characterization revealed that the kinetics of folding of these three mutant proteins are affected only in the reduced form. In contrast, the three mutant proteins have either an enhanced or unaltered conformational stability compared to that of wild-type thioredoxin. Taken together with our kinetic analysis, these results suggest that it is the rate of folding, rather than its stability, that is the more important factor causing an increased level of export of thioredoxin in vivo.

D13 and D15, along with D9 and D10, are part of series of negatively charged surface-exposed residues in the first helix of thioredoxin and, with the exception of D9 (*6*), do not appear to contribute substantially to the thermostability of the protein. Thus, the finding that substitutions at these residues could affect

folding of thioredoxin was unexpected. Indeed, in our previous study, we did not observe an effect on folding of the D15N substitution (*11*). However, an effect on the folding of reduced thioredoxin became apparent in this study with more measurements and at longer refolding times. One explanation for the observed effect on folding for these substitutions is that these residues could be involved in interactions required for the stabilization of an important folding step, interactions that no longer exist in the native structure. For example, according to our structural analysis, D13 forms a fully hydrated salt bridge with K18 in the same helix. The elimination of this interaction by the introduction of an asparagine residue does not appear to affect the thermostability of thioredoxin significantly but could slow the formation of this helix. Similarly, D15 could be involved in a transient interaction required for some step in thioredoxin folding, which is no longer present in the fully folded conformation. In addition, D15 is located above a hydrophobic pocket in the thioredoxin structure, and substitution with valine could result in the burying of the amino acid side chain. This artificial interaction could stabilize the fully folded conformation but may interfere with folding of thioredoxin. This possibility is supported by the small, but significant, increase in the ΔH_{T_M}, which suggests an increase in the degree of order of the protein that should result in a negative difference in the $T\Delta S$ terms between the D15V mutant and the WT proteins. Indeed, the $\Delta(T\Delta S)$ calculated from the experimental values of ΔH_{T_M} and ΔC_p deduced from the fits of the thermal transitions (see Table 2) is negative for all temperatures up to 64 °C (unpublished results).

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SUPPORTING INFORMATION AVAILABLE

A more detailed description of the materials and methods. This material is available free of charge via the Internet at http://pubs.acs.org.

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