A selection for mutants that interfere with folding of *Escherichia coli* thioredoxin-1 *in vivo*

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Contributed by Jon Beckwith, November 9, 2005

***Escherichia coli* thioredoxin is normally a cytoplasmic protein involved in the reduction of disulfide bonds. However, thioredoxin can be translocated to the periplasm when it is attached to a cotranslational signal sequence. When exported to the periplasm, it can partially replace the activity of DsbA in promoting the formation of disulfide bonds. In contrast, when thioredoxin is fused to a posttranslational signal sequence, very little of it appears in the periplasm. We propose that this absence of posttranslational export is due to the rapid folding of thioredoxin in the cytoplasm. We sought mutants of thioredoxin that retarded its folding in the cytoplasm, which we accomplished by fusing thioredoxin to a posttranslational signal sequence and selecting for mutants in which thioredoxin was exported to the periplasm, where it could replace DsbA. The collection of mutants obtained represents a limited number of amino acid changes in the protein. *In vitro* studies on purified mutant proteins show that all but one are defective in the kinetics and thermodynamics of protein folding. We propose that the slower folding of the thioredoxin mutant proteins in the cytoplasm allows their export by a posttranslational pathway. We discuss some implications of this class of mutants for aspects of the folding pathway of thioredoxin and for its mechanism of export. In particular, the finding that a folding mutant that allows protein translocation alters an amino acid at the C terminus of the protein suggests that the degree to which thioredoxin folds during its translation must be severely restricted.**

in vivo protein folding | protein folding | protein translocation | thioredoxin folding

The issue of protein folding is intimately connected with mechanisms of protein secretion. In *Escherichia coli*, proteins that are exported to the periplasm by the general secretory pathway must be in an unfolded state to pass through the membrane-embedded secretion machinery (1). Yet most of these proteins are exported only after much of the polypeptide chain has been synthesized (i.e., posttranslationally) (2). Without some mechanism for maintaining these proteins in a secretion-competent state, there is ample time for these proteins to fold and become trapped in the cytoplasm. To avoid this problem, the cell has evolved a number of means to prevent premature folding of exported proteins in the cytoplasm. For example, the dedicated chaperone, SecB, specifically recognizes a subset of proteins and holds them in an unfolded state until it can target them to the secretion machinery (3). In some cases, the signal sequence itself can slow the folding process enough to allow a productive interaction with the secretion machinery (4). Whether or not a protein folds into an export-incompetent state can also be influenced by the presence or absence of certain covalent modifications. Some proteins that are destined to be exported to the cell envelope require disulfide bonds to fold stably. However, because they cannot acquire these bonds in the cytoplasm, their cysteines remain reduced, and the proteins are maintained in a secretion-competent state (5). Another means by which the problem of cytoplasmic folding is avoided in the secretion process is by exporting proteins cotranslationally via the signal-recognition particle (SRP) pathway, thereby preventing the formation of cytoplasmic folding intermediates altogether (6).

Mutant analysis of exported proteins further supports the correlation between the folded state of the protein and its ability to be exported posttranslationally. Certain mutations in the signal sequence of maltose-binding protein or ribose-binding protein kinetically slow the process of export. In such mutants, much of the maltose-binding or ribose-binding protein folds before it can be exported and becomes trapped in the cytoplasm. However, protein folding mutants of these two proteins appeared in selections for restored export of the signal-sequence defective versions (7–9).

In contrast to exported proteins, proteins that are destined to remain in the cytoplasm presumably do not require any anti-folding factors. Consequently, attempts to export cytoplasmic proteins by a posttranslational mechanism have met with variable success. For example, efforts to efficiently export the cytoplasmic protein thioredoxin-1 to the periplasm by a posttranslational pathway have been unsuccessful (10, 11). Attaching the alkaline phosphatase (PhoA) signal sequence (a posttranslational signal sequence) to thioredoxin-1 of *E. coli* results in only a very small amount of the protein reaching the periplasm. We have proposed that this poor export of thioredoxin is due to the rapid folding of the protein in the cytoplasm, preventing its posttranslational translocation across the cytoplasmic membrane (11). In support of this model, thioredoxin is efficiently exported by the cotranslational pathway. which bypasses cytoplasmic folding intermediates (11).

We sought to isolate folding mutants of thioredoxin because it is an attractive subject for protein folding studies *in vivo* and *in vitro*: It is a small, abundant protein, is easily purified, and has a well defined activity. Its structure has been determined by several methods (12, 13), and it is not essential for viability in *E. coli*, making it amenable to genetic manipulation. Because of these characteristics, thioredoxin has been the subject of protein folding studies (14–17). In this paper, we describe a means of isolating folding mutants of thioredoxin. The genetic selection for such mutants is based on our proposal that the poor export of thioredoxin to the periplasm when it is fused to the PhoA signal sequence is due to rapid cytoplasmic folding. Previously, we have shown that the small amount of thioredoxin that is exported to the periplasm from this construct very weakly

Conflict of interest statement: No conflicts declared.

Abbreviations: PhoA, alkaline phosphatase; SRP, signal-recognition particle; T_M, transition temperature.

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substitutes for the protein DsbA, the protein responsible for disulfide bond formation (10). This activity occurs because DsbB, the dedicated oxidant of DsbA, is able to oxidize exported thioredoxin, thus allowing it to donate disulfide bonds to periplasmic substrates (18, 19). In a *dsbA* deletion strain, this small amount of exported thioredoxin is able to oxidize small amounts of substrates of DsbA such as FlgI, a component of the flagella required for motility (20). To seek mutants defective in thioredoxin folding, we selected for mutants with increased motility starting with a strain carrying a *dsbA*-null mutation and expressing thioredoxin fused to the PhoA signal sequence. All of the mutants we obtained result in a substantial increase in the export of thioredoxin to the periplasm. Characterization of the purified mutant proteins shows that all but one of the mutant proteins have significant defects in their folding properties *in vitro*. The study of these mutants reported here and further characterization should contribute to an understanding of the *in vivo* folding pathway for thioredoxin.

Materials and Methods

Strain and Plasmid Construction and Growth Conditions. Strains and plasmids are listed in Table 3, which is published as supporting information on the PNAS web site, and were constructed by using standard genetic and molecular techniques (21, 22). All restriction enzymes were obtained from New England Biolabs. The mutant *trxA* genes were placed under *lac* control by amplifying the DNA corresponding to the *trxA* gene from the mutant plasmids by PCR using primers that introduce a BspHI site at the 5′ end of the gene and ligating into pCFS122 cut with NcoI and XbaI. Cells were generally grown at 37°C in NZ medium (23). When necessary, 200 μg/ml ampicillin, 10 μg/ml chloramphenicol, 40 μg/ml kanamycin, and 15 μg/ml tetracycline were added. Induction of *lac* promoter constructs was accomplished by addition of isopropyl β-D-thiogalactoside to a final concentration of 10 μM.

Mutagenesis. Plasmid pLMD82 carrying the gene encoding the PhoAss-TrxA fusion was mutagenized by passage through a *mutD5* mutator strain in two separate rounds of mutagenesis. Initially, 24 Mal^+ transformants were used to start independent cultures in NZ medium and grown at 37°C. Plasmid DNA was prepped (Qiagen, Valencia, CA) from cultures that contained mixed Mal^+ and Mal^- cells as determined by plating on MacConkey medium containing 1% maltose. The preps were then pooled to be used in the genetic screen. In a second round of mutagenesis, individual Mal^+ transformants were used to start 24 independent cultures in NZ medium and 24 independent cultures in M63 minimal medium supplemented with 0.2% glucose. Plasmid DNA from each of these cultures was prepped and used as independent mutagenized pools in the genetic screen.

Genetic Screen for Thioredoxin Folding Mutants. The mutagenized pools were transformed into either strain RI249 (*dsbA*::Kan^R Δ*dsbC*::Cam^R *dsbD*::miniTn*10*Cam^R.*cadC1*::Tn10) or strain DRH119 (Δ*dsbA*::Kan^R Δ*dsbC dsbD*::miniTn*10*Cam^R) to have between 5,000 and 10,000 colonies in 25 μl spotted in the center of a NZ motility plate (0.35% agar) with ampicillin. After 3–4 days of incubation at 30°C, the fringe of the growth on each plate exhibiting greater motility than the control was picked and streaked onto a NZ plate with ampicillin. Independent colonies picked from these streaks were tested for the increased motility phenotype. Plasmid from colonies exhibiting increased motility was miniprepped and retransformed into the parent strain (RI249 or DRH119). Plasmids that retained the increased motility phenotype were sequenced. The AgeI–XbaI fragment from each of the mutants was subcloned into the unmutagenized parent vector (pLMD82) and retested for motility.

Subcellular Fractionation. Fractionations were performed as described in ref. 11. DRH245 cells (*degP41* Δ*ompT ptr-32* Δ*tsp3* Δ*trxA*) containing the appropriate plasmid were grown to saturation and then subcultured 1:100 and grown to an OD_{600} of 0.5. Cultures were centrifuged, and pellets were resuspended in 50 mM Tris, pH 8/18% sucrose/1 mM $CaCl_2$·EDTA (0.5 mM) and lysozyme (0.5 μg/ml) were added, and samples were left on ice for 30 min before centrifugation at 3,100 × *g* in a bench-top centrifuge for 5 min. Supernatants and pellets were used as periplasmic and spheroplast fractions, respectively.

Western Blots. Cell extracts were subjected to SDS/PAGE and transferred to nitrocellulose membrane by using a semidry apparatus from Bio-Rad. Rabbit anti-thioredoxin-1 and anti-β-lactamase antibodies for probing membranes were obtained from Sigma and 5 Prime → 3 Prime, respectively. Immunodetection was done according to ECL protocol (Amersham Pharmacia) with streptavidin–horseradish peroxidase-linked anti-rabbit antiserum (Amersham Pharmacia).

Expression and Purification of Thioredoxin Mutant Proteins. Thioredoxin was purified according to a method derived from that of Kemple *et al.* (24). WT thioredoxin (without the PhoA signal sequence) was expressed from the expression plasmid pTK100. Mutants of thioredoxin were introduced into pTK100 by QuikChange site-directed mutagenesis (Stratagene). Plasmid pTK100 bearing the mutated thioredoxin genes were transformed into *E. coli* JF521 [Δ(*lac, pro*) *thi supE metE46 srl300*::Tn*10 trxA2*(*7004*) *recA*/F′ *traD36 proAB lacI*^{Q} ΔlacZ(M15)] and grown overnight in superbroth (25) for protein expression. Each variant was purified to apparent homogeneity by gel filtration chromatography on a XK 50/100 Sephacryl S-100 column (Amersham Pharmacia) using 30 mM Tris·HCl, pH 8.3/1 mM EDTA as the mobile phase. Purified proteins were stored as ammonium sulfate precipitates. The homogeneity of each protein sample was characterized by SDS/PAGE silver staining and analytical gel filtration. Protein concentration was determined spectrophotometrically by using the average molecular mass and the extinction coefficient ε_{280} = 14,100 M^{-1} cm^{-1}. Precipitates were resuspended in 7 M GuHCl/10 mM KP_i buffer at pH 7.5. The buffer of each protein sample was exchanged to 10 mM KP_i buffer at pH 7.5 or 6.5 by using a Sephadex G25 column and further concentrated as required.

Thioredoxin Activity Assays. Thioredoxin activity was determined by using 5,5′-di-thiobis(2-nitrobenzoic acid) (DTNB) as described in ref. 26. Thioredoxin (0.2 μM) was added to reaction buffer (0.1 M Tris·HCl, pH 8.0/2 mM EDTA/0.1 mg/ml BSA/0.5 mM DTNB/0.24 mM NADPH) containing 40 nM thioredoxin reductase (Sigma). Reduction of DTNB to 2-nitro, 5-thiobenzoic acid (TNB) was measured spectrophotometrically by absorbance at 412 nm. The concentration of TNB was calculated by using an extinction coefficient ε_{412} = 13.6 mM^{-1} cm^{-1}. The activity was converted into mmol NADPH per min by assuming 2 mol of TNB formed per 1 mol of NADPH consumed.

Circular Dichroism Spectroscopy. Far- and near-UV circular dichroism spectra were obtained on an Aviv 215 spectropolarimeter (Aviv Associates, Lakewood, NJ). Samples (0.5–1.0 mg/ml) were dialyzed against 10 mM phosphate buffer (pH 7.0) before recording the spectrum. Corresponding baselines were obtained with dialysis buffer under the same conditions and then subtracted from the sample spectrum.

1D-^{1}H-NMR Spectroscopy. Samples containing ≈0.3 mM Trx variants in 10 mM KP_i/90% H_2O/10% D_2O, pH 6.5 (uncorrected) were used to acquire data on a 600-MHz Varian Unity Plus spectrometer (Varian) at 25°C. Data processing was carried out

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on a Silicon Graphics computer by using FELIX 2.3 (Biosym Technologies, San Diego).

Stopped-Flow Experiments. Refolding kinetics were recorded in a SF 300 stopped-flow device from Biologic (Grenoble, France) equipped with fluorescence detection. The excitation wavelength was 295 nm (8-nm bandwidth), and the fluorescence emission was recorded through a 320-nm high-pass filter. Typically, WT and mutant thioredoxin were initially unfolded at 2 mg/ml by overnight dialysis against 3.5 M GdmCl in 10 mM potassium phosphate, pH 7.0. Refolding kinetics were initiated in the stopped-flow apparatus by mixing 10 μl of unfolded protein from a 3-ml syringe with 195 μl of refolding buffer (10 mM potassium phosphate) from each of the two 10-ml syringes. Where noted, 1 mM DTT was added to the dialysis and refolding buffers. The final concentration of GdmCl thus was 0.087 mM, and the final concentration of protein was 50 μg/ml.

Analysis of Kinetic Data. The kinetic traces were analyzed by using FIGP software from Biosoft (Cambridge, U.K.) by nonlinear least squares fitting to the equation $F(t) = \Sigma F_i \exp(-k_i t) + F_\infty$ where $F(t)$ is the fluorescence at time t, F_i and k_i are the amplitude and the rate constant of the phase i, respectively, and F_∞ is the fluorescence at infinite time. The number i of phases was increased until no significant improvement of the fit was observed as judged from the χ^2 value.

Differential Scanning Calorimetry. Calorimetric measurements of Trx mutants were performed by using a VP-DSC microcalorimeter (MicroCal, Northampton, MA) with a scan rate of 1.5°/min as described in ref. 27. The concentration of Trx mutants in potassium phosphate buffer (pH 6.5) varied between 0.3 and 1.8 mg/ml. Samples were filtered and centrifuged at $>15{,}000 \times g$ before measurements. The reversibility of the differential scanning calorimetry (DSC) scan was tested by heating and reheating the sample. In all cases, the reversibility was >75%. DSC measurements were analyzed according to a two-state transition model based on previous reports (27) to obtain the transition temperature (T_M) and the enthalpy change of unfolding (ΔH) at the T_M.

Results

A Genetic Screen for Folding Mutants. To seek mutants potentially defective in thioredoxin folding, we mutagenized a plasmid expressing the PhoAss–thioredoxin fusion protein (pLMD82) by passaging it through a *mutD5* mutator strain. Mutagenized plasmid was transformed into a nonmotile Δ*dsbA* screening strain (RI249 or DRH119), and motile mutants were selected. The screening strains also contain mutations in *dsbC* and *dsbD* to eliminate those motile derivatives that were due to alterations of the DsbD–DsbC pathway (23). We performed a total of 49 independent selections.

To confirm that suppression of the motility defect was caused by the plasmid, we retransformed our screening strain with plasmid DNA purified from isolated mutants. We sequenced all of the mutants that retained the increased motility phenotype upon retransformation. To confirm that the mutations in the *trxA* coding sequence caused the observed phenotype, we subcloned the portion of the fusion corresponding to the *trxA* gene into an unmutagenized parent vector and demonstrated the ability of these transformants to promote increased motility.

By this process, we isolated the following mutations in thioredoxin (numbered according to the amino acid sequence of thioredoxin): D9N (G**A**C→**A**AC), D15N (G**A**T→**A**AT), A29V (G**C**A→G**T**A), P34S (C**C**G→C**T**G), I41T (A**T**T→A**C**T), I75T (A**T**C→A**C**C), S95P (**T**CT→**C**CT), and L107P (C**T**G→C**C**G) (Fig. 1; see also Fig. 4, which is published as supporting information on the PNAS web site). In addition, we isolated a proline-to-leucine (C**C**G→C**T**G) change in the PhoA signal sequence at position −10 relative to the start of thioredoxin. This mutation makes the signal sequence significantly more hydrophobic than the cutoff for SRP-dependent signal sequences as determined by Huber *et al.* (6) and likely causes thioredoxin to be exported by the SRP pathway (see *Discussion*). We independently isolated the A29V change at least seven times, the I75T change four times, and the P(−10)L change twice.

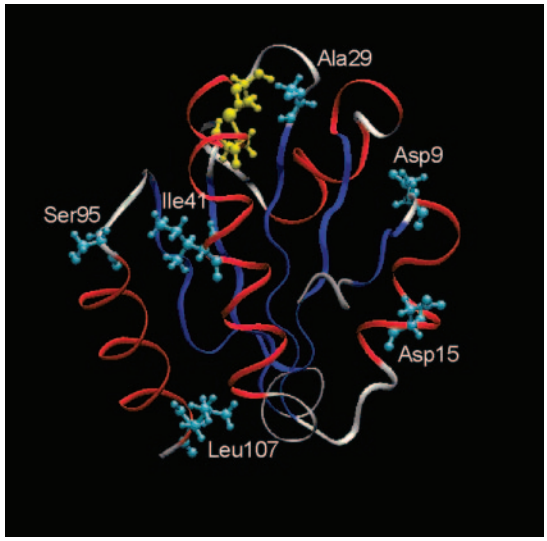

Fig. 1. Location of thioredoxin mutants in the three-dimensional structure. Shown is a ribbon diagram of thioredoxin from Protein Data Bank entry 1XOA. Sheets are blue, loops are white, and helices are red. Residues of the WT protein that were changed in our mutants are labeled and in cyan in stick diagrams. The active site of thioredoxin is yellow and in a stick diagram. The view is with the N terminus of thioredoxin in the foreground. The figure was generated by using VMD.

Assay for Increased Export of Mutants. We reasoned that our mutants could cause increased motility by one of at least three mechanisms: (*i*) by slowing folding or causing unfolding of thioredoxin enough to allow a more productive interaction with the secretion machinery, (*ii*) by increasing the fusion protein's affinity for or kinetics of interaction with the secretion machinery (e.g., signal sequence mutants), or (*iii*) by altering the enzymatic properties of the small amount of exported protein so that it is better able to substitute for DsbA. Mutants of classes *i* and *ii* should result in increased accumulation of thioredoxin in the periplasm and should be detectable by fractionating spheroplasts into a periplasmic and a cytoplasm plus membrane fraction and Western blotting against thioredoxin antibody. However, a number of our mutants were expressed at very low levels in strain DRH120, making it difficult to detect increased amounts in the periplasmic fractions. We assumed this low expression to be due to decreased stability and proteolysis because all of the mutations occur in the coding sequence for thioredoxin. To avoid this problem, we expressed all of the mutant proteins in a strain deficient for multiple periplasmic proteases, DRH245. In addition, to gain tighter control over expression, we placed our mutants under control of the *lac* promoter on plasmid pDSW206. Because we assumed that signal sequence mutant P(−10)L caused suppression by mechanism *ii*, we did not study it further. For the remaining mutants, more thioredoxin accumulated in the periplasm in the protease-deficient strain than was seen with the WT protein, indicating that export of the mutant proteins had been substantially increased (Fig. 2).

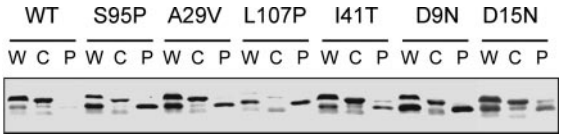

Fig. 2. Thioredoxin mutants result in increased export to the periplasm. Cells deficient for multiple periplasmic proteases (DRH245) expressing WT (pCFS126), S95P (pDH10), A29V (pDH11), L107P (pDH12), I41T (pDH312), D9N (pDH313), or D15N (pDH314) thioredoxin fused to the PhoA signal sequence were fractionated by spheroplasting and analyzed by Western blot against thioredoxin. A cross-reacting cytoplasmic protein running just below thioredoxin is present in all cases. W, whole cell extract; C, cytoplasm plus membrane fraction; P, periplasm fraction.

Purification of Mutant Proteins. To characterize the *in vitro* properties of our mutants, we purified WT thioredoxin and the following mutant proteins to near homogeneity: D9N, D15N, A29V, I41T, S95P, and L107P. Because we were interested only in mutations that altered amino acids within thioredoxin that specifically affect thioredoxin folding, we expressed the proteins (in the cytoplasm) without the PhoA signal sequence. Analytical gel filtration showed that all of the purified proteins were monomeric. In addition, circular dichroism and 1D-^{1}H-NMR experiments (Fig. 5, which is published as supporting information on the PNAS web site) showed that the proteins are able to adopt a native structure similar to that of WT thioredoxin (data not shown).

To characterize the activity of our mutant proteins, we assayed their ability to reduce 5,5′-di-thiobis(2-nitrobenzoic acid) (DTNB) in the presence of NADPH and thioredoxin reductase. The activities of the unfolded and renatured proteins in units of mM NADPH per min^{-1} for each variant are as follows: WT, 0.3; D9N, 0.28; D15N, 0.29; A29V, 0.55; I41T, 0.26; S95P, 0.29; L107P, 0.26. The activity of the A29V mutation was roughly twice that of WT, suggesting that both increased export and increased activity may have contributed to the increased motility. However, the activity measurement may not be relevant because it measures activity toward an oxidized substrate, whereas, in our selection for mutants, the mutant protein must act to oxidize a reduced substrate (FlgI).

Folding Kinetics of Mutant Proteins. Although, *in vivo*, thioredoxin is synthesized and maintained in a reduced state in the cytoplasm, we chose to analyze the folding of the oxidized form of the protein in the following experiments because it is much better characterized than the reduced form (nearly all protein folding studies of thioredoxin have been performed on the oxidized species).

Oxidized thioredoxin folds by five distinct phases observable by tryptophan fluorescence after the protein has first been denatured in guanidinium hydrochloride and is then rapidly diluted to renaturing conditions. These phases have been termed the burst, very fast, fast, medium, and slow phases (16). All of these phases have negative amplitudes, i.e., a loss of fluorescence. They have been well characterized and fit into a proposed folding pathway (16). Three of these phases (the very fast, fast, and medium phases) can be measured using stopped-flow mixing. The slowest phase is due to a trans→cis proline isomerization and is unlikely to be affected by any of our mutants because neither of the two residues adjacent to the proline were changed in any of our purified mutants (15, 16, 28, 29). We determined the refolding kinetics of our mutant proteins by tryptophan fluorescence by using stopped-flow mixing (see Fig. 3 for an example). The burst phase is a rapid fluorescence quenching that occurs in the 3.2-ms "dead time" between mixing and fluorescence measurement. This phase may consist of one or multiple phases, but it is too rapid for the corresponding rate constant(s)

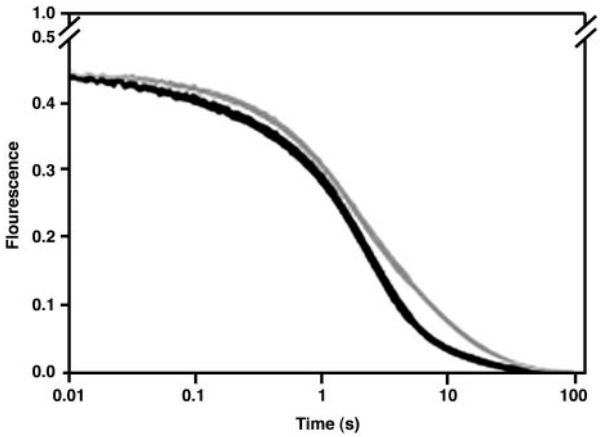

Fig. 3. Example of stopped-flow kinetic trace. Tryptophan fluorescence was measured after denatured thioredoxin (3.5 M guanidinium) was rapidly diluted to renaturing conditions by using stopped-flow mixing. Shown is the average of five independent experiments consisting of 10 traces each for WT (black) and L107P (gray) thioredoxin. Units of fluorescence are given in terms of the fraction of the total change in fluorescence between the denatured and fully renatured states with an excitation wavelength of 295 nm and an emission wavelength of 320 nm.

to be measured. The half-lives of the very fast, fast, and medium phases for the WT protein in our experiments were 37 ms, 1.6 s, and 11.6 s, respectively. All of our mutant proteins exhibited a similarly sized burst phase. However, all but one of the mutant proteins (D15N) exhibited a significant slowing in folding compared with the WT protein in the first two observable phases (the very fast and fast phases) (Table 1; see also Table 4, which is published as supporting information on the PNAS web site). The most dramatic change was seen for the L107P protein, in which the half-life of the very fast phase increased to ≈1 s: an almost 30-fold change. The rate constant for the medium phase was not significantly affected in any of the mutants. Two of the mutant proteins (A29V and I41T) exhibit an additional short-lived intermediate ($t_{1/2}$ = 35 ms and 110 ms, respectively). The implication of the additional folding phase for these proteins is treated in *Discussion*. With the exception of D15N thioredoxin, the severity of the folding defect *in vitro* roughly paralleled the ability to suppress the motility defect of a *dsbA* mutant *in vivo*.

To eliminate the possibility that the folding defects we observed were specific to the oxidized form of the protein, we analyzed the folding kinetics of WT, D15N, and L107P thioredoxin in the presence of 1 mM DTT. Under reducing conditions, WT thioredoxin exhibits five phases of folding observable by stopped-flow fluorescence. Consistent with our observations for

Table 1. Folding kinetic rate constants for oxidized proteins

Protein	Burst amplitude*	Rate constants,† s^{-1}			
		k_N	k_{VF}	k_F	k_M
WT	0.56		18.6	0.429	0.0597
D9N	0.62		3.22	0.312	0.0496
L107P	0.56		0.735	0.178	0.0529
A29V	0.52	20.0	1.04	0.0816	0.0240
D15N	0.54		18.1	0.469	0.0536
I41T	0.55	6.316	6.38	0.122	0.0329
S95P	0.53		1.13	0.215	0.0483

*Given as a fraction of the total amplitude normalized to a value of 1.
†k_{VF}, k_F, and k_M are the rate constants for the very fast, fast, and medium phases, respectively. k_N corresponds to the novel phase of positive change in fluorescence for the A29V and I41T mutants.

Table 2. Thermodynamic measurements

Variant	T_M, °C	$\Delta H(T_M)$, kJ/K·mol
WT*	86.6	477
D9N	79.4	375
D15N	88.2	459
A29V	75.9	386
I41T	80.8	404
S95P	81	393
L107P	72.9	359

*As reported by Mancusso *et al.* (27).

the oxidized form, L107P thioredoxin is significantly slowed in three of the five folding phases, whereas D15N thioredoxin exhibits folding properties nearly identical to the WT protein (Table 5, which is published as supporting information on the PNAS web site).

Differential Scanning Calorimetry. To study the thermodynamics of the folding of our mutant proteins, we subjected them to differential scanning calorimetry. We analyzed the results under the assumption of a two-state unfolding process. According to this analysis, all of the mutant proteins have lowered unfolding enthalpies at the T_M, indicating that a number of intramolecular contacts in each of the proteins has been altered or eliminated, resulting in a decreased net number of favorable interactions in the folded state (Table 2; see also Fig. 6, which is published as supporting information on the PNAS web site). In addition, all but one of the mutant proteins have decreased thermostability compared with the WT protein, as indicated by the change in T_M.

Discussion

We have isolated a number of mutants that alter the kinetics of folding of thioredoxin. These mutants were obtained by selecting for mutant thioredoxins that, when fused to the PhoA signal sequence, allow export of thioredoxin to the periplasm. In the periplasm, the exported thioredoxin is able to suppress the motility defect of a *dsbA* mutant. We were able to obtain such mutants presumably because of the intimate connection between the folded state of a protein and its translocation *in vivo*; proteins must be in an unfolded state to pass through the translocon. Although we anticipated that some mutants with increased motility might be due to alterations of thioredoxin that made the small amount of it exported a better oxidant, the fact that all of the mutants showed substantially increased amounts of the protein in the periplasm indicates that increased export is the primary explanation for the properties of the mutants. We point out that this selection requires that, even if the mutants slow down the folding of thioredoxin, the proteins must still fold efficiently and stably enough to retain their redox activity.

The properties of our mutants lead us to consider how the protein folding and protein translocation of thioredoxin are connected. We can conceive of at least three possible mechanisms by which our mutants reduce the interference of protein folding with translocation: (*i*) by kinetically slowing down the folding of thioredoxin, (*ii*) by shifting the equilibrium between the folded and unfolded state more toward unfolding *in vivo*, or (*iii*) by lowering the energy required to unfold the protein sufficiently to allow the protein secretion machinery to actively unfold the protein. Although our results do not distinguish between these possibilities, we prefer the first of these explanations. Even the most defective of the mutant proteins (L107P) is stably and fully folded at room temperature and has a melting temperature in the oxidized form of ≈73°C, suggesting that the equilibrium *in vivo* is still much in favor of the folded form.

Our data for the L107P mutant protein also suggest that the C terminus of the polypeptide chain is involved in the kinetic folding steps for the protein *in vivo*. Leucine-107 is the penultimate amino acid of thioredoxin. Obviously, by the time leucine-107 (or the proline substitution) is added to the growing chain, substantial amounts of the polypeptide chain should be exposed to the cytoplasm (including the signal sequence). We point out that, for changes at both the very C terminus and the N terminus of the protein (L107P and D9N) to affect protein translocation, the translation of thioredoxin must be complete before translocation begins. That is, translocation is fully posttranslational. This conclusion in turn implies that folding of thioredoxin *in vivo* must be very nearly posttranslational, i.e., that there is not significant enough folding of the nascent polypeptide while it is being synthesized to prevent translocation.

We can suggest explanations for the effects of our mutants based on the structure of the protein and on previous *in vitro* studies on thioredoxin folding. We use the examples of the D9N and L107P mutants to illustrate. The D9N change is identical to one previously constructed to determine whether charged surface residues (aspartates in particular) are important for thermostability (27). Aspartate-9 is located in a pocket on the surface of the protein and makes a number of important interactions (both polar and nonpolar) with other surface residues. We independently isolated the D9N mutant in our genetic screen, confirming the importance of this residue for folding *in vivo*, and we showed that this residue is important for the kinetics of thioredoxin folding, validating the approach we have taken.

An examination of the three-dimensional structure of thioredoxin as determined by NMR (Protein Data Bank entry 1XOA) reveals that leucine-107 forms a hydrophobic cluster composed of residues distributed throughout the primary sequence of the protein including residues alanine-22, tyrosine-49, lysine-52, leucine-103, and leucine-107. Changing leucine-107 to proline would cause it to become significantly more solvent-exposed, which could destabilize this cluster in the folded state. The formation of this cluster may be important in the early steps of protein folding. The folding defect in this mutant does not appear to be due to a slow cis/trans prolyl isomerization. The L107P change has substantial effects on the fastest observable folding phase, resulting in a $t_{1/2}$ of ≈1 s, whereas prolyl isomerizations are slow steps, occurring with a $t_{1/2}$ on the order of hundreds of seconds. Although leucine-107 is located within helix 4, the introduction of a proline is also unlikely to destabilize the structure because of a disruption of an α-helix (see Fig. 1). Changing this residue to proline would disrupt the hydrogen bonding pattern of the α-helix, but it is not likely to significantly disrupt the helix because leucine-107 is located at its very C terminus (indeed, thioredoxin itself is only 108 aa in length). Consistent with our interpretation, we have since isolated an additional mutant that increases export of PhoAss–thioredoxin that changes leucine-107 to arginine, a change that would not disrupt the α-helix (unpublished results).

Two of our mutant proteins (A29V and I41T) exhibited an additional short-lived intermediate with positive fluorescence amplitude in the stopped-flow refolding kinetics. These intermediates may be specific to these mutant proteins and do not occur on the folding pathway for the WT protein. However, we suggest it is also possible that these intermediates belong to the folding pathway of thioredoxin but are so transient for the WT protein that they are incorporated in the so-called burst phase, which occurs during the 3.5-ms time delay between mixing and observation in the stopped flow. The effect of these two substitutions would be to slow down the kinetics of formation of these intermediates so as to uncouple their accumulation with that of the burst intermediate.

In contrast to the other mutant proteins, the D15N change has a minimal effect on the *in vitro* folding of thioredoxin. This

residue is located on a protrusion on the surface of the protein and makes few important short-range interactions. Thus, it is not predicted to be disruptive of protein folding. Our *in vitro* data agree with this prediction. We found a small decrease in the enthalpy of unfolding, but the T_M and folding kinetics of the oxidized and reduced forms of the D15N mutant protein are nearly identical to the WT. Because it is difficult to envision how such a very minor change in the enthalpy of folding at the T_M could affect export of thioredoxin so dramatically, we have tested several alternative explanations for the increased export of the protein to the periplasm *in vivo*. The D15N mutant does not appear to be exported by the cotranslational SRP pathway, because there was no difference in the export of the mutant protein in an SRP mutant (*ffh77*) (data not shown). Another possibility is that translational pausing early in the synthesis of the protein could provide more time for the nascent polypeptide to engage the secretion machinery cotranslationally. The asparagine codon used in our mutant (AAT) has no corresponding tRNA anticodon and is relatively infrequently used (30), suggesting that a pausing event could occur at this position. However, when we introduced a mutation changing this codon to the only alternative asparagine codon (AAT to AAC), we did not see any change in the motility of a strain expressing this mutant, indicating that the change is not due to the specific asparagine codon (data not included). Although it is possible that the methods we used to analyze protein folding *in vitro* are simply not sensitive enough to detect the folding defect, we can also think of several other explanations. For example, the change may create a binding site for a chaperone that could hold the protein in an unfolded state. Alternatively, an asparagine at this position may allow an increased interaction with the PhoA signal sequence, which would slow its folding in a mechanism similar to that of maltose-binding protein (31, 32) (we analyzed only the signal sequenceless versions of our mutant proteins *in vitro*).

We point out that our genetic selection places certain restrictions on which amino acid changes we could obtain. Because the mutant proteins must be reasonably functional in the periplasm for this selection to work, the amino acid replacements could not have too severe an effect on the folding or the activity of the protein. We isolated mutations at a small number of positions in thioredoxin with a number of mutations arising multiple times independently. It is possible that a different mutagenesis technique will yield mutations at other positions. However, our results suggest that there may be a limited number of mutable positions in thioredoxin that cause folding defects sufficient to allow export but not so disruptive to the structure that the protein never folds properly at all. Mutations at other positions either may have no or very small effects on protein folding or may be too deleterious to folding or activity.

By using genetic approaches in combination with already existing *in vitro* methods, it may be possible to answer other key questions regarding thioredoxin folding. For example, the β-sheet backbone of thioredoxin forms the hydrophobic core of the protein. Tasayco *et al.* (33) proposed that folding of thioredoxin begins with the formation of the rather hydrophobic central β-strands. From this, one might expect the amino acids that form the β-strands (especially their hydrophobicity) to play a critical role in protein folding. Of the several mutants we isolated, the majority were in the loops between regular secondary structure, and none were in the β-sheet, despite its being a sizable proportion of the overall sequence (Fig. 1). Although our results do not reveal whether changes to the sequence of the β-sheet have no effect on folding or are detrimental to it, they do suggest that such changes may not have an intermediate effect; they may either have no effect or be strongly detrimental.

We thank members of the J.B. laboratory; Tom Rapoport, Steve Blacklow, and Andrew Gray for helpful conversations; and George Georgiou (University of Texas) for providing strains. J.B. is an American Cancer Society Professor, and D.H. is a Harold Amos Fellow. This work was supported by National Institutes of Health Grant GM41883 (to J.B.), grants from the Institut Pasteur and Centre National de la Recherche Scientifique Grant URA 2185 (to A.C.), and National Science Foundation Grant MCB-0118252 (to M.L.T.).

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