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Biochemistry

Thioredoxin-catalyzed refolding of disulfide-containing proteins

(protein folding/disulfide exchange)

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ABSTRACT **Thioredoxin, a known catalyst for reducing protein disulfides, was shown to catalyze efficiently the refolding of pancreatic RNase either from the reduced, denatured form or from the scrambled form containing oxidized but incorrectly paired disulfides. Thioredoxin was 1000-fold more efficient on a molar basis than the model dithiol, dithiothreitol, in reactivating reduced, denatured RNase, suggesting that thioredoxin acts as an efficient catalyst for disulfide interchange. Starting with reduced, denatured RNase, enzyme activity was recovered quantitatively with a $t_{1/2}$ of 30 hr with 100 μM thioredoxin compared to only a 10–20% recovery of activity in the control using air oxidation. Oxygen further stimulated the effectiveness of thioredoxin severalfold. Thioredoxin was most effective in reactivating inactive scrambled RNase, which contained mispaired disulfides, showing a $t_{1/2}$ of 2 hr. Reduced thioredoxin was optimal for catalyzing disulfide interchange in scrambled RNase, whereas oxidized thioredoxin was required for reactivation of the reduced, denatured species. Optimal reactivation of scrambled RNase required a mixture of reduced and oxidized thioredoxin. Addition of reduced thioredoxin after initiating refolding of reduced denatured RNase with oxidized glutathione effected a rapid reactivation of RNase, suggesting a two-step model for protein refolding in which the monothiol catalyzes the rapid initial formation of protein disulfides and thioredoxin catalyzes the second step of disulfide interchange. Arguments are presented suggesting that thioredoxin may serve an *in vivo* role analogous to the protein disulfide-isomerase (EC 5.3.4.1).**

Thioredoxin(s) have been implicated in a variety of physiological roles, including serving as the hydrogen donor for the reduction of ribonucleotides and sulfoxides (1, 2) and for the activation of enzymes (3) and serving as a co-factor for T_7 phage DNA polymerase (4) and assembly of filamentous phage (5). Although the mechanism of action of thioredoxin in the latter two examples is not yet clear, in general, thioredoxin functions as an efficient protein disulfide reductant. For example, thioredoxin can reduce the interchain disulfide of insulin at a rate 10^4-fold greater than dithiothreitol at physiological pH (6). Efficient catalysis of disulfide reduction apparently depends on the presence of a reactive thiol to act as a nucleophile and on the formation of a stable intrachain disulfide.

The ability of thioredoxin to reduce reversibly a variety of protein disulfides suggested the possibility that it also catalyzes the formation of correct disulfides during protein folding. Starting from the fully reduced state, folding is a two-stage reaction requiring oxidation to disulfides coupled to reversible disulfide interchange (7). Acquisition of the correctly folded state occurs concomitantly with the formation of the correct disulfide pattern and is driven by the thermodynamic stability of the native peptide configuration. Since protein folding has been shown to follow a defined kinetic path, including the obligate formation of nonnative disulfides (7–10), disulfide interchange is necessary to achieve the correctly folded form. Given the ability of thioredoxin to oxidize and reduce protein disulfides reversibly, we explored the possibility that it catalyzes refolding of a disulfide-containing protein, ribonuclease, from either its unfolded, reduced form or from the randomly oxidized scrambled forms.

MATERIALS AND METHODS

Materials. Ampicillin, bovine pancreatic ribonuclease A (RNase), cytidine 2′:3′-cyclic monophosphate, oxidized and reduced dithiothreitol, and oxidized and reduced glutathione were obtained from Sigma. Electrophoresis-grade agarose was purchased from Bio-Rad. 3-(*N*-Morpholino)propanesulfonic acid, sodium salt (Mops), was the Ultrol grade from Calbiochem. Sephadex G-25 Superfine was also obtained from Sigma. All other chemicals were reagent grade.

Isolation of Thioredoxin. Thioredoxin was isolated as described by Lunn *et al.* (11) from the strain SK3981, a derivative of *Escherichia coli* K-12 (SK3967).

Protein Concentration. Thioredoxin protein was assayed either spectrophotometrically at 280 nm using an extinction coefficient of 13,600 $cm^{-1} \cdot M^{-1}$ (12) or immunologically using quantitative rocket immunoelectrophoresis (13). The concentration of native RNase was determined using a molar extinction coefficient of 9800 $cm^{-1} \cdot M^{-1}$ measured at 277.5 nm (7). Fully reduced enzyme was determined at 275 nm using an extinction coefficient of 9200 $cm^{-1} \cdot M^{-1}$ (9).

RNase Activity. RNase activity was determined according to Crook *et al.* (14) replacing 0.1 M cacodylate (pH 7.0) with 0.1 M Mops (pH 7.0). The final assay mixture consisted of 0.1 M Mops (pH 7.0), 7 mM cytidine 2′:3′-cyclic monophosphate, and 10–30 μg of RNase A per ml.

Reduction and Denaturation of RNase. Reduced, denatured RNase was prepared by incubating the native enzyme (30 mg) overnight in 1.5 ml of 0.1 M Tris·HCl (pH 8.6) containing 0.15 M dithiothreitol and 6 M guanidine hydrochloride (15). The reduced RNase was separated from excess dithiothreitol and guanidine hydrochloride chromatographically using Sephadex G-25 equilibrated with 0.01 M HCl. RNase A fractions were stored under argon at −20°C.

Reactivation of Reduced and Denatured RNase. Reoxidation of RNase was initiated by diluting the reduced enzyme in 0.1 M Tris, pH 7.4 or pH 9.0, with 1 mM EDTA containing 0–500 μM thioredoxin and/or either oxidized dithiothreitol or glutathione at 25°C. At various times aliquots were assayed and the percentage of refolding was calculated relative to a control sample of native RNase.

Scrambled RNase. Reduced, denatured RNase was made 6 M in guanidine hydrochloride and the pH was adjusted to 8.6

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Abbreviation: Mops, 3-(*N*-morpholino)propanesulfonic acid.

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with solid Tris. The sample was then sparged with oxygen and then incubated at room temperature in the dark for 3–4 days. Free thiol was <0.1 mol/mol of RNase (16). RNase activity was 0–5%.

Reactivation of Scrambled RNase. Reactivation of scrambled RNase was initiated by a 1:10 dilution in 0.1 M Tris (pH 7.4) with 1 mM EDTA containing various amounts of thioredoxin and/or either dithiothreitol or glutathione.

RESULTS

Reactivation of Scrambled RNase with Reduced Thioredoxin. In the early stages of refolding denatured and reduced proteins, oxidation produces a heterogeneous population of molecules containing random disulfide bonds (17, 18). These disulfide-containing molecules have acquired some three-dimensional structure but they are not active and require interchange of disulfide bonds to form the thermodynamically stable native and active conformation. The ability of thioredoxin to catalyze the interchange of these incorrectly paired disulfides was assessed, using "scrambled" RNase containing no free thiols. As shown in Fig. 1 at pH 7.4 thioredoxin efficiently reactivated scrambled, inactive RNase to yield a quantitative recovery of active enzyme.

Reduced thioredoxin was required to stimulate this reactivation. When 10 μM reduced thioredoxin was added to scrambled RNase, the $t_{1/2}$ (i.e., the time required to obtain 50% of maximal activity) was 8 hr, and recovery of activity was 100% within 48 hr. Thioredoxin was reduced either directly in the refolding mixture by preincubating with a stoichiometric amount of reduced dithiothreitol or by pretreatment with an excess of reduced dithiothreitol followed by chromatographic isolation. Results were identical with both procedures (Fig. 1), suggesting that reduced thioredoxin does not require dithiothreitol for catalyzing disulfide exchange. The optimal thioredoxin system for reactivating scrambled RNase was oxidized and reduced thioredoxin at a ratio of approximately 10:1. Reactivation in the presence of 100 μM oxidized and 10 μM reduced thioredoxin had a $t_{1/2}$ of 2 hr and 100% activity was recovered within 24 hr. Higher concentrations of thioredoxin further reduced the reactivation half-time, but the requirement for a 10:1 redox ratio was maintained. The requirement for a redox balance of thioredoxin for optimal effectiveness is consistent with the initial need to reduce mispaired disulfides, coupled to the subsequent reaction of catalyzing disulfide interchange.

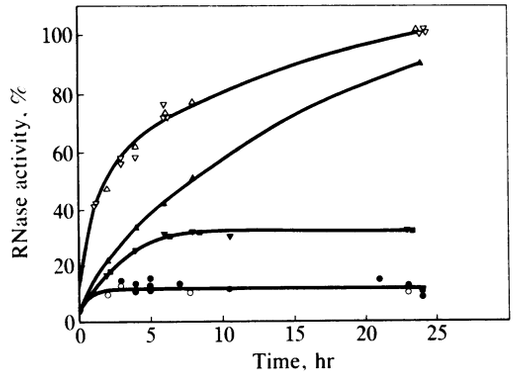

FIG. 1. Kinetics of the reactivation of scrambled RNase with thioredoxin. Thioredoxin (100 μM) either was preincubated with 10 μM dithiothreitol for 30 min (▽), or 10 μM reduced thioredoxin (△) was added to 100 μM thioredoxin immediately prior to addition of 25 μM RNase. Other samples contained 10 μM reduced thioredoxin (▲), 100 μM oxidized thioredoxin (○), 10 μM dithiothreitol (■), 100 μM glutathione disulfide with 10 μM glutathione (▼), or air (●). Reactivation was carried out in 0.1 M Tris, pH 7.4/1 mM EDTA at 25°C in the presence of air.

Reduced dithiothreitol, reduced glutathione, and air were all ineffective in reactivating of scrambled RNase (Fig. 1), presumably because these simple thiols lack the necessary combined characteristics for a good reducing agent and a good disulfide interchange agent. A maximum yield of only 30% RNase activity was recovered using either 10 μM reduced dithiothreitol or a mixture of 100 μM oxidized and 10 μM reduced glutathione. Thioredoxin is an excellent protein disulfide reductant (19) and as such is effective in catalyzing the first step in the reactivation of the scrambled protein. In contrast, glutathione is a poor protein reductant (20, 21). The ineffectiveness of glutathione using scrambled RNase supports the notion that the critical kinetic step in refolding the scrambled substrate is reduction of incorrect disulfides. The poor efficiency of reduced dithiothreitol in reactivating scrambled RNase is likely due to its being a relatively poor protein reductant at neutral pH (compared to thioredoxin; ref. 19) combined with its inability to catalyze protein disulfide interchange (see below).

Reactivation of Reduced, Denatured RNase with Oxidized Thioredoxin. The first step in folding a reduced, denatured protein is the formation of random disulfides followed by rearrangement of the disulfides to the native conformation (7). In the absence of any thiol reagent there is a very slow recovery of activity in the refolding of reduced, denatured RNase in the presence of air (Table 1, Fig. 2). In the presence of air, the $t_{1/2}$ for RNase reactivation was >100 hr (at pH 7.4) and 22 hr at pH 9.0 (Table 1). The increased rate of reactivation at the higher pH was most likely due to the increased concentration of RNase thiols in the thiolate form that catalyze intramolecular disulfide interchange (7, 8), although reactivation also may be stimulated by more efficient disulfide formation at the higher pH. This observation is consistent with the findings of Anfinsen *et al.* (9) that disulfide formation occurred much more rapidly than the recovery of enzymatic activity, suggesting that interchange of incorrect disulfides and not disulfide formation is rate limiting.

Thioredoxin catalyzed the reactivation of reduced, denatured RNase at a rate significantly greater than air oxidation. For example, the $t_{1/2}$ for reactivation with 20–500 μM oxidized thioredoxin ranged from 50 to 8 hr, respectively, at pH 7.4 at an RNase concentration of 23 μM (Fig. 2). For air oxidation the $t_{1/2}$ was >100 hr and the recovery of activity was usually far less than 100% (Fig. 2). In the presence of oxidized thioredoxin (≥20 μM) the recovery of RNase activity was quantitative. The ability of oxidized thioredoxin to catalyze RNase refolding was concentration dependent, with a greater rate of reactivation as oxidized thioredoxin concentration

Table 1. Relative ability of thioredoxin and thiols to refold reduced RNase

Sample	Concentration, mM	Reaction $t_{1/2}$, hr	
		pH 7.4	pH 9.0
Control		>100	22
Thioredoxin	0.02	50	17
	0.1	27	13
Dithiothreitol (oxidized)	0.1	>100	21
	20	52	18
	100	30	10
GSSG	0.02*	48	16
	0.1*	8	4
	20	—†	—†

Refolding conditions were as described in the legend to Fig. 1. The recovery of RNase activity was 100% for all samples except those containing glutathione disulfide (GSSG).

*Total recovery of RNase activity was <80%.

†After 50 hr, the total recovery of RNase activity was 15% at pH 7.4 and 30% at pH 9.0.

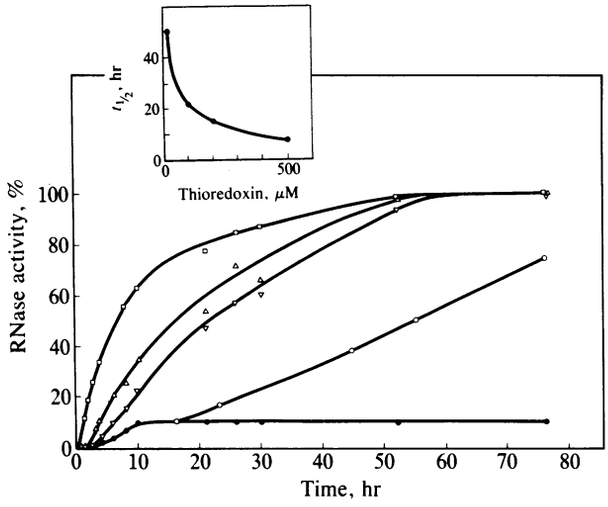

FIG. 2. Kinetics of the reactivation of reduced, denatured RNase in the presence of thioredoxin. Thioredoxin concentrations were 0 (●), 20 (○), 100 (▽), 200 (△), and 500 (□) μM in a 2-ml reaction mixture containing 23 μM RNase. (*Inset*) Plot of time for 50% reactivation vs. thioredoxin concentration.

was increased (Fig. 2 *Inset*). The lag characteristic of the initial phase of reactivation was also dependent on the concentration of oxidized thioredoxin. As the concentration of thioredoxin increased, the lag phase decreased and was eliminated at 500 μM oxidized thioredoxin (Fig. 2). The effect of thioredoxin on reducing the overall half-time for reactivation and elimination of the lag phase suggests a 2-fold role of thioredoxin in the refolding of reduced, denatured RNase. Initially, oxidized thioredoxin may catalyze the formation of RNase disulfides by a simple process of thiol–disulfide interchange. The reduced thioredoxin produced in the oxidation of RNase thiols can then catalyze RNase disulfide interchange as occurs with scrambled RNase (Fig. 1). At low concentrations of oxidant, the initial formation of protein disulfides is rate limiting. As thioredoxin is increased, disulfide interchange then becomes the rate-limiting step.

The proposed dual role of thioredoxin is supported by the increased catalytic efficiency of thioredoxin in refolding reduced, denatured RNase in the presence of oxygen (Fig. 3). In the presence of 100 μM oxidized thioredoxin and oxygen there was an increase in the reactivation rate as compared to either oxidized thioredoxin and air or oxygen alone. The $t_{1/2}$ for reactivating RNase with 100 μM oxidized thioredoxin decreased to 8.5 hr in the presence of oxygen (Fig. 3) from 27 hr in air (Fig. 2). This result suggests that oxygen is recycling

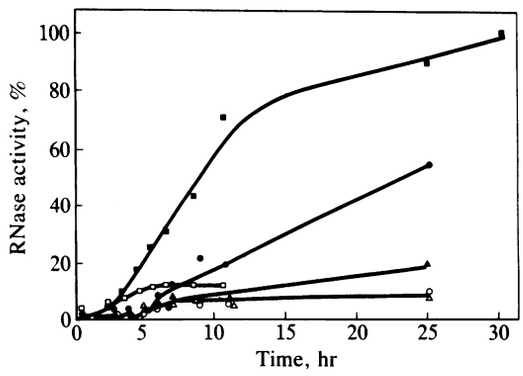

FIG. 3. Reactivation of reduced, denatured RNase in the presence of thioredoxin under different atmosphere. The reactivation of RNase was carried out under air (○, ●), oxygen (□, ■), or nitrogen (△, ▲) in the presence (●, ■, ▲) or absence (○, □, △) of 100 μM thioredoxin.

thioredoxin back to the oxidized form. The regeneration of oxidized thioredoxin by oxygen is further supported by the presence of a lag in the reactivation of RNase with thioredoxin and oxygen. If oxygen catalyzed the direct formation of RNase disulfides and not the regeneration of oxidized thioredoxin, the lag phase should either decrease or be eliminated, as observed by increasing thioredoxin concentration (Fig. 2). However, the lag phase in the presence of thioredoxin and oxygen (Fig. 3) is comparable to the lag observed in the presence of thioredoxin and air (Fig. 2).

The amount of RNase activity recovered in the presence of oxygen attained a maximum of only 14% after 5 hr. It is presumed that the majority of RNase in this sample contains incorrect disulfide bonds. In contrast, in the presence of thioredoxin and oxygen, the rate of reactivation was rapid and the recovery of active RNase was 100% (Fig. 3), supporting the role of thioredoxin as a catalyst for protein disulfide interchange. Regeneration of oxidized thioredoxin may affect the redox ratio aiding disulfide interchange, as observed for scrambled RNase.

The catalytic effect of oxidized thioredoxin on refolding reduced, denatured RNase was compared to that of a chemical dithiol, oxidized dithiothreitol, and a monothiol, oxidized glutathione (Table 1). Dithiothreitol forms an unstable, transient mixed disulfide during protein folding (22), so the rate of disulfide formation is generally determined by the rate of the intramolecular conformational transition in which a second cysteine residue displaces the mixed disulfide (23). As a dithiol, thioredoxin should catalyze the refolding of reduced, denatured RNase by the same mechanism. Oxidized thioredoxin, however, was significantly more effective than oxidized dithiothreitol in catalyzing the refolding of reduced, denatured RNase (Table 1). At pH 7.4 and at pH 9.0 the half-time for recovery of RNase activity for 100 μM thioredoxin was comparable to that of 100 mM oxidized dithiothreitol. One hundred micromolar oxidized dithiothreitol showed no increase in refolding above control levels. Creighton reported an accumulation of the one and two disulfide intermediates of RNase and very little recovery of native activity in the presence of oxidized dithiothreitol (18). A likely explanation for the greater efficiency of thioredoxin as compared to dithiothreitol is that reduced thioredoxin formed in the initial oxidation of RNase thiols catalyzes the second step of refolding, protein disulfide interchange. As shown with the reactivation of scrambled RNase, thioredoxin effectively catalyzes disulfide interchange, whereas dithiothreitol is ineffective (Fig. 1).

In contrast to thioredoxin and dithiothreitol, for which the rate-limiting step is an intramolecular conformation transition of RNase, with glutathione the rate-limiting step is the formation of a stable glutathione RNase mixed disulfide intermediate (23). At a concentration of 20 μM, oxidized glutathione and oxidized thioredoxin had comparable half-times for the reactivation of reduced, denatured RNase (Table 1). At 100 μM, however, oxidized glutathione showed a half-time of reactivation 3-fold better than that observed using oxidized thioredoxin. When oxidized glutathione was increased further to 20 mM, the recovery of activity was only 15–30%. This reduced recovery is due to the relatively stable glutathione-RNase mixed disulfide intermediate, which is favored over the transient intermediate formed by dithiols such as oxidized dithiothreitol and oxidized thioredoxin. As a result, at high concentrations of oxidized glutathione (i.e., 20 mM) protein disulfide formation must compete with the formation of the glutathione-RNase mixed disulfide (18, 23).

Although oxidized glutathione at 100 μM increased the rate of reactivation 3-fold compared to thioredoxin, the final yield of RNase activity was never >80%. In contrast, the yields with oxidized thioredoxin were always 100%. Creighton also reported low yields of native RNase when refolding in the

presence of oxidized glutathione (18), consistent with our observation that glutathione is not an effective protein disulfide interchange reagent for scrambled RNase (Fig. 1).

Temporal Addition of Thioredoxin. The data presented above suggest that a monothiol such as glutathione is most effective in catalyzing the formation of random disulfides in a reduced, denatured protein, whereas thioredoxin is more effective in catalyzing disulfide rearrangement to the native conformation (Table 1 and Fig. 1). This model is further supported by the results shown in Fig. 4. When the refolding of reduced, denatured RNase was initiated by addition of 100 μM oxidized glutathione there was an initial rapid increase in the reactivation of RNase. After 4 hr, however, the rate of reactivation declined. When reduced thioredoxin was added to the reaction initiated with oxidized glutathione there was an increase in the rate of reactivation, and this effect was more dramatic at later addition times (Fig. 4). When reduced thioredoxin was added 30 min after initiating the reaction there was only a small increase in the reactivation rate, whereas addition of thioredoxin after 1 hr produced a significant increase in the rate of reactivation. When thioredoxin was added 5 hr after the initiation of refolding, 96% of RNase activity was recovered 3 hr later compared to 56% for the sample without thioredoxin. This reflects a 100% increase in the recovery of RNase activity from the time of addition of thioredoxin as compared to only a 17% increase in recovery of RNase activity without the addition of thioredoxin.

DISCUSSION

Previously thioredoxin was shown to reduce effectively a variety of protein disulfides (24, 25), and these data have served as the basis for postulating a role for thioredoxin as a biological protein reducing agent. Data presented here have demonstrated that thioredoxin can act as a protein disulfide isomerase, catalyzing the formation and the rearrangement of protein disulfide bonds in RNase. These data raise the interesting possibility that thioredoxin may have an additional *in vivo* role in the formation and interchange of protein disulfide bonds.

Studies with bovine serum albumin (26), procollagen (27), and transferrin (28) have shown that disulfide bond formation is an early event in protein synthesis, occurring during and immediately after synthesis. For proalbumin, 7 of a possible 17 disulfides are formed in the nascent chain; after the polypeptide is completed and detached from the ribosome, 11 disulfides are present (26). The disulfides formed are not thought to be the correct ones implying the existence of a cellular mechanism for isomerizing of protein disulfides. The protein postulated to catalyze the interchange of protein disulfide bonds *in vivo* is protein disulfide-isomerase (EC 5.3.4.1). This protein catalyzes the interchange of incorrect disulfides in a scrambled protein substrate and catalyzes refolding of reduced substrates in the presence of an oxidant, such as dithiothreitol (20). Evidence presented here supports the ability of thioredoxin to catalyze these two reactions characteristic of protein disulfide-isomerase.

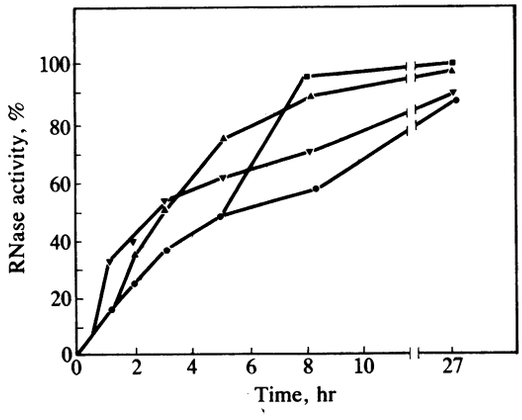

FIG. 4. Effect of thioredoxin on the glutathione-catalyzed reactivation of reduced, denatured RNase. The initial reaction mixture consisted of 25 μM reduced, denatured RNase and 100 μM oxidized glutathione (●). Reduced thioredoxin (10 μM) was added to aliquots at 0.5 (▼), 1.0 (▲), and 5.0 (■) hr after starting the refolding reaction.

Like protein disulfide-isomerase, thioredoxin requires the proper redox state to effectively catalyze protein refolding. Starting with reduced, denatured proteins, the oxidized form of thioredoxin is required, and the reaction is concentration dependent. Addition of oxidant (i.e., oxygen) enhances the rate of refolding, possibly by regenerating the oxidized form of thioredoxin. Reactivation of a scrambled protein requires that either thioredoxin or protein disulfide-isomerase be in the reduced form. The substantially faster recovery of activity from scrambled RNase compared to the reduced, denatured substrate supports the logic that thioredoxin (like protein disulfide-isomerase) works primarily as a catalyst for disulfide interchange, and the oxidation of thiols to the disulfide form may be a secondary role. The ability of thioredoxin to perform efficiently the same set of reactions as protein disulfide-isomerase is, perhaps, not surprising since the active site sequence of these proteins shows a high degree of homology (29).

Thioredoxin	Trp–Cys–Gly–Pro–Cys–Lys
Protein disulfide-isomerase	Trp–Cys–Gly–His–Cys–Lys

The tissue and intracellular localization of thioredoxin also suggests a role for thioredoxin in protein folding. Thioredoxin is found in high concentrations in liver, kidney, and thymus (30) and is predominant in the secretory cells of these organs (31). Within liver cells, thioredoxin is present in various subcellular fractions, including plasma membrane and microsomal fractions. Protein disulfide-isomerase is predominantly found in the microsomal fraction as well (32). Disulfide-containing proteins are predominantly extracellular and cell-surface proteins that interact with the endoplasmic reticulum and plasma membranes before being released from the cell. Disulfide rearrangement *in vivo* is thought to occur at the rough endoplasmic reticulum for several proteins (17, 33). The presence of thioredoxin in these organelles and its ability to catalyze disulfide exchange *in vitro* suggest that along with protein disulfide-isomerase it may be responsible for catalyzing disulfide exchange *in vivo*.

Folding reduced, denatured proteins involves two steps. Hantgan *et al.* (7) have shown that the first step in refolding RNase in the presence of glutathione is the rapid formation of disulfide bonds. Monothiols such as oxidized glutathione are effective in catalyzing this first step because they form a stable mixed disulfide intermediate (7, 17). Dithiols such as dithiothreitol or thioredoxin are less effective in this first step because the second thiol competes with RNase thiols to form an intramolecular disulfide. As a result, the mixed disulfide intermediate is not stable and the rate of RNase disulfide formation is dependent on the rate of an intramolecular conformational transition of RNase (23). Thioredoxin, like protein disulfide-isomerase, is efficient in catalyzing the second step of the process, disulfide interchange. Catalysis of this second step requires the initial reduction of RNase disulfides. These results are consistent with the substantially greater ability of thioredoxin to catalyze effectively the reduction of protein disulfides as compared to dithiothreitol (19) or glutathione (20). This proposed two-step model is supported by the fact that the best condition for refolding

reduced, denatured RNase is to initiate the reaction with oxidized glutathione and subsequently add reduced thioredoxin after initial disulfide formation is essentially completed.

The greater efficiency of thioredoxin to act as a protein reductant at pH 7.4 as compared to glutathione or dithiothreitol may be explained in part by the low pK_a (6.8) of one of thioredoxin's thiols. The pK_a of Cys-32 of thioredoxin is 6.8 (33), whereas the thiols of dithiothreitol and glutathione have pK_a values of 9.2 and 8.7, respectively (34). Since disulfide reduction requires that the reducing thiol be in the thiolate form, and the most rapidly reducing thiols are those having pK_a values closest to the solution pH (21), thioredoxin should be the better reducing agent at pH 7.4. However, pK_a alone cannot account solely for this increased effectiveness of thioredoxin. If so, thioredoxin, dithiothreitol, and glutathione would be equally effective protein reductants at a more alkaline pH. In fact, at pH 7.0, thioredoxin reduces cystine and oxidized glutathione $\approx$50 times faster than dithiothreitol at pH 8.7 (19). Shaked *et al.* (21) observed that the rate of reduction of proteins by glutathione is significantly lower than expected from its pK_a. These observations suggest that thioredoxin, like protein disulfide-isomerase, is a unique protein reductant and not simply a macromolecular dithiol. Creighton *et al.* (20) have shown that protein disulfide-isomerase does not behave like a simple thiol or disulfide reagent as it has no effect on thiol–disulfide exchange between reduced dithiothreitol and glutathione. As with protein disulfide-isomerase, it seems probable that thioredoxin could have an affinity for its protein substrates (20). This would be consistent with Holmgren's observation that thioredoxin is more effective in reducing insulin disulfides than in reducing small linear disulfides such as cystine and oxidized glutathione (19). If thioredoxin is involved in protein–protein interactions with its substrates, thioredoxin could be envisaged as catalyzing protein conformational transitions as well as being an effective protein reductant. This mechanism would be consistent with the ability of thioredoxin to effectively catalyze disulfide interchange in scrambled proteins.

As a practical consideration, the disulfide isomerization activity of thioredoxin can be applied to the refolding of recombinant mammalian proteins produced in bacteria. Mammalian disulfide proteins cloned and expressed in *E. coli* are often produced in inclusion bodies of insoluble protein and may be in a scrambled, partially oxidized form (35, 36). Perhaps this is not surprising since *E. coli* generally do not secrete proteins or produce disulfide-containing proteins. Nonetheless, there are numerous advantages of using *E. coli* for cloning and expression of heterologous proteins if the technical problems of refolding could be solved.

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