A Pro to His Mutation in Active Site of Thioredoxin Increases Its Disulfide-Isomerase Activity 10-fold

NEW REFOLDING SYSTEMS FOR REDUCED OR RANDOMLY OXIDIZED RIBONUCLEASE*

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Thioredoxin (Trx) from Escherichia coli was compared with bovine protein disulfide-isomerase (PDI) for its ability to catalyze native disulfide formation in either reduced or randomly oxidized (scrambled) ribonuclease A (RNase). On a molar basis, a 100-fold higher concentration of Trx than of PDI was required to give the same rate of native disulfide formation measured as recovery of RNase activity. A Pro-34 to His (P34H Trx) mutation in the active site of E. coli Trx (WCGHC), mimicking the two suggested active sites in PDI (WCGHC), increased the catalytic activity in disulfide formation about 10-fold. The mutant P34H Trx displayed a 35-mV higher redox potential (E*) of the active site disulfide/dithiol relative to wild type Trx, making it more similar to the redox potential observed for PDI. This higher redox potential correlates well with the enhanced activity and suggests a role for the histidine side chain.

Enzymatic isomerization of disulfides in scrambled, oxidized RNase requires the presence of a catalytic thiol such as GSH to initiate the thiol-disulfide interchange. Bovine thioredoxin reductase, together with NADPH, could replace GSH. For oxidative folding of reduced RNase in air with Trx, P34H Trx, or PDI, catalytic amounts of sodium selenite (1 μM) resulted in rapid disulfide formation and high yields of ribonuclease activity equivalent to previously known reduced RNase buffers of GSH and GSSG. These results demonstrate no obligatory role for glutathione in disulfide formation. A possible mechanism for the unknown thiol oxidative process accompanying folding and protein disulfide formation in vivo is discussed.

Protein disulfide-isomerase (PDI) is a major protein (4%) in the lumen of the endoplasmic reticulum, where it is implicated as a catalyst of native disulfide bond formation in the biosynthesis of secretory and cell surface proteins (Freedman, 1989; Freedman et al., 1989). Mammalian PDI (57 kDa) contains two internally homologous domains that are 30% identical with thioredoxin, a ubiquitous 12-kDa protein having a redox-active disulfide/dithiol catalyzing thiol-disulfide exchange reactions (Edman et al., 1985; Holmgren, 1985, 1989). In a previous paper (Lundström and Holmgren, 1990), we have shown that the thioredoxin-like domains of PDI are substrates for NADPH and thioredoxin reductase and that PDI has thioredoxin-like activity and thus a similar mechanism of action. Previously, Escherichia coli thioredoxin has been shown to have powerful protein disulfide reductase activity (Holmgren, 1979a, 1979b) and also a low protein disulfide isomerase activity (Figet and Schuster, 1986; Hawkins et al., 1991a).

Ribonuclease A (RNase) is a classic protein to study folding and native disulfide formation in vitro (Anfinsen, 1961; Anfinsen and Scheraga, 1975). The fully reduced molecule with eight thiol groups is enzymatically inactive, as is the randomly oxidized or scrambled (sRNase) product with incorrect disulfides formed during oxidative folding under denaturing conditions. PDI catalyzes formation of native disulfides in sRNase in the presence of a catalytic thiol or oxidative folding of reduced RNase in a redox buffer of GSH and GSSG. This follows a largely unknown multistep pathway of isomerization of the nonnative disulfides as a result of disulfide reduction and dithiol oxidation. Whether a similar redox interchange with nonnative disulfides also occurs during folding of a reduced polypeptide is presently under debate (Creighton, 1988, Weissman and Kim, 1991).

Thioredoxin and thioredoxin reductase (TR) catalyze reduction of protein disulfides by NADPH according to Reactions 1 and 2.

$$\text{Trx-S}_2 + \text{NADPH} + \text{H}^+ \rightleftharpoons \text{Trx-(SH)}_2 + \text{NADP}^+$$ (1)

$$\text{Protein-S}_2 + \text{Trx-(SH)}_2 \rightleftharpoons \text{protein-(SH)}_2 + \text{Trx-S}_2$$ (2)

Many different protein disulfides (e.g. those found in insulin, trypsin, factor VIII, elongation factor 2, and photosynthetic enzymes) are good substrates (Holmgren, 1985), whereas structurally buried disulfides like those found in RNase (four) or bovine serum albumin (17) are not reactive. The active site of E. coli Trx-(SH)$_2$, Trp-Cys-Gly-Pro-Cys, is located in a protruding loop near a hydrophobic surface area including Ile-75, Pro-76, Gly-92, and Ala-93 (Dyson et al., 1990); a similar structure can be modeled for the thioredoxin domains of PDI (Eklund et al., 1991). Furthermore, in Trx-(SH)$_2$, only the exposed sulfhydryl group of Cys-32 is alkylated with iodoacetic acid at neutral pH and shows a low apparent pK$_a$ value of 6.7, which is consistent with its role as the attacking thiolate in thiol-disulfide exchange (Kallis and Holmgren, 1980). Reduced PDI has recently been shown to have the same alkylating properties and apparent Cys pK$_a$ value.
Disulfide Isomerization by Pro to His Mutated Thioredoxin

(Hawkins and Freedman, 1991). However, whereas the redox potential of the dithiol/disulfide in Trx is $-270$ mV (Holmgren, 1985; Krause et al., 1991), PDI has a much higher redox potential as estimated from equilibrium studies with Trx (Lundström and Holmgren, 1990) or from the equilibrium with GSH/GSSG redox buffers (Hawkins et al., 1991b). Consequently, PDI is more oxidizing than Trx in thiol-disulfide interchange reactions.

The difference between the active site of Trx and the two thioredoxin domains in PDI is replacement of Pro-34 in E. coli Trx by His in PDI (Edman et al., 1985). We have previously generated P34H Trx by site-directed mutagenesis (Krause et al., 1991). This Trx had a 35-mV higher redox potential and is a slower insulin disulfide reductant than wt Trx.

The purpose of the present investigation was to analyze the effect of the Pro to His exchange in the catalysis of disulfide formation and isomerization. The catalytic activities of P34H Trx, wt Trx, and PDI were compared in oxidative refolding of RNase using established conditions with GSSG and in isomerization reactions using GSH. In addition, we developed novel methods for oxidative folding of reduced RNase employing catalysis by selenite, as well as for refolding of sRNase employing NADPH and thioredoxin reductase in place of thiol. In all these systems, P34H Trx showed a large increase in activity compared with wt Trx, making it useful for folding of disulfide-containing proteins in vitro and possibly in vivo.

EXPERIMENTAL PROCEDURES

Materials—E. coli thioredoxin reductase, wt E. coli Trx, P34H Trx (Krause et al., 1991), bovine PDI (Lundström and Holmgren, 1990), and bovine thioredoxin reductase (Luthman and Holmgren, 1982) were purified to homogeneity according to published procedures. NADPH, dithiothreitol, Na$_2$SeO$_3$, RNase, and 5,5'-dithiobis-(nitrobenzoic acid) was prepared from Sigma; GSH and GSSG were from Boehringer Mannheim. Stock solutions of 0.10 M GSH were titrated to pH 5 with NaOH and stored at $-70^\circ$C in aliquots sufficient for one experiment. Urea and potassium phosphate were from Merck, and PD10 columns for gel filtration were from Pharmacia LKB Biotechnology Inc.

Spectrophotometric Determinations—Spectrophotometric determinations were performed at room temperature ($25^\circ$C) in a Shimadzu U2100 spectrophotometer equipped with a multicuvette holder and automatic cuvette control. Six samples could be measured simultaneously with recordings every 30 s.

Preparation of Fully Reduced RNase—A solution of RNase (30 mg/ml) was prepared in Na$_2$S$_2$O$_3$-equilibrated 9 M urea and 130 mM dithiothreitol, adjusted to pH 8.6 by Tris base. After incubation for 60 min at 37°C, the pH was adjusted to 4.0 with glacial acetic acid, and the sample was desalted on Sephadex G-25 (Pharmacia P10) equilibrated with 0.1 M acetic acid. The reduced RNase was further dialyzed for 2 h against 0.1 M acetic acid at +4°C and then stored at $-20^\circ$C in aliquots (4 mg/ml) to avoid repeated freezing and thawing.

Preparation of sRNase—The substrate for disulfide isomerization assays was prepared essentially as described by Hillson et al., 1984. Fractions containing reduced RNase from chromatography on Sephadex G-25 as described above were pooled and diluted to 0.5 mg/ml in 9 M urea, 0.1 M sarcosine HCl. The pH was adjusted to 8.5 by addition of 1 M Tris base, and the mixture was oxidized in the dark by exposure to air at ambient temperature until it contained less than 0.1 mol of free SH groups/mol of RNase molecule (about 3 days). The thiol content was determined using 1 mM 5,5'-dithiobis (nitrobenzoic acid) in 6 M guanidine HCl, pH 8.0 (Ellman, 1959). sRNase was dialyzed against 50 mM (NH$_4$)$_2$CO$_3$ with extensive buffer changes and then lyophilized. The lyophilized material was dissolved in 0.1 M acetic acid to 4 mg/ml and stored frozen in aliquots.

Preparation of sRNase—Assays for protein disulfide-isomerase activity were performed by following reactivation of inactive reduced RNase or sRNase. To a N$_2$-saturated buffer containing 0.1 M potassium phosphate, pH 7.0, 1 mM EDTA was first added the required amount of GSH, GSSG, SeO$_3^2$- or NADPH and thioredoxin reductase (less than 20 µl), followed by PDI or one of the thioredoxins (less than one-tenth of the final volume). Refolding was then started by addition of RNase from stock solutions of reduced or sRNase to a final concentration of 0.5 mg/ml and incubation at 37°C. No attempt was made to exclude air from the samples, which contained 0.3-0.8 ml in closed Eppendorf tubes. Aliquots of 100 µl were removed at sequential times from the reactivation mixture and immediately assayed for RNase activity by mixing with 400 µl of 50 mM Tris-Cl, pH 7.5, 25 mM KCl, 5 mM MgCl$_2$, containing 200 µg/ml 2',3'-cCMP (compiled from Crook et al. (1980) and Hillson et al. (1984)). The enzyme concentration was previously used by Pigiet and Shuster, 1986, who found that 500 µM E. coli Trx gave maximal reactivation of RNase activity after 30-50 h of incubation. A concentration of 25 µM is also 3-5 times higher than the apparent $K_m$ value of PDI for RNase recently reported by Hawkins et al. (1991b) and Lyles and Gilbert (1991). Incubations were done at $37^\circ$C at pH 7.0 in the presence of 1 mM EDTA in air. In incubations with only the enzymes (1-100 µM Trx or P34H Trx or 1-10 µM PDI) in the absence of any exogenously added thiol oxidant, significant recovery of RNase activity could only be detected in incubations containing 100 µM P34H Trx (15%, data not shown).

Other investigators have shown that low molecular weight disulfides enhance reactivation; Creighton et al. (1980) used 100 µM GSSG as an oxidant in RNase refolding, and Lyles and Gilbert (1991) showed that a slightly better reoxidation buffer for refolding at a lower RNase concentration consisted of 1 mM GSH and 0.1 mM GSSG. The rate of recovery of RNase activity with 100 µM GSSG in one typical experiment is shown in Fig. 1. PDI was the best catalyst and about 100-fold more active than wt Trx on a molar basis. Most importantly, P34H Trx was much more active than wt Trx; in fact, 10 µM P34H Trx appeared more active than 100 µM wt Trx. There is also a factor of 10 difference in activity between PDI and P34H Trx. Thus, the exchange of Pro-34 for His in Trx increased the disulfide isomerase activity dramatically.

We found that selenite at 0.1-10 µM was a useful nonglucose oxidizing agent for protein disulfide formation. As seen in Fig. 2, sodium selenite at the catalytic concentration of 1 µM resulted in a final yield at 6 h that was similar to experiments in the presence of 100 µM GSSG. Selenite is an efficient oxidant of the active-site diithiole in thioredoxin and will promote nonstoichiometric oxidation by redox cycling with oxygen in air (Holmgren and Kumar, 1989).³ The relative

³ J. Lundström and A. Holmgren, manuscript in preparation.
Fig. 1. Kinetics of recovery of RNase activity from completely reduced RNase catalyzed by PDI or thioredoxins in the presence of 100 μM GSSG. RNase, 25 μM, was incubated at 37 °C in 100 mM potassium phosphate pH 7.0, 1 mM EDTA. X, in the absence of thioredoxin or PDI; ○, ○, 10 and 100 μM E. coli wt Trx, respectively; ▲, ▼, 10 and 100 μM P34H Trx, respectively. Aliquots were removed from the reactivation mixtures and assayed for RNase activity with 160 μg/ml 2',3'-cCMP in 50 mM Tris-Cl, pH 7.5, 25 mM KCl, 5 mM MgCl₂. Hydrolysis of the nucleotide was followed spectrophotometrically at 288 nm, and the recovery was calculated from a standard curve of native RNase.

Fig. 2. Refolding of fully reduced RNase in the presence of a catalytic amount of selenite. Assay conditions were as in Fig. 1 except for the addition of 1 μM SeO₃²⁻ in place of GSSG. X, in the absence of PDI or thioredoxin; ○, ○, 10 and 100 μM E. coli wt Trx, respectively; ▲, ▼, 10 and 100 μM P34H thioredoxin, respectively; ■, □, 1 and 10 μM PDI, respectively.

Efficiency of 10 μM P34H Trx with selenite was equal to or higher than that of 100 μM wt Trx. Thus, again in this system, P34H Trx was about 10-fold more effective than wt thioredoxin.

Refolding of Scrambled RNase (sRNase)—Recovery of RNase activity from the inactive, randomly oxidized molecule requires isomerization of disulfide bonds by thiol-disulfide exchange. This involves reduction and oxidation cycles. The process has an obligatory requirement for a thiol such as GSH or DTT to initiate reduction. We compared PDI, wt Trx, and P34H Trx using 100 μM GSH as the thiol. Under the conditions employed (Fig. 3), wt Trx showed almost no effect on regain of activity at either 10 or 100 μM, as compared with a control without enzyme. In contrast, P34H Trx gave considerable activity at 10 μM. However, the most rapid activation was obtained with 1 or 10 μM PDI. In contrast to P34H Trx, wt Trx is not reduced by 100 μM GSH due to its lower redox potential (Lin and Kim (1989)). As a result, Trx can only participate in thiol-disulfide interchange with RNase thioles in this system.

Since PDI is a substrate for mammalian thioredoxin reductase, we tried to use NADPH and thioredoxin reductase as a source of reducing equivalents for thiol-disulfide exchange. As shown in Fig. 4, 100 μM NADPH and a very low concentration of thioredoxin reductase (10 nM) worked efficiently. Regain of activity was faster and generally higher than with 100 μM GSH. Under these conditions, PDI was the best catalyst followed by P34H Trx. The difference between wt Trx and P34H Trx was again a factor of 10, as illustrated by the similar activation with 100 μM Trx and 10 μM P34H (Fig. 4). Thus, with all three proteins, NADPH and thioredoxin reductase is an excellent source of reducing equivalents for disulfide interchange. In theory, 100 μM NADPH is sufficient for complete reduction of 25 μM sRNase with four disulfides. These results demonstrate that there is no obligatory requirement for glutathione or another small thiol such as cysteamine in protein disulfide isomerization reactions. In addition, with a lower redox potential of the reducing equivalents (E° NADPH/NADP (-320 mV) compared with GSH/GSSG (-260 mV) (Gilbert, 1990), wt Trx catalyzes disulfide isomerization at a low but significant rate.

Final Yield of Ribonuclease Activity—In each experiment, a

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Footnote:

a J. Lundström and A. Holmgren, unpublished observations.
single batch of fully reduced RNase or sRNase was used. However, as noted by others (Hawkins and Freedman, 1991), different batches showed different final recoveries of activity, varying between 60 and 95%. The experiments in Figs. 1-4 show the results of separate, but typical experiments. The absolute values of RNase activity recovered at different time points varied in parallel to the final recovery of the particular batch of RNase used. The relative activities of the three enzymes was completely reproducible. In a separate series of experiments, several preparations were used, and the average final yield of active ribonuclease was determined by removing samples at 2 h (data not shown) and 24 h in two to five different experiments. With reduced RNase, long incubations with 1 μM selenite (Fig. 5C) generally resulted in better recovery of activity than with 100 μM GSSG (Fig. 5B), which unlike selenite is stoichiometrically consumed. Thus, P34H Trx and selenite constitute an excellent refolding system for a fully reduced protein. Only exposure to air for 24 h resulted in a high recovery of activity in the presence of 100 μM P34H Trx or 10 μM PDI (Fig. 5A).

Similar incubations with sRNase (Fig. 6) showed essentially no recovery of activity without addition of a reductant (Fig. 6A). However, addition of PDI gave a low but significant recovery of activity, possibly due to the fact that isolated PDI contains about one free sulfhydryl group (Lyles and Gilbert, 1991). By using 100 μM GSH, considerable activity was re-

![Figure 5](image_url)

**FIG. 5. Effects of different concentrations of PDI, wt Trx, or P34H Trx on recovery of RNase activity from fully reduced RNase after 24-h incubation at 37 °C.** A, only air; B, in the presence of 100 μM GSSG; C, in the presence of 1 μM SeOl⁻. The values are averages of two to five different experiments.

covered without enzyme. Low concentrations of wt Trx showed no effect in comparison with P34H Trx and PDI (Fig. 6B). The 24-h yields using 100 μM NADPH and 10 nM bovine thioredoxin reductase and 100 μM NADPH. The values are averages of two to five different experiments.

![Figure 6](image_url)

**FIG. 6. Effects of different concentrations of PDI, wt Trx, or P34H Trx on recovery of RNase activity from sRNase after 24-h incubation at 37 °C.** A, in the absence of a catalytic thiol; B, in the presence of 100 μM GSH; C, in the presence of 10 nM bovine thioredoxin reductase and 100 μM NADPH. The values are averages of two to five different experiments.

**DISCUSSION**

Thiol-disulfide interchange between thioredoxin and proteins occur 10⁹ to 10¹⁰ times faster than reactions between low molecular weight thiols and protein disulfides in water solution (10⁹ M⁻¹ min⁻¹ (Holmgren, 1979a) versus 0.1 M⁻¹ min⁻¹ (Shaked et al., 1980)). This difference in rate may be explained by interaction of a hydrophobic surface at the active site of thioredoxin (Holmgren, 1985) with other protein surfaces that bring the reacting thiols and disulfides into proximity of each other in a hydrophobic environment. Thiol-disulfide interchange via a mixed disulfide intermediate occurs orders of magnitude faster in gas phases and in organic solvents, as compared with the rate in water solution (Singh and Whitesides, 1990a, 1990b). For this reason, enzymatic catalysis of thiol/disulfide interchange may prove very useful for formation of native disulfides in vitro, e.g. in refolding of recombinant proteins.

PDI has been assumed to be the in vivo catalyst of protein...
disulfide formation (Freedman, 1989). Our results are in agreement with Hawkins et al. (1991a) showing that PDI on a weight basis is about 25-fold more efficient than thioredoxin in disulfide formation and isomerization in vitro. However, the presence of two thioredoxin-like domains in PDI raises the question of how and why PDI differs in its catalytic activity from thioredoxin. We have demonstrated that part of the explanation is in the active site sequence. P34H Trx, with a PDI-like active site, has about 10% of PDI activity in protein refolding compared on a molar basis. The intermediate redox potential of P34H Trx (−270 mV for wt E. coli Trx and −235 mV for P34H Trx (Krause et al., 1991) and values for PDI of −190 mV (Lundström and Holmgren, 1990) or −110 mV (Hawkins et al., 1991b)) correlates well with the intermediate activity of the mutant in protein thiol-disulfide interchange.

Selenite has been shown to be a substrate for mammalian thioredoxin reductase and for the reduced form of E. coli and mammalian thioredoxin through efficient redox cycling with oxygen (Holmgren and Kumar, 1989). In addition, the two thioredoxin-like domains of reduced PDI are rapidly oxidized by selenite. The rate of the oxidation of reduced thioredoxin and selenite is of the same order as the previously characterized reaction with insulin (10^4 M^-1 s^-1) (Krause et al., 1991). Our results show that selenite effectively replaced GSSG as a dithiol oxidant in the in vitro disulfide formation experiments, but the mechanism by which selenite participates in oxidative folding is not quite clear. A direct reaction between high selenite concentrations and the reduced polypeptide may occur with incorporation of selenium between thiols (Ganter and Corcoran, 1969). This will inactivate RNase, and it is more likely that selenite in our system acts to catalyze oxidation of PDI or thioredoxin. The concentration of PDI in the lumen of the endoplasmic reticulum is probably millimolar (Hillson et al., 1984), and selenite may exist at micromolar concentrations in cells (Stadtman, 1979). Thus, the combined effects of selenite and PDI at physiological concentrations extrapolated from the in vitro rates of reactivation of reduced RNase may approach or even be faster than the rates assumed for in vivo disulfide formation.

In the presence of molecular oxygen but in the absence of any exogenously added oxidant, the oxidation of thiols is clearly the rate-limiting step in oxidative folding of reduced RNase. The mechanism for enzymatic catalysis of disulfide formation in RNase is intimately connected to the nature of the oxidant of the reaction. Early experiments by Anfinsen and co-workers (Givol et al., 1964) where dihydroascorbate was used as the oxidant suggested that disulfide formation proceeds in two steps. First, random, spontaneous oxidation of thiols in the reduced polypeptide followed by isomerization of nonnative disulfides by an enzyme containing active site thiols. This led to the established use of scrambled RNase as a substrate for PDI.

In view of the findings with selenite, we believe that an initial, direct oxidation of a reduced polypeptide by PDI or Trx is an important first event in disulfide formation (Fig. 7) in folding systems containing oxidants that do not form stable mixed disulfides as with GSSG. A reaction intermediate (I) in such a reaction is a mixed disulfide between the two proteins. The rate of formation of disulfides is then determined by the extent to which the reaction proceeds via pathway a or b, a choice closely connected to the redox potential of the catalyst. The yield of correct disulfide is likely to be influenced by the relative stability of I. A long lived intermediate allows for thiols in the reduced polypeptide to search for the correct conformation before attacking the mixed disulfide. The reduced catalyst is then recycled back to its oxidized state after direct interaction with an oxidant.

Oxidation of the catalyst, rather than oxidation of the reduced polypeptide, is supported by the fact that both thioredoxin and PDI catalyze the following reaction.4

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\text{RNase-(SH)}_2 + \text{insulin-(SS)} \rightarrow \text{RNase-(SS)} + \text{insulin-(SH)}_2
\]

Furthermore, equilibrium studies between reduced RNase and Trx where the redox state of thioredoxin was followed as a change in fluorescence quantum yield revealed that equilibrium concentrations of RNase and thioredoxin (1 μM reduced RNase corresponding to 8 μM thiols and 1 μM wt thioredoxin) resulted in 30% reduction of thioredoxin.4 This shows that with RNase thiols in excess, pathway a (Fig. 7) is favored relative to b for thioredoxin. This is even more plausible for a protein with a higher redox potential such as P34H Trx or PDI.

Recently, Bardwell et al. (1991) have isolated a 25-kDa protein, DsbA, from E. coli required for disulfide formation. Interestingly, this protein also contains a histidine in the proposed active site, Cys-Pro-His-Cys. The redox potential of the dithiol/disulfide in this new protein, is with RNase thiols in excess, pathway a (Fig. 7) is favored relative to b for thioredoxin. This is even more plausible for a protein with a higher redox potential such as P34H Trx or PDI.

![Fig. 7. Hypothetical scheme of the mechanism for enzyme-catalyzed oxidative folding of a reduced polypeptide. A reduced polypeptide reacts with an active-site disulfide of PDI or thioredoxin, forming an intermediate, x. A nucleophile attack by a thiolate anion of the reduced polypeptide (pathway a) results in formation of a disulfide bond and reduced PDI or thioredoxin. Thioredoxin or PDI is then reoxidized by an oxidant, X.](https://example.com/fig7.png)
feature responsible for the higher activity of PDI, as compared with thioredoxin. Different combinations of thiols and oxidants and P34H Trx showed intermediate activity. Selenite and NADPH plus thioredoxin reductase were shown to replace GSH/GSSG redox buffers as “redox environment” for PDI and thioredoxin. It remains to be clarified if PDI, together with the thioredoxin system and selenite or other selenium compounds, is involved in disulfide formation in vivo.

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