Catalysis of Oxidative Protein Folding by Mutants of Protein Disulfide Isomerase with a Single Active-Site Cysteine†

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ABSTRACT: Protein disulfide isomerase (PDI), a very abundant protein in the endoplasmic reticulum, facilitates the formation and rearrangement of disulfide bonds using two nonequivalent redox active-sites, located in two different thioredoxin homology domains [Lyles, M. M., & Gilbert, H. F. (1994) *J. Biol. Chem. 269,* 30946−30952]. Each dithiol/disulfide active-site contains the thioredoxin consensus sequence CXXC. Four mutants of protein disulfide isomerase were constructed that have only a single active-site cysteine. Kinetic analysis of these mutants show that the first (more N-terminal) cysteine in either active site is essential for catalysis of oxidation and rearrangement during the refolding of reduced bovine pancreatic ribonuclease A (RNase). Mutant active sites with the sequence SGHC show no detectable activity for disulfide formation or rearrangement, even at concentrations of 25 μM. The second (more C-terminal) cysteine is not essential for catalysis of RNase disulfide rearrangements, but it is essential for catalysis of RNase oxidation, even in the presence of a glutathione redox buffer. Mutant active sites with the sequence CGHS show 12%−50% of the k_{cat} activity of wild-type active sites during the rearrangement phase of RNase refolding but <5% activity during the oxidation phase. In addition, mutants with the sequence CGHS accumulate significant levels of a covalent PDI−RNase complex during steady-state turnover while the wild-type enzyme and mutants with the sequence SGHC do not. Since both active-site cysteines are essential for catalysis of disulfide formation, the dominant mechanism for RNase oxidation may involve direct oxidation by the active-site PDI disulfide. Although it is not essential for catalysis of RNase rearrangements, the more C-terminal cysteine does contribute 2−8-fold to the rearrangement activity. A mechanism for substrate rearrangement is suggested in which the second active-site cysteine provides PDI with a way to "escape" from covalent intermediates that do not rearrange in a timely fashion. The second active-site cysteine may normally serve the wild-type enzyme as an internal clock that limits the time allowed for intramolecular substrate rearrangements.

The folding of disulfide-containing proteins from their reduced, cysteine-containing state is linked to the relatively slow chemical conversion of thiols to disulfides (oxidative folding); consequently, the spontaneous folding of disulfide-containing proteins can be a slow process (Creighton, 1984a; Gilbert, 1994). This realization led Anfinsen and his colleagues to search for and isolate a biological catalyst that accelerates the oxidative folding of disulfide-containing proteins (Goldberger *et al.,* 1963). This catalyst, protein disulfide isomerase (PDI),[1] is a highly conserved, 55 kDa protein that resides principally in the lumen of the endoplasmic reticulum where it contributes to the rapid folding of disulfide-containing proteins (Noiva & Lennarz, 1992; Freedman *et al.*, 1994; LaMantia & Lennarz, 1993).

PDI is a member of the thioredoxin superfamily of proteins (Freedman *et al.*, 1994), a family of enzymes and redox catalysts distinguished by an active site that contains a pair of cysteine residues in the sequence motif CXXC (Holmgren, 1985). In all of the family members, the biological activity involves the redox cycling of these vicinal cysteines between disulfide and dithiol redox states in order to mediate the transfer of redox equivalents between protein substrates. Among the various family members, the active sites vary greatly with respect to redox potential and effectiveness in catalyzing thiol/disulfide exchange (Freedman *et al.*, 1994). Mature PDI (491 amino acids) has two thioredoxin homology domains, each of which contains an active-site CGHC motif. One domain is located near the amino terminus (residues 7−97) while the second is located near the C-terminus (amino acids 351−432) (Edman *et al.*, 1985). Although the two active sites can function independently (Vuori *et al.*, 1992), they do not have equivalent catalytic properties (Lyles & Gilbert, 1994). For the oxidative folding of reduced, denatured RNase, the amino-terminal domain is capable of providing almost all of the catalytic activity at saturating concentrations of substrate, while the carboxy-terminal domain contributes more to the steady-state binding of the substrate. At substrate concentrations near the K_m, both active sites contribute almost equally to catalysis.

A common feature of both catalyzed and uncatalyzed oxidative protein folding is that some of the native or non-native disulfide bonds formed early in the folding reaction must be rearranged before folding can proceed to the native structure (eq 1).

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[1] Abbreviations: PDI, protein disulfide isomerase; GSH, glutathione; GSSG, glutathione disulfide; RNase, bovine pancreatic ribonuclease A; rRNase, reduced RNase; sRNase, scrambled RNase; BPTI, bovine pancreatic trypsin inhibitor; ER, endoplasmic reticulum; Gdn-HCl, guanidinium hydrochloride; DTT, dithiothreitol; DTNB, 5,5′-dithiobis-(2-nitrobenzoic acid); SDS−PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis.

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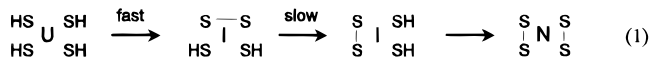


(1)

These oxidized folding intermediates may represent kinetic traps (States *et al.*, 1984; Creighton & Goldenberg, 1984; Weissman & Kim, 1993; Walker & Gilbert, 1995) containing native disulfide bonds (Weissman & Kim, 1991) or disulfide bonds that pair incorrect cysteine residues (Creighton, 1984b; Konishi *et al.*, 1982). In the cases where it has been examined, the initial formation of one or more disulfides is generally faster than the disulfide rearrangements leading to the native structure (Creighton, 1984b; Konishi *et al.*, 1982; Schaffer *et al.*, 1975; Lyles & Gilbert, 1991a). Because of the requirement for oxidizing equivalents to form disulfides in the substrate and the necessity of maintaining free sulfhydryl groups to mediate rearrangements, oxidative folding exhibits an optimum with respect to the thiol/disulfide redox state that is maintained during the reaction (Saxena & Wetlaufer, 1970; Konishi *et al.*, 1982; Lyles & Gilbert, 1991a). The redox state is usually maintained by a thiol/disulfide redox buffer, often reduced glutathione and its disulfide. For RNase oxidative folding, the redox optimum occurs at a GSH concentration of 1 mM and a GSSG concentration of 0.2 mM (Lyles & Gilbert, 1991a).

PDI is the most effective catalyst of oxidative folding among all of the thioredoxin family members isolated to date; it catalyzes both the oxidation and rearrangement phases of the reaction (Lyles & Gilbert, 1991a; Weissman & Kim, 1993). Recent reports show that PDI is also capable of catalyzing the refolding of proteins in which the cysteines have been converted to intermolecular mixed disulfides with glutathione (Ruoppolo & Freedman, 1995), showing that glutathione mixed disulfides can be intermediates in the folding reaction. In fact, the predominant pathway for forming a disulfide in a model peptide involves PDI catalysis of the formation of a mixed disulfide between the peptide and glutathione rather than a direct oxidation of the peptide dithiol by a disulfide active site (Darby *et al.*, 1994).

Although PDI, like other members of the thioredoxin family, has two cysteines at each of the active sites, catalysis of both rearrangements and intermolecular reactions involving the glutathione redox buffer could be accomplished using only one cysteine per active site (Holmgren, 1985). Mutants of *Dsb*A, a thiol/disulfide redox catalyst of the periplasmic space of *Escherichia coli*, catalyze both peptide oxidation and disulfide rearrangements with only a single active-site cysteine (Wunderlich *et al.*, 1995; Zapun *et al.*, 1994). *Eug*1, a thioredoxin-family member from yeast, has only one cysteine at each of two active sites, with the sequences CLHS and CIHS (Tachibana & Stevens, 1992). In the experiments described below, we have examined the catalysis of oxidative folding by mutants of PDI with only a single cysteine at one or both of the two active sites. The results suggest that efficient oxidation of the substrate requires an active-site with two cysteines—all single-cysteine active sites are virtually inactive (less than 5% of wild-type) as catalysts of disulfide formation. However, mutant active sites of the type CXXS display reasonably high activity in catalyzing disulfide rearrangement (12%−50% depending on the context of the mutation). The lower activity of single-cysteine mutants is also associated with the accumulation of covalent intermediates between PDI and its substrate, suggesting that the second active-site cysteine may provide PDI with a route to avoid the accumulation of PDI in intermediates that rearrange slowly.

MATERIALS AND METHODS

Materials. Pancreatic ribonuclease A (RNase), trypsin, HEPES, glutathione (GSH), glutathione disulfide (GSSG), *N*-ethylmaleimide (NEM), 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), and dithiothreitol (DTT) were obtained from Sigma (St. Louis, MO). Guanidine hydrochloride (Gdn-HCl) was from Pierce (Rockford, IL), and urea was from Kodak International Biotechnologies Inc. (New Haven, CT). EDTA and 2-mercaptoethanol were from J. T. Baker (Phillipsburg, NJ). Tris base and SDS were from Boehringer Mannheim (Indianapolis, IN). Bio-Gel P-4 and Bio-Gel P-6 were from Bio-Rad Laboratories (Richmond, CA). Oligonucleotides were obtained from Genosys (The Woodlands, TX), and restriction enzymes were from New England Biolabs (Beverly, MA). The T7 expression plasmid for rat PDI (pET-PDI.2) is from a previous study (Gilbert *et al.*, 1991). Wild-type and mutant PDI were expressed in *E. coli* strain BL21 (DE3) obtained from Novagen Inc. (Madison, WI).

Site-Directed Mutagenesis and Purification of PDI. The amino-terminal active-site mutants C38S and C35S ($\mathbf{N}_{SO}$ and $\mathbf{N}_{OS}$)[2] were generated using a M13 oligonucleotide-directed mutagenesis system purchased from Amersham Corp. (Arlington Heights, IL). An M13mp18 template with the 1160-bp *Xba*I−*Eco*RI pET-PDI.2 fragment was used along with a degenerate oligonucleotide TATGCCCCATGG-TSTGGCCA-CTSCAAAGCACTGG (S = G and C) to generate both amino-terminal mutants, but it failed to yield the $\mathbf{N}_{OS}$ mutation; therefore, the oligo GTTCTATGC-CCCATGGTCTGGCC-AACTGCAAAGCACTG was used to generate the $\mathbf{N}_{OS}$ mutation. The *Xba*I−*Eco*RI fragment was then subcloned back into a digested pET-PDI.2 vector containing the $\mathbf{C}_{OO}$ mutations (Lyles & Gilbert, 1994) to produce $\mathbf{N}_{SO}$:$\mathbf{C}_{OO}$ and $\mathbf{N}_{OS}$:$\mathbf{C}_{OO}$.

The carboxy-terminal mutations C379S ($\mathbf{C}_{OS}$) and C382S ($\mathbf{C}_{SO}$) were produced using the polymerase chain reaction (PCR) (Mullis et al., 1986). The forward primers, TTGT-TGAATTCTATGCTCCCTGGTGTGGTCAC-TCCAAGCAGCTAG and GTTGAATTCTATGCTCCCTG-GAGTGGTCACTCCAAGCAG contain the mutations and an *Eco*RI site, and the reverse primer, TCTGGCTCTA-AAGCTTCTTCTAGGT contains a *Hin*dIII site. The PCR product containing $\mathbf{C}_{OS}$ and $\mathbf{C}_{SO}$ was digested with *Eco*RI and *Hin*dIII, and the fragment containing the carboxy-terminal active site was subcloned into a digested pET-PDI.2 vector containing the $\mathbf{N}_{OO}$ mutations to produce $\mathbf{N}_{OO}$:$\mathbf{C}_{SO}$ and $\mathbf{N}_{OO}$:$\mathbf{C}_{OS}$. The $\mathbf{N}_{SO}$:$\mathbf{C}_{SO}$ mutant was produced by subcloning the *Xba*I−*Eco*RI fragment of $\mathbf{N}_{SO}$:$\mathbf{C}_{OO}$ into the $\mathbf{N}_{OO}$:$\mathbf{C}_{SO}$ vector. The sequences of all mutants were confirmed in the expression vectors by double-stranded DNA sequencing using the United States Biochemical (Cleveland, OH) sequenase version 2.0 DNA sequencing kit.

PDI was purified to >90% homogeneity, determined by SDS−PAGE, as described previously (Gilbert *et al.*, 1991).

[2] The following nomenclature will be used to refer to the different mutants of PDI: $\mathbf{N}_{SO}$ refers to the amino-terminal active site with the first (amino-most) cysteine intact (indicated by "S" for sulfur) and the second (carboxy-most) cysteine mutated to Ser (indicated by "O" for oxygen). $\mathbf{C}_{OS}$ refers to the carboxy-terminal active site with a cysteine to serine mutation at the first cysteine with the second cysteine unchanged.

The concentration of PDI was determined by absorbance at 280 nm using an $E^{0.1\%}$ of 0.94 $(mg/mL)^{-1}$ cm^{-1} (Lyles & Gilbert, 1991a), as confirmed by quantitative amino acid composition analysis. All absorbance measurements were performed using a Beckman DU-70 spectrophotometer.

Limited Proteolysis of PDI. Wild-type and mutant PDI (30 μM) were incubated with 6.9 μg/mL or 3.5 μg/mL of L-1-(tosylamido)-2-phenylethyl chloromethyl ketone-treated trypsin in 25 mM Tris-HCl, pH 8.0 for 1 h at 25 °C. Digestion was then stopped by adding SDS−PAGE sample buffer (2% SDS, 1.4 M 2-mercaptoethanol, 138 mM tris HCl, 22% glycerol, 56 μg of bromophenol blue/mL, pH 6.8) and immediately boiling for 5 min. The fragments were analyzed using 12.5% SDS−PAGE.

Preparation of Reduced and Scrambled Ribonuclease. Reduced RNase (rRNase) was produced as described previously (Lyles & Gilbert, 1991a) by incubating RNase (10 mg/mL) with DTT (0.14 M) in 0.1 M Tris-HCl, 2 mM EDTA, 6 M Gdn-HCl, pH 8.0 for 1 h at 37 °C. The denaturing buffer was removed by centrifugal gel filtration using Bio-Gel P-4 swollen in 0.1% acetic acid. The rRNase concentration was determined by absorbance at 280 nm using $\epsilon = 9300$ M^{-1} cm^{-1}.

Scrambled RNase (sRNase) (Hillson *et al.*, 1984) was produced by reducing native RNase (30 mg/mL) (50 mM Tris-HCl, 9 M urea, 130 mM DTT) followed by extensive dialysis against 0.1 M acetic acid. The RNase was then diluted into 50 vol of scrambling buffer (8 M urea, 100 mM sarcosine HCl, 100 mM acetic acid, pH 8.5), stirred in the dark for 3 days at 25 °C, and dialyzed extensively against 25 mM Tris-HCl, pH 8.0. The sRNase concentration was determined by absorbance at 280 nm using $\epsilon = 9800$ M^{-1} cm^{-1}. Thiol content was analyzed using Ellman's assay (Ellman, 1959) and was determined to be <4% of the RNase cysteine residues.

Ribonuclease Refolding Assay. Renaturation of RNase was followed in a continuous assay (Lyles & Gilbert, 1991a); the formation of active RNase was measured spectrophotometrically by monitoring hydrolysis of the RNase substrate, cCMP, at 296 nm. Typical assays contained 4.5 mM cCMP, 1 mM GSH, 0.2 mM GSSG, 100 mM Tris-HCl, 2 mM EDTA, 0−40 μM RNase, and 0−25 μM PDI. Assays were performed at 25 °C and initiated by the addition of RNase. The concentration of native RNase at any time was calculated from the first derivative of the absorbance versus time data and corrected for the depletion of cCMP and the product inhibition of RNase by CMP. The initial velocity of RNase folding was determined from the steady-state slope of RNase concentration versus time after the lag period.

Cysteine Reactivity. Wild-type and all mutants of PDI were reduced in 10 mM DTT for 1.5 h at 37 °C. The DTT was removed by centrifugal gel filtration using Bio-Gel P-6 equilibrated in 50 mM HEPES, pH 7.0. The total cysteine content of PDI was determined by reacting PDI with 500 μM DTNB in 3.5 M Gdn-HCl, 100 mM potassium phosphate, 5 mM EDTA, pH 7.0 at 25 °C, using a $\Delta\epsilon$ for TNB^- at 412 nm of 13 600 M^{-1} cm^{-1} (Ellman, 1959). The rate of reaction of DTNB with the slow-reacting PDI cysteines was measured at 25 °C using 7 μM PDI, 500 μM DTNB, 75 mM Tris-HCl, 2.25 mM EDTA, pH 8.0. The first absorbance point, taken approximately 1 min after mixing, was treated as the absorbance change due to fast-reacting cysteines. The number of slow reacting cysteines was determined from the difference in the initial absorbance and the absorbance upon completion of the reaction.

Intermediate Trapping Reaction. Wild type and mutant PDI's were reduced as described above. Reduced PDI (5 μM) was incubated with 16 μM rRNase, 1 mM GSH, 0.2 mM GSSG, 2 mM EDTA, 100 mM Tris-HCl, pH 8.0, for 30 min at 25 °C. The reaction was quenched by adding NEM (5 mM final concentration) and incubating 5 min at 25 °C. The samples were analyzed on 12.5% SDS−PAGE under reducing and non-reducing conditions.

RESULTS

Mutagenesis of PDI. In order to determine the role that each cysteine in each active site plays in oxidative folding, four PDI mutants were created by site-directed mutagenesis, each of which has only one of the four active-site cysteines. The other active-site cysteines were mutated to serine in order to preserve the hydrogen-bonding capability of the side chain while destroying the capacity for thiol/disulfide exchange involving the mutated amino acid. An additional mutant with a single cysteine at each active site was also created to analyze the interaction between the two active sites. All mutations were verified in the expression vector using double-stranded sequencing, and all mutants expressed at levels comparable to wild-type PDI. Using "O" and "S" to represent an oxygen (Ser) or sulfur (Cys) atom at a given position in the N-terminal (N) or C-terminal (C) thioredoxin domain, the four single-cysteine mutants are $\mathbf{N}_{SO}$:$\mathbf{C}_{OO}$, $\mathbf{N}_{OS}$:$\mathbf{C}_{OO}$, $\mathbf{N}_{OO}$:$\mathbf{C}_{SO}$, and $\mathbf{N}_{OO}$:$\mathbf{C}_{OS}$, and the two-cysteine mutant is $\mathbf{N}_{SO}$:$\mathbf{C}_{SO}$.[2]

Cysteine Reactivity. The effect of the mutations on enzyme stability, structure, and cysteine availability was examined by measuring the reactivity of each mutant with DTNB. In addition to the four active-site cysteines, PDI has two additional cysteine residues in the region linking the two thioredoxin domains (Edman *et al.*, 1985). In the presence of 3.5 M Gdn-HCl, all PDI cysteines reacted rapidly with DTNB, and the total number of cysteine residues agreed with the number expected from the sequence (±0.5 cysteines). In the absence of denaturant, the reaction of PDI with DTNB is distinctly biphasic (data not shown). A fraction of the cysteines reacts very quickly (half-life, <30 s) while the remainder reacts considerably more slowly (half-life, 5.1−9.7 min). The number of fast-reacting cysteines corresponds closely to the number of active-site cysteines in each of the PDI mutants. All six PDI's, including the wild-type enzyme, also have two slow-reacting cysteines, corresponding to the number of non-active-site cysteines. The reactivity of the slowly reacting cysteines ($k \approx 0.1$ min^{-1}) is approximately 10^4-fold slower than that expected for a typical exposed cysteine (Wilson *et al.*, 1977), suggesting that the tertiary structure of PDI significantly hinders the reactivity of the non-active-site cysteines. Since the slow cysteine reaction rate for the various mutants of PDI does not differ greatly from the wild-type reactivity (±33%), the active-site mutations are not likely to have induced major structural changes in PDI that alter the exposure of these cysteines to DTNB. In addition, limited proteolysis with trypsin indicates less than a 2-fold difference in proteolysis rates for the various mutants and wild-type proteins (data not shown), again indicating that no major structural changes are induced by the mutations.

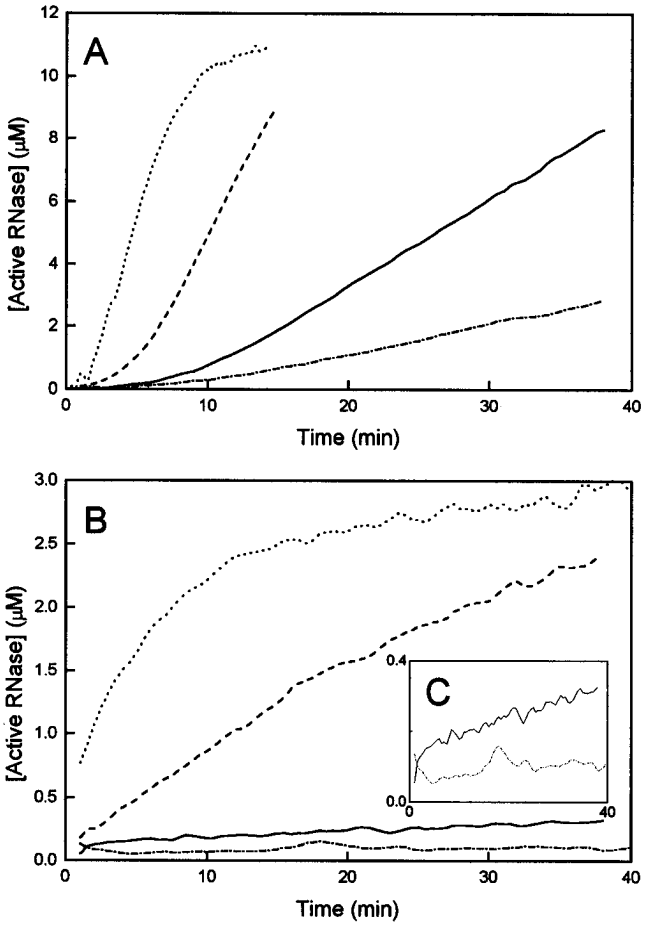


FIGURE 1: Assay of PDI-catalyzed folding of rRNase and sRNase. All assays were performed in 4.5 mM cCMP, 100 mM Tris-HCl, 2 mM EDTA, pH 8.0, 25 °C. The concentration of RNase versus time plot was obtained by taking the first derivative of the absorbance at 296 nm versus time data correcting for changing cCMP concentration and product inhibition by CMP (Lyles & Gilbert, 1991a). (A) 8 μM rRNase was used as the substrate with the following concentrations of the indicated mutants: (—) 8 μM $\mathbf{N}_{SO}$:$\mathbf{C}_{OO}$; (- - -) 8 μM $\mathbf{N}_{SO}$:$\mathbf{C}_{SO}$; (···) 7 μM $\mathbf{N}_{SS}$:$\mathbf{C}_{SS}$; (·- ·- ·) no PDI. (B) 8 μM sRNase was use as the substrate with the following concentrations of the indicated mutants: (—) 8 μM $\mathbf{N}_{SO}$:$\mathbf{C}_{OO}$; (- - -) 8 μM $\mathbf{N}_{SO}$:$\mathbf{C}_{SO}$; (···) 8 μM $\mathbf{N}_{SS}$:$\mathbf{C}_{SS}$; (·- ·- ·) no PDI. (C) Enlarged view of the data shown in Figure 1B for the mutant $\mathbf{N}_{SO}$:$\mathbf{C}_{OO}$ (—) and no PDI (·- ·- ·).

Effect of PDI Concentration on Catalysis of RNase Oxidation. Spontaneous refolding of rRNase in a glutathione redox buffer shows a distinct lag period (14 ± 2 min) in the generation of native RNase (Figure 1A). This lag is attributed to the formation of inactive, oxidized folding intermediates of RNase with disulfide bonds that must be broken or rearranged in a second, slow step before the RNase attains its native structure (Creighton, 1984b; Konishi *et al.*, 1982; Schaffer *et al.*, 1975; Lyles & Gilbert, 1991a). To verify that oxidation is the cause of the lag period, preoxidized, scrambled RNase (sRNase) was also used as a substrate. As expected, none of the active forms of PDI tested showed a lag using sRNase as the substrate (Figure 1B), even at much lower concentrations of wild-type PDI (2 μM) (data not shown).

To determine if the various mutants of PDI are capable of catalyzing the oxidation step and reducing the lag period, the lag time was measured as a function of PDI concentration for the various PDI species. Increasing concentrations of wild-type PDI substantially reduce the lag (0.9 min at 20 μM PDI) (Figure 2). Mutants of PDI with at least one active site intact ($\mathbf{N}_{SS}$:$\mathbf{C}_{OO}$ and $\mathbf{N}_{OO}$:$\mathbf{C}_{SS}$) also decrease the lag significantly (42% ± 2% and 75% ± 2% of wild-type activity, respectively), indicating that either of the two active sites can catalyze effective oxidation as long as both cysteine residues are present (Table 1). However, mutant active sites with only one cysteine ($\mathbf{N}_{SO}$:$\mathbf{C}_{OO}$, $\mathbf{N}_{OO}$:$\mathbf{C}_{SO}$, and $\mathbf{N}_{SO}$:$\mathbf{C}_{SO}$) have only minor effects on the lag period (Table 1), reflecting a low activity for catalysis of oxidation.

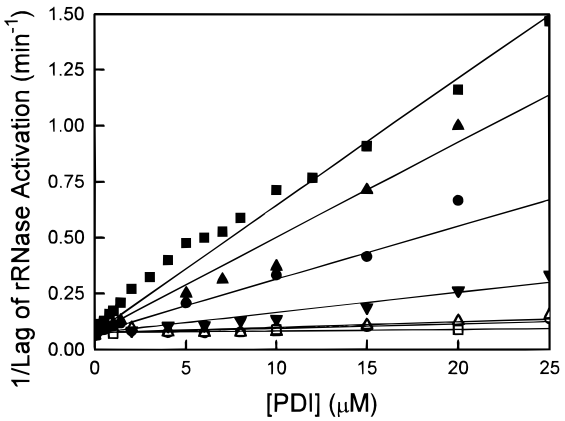


FIGURE 2: The effect of PDI type and concentration on the lag in rRNase folding. All assays were performed using 8 μM rRNase in 4.5 mM cCMP, 100 mM Tris-HCl, 2 mM EDTA, pH 8.0, 25 °C. The lag was determined from the *x*-intercept defined by a line following the steady-state rate of RNase concentration versus time. (■) $\mathbf{N}_{SS}$:$\mathbf{C}_{SS}$; (▲) $\mathbf{N}_{SS}$:$\mathbf{C}_{OO}$; (●) $\mathbf{N}_{OO}$:$\mathbf{C}_{SS}$; (▼) $\mathbf{N}_{SO}$:$\mathbf{C}_{SO}$; (△) $\mathbf{N}_{OO}$:$\mathbf{C}_{SO}$; (○) $\mathbf{N}_{SO}$:$\mathbf{C}_{OO}$; (□) $\mathbf{N}_{OO}$:$\mathbf{C}_{OO}$. Lines are drawn with the slopes indicated in Table 1 determined by a least-squares linear fit with a forced common *y*-intercept at the lag^{-1} in the absence of PDI.

Table 1: Effect of PDI Type and Concentration on the Lag of rRNase Folding[a]

PDI type	[PDI]/lag[b] [10^{-2} × (μM^{-1} min^{-1})]	[PDI]/lag (% wt)
$\mathbf{N}_{SS}$:$\mathbf{C}_{SS}$	5.7 ± 0.1	100
$\mathbf{N}_{SS}$:$\mathbf{C}_{OO}$	4.3 ± 0.1	75
$\mathbf{N}_{OO}$:$\mathbf{C}_{SS}$	2.4 ± 0.1	42
$\mathbf{N}_{OO}$:$\mathbf{C}_{OO}$	0.068 ± 0.021	1.2
$\mathbf{N}_{SO}$:$\mathbf{C}_{OO}$	0.19 ± 0.03	3.3
$\mathbf{N}_{OO}$:$\mathbf{C}_{SO}$	0.24 ± 0.04	4.2
$\mathbf{N}_{SO}$:$\mathbf{C}_{SO}$	0.89 ± 0.06	16

[a] See Figure 2 legend for experimental details. [b] [PDI]/lag is the slope of the least-squares linear fit to the data in Figure 2.

Effect of PDI Concentration on RNase Disulfide Rearrangement. The steady-state rate of rRNase folding after the lag was used to measure the effectiveness of single-cysteine mutants of PDI in catalyzing substrate rearrangements. Mutants containing the first cysteine in each active site ($\mathbf{N}_{SO}$:$\mathbf{C}_{OO}$ and $\mathbf{N}_{OO}$:$\mathbf{C}_{SO}$) increase the rate of rRNase refolding after the lag. The rate becomes nonlinear with respect to PDI concentration as the concentration of PDI exceeds that of the RNase substrate and saturates at high PDI concentrations (Figure 3). At saturating PDI, the rates correspond to 12% and 24%, respectively, of the activity of the corresponding wild-type active site (Table 2). By contrast, mutants containing only the second cysteine in each active site ($\mathbf{N}_{OS}$:$\mathbf{C}_{OO}$ and $\mathbf{N}_{OO}$:$\mathbf{C}_{OS}$) fail to measurably increase rRNase folding rates even at high concentration (Figure 3). Thus, the first cysteine in each active site is essential for catalysis of disulfide rearrangement while the second cysteine is not. However, it should be noted that the active mutants,

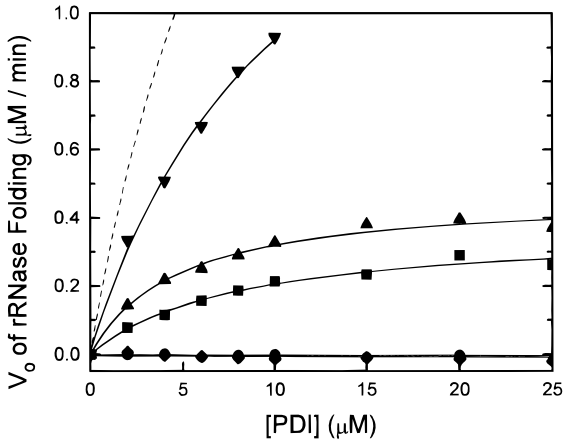


FIGURE 3: The effect of PDI type and concentration on the rate of rRNase folding. All assays were performed using 8 μM rRNase in 4.5 mM cCMP, 100 mM Tris-HCl, 2 mM EDTA, pH 8.0, 25 °C. (▼) $\mathbf{N}_{SO}$:$\mathbf{C}_{SO}$; (▲) $\mathbf{N}_{OO}$:$\mathbf{C}_{SO}$; (■) $\mathbf{N}_{SO}$:$\mathbf{C}_{OO}$; (●) $\mathbf{N}_{OO}$:$\mathbf{C}_{OS}$; (◆) $\mathbf{N}_{OS}$:$\mathbf{C}_{OO}$. The dashed line (- - -) shows the activity of the wild-type enzyme ($\mathbf{N}_{SS}$:$\mathbf{C}_{SS}$) drawn according to previously published kinetic constants (Lyles & Gilbert, 1994). Solid curves (—) are the least squares nonlinear hyperbolic fits to the data as shown in Table 2.

Table 2: Effect of PDI Type and Concentration on rRNase Folding[a]

PDI type	k_{cat}[c] (min^{-1})	K_m[c] (μM)	k_{cat}/K_m[d] [$\times 10^{-4}$ ($M\ min)^{-1}$]	k_{cat} (%)	K_m (%)	k_{cat}/K_m (%)
$\mathbf{N}_{SS}$:$\mathbf{C}_{SS}$[b]	0.49 ± 0.02	6.6 ± 0.6	7.4	100	100	100
$\mathbf{N}_{SO}$:$\mathbf{C}_{SO}$	0.24 ± 0.03	11 ± 2	2.2	50	170	30
$\mathbf{N}_{OO}$:$\mathbf{C}_{OO}$[b]	ND	ND	0.028	ND	ND	0.38
$\mathbf{N}_{SS}$:$\mathbf{C}_{OO}$[b]	0.39 ± 0.05	18 ± 5	2.2	100	100	100
$\mathbf{N}_{SO}$:$\mathbf{C}_{OO}$	0.046 ± 0.003	7.9 ± 1.4	0.58	12	44	27
$\mathbf{N}_{OS}$:$\mathbf{C}_{OO}$	ND	ND	<0.0036	ND	ND	0.62
$\mathbf{N}_{OO}$:$\mathbf{C}_{SS}$[b]	0.25 ± 0.03	15 ± 4	1.7	100	100	100
$\mathbf{N}_{OO}$:$\mathbf{C}_{SO}$	0.059 ± 0.003	4.7 ± 0.6	1.3	24	31	75
$\mathbf{N}_{OO}$:$\mathbf{C}_{OS}$	ND	ND	<0.0036	ND	ND	0.22

[a] See Figure 3 legend for experimental details. [b] Data from Lyles and Gilbert 1994. [c] Determined by fitting the data in Figure 3 using nonlinear least-squares fitting to the equation of a rectangular hyperbola. The error is the standard deviation estimated for the parameter. [d] Calculated from k_{cat} and K_m data, except for $\mathbf{N}_{OS}$:$\mathbf{C}_{OO}$ and $\mathbf{N}_{OO}$:$\mathbf{C}_{OS}$. For these two mutants, the detection limit was set at 10% above the background folding rate.

$\mathbf{N}_{SO}$:$\mathbf{C}_{OO}$ and $\mathbf{N}_{OO}$:$\mathbf{C}_{SO}$, are less efficient at catalyzing rearrangements than a wild-type active-site; the second active-site cysteine makes a significant contribution to the rate of RNase rearrangement. A mutant with the first cysteine at both active sites ($\mathbf{N}_{SO}$:$\mathbf{C}_{SO}$) has 50% ± 11% the activity of wild-type PDI (Table 2). Since this activity is greater than either single site mutant, the two active sites must be able to simultaneously catalyze RNase rearrangements.

Effect of RNase Concentration on Catalysis of Disulfide Rearrangement. Variable substrate (rRNase) concentrations were used to compare the steady-state kinetic parameters of the active mutants of PDI to the wild-type enzyme (Figure 4). The mutants with an active C-terminal domain, $\mathbf{N}_{OO}$:$\mathbf{C}_{SO}$ and $\mathbf{N}_{SO}$:$\mathbf{C}_{SO}$, have K_m's similar to wild-type PDI; however, the mutant with an inactive carboxy-terminal domain ($\mathbf{N}_{SO}$:$\mathbf{C}_{OO}$) has a K_m that is 7.4-fold greater than wild-type PDI (Table 3). Mutation of the C-terminal active site to remove both cysteines results in poorer steady-state binding of rRNase to PDI, consistent with previous findings that $\mathbf{N}_{OO}$:$\mathbf{C}_{SS}$ has a similar K_m (7.1 μM) to that of wild-type,

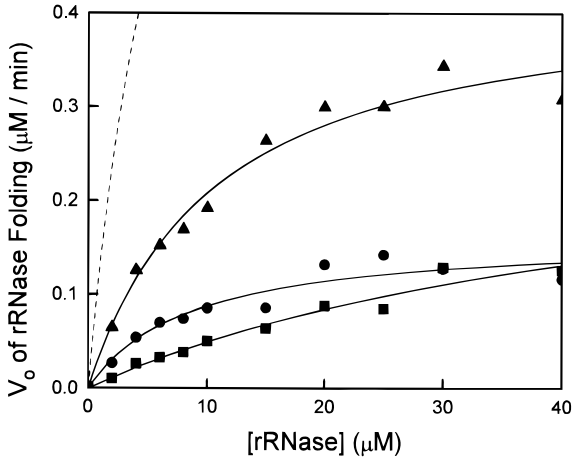


FIGURE 4: The effect of rRNase concentration on PDI-catalyzed rRNase folding. All assays were performed using 2 μM PDI in 4.5 mM cCMP, 100 mM Tris-HCl, 2 mM EDTA, pH 8.0, 25 °C. (▲) $\mathbf{N}_{SO}$:$\mathbf{C}_{SO}$; (●) $\mathbf{N}_{OO}$:$\mathbf{C}_{SO}$; (■) $\mathbf{N}_{SO}$:$\mathbf{C}_{OO}$. The dashed line (- - -) shows the activity of the wild-type enzyme ($\mathbf{N}_{SS}$:$\mathbf{C}_{SS}$) drawn according to previously published kinetic constants (Lyles & Gilbert, 1994). Solid curves (—) are the nonlinear least-squares hyperbolic fits to the data as shown in Table 3.

Table 3: Effect of RNase Concentration on PDI-Mediated RNase Folding[a]

PDI type	k_{cat}[b] (min^{-1})	K_m[b] (μM)	k_{cat}/K_m[c] [$\times 10^{-4}$ ($M\ min)^{-1}$]	k_{cat}[d] (%)	K_m (%)	k_{cat}/K_m (%)
$\mathbf{N}_{SS}$:$\mathbf{C}_{SS}$[e]	0.76 ± 0.02	6.9 ± 0.8	11	100	100	100
$\mathbf{N}_{SO}$:$\mathbf{C}_{SO}$	0.22 ± 0.01	10 ± 2	2.0	28	160	18
$\mathbf{N}_{SS}$:$\mathbf{C}_{OO}$[e]	0.72 ± 0.06	29 ± 4	2.5	100	100	100
$\mathbf{N}_{SO}$:$\mathbf{C}_{OO}$	0.15 ± 0.04	51 ± 19	0.30	21	180	12
$\mathbf{N}_{OO}$:$\mathbf{C}_{SS}$[e]	0.24 ± 0.01	7.1 ± 1.1	3.4	100	100	100
$\mathbf{N}_{OO}$:$\mathbf{C}_{SO}$	0.082 ± 0.011	8.7 ± 2.6	0.95	34	120	28

[a] See Figure 4 legend for experimental details. [b] Determined by fitting the data in Figure 4 using nonlinear least-squares fitting to the equation of a rectangular hyperbola. The error is the standard deviation estimated for the parameter. [c] Data calculated from k_{cat} and K_m values. [d] The % activity of the single-cysteine mutants is calculated relative to the corresponding species with a wild-type active site in only one of the two thioredoxin domains. [e] Data from Lyles and Gilbert, 1994.

but $\mathbf{N}_{SS}$:$\mathbf{C}_{OO}$ has a K_m that is 4.2-fold greater than wild-type PDI (Lyles & Gilbert, 1994).

The k_{cat} for the active mutants ($\mathbf{N}_{SO}$:$\mathbf{C}_{OO}$ and $\mathbf{N}_{OO}$:$\mathbf{C}_{SO}$) reveals substantial activity (21% and 34%, respectively) when compared directly to the corresponding wild-type active site ($\mathbf{N}_{SS}$:$\mathbf{C}_{OO}$ and $\mathbf{N}_{OO}$:$\mathbf{C}_{SS}$) (Table 3). The mutant with two single-cysteine active sites ($\mathbf{N}_{SO}$:$\mathbf{C}_{SO}$) has about 30% the activity of the wild-type enzyme ($\mathbf{N}_{SS}$:$\mathbf{C}_{SS}$), which is similar to that expected from summing the activities of the $\mathbf{N}_{SO}$:$\mathbf{C}_{OO}$ and $\mathbf{N}_{OO}$:$\mathbf{C}_{SO}$ mutants. Thus, PDI's active sites appear to react independently at high substrate concentrations. Experiments with varying concentrations of sRNase also show that the single-cysteine mutants of PDI have substantial activity in catalyzing the rearrangements of this substrate. However, sRNase is a very heterogeneous substrate, and we noted that less than half of the RNase could be recovered as active enzyme. At the high total sRNase concentrations required for saturation of the wild-type and mutant enzymes, the assay with this substrate was not very reproducible. Consequently, data for the k_{cat} and K_m are reported only for rRNase as a substrate.

Detection of PDI−RNase Intermediates. Although mutants with a single cysteine are quite active in catalyzing

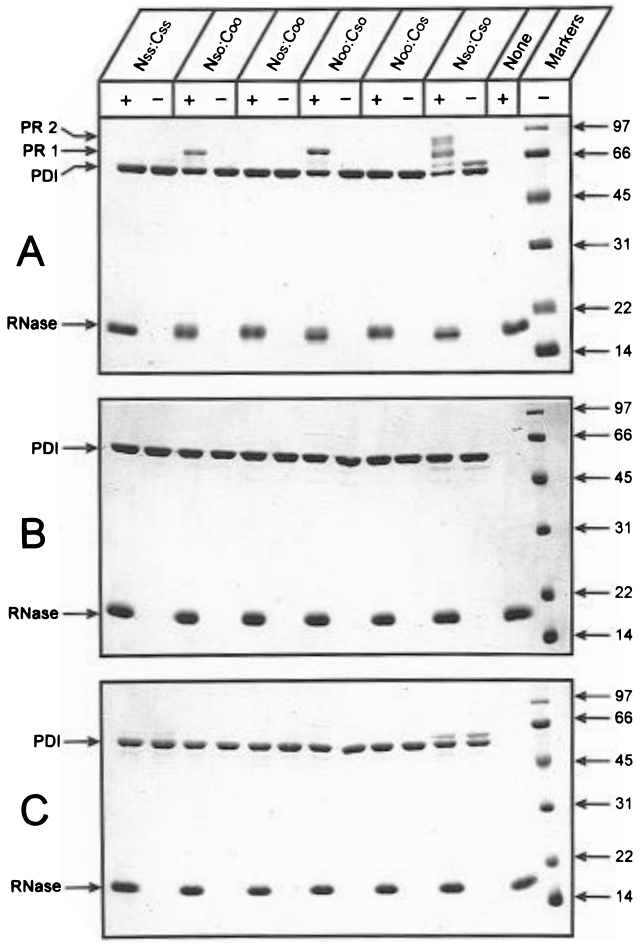


FIGURE 5: Accumulation of PDI−RNase folding intermediates. Coomassie-stained 12.5% SDS−PAGE. All assays were performed using 5 μM PDI, 1 mM GSH, 0.2 mM GSSG, 100 mM Tris-HCl, 2 mM EDTA, pH 8.0, 25 °C. All lanes marked with "+" have 16 μM rRNase present, and all lanes marked with "−" have no RNase present. The molecular masses of the standards are shown on the right in kDa. PR1 is a covalent complex between PDI and RNase while PR2 is a complex of two RNase molecules and PDI. (A) Samples were alkylated and displayed under non-reducing conditions. (B) Alkylated samples under reducing conditions. (C) Samples after completion of the folding reaction and alkylation run under non-reducing conditions.

RNase rearrangement, the second cysteine at each active site makes a substantial (2−8-fold) contribution to the rearrangement rate. All three mutant forms of PDI that are active in catalyzing RNase rearrangements ($\mathbf{N}_{SO}$:$\mathbf{C}_{OO}$, $\mathbf{N}_{OO}$:$\mathbf{C}_{SO}$, and $\mathbf{N}_{SO}$:$\mathbf{C}_{SO}$) form a higher molecular weight complex (PR1) on non-reducing SDS−PAGE (Figure 5A); however, the wild-type enzyme and the inactive mutants ($\mathbf{N}_{OS}$:$\mathbf{C}_{OO}$ and $\mathbf{N}_{OO}$:$\mathbf{C}_{OS}$) do not form this intermediate. The PR1 complexes are not present after reduction (Figure 5B), indicating that they are connected by intermolecular disulfides. After being trapped by high concentrations of NEM, purification of PR1 by DEAE HPLC followed by reduction and SDS−PAGE shows that that PR1 is a disulfide-bonded complex between PDI and RNase (Figure 6). In addition to PR1, the $\mathbf{N}_{SO}$:$\mathbf{C}_{SO}$ mutant forms a second, higher molecular weight intermediate, PR2 (Figure 5A). On SDS−PAGE, PDI has an apparent molecular weight of 59 000 (55 000 actual molecular weight) and the apparent molecular weight of PR1 is 71 000. PR2 has an apparent molecular weight of 83 000. The 12 kDa shifts between PDI and PR1 and between PR1 and PR2 are consistent with disulfide-linked complexes

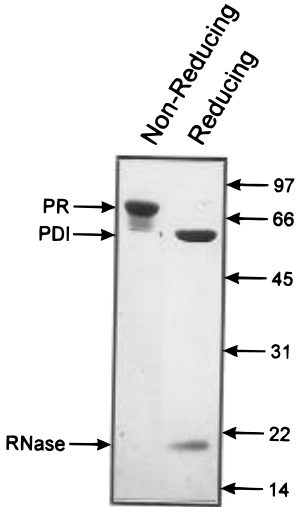


FIGURE 6: Conversion of PDI−RNase complex into PDI and RNase. Coomassie-stained 12.5% SDS−PAGE. The sample was prepared using 5 μM PDI, 16 μM rRNase, 1 mM GSH, 0.2 mM GSSG, 100 mM Tris-HCl, 2 mM EDTA, pH 8.0, 25 °C for 30 min followed by NEM alkylation. The PDI−RNase complex (PR) was purified on HPLC using a DEAE 5PW column. Lane 1. Alkylated intermediate. Lane 2. Alkylated intermediate treated with 2-mercaptoethanol prior to electrophoresis. The numbers on the right of the figures indicate the molecular masses in kDa and location of the molecular weight markers.

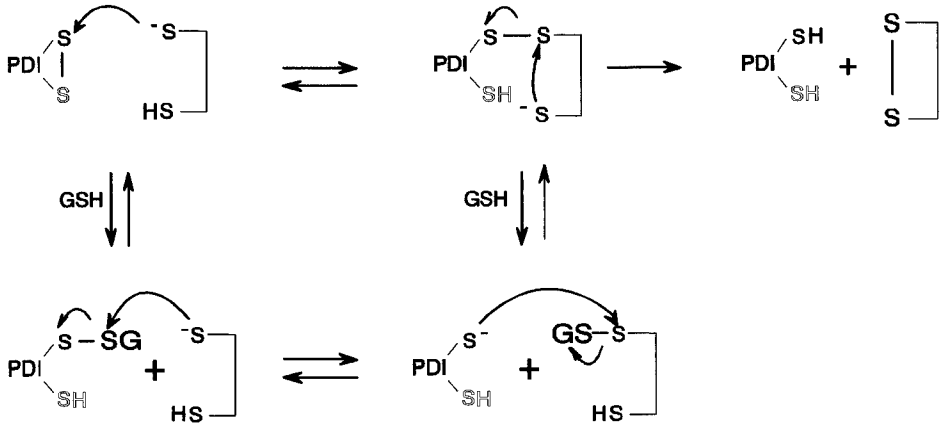
between PDI and one and two molecules of RNase (13.7 kDa), respectively. After 20 h of incubation, the PR1 and PR2 complexes disappear (Figure 5C), indicating that PR1 (and PR2) formation is not irreversible. Furthermore, after completion of the folding reaction, 100% ± 8% of the RNase is recovered (data not shown), showing that the RNase in the covalent complexes is chemically competent to be converted to native RNase.

DISCUSSION

Mutation of the individual cysteine residues in PDI's conserved CXXC sequences reveals different roles for the two active-site cysteines. Effective formation of disulfide bonds during the lag of RNase refolding requires an active site with both cysteines. The first cysteine[3] in either the N-terminal or C-terminal thioredoxin domain is essential for the disulfide isomerase activity of that domain and for the formation of covalent complexes with the substrate; mutants $\mathbf{N}_{OS}$:$\mathbf{C}_{OO}$ and $\mathbf{N}_{OO}$:$\mathbf{C}_{OS}$ have undetectable (<2%) activity in catalyzing RNase refolding even at concentrations as high as 25 μM. Using a single concentration of scrambled RNase as a substrate and a single concentration of PDI, Vuori *et al.* (1992) reported that the mutants $\mathbf{N}_{OS}$:$\mathbf{C}_{SS}$ and $\mathbf{N}_{SS}$:$\mathbf{C}_{OS}$ were half as active as wild-type PDI and that the mutant $\mathbf{N}_{OS}$:$\mathbf{C}_{OS}$ had no measurable activity. Our results are consistent with these findings and further suggest that the lack of activity results from the inability of the second cysteine in either active site to form covalent intermediates with the substrates, most likely due to steric constraints at the active site similar to that observed for the *E. coli* homologue, *DsbA* (Wunderlich *et al.*, 1995; Zapun *et al.*,

[3] The "first" cysteine is used to denote the more N-terminal cysteine at each active site (CXXC), and the "second" cysteine is used to designate the more C-terminal cysteine (CXXC). This designation was adopted to avoid confusion with the location of the cysteines within the N- and C-terminal thioredoxin domains.

Scheme 1: Catalysis of Substrate Disulfide Formation by PDI[a]



[a] In the top pathway, PDI's active-site disulfide is transferred to the substrate through thiol/disulfide exchange. This pathway requires the second active-site cysteine. The bottom pathway represents the formation of a substrate disulfide through thiol/disulfide exchange involving the substrate, PDI, and the redox buffer (GSH and GSSG). Oxidation mediated by the redox buffer does not specifically require the participation of the second active-site cysteine.

1994). In contrast, CXXS mutants ($\mathbf{N}_{SO}$:$\mathbf{C}_{OO}$, $\mathbf{N}_{OO}$:$\mathbf{C}_{SO}$, and $\mathbf{N}_{SO}$:$\mathbf{C}_{SO}$) retain significant but not complete activity in catalyzing the conversion of folding intermediates to the native enzyme. In all cases, mutation of the second active-site cysteine to serine is associated with the accumulation of substantial quantities of a covalent intermediate between PDI and the RNase substrate.

RNase Oxidation. The lag observed during the oxidative refolding of reduced RNase in a glutathione redox buffer can be attributed to the slow accumulation of inactive folding intermediates that must precede the formation of native RNase (Schaffer *et al.*, 1975; Lyles & Gilbert, 1991a). These intermediates are a complex mixture consisting of nearly random intramolecular disulfides (Creighton, 1984b) as well as mixed disulfides with the glutathione redox buffer (Konishi *et al.*, 1982). The amount of GSH formed during the lag suggests that the lag is associated with extensive RNase disulfide formation, >3.5 disulfide equiv per RNase (Lyles & Gilbert, 1991a). In addition, the refolding of scrambled RNase proceeds without a lag (Figure 1B), suggesting that the lag is not the result of slow disulfide rearrangements.

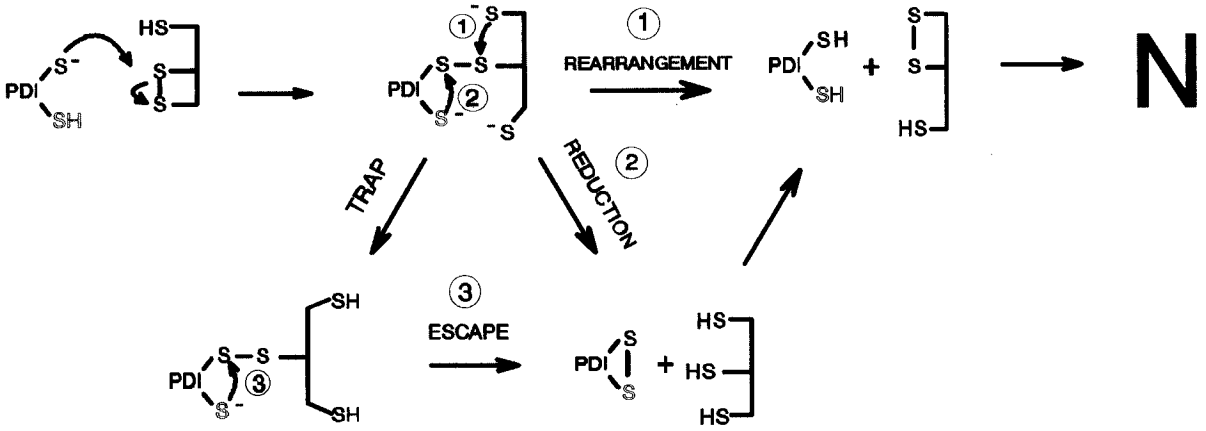
Wild-type PDI is an effective catalyst of disulfide formation, including the formation of glutathione mixed disulfides. Because only one active-site cysteine is formally required to catalyze disulfide formation through a protein−SSG intermediate (Scheme 1) (Zapun *et al.*, 1994; Wunderlich *et al.*, 1995), we anticipated that single-cysteine mutants of PDI might catalyze disulfide formation in a glutathione redox buffer. However, the single-cysteine mutants of PDI are ineffective in decreasing the lag during the refolding of reduced RNase, even when a glutathione redox buffer is present. Species that contain both active-site cysteines, $\mathbf{N}_{SS}$:$\mathbf{C}_{OO}$, $\mathbf{N}_{OO}$:$\mathbf{C}_{SS}$, and $\mathbf{N}_{SS}$:$\mathbf{C}_{SS}$, are much more effective in decreasing the lag. The simplest interpretation is that the predominant pathway for forming disulfide-containing intermediate(s) during the refolding of reduced RNase involves the direct oxidation of the substrate by an active-site disulfide formed between the two cysteines in each CGHC sequence. PDI can participate in the formation and rearrangement of glutathione mixed disulfides (Darby *et al.*, 1994; Ruoppolo & Freedman, 1995), but glutathione mixed disulfides are not essential intermediates. Although we cannot rigorously eliminate the possibility that the second cysteine residue at each active site might facilitate reactions involving glutathione through hydrogen-bonding mechanisms analogous to those proposed by Jeng *et al.* (1995), wild-type PDI can stoichiometrically provide disulfide equivalents and catalyze isomerization in the absence of any redox buffer (Lyles & Gilbert, 1991b).

LaMantia and Lennarz (1993) constructed mutants of the type $\mathbf{N}_{SO}$:$\mathbf{C}_{SS}$, $\mathbf{N}_{SS}$:$\mathbf{C}_{SO}$, and $\mathbf{N}_{SO}$:$\mathbf{C}_{SO}$ that showed diminished activity in catalyzing the oxidative folding of reduced bovine pancreatic trypsin inhibitor (BPTI) as measured by the gain in trypsin inhibitor activity. The $\mathbf{N}_{SO}$:$\mathbf{C}_{SO}$ mutant displayed less than 5% of the wild-type activity in this assay. Since folding intermediates of BPTI with only one disulfide bond are active trypsin inhibitors (Staley & Kim, 1992), this assay should detect the oxidation activity of PDI but may be less sensitive to disulfide isomerization (isomerization will only convert one active BPTI species to another active BPTI species). Thus, the results of LaMantia and Lennarz (1993) are consistent with our findings that mutant active sites lacking the more C-terminal cysteine are inactive in catalyzing disulfide bond formation.

RNase Rearrangements. After accelerating the formation of highly oxidized folding intermediates during the lag, PDI also catalyzes the subsequent rearrangements of these intermediates to native RNase. Although the second active-site cysteine is not essential for catalysis of RNase rearrangement, it does contribute significantly to catalysis. Removing the second active-site cysteine results in mutant PDI's that have only 12%−50% the k_{cat} of the corresponding wild-type active-site.

For intramolecular rearrangement of the substrate (Scheme 2, pathway 1), there is no obvious need for a PDI active site with two cysteine residues; yet, a dithiol active site is 3−5-fold more effective as a rearrangement catalyst than a monothiol active-site. The second active-site cysteine might contribute to catalysis of RNase "rearrangements" through a reduction−oxidation mechanism (Scheme 2, path 2) involving net reduction of the substrate and formation of an active-site disulfide. Alternatively, the second cysteine might provide an "escape" route by which PDI could extricate itself from covalent complexes that fail to rearrange (Scheme 2, path 3) in a timely manner. In these reduction−oxidation

Scheme 2: Catalysis of Disulfide Rearrangements by PDI[a]



[a] Net disulfide rearrangement may proceed by a rearrangement involving only one active-site thiol (pathway 1), or by a reduction/reoxidation that requires both active-site thiols (pathway 2). In addition, substrate that becomes trapped on PDI and unable to undergo rearrangement may use the second active-site cysteine to provide an escape (pathway 3) mechanism.

mechanisms, the second active-site cysteine could serve as an internal clock. When PDI−substrate complexes fail to rearrange fast enough, displacement of the substrate by the second active-site cysteine would free up both substrate and PDI for another attempt at rearrangement or oxidation. When these "escape" pathways are blocked by mutation, alternative pathways such as slower rearrangements or glutathione-dependent processes could provide for complete substrate refolding, albeit at a slower rate.

In yeast, PDI is an essential protein; however, the lethal mutation can be rescued by a plasmid containing a yeast PDI mutant of the type $\mathbf{N}_{SO}$:$\mathbf{C}_{SO}$ (LaMantia & Lennarz, 1993). Our finding that the corresponding mutant of the rat enzyme has a relatively high level of disulfide isomerase activity (30%−50% of wild-type) and measurable oxidase activity (15% of wild-type) would suggest that these activities could indeed be essential for yeast viability. Gunther *et al.* (1993) were able to rescue a lethal mutation in the yeast PDI gene by overexpressing rat *Erp*72, a thioredoxin family member with three thioredoxin domains, even though this protein has only a fraction of the disulfide isomerase activity of PDI (Rupp *et al.*, 1994).

Accumulation of Intermediates. According to the mechanisms of Scheme 2, slowing the reduction and escape pathways by mutating the second active-site cysteine should increase the steady-state levels of covalent PDI−substrate complexes if these pathways make a significant contribution to catalysis by the wild-type enzyme. Under the conditions that we examined, wild-type PDI does not accumulate detectable PDI−RNase covalent intermediates, presumably because of rapid displacement of the intermediate by attack of the second active-site cysteine. The PDI−RNase complex that accumulates when the second cysteine is absent may represent species that would normally escape from the complex through rapid displacement mediated by the second cysteine. In a glutathione redox buffer, the intermediates, which represent as much as 30%−70% of the total PDI, form early during the reaction and are chemically competent to form native RNase. The kinetic competence of the various PDI−RNase covalent complexes and whether or not they represent on-pathway or off-pathway species will be the subject of further studies.

Interestingly, the $\mathbf{N}_{SO}$:$\mathbf{C}_{SO}$ mutant displays two different disulfide-linked species, one with a single RNase bound per PDI monomer and one with two RNase molecules bound. Thus, the two PDI active sites appear to be capable of interacting with substrate simultaneously. The fact that the k_{cat} for the mutant $\mathbf{N}_{SO}$:$\mathbf{C}_{SO}$ is similar to sum of the k_{cat} for the two mutants $\mathbf{N}_{SO}$:$\mathbf{C}_{OO}$ and $\mathbf{N}_{SO}$:$\mathbf{C}_{SO}$ is also consistent with the simultaneous functioning of both active sites.

REFERENCES

Bardwell, J. C. A., Lee, J-O., Jander, Martin N., Belin, D., & Beckwith, J. (1991) *Proc. Natl. Acad. Sci. U.S.A. 90*, 1038−1042.

Bergman, L. W., & Kuehl, W. M. (1979) *J. Biol. Chem. 254*, 8869−8876.

Creighton, T. E. (1984a) *Methods Enzymol. 107*, 305−329.

Creighton, T. E. (1984b) *J. Mol. Biol. 129*, 411−434.

Creighton, T. E., & Goldenberg, D. P. (1984) *J. Mol. Biol. 179*, 497−526.

Darby, N. J., Freedman, R. B., & Creighton, T. E. (1994) *Biochemistry 33*, 7937−7947.

Edman, J. C., Ellis, L., Blacher, R. W., Roth, R. A., & Rutter, R. J. (1985) *Nature 317*, 267−270.

Ellman, G. L. (1959) *Arch. Biochem. Biophys. 82*, 70−77.

Freedman, R. B., Hirst, R. R., & Tuite, M. F. (1994) *Trends Biol. Sci. 19*, 331−336.

Gilbert, H. F. (1989) *Biochemistry 27*, 7298−7305.

Gilbert, H. F. (1990) *Adv. Enzymol. 63*, 69−172.

Gilbert, H. F. (1994) in *Protein Folding* (Pain, R., Ed.) pp 104−136, Oxford.

Gilbert, H. F., Kruzel, M. L., Lyles, M. M., & Harper, J. W. (1991) *Protein Expression Purif. 2*, 194−198.

Goldberger, R. F., Epstein, C. J., & Anfinsen, C. B. (1963) *J. Biol. Chem. 238*, 628−635.

Gunther, R., Srinivasan, M., Haugejorden, S., Green, M., Ehbrecht, I. M., & Kuntzel, H. (1993) *J. Biol. Chem. 268*, 7728−7732.

Hillson, D. A., Lambert, N., & Freedman, R. B. (1984) *Methods. Enzymol. 107*, 281−292.

Holmgren, A. (1985) *Annu. Rev. Biochem. 54*, 237−271.

Hu, C. H., & Tsou, C. L. (1991) *FEBS Lett. 290*, 87−89.

Jeng, M-F., Holmgren A., & Dyson, H. J. (1995) *Biochemistry 34*, 10101−10105.

Konishi, Y., Ooi, T., & Scheraga, H. A. (1982) *Biochemistry 21*, 4734−4740.

LaMantia, M., & Lennarz, W. J. (1993) *Cell 74*, 899−908.

Lundstrom-Ljung J., & Holmgren, A. (1995) *J. Biol. Chem. 270*, 7822−7828.

Lyles, M. M., & Gilbert, H. F. (1991a) *Biochemistry 30*, 613−619.

Lyles, M. M., & Gilbert, H. F. (1991b) *Biochemistry 30*, 619−625.

Lyles, M. M., & Gilbert, H. F. (1994) *J. Biol. Chem. 269,* 30946−30952.
Martin, J. L., Bardwell, J. C., & Kuriyan, J. (1993) *Nature 365*, 464−468.
Mullis, K., Faloona, F., Schart, S., Saki, R., Horn, G., & Erlich, H. (1986) *Cold Spring Harbor Symp. Quant. Biol. 51,* 263−272.
Noiva, R., & Lennarz, W. J.(1992) *J. Biol. Chem. 267*, 3553−3556.
Ruoppolo, M., & Freedman, R. B. (1995) *Biochemistry 34*, 9380−9388.
Rupp, K., Birnbach, U., Lundstrom, J., Van, P. N., & Soling, H. D. (1994) *J. Biol. Chem. 269*, 2501−2507.
Saxena, V P., & Wetlaufer, D. B. (1970) *Biochemistry 9*, 5015−5022.
Schaffer, S. W., Ahmed, A. K., & Wetlaufer, D. B. (1975) *J. Biol. Chem. 250*, 8483−8486.
Staley, J. P., & Kim, P. S. (1992) *Proc. Nat. Acad. Sci. U.S.A. 89*, 1519−1523.
States, D. J., Dobson, C. M., Karplus, M., & Creighton, T. E. (1984) *J. Mol. Biol. 174*, 411−418.
Tachibana, C., & Stevens, T. H. (1992) *Mol. Cell. Biol. 12*, 4601−4611.
Vuori, K., Myllyla, R., Pihlajaniemi, T., & Kivirikko, R. I. (1992) *J. Biol. Chem. 267*, 7211−7214.
Walker, K. W., & Gilbert, H. F. (1995) *Biochemistry 34*, 13642−13650.
Weissman, J. S., & Kim, P. S. (1991) *Science 253*, 1386−1390.
Weissman, J. S., & Kim, P. S. (1993) *Nature 365*, 185−188.
Wilson, J. M., Bayer, R. J., & Hupe, D. J. (1977) *J. Am. Chem. Soc*. *99*, 7922−7927.
Wunderlich, M., Otto, A., Maskos, K., Mucke, M., Seckler, R., & Glockshuber, R. (1995) *J. Mol. Biol. 247*, 28−33.
Zapun, A., Cooper, L, & Creighton, T. E. (1994) *Biochemistry 33*, 1907−1914.

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