Catalysis of Thiol/Disulfide Exchange

GLUTAREDOXIN 1 AND PROTEIN-DISULFIDE ISOMERASE USE DIFFERENT MECHANISMS TO ENHANCE OXIDASE AND REDUCTASE ACTIVITIES*

Received for publication, October 7, 2004, and in revised form, March 16, 2005
Published, JBC Papers in Press, April 6, 2005, DOI 10.1074/jbc.M411476200

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Glutaredoxin (Grx) and protein-disulfide isomerase (PDI) are members of the thioredoxin superfamily of thiol/disulfide exchange catalysts. Thermodynamically, rat PDI is a 600-fold better oxidizing agent than Grx1 from Escherichia coli. Despite that, Grx1 is a surprisingly good protein oxidase. It catalyzes protein disulfide formation in a redox buffer with an initial velocity that is 30-fold faster than PDI. Catalysis of protein and peptide oxidation by the individual catalytic domains of PDI and by a Grx1-PDI chimera show that differences in active site chemistry are fundamental to their oxidase activity. Mutations in the active site cysteines reveal that Grx1 needs only one cysteine to catalyze rapid substrate oxidation, whereas PDI requires both cysteines. Grx1 is a good oxidase because of the high reactivity of a Grx1-glutathione mixed disulfide, and PDI is a good oxidase because of the high reactivity of the disulfide between the two active site cysteines. As a protein disulfide reductase, Grx1 is also superior to PDI. It catalyzes the reduction of nonnative disulfides in scrambled ribonuclease and protein-glutathione mixed disulfides 30–180 times faster than PDI. A multidomain structure is necessary for PDI to catalyze effective protein reduction; however, placing Grx1 into the PDI multidomain structure does not enhance its already high reductase activity. Grx1 and PDI have both found mechanisms to enhance active site reactivity toward proteins, particularly in the kinetically difficult direction: Grx1 by providing a reactive glutathione mixed disulfide to supplement its oxidase activity and PDI by utilizing its multidomain structure to supplement its reductase activity.

Members of the thioredoxin superfamily of redox proteins transfer reducing and oxidizing equivalents between proteins through thiol-disulfide exchange. With overall structural similarity, the classical thioredoxin and glutaredoxin members of the family all have an active site with the sequence CXXC (1). The two active site cysteines are able to cycle between dithiol and disulfide redox states. Despite the similarities in the active sites and a general mechanism involving thiol-disulfide exchange, thioredoxin family proteins serve a diverse set of biological functions. The family patriarch is thioredoxin, the principal function of which is to provide reducing equivalents for deoxyribonucleotide synthesis by reducing the active site disulfide of ribonucleotide reductase (2). Glutaredoxins (Grx),1 which have a GSH binding site (3), serve as reductants of protein-SG mixed disulfides and also provide reducing equivalents to ribonucleotide reductase apart from their many other functions (4). Protein-disulfide isomerase (PDI) and its prokaryotic counterpart, DsbA, function primarily to introduce disulfides into protein substrates during oxidative protein folding (5, 6).

In thioredoxin and PDI, the more N-terminal cysteine, CXXC, of the active site is exposed to solution (7) and acts as a nucleophile to attack substrate disulfides (8). The second active cysteine, CXXX, is buried and constrained to react with only the N-terminal active site cysteine (7). The reduction potentials of the active site disulfides of the thioredoxin family span an extremely large range, depending, in part, on the identity of the two XX residues and the protein context in which the CXXC sequence occurs. The reduction potential of the best oxidant, DsbA (−124 mV) differs from the reduction potential of the worst oxidant, thioredoxin (−270 mV) by more than 146 mV, corresponding to a ratio of nearly 105 in thermodynamic stability during the disulfide/dithiol equilibrium (9). Within the family, there is a strong correlation between the identity of the two intervening residues and the redox potentials (10–12). Both Raines and co-workers (12) and Creighton and co-workers (13) have argued that much of the difference in reduction potential can be attributed to factors that stabilize the thiolate anion of the reduced active site. The active site cysteines have been suggested to form a thiol-thiolate hydrogen bond network, which stabilizes the more N-terminal active site thiolate in the reduced form of the proteins (14–16).

In general, the kinetic properties of the thioredoxin family proteins parallel their thermodynamic ones. In its reduced state, thioredoxin and glutaredoxin are exceptional reductants of both protein and nonprotein disulfides (2, 4). By contrast, the poorest reductants thermodynamically, PDI and DsbA, are also poor reductants kinetically. When comparing the kinetics of substrate disulfide formation using the oxidized (disulfide) active site, DsbA and protein-disulfide isomerase are the best oxidants kinetically. A link between thermodynamics and kinetic reactivity is not unexpected, given the overall mechanism of thiol-disulfide exchange, where the fundamental chemical process is the same among all the family members. For thiol/disulfide exchange, the transition state is symmetrical (18),

1 The abbreviations used are: Grx, glutaredoxin; Grx1, glutaredoxin 1 from E. coli; PDI, protein-disulfide isomerase; SG-RNase, RNase with its cysteines in mixed disulfides with glutathione.
suggested that factors affecting the stabilities of either the thiolate or disulfide will also be reflected in the transition state for thiol-disulfide exchange.

Glutaredoxins, also known as thioredoxins, are predominately localized to the cytoplasm but also have been detected in the nucleus and mitochondria (19–21). Glutaredoxins catalyze the reduction of protein-SG mixed disulfides, preventing their accumulation in the reducing environment of the cytoplasm (4). By contrast, PDI and DsbA are localized in the more oxidizing environment of the endoplasmic reticulum and periplasm, respectively, in keeping with their function as oxidases.

*Escherichia coli* Grx1 is a single domain protein, but mammalian PDI is a multidomain catalyst (22). PDI is composed of four structural domains, all of which adopt a thioredoxin fold. The two catalytic domains of PDI (a and a') are separated by two intervening structural domains (b and b'). The multidomain structure is important in the catalysis of disulfide isomerization during protein folding, but it is not essential for PDI oxidase activity. PDI catalyzes disulfide isomerization *in vivo*, but its principal function in yeast is to serve as an oxidase for disulfide formation in the endoplasmic reticulum (6).

In light of the expected linkage of kinetics and thermodynamics in the thioredoxin family, we were struck by the observation that Grx1 is a much better oxidase for protein disulfides than PDI despite the fact that PDI is a 600-fold better oxidant, thermodynamically (9). To investigate the basis of the unusual versatility of glutaredoxin as a protein oxidase and reductase, we have compared the kinetics of disulfide formation and reduction catalyzed by Grx1 and PDI. We also constructed a chimerical protein with the Grx1 structure in place of one of the active site domains in PDI.

Mutation of the more C-terminal active site cysteines of Grx1 and PDI shows that Grx1, but not PDI, can utilize a single active site cysteine to catalyze protein oxidation through the formation of a Grx-SG mixed disulfide. As an oxidase, Grx1 provides oxidizing equivalents to its substrates through a re-active mixed disulfide with glutathione. As protein-disulfide reductases, both Grx1 and PDI require two active site cysteines. For PDI, its multidomain structure is needed to catalyze protein disulfide reduction, whereas multiple domains are not needed to catalyze substrate oxidation. It is suggested that both Grx1 and PDI have developed specialized mechanisms to enhance catalysis of reactions that would normally be difficult because of the thermodynamic stability of the active site thiols and disulfides.

**EXPERIMENTAL PROCEDURES**

**Materials**—Bovine pancreatic ribonuclease A (RNase), GH, GSSG, NADPH, CMP, and glutathione reductase were from Sigma. The peptide substrate used in this study was purchased from Genemed Synthesis (South San Francisco, CA). It has the sequence NRCSQGSCWN, and its oxidation by PDI provides a way to follow the catalytic cycle of the enzyme. The peptide substrate used in this study was purchased from Genemed Synthesis (South San Francisco, CA). It has the sequence NRCSQGSCWN, and its oxidation by PDI provides a way to follow the catalytic cycle of the enzyme. The peptide-substrate used in this study was purchased from Genemed Synthesis (South San Francisco, CA). It has the sequence NRCSQGSCWN, and its oxidation by PDI provides a way to follow the catalytic cycle of the enzyme. The peptide-substrate used in this study was purchased from Genemed Synthesis (South San Francisco, CA). It has the sequence NRCSQGSCWN, and its oxidation by PDI provides a way to follow the catalytic cycle of the enzyme.

**Plasmids and Mutagenesis**—Plasmids and mutants with a single active site cysteine of Grx1 were produced previously (8). Cys14 of Grx1 was mutated to Ser using the QuikChange® site-directed mutagenesis kit from Stratagene to create Grx1C14S. The method of Xiao et al. (24) was employed to create the Grx1-PDI chimera, Grx-b-b'-a (SGSHC). The vector containing an N-terminal Grx1 domain with the b-b'-a' domains of PDI was expressed in a strain of *E. coli* Grx1 with a GlySer linker. The cysteines in the C-terminal, a', catalytic domain of PDI were both mutated to serine so that the only catalytic contribution must come from the Grx1 domain. For PDI, Grx1, and their mutants, proteins were expressed from pET15Ixi with an N-terminal His6 tag (24). Proteins were purified using a HitTrap Cheating column (Amersham Biosciences) as described (24) and were greater than 90% pure by SDS-PAGE.

**Preparation of RNase Variants**—Fully reduced RNase was prepared by reduction of the native protein in urea as described (25). The mixed disulfide of RNase and glutathione (SG-RNase) was prepared as described in Ref. 26, but without HPLC purification. Scrambled RNase A was prepared as described in Ref. 27.

**Peptide Oxidase Activity**—The reaction of thioldisulfide exchange reactions between peptide, Grx1, and PDI or its domains were carried out in 0.1 M Tris-HCl, pH 7.4, 0.2 mM KC1, 1 mM EDTA, 0.5 mM GSSG, and 2 mM GH at 25 °C with a peptide concentration of 20 μM and at an enzyme concentration of 1 μM. Aliquots of 100 μl were removed after different times, and thioldisulfide exchange was quenched by the addition of HCl to a final concentration of 0.2 M. oxidized and reduced peptide species in the quenched reaction mixture were separated and analyzed on Sephasil C18 SC 2.1/1.0 column (SMART system; Amersham Biosciences) using a linear gradient of 5–25% (v/v) acetonitrile in 0.1% (v/v) trifluoroacetic acid in 25 min at a flow rate of 100 μl min⁻¹. At the end of this gradient, the acetonitrile concentration was increased to 75% (v/v) to elute the absorbed enzyme. Peptides were detected and quantified by absorption at 215 nm. The initial velocity of the peptide oxidation was determined from the initial rate of formation of the oxidized peptide.

**Oxidase Activity toward Reduced RNase**—In 0.1 M Tris-HCl, pH 8.0, the renaturation of RNase activity was followed by the concurrent hydrolysis of cCMP due to the gain of RNase activity as described by Lyles and Gilbert (28). A glutathione redox buffer (1 mM GH, 0.2 mM GSSG) was added to minimize the minimum redox potential of reduced RNase was 8 μM. Plots of the concentration of active RNase against time show a characteristic lag, during which time the reduced RNase is oxidized to a mixture of nearly random disulfides (25, 29). The lag time was determined by extrapolating the linear gain in RNase activity after the lag back to zero active RNase formed. The initial velocity of diethiol oxidation was estimated from the lag time by assuming that lag represents approximately three half-lives for the oxidation of the four disulfides of RNase. Wherever this is a rough approximation of the initial velocity, the rate is proportional to the concentration of the oxidase (8), and the lag can be measured reproducibly. The background rate in the absence of catalyst was subtracted. For catalysts such as Grx1 that do not display any isomerase activity, a small amount of wild type PDI or an oxidase-deficient mutant with only a single cysteine active site (PDI NCGHS:CCGHS) was included at a concentration of 1 μM. In this case, the background velocity was that observed in the absence of Grx1.

**Reduction of Scrambled RNase and SG-RNase**—The reduction of SG-RNase was followed by coupling the formation of GSSG to the oxidation of NADPH using glutathione reductase. Reaction mixtures contained 100 mM potassium phosphate buffer, pH 7.0, 1 mM EDTA, 1 mM GH, 100 μg/ml bovine serum albumin, 100 μM NADPH, yeast glutathione reductase (0.3 units/mg), and 0.5 M GSSG. Reactions were started by adding PDI or Grx1. Rates were corrected for the slow uncatalyzed reduction that occurs in the absence of PDI or Grx1. The reduction of 25 μM scrambled RNase was carried out in the presence of 3 mM GH in 0.1 M Tris-HCl, pH 8.0, 0.15 mM NADPH, and 1 unit/ml glutathione reductase. The oxidation of NADPH was followed at 340 nm in the presence of 0–10 μM Grx1 or PDI, Grx1C14S, or PDI NCGHS:CCGHS, using the molar extinction coefficient of 6300 M⁻¹ cm⁻¹ for NADPH.

**Reduction of Grx1**—The stoichiometric reduction of Grx1 by GH was observed by coupling the formation of GSSG to the oxidation of NADPH with glutathione reductase. The reaction was monitored by the change in fluorescence of NADPH using a cut-off filter of 395 nm (excitation 340 nm). The reactions were initiated by mixing equal molar concentrations of oxidized Grx1 (3 μM final concentration) with a solution of NADPH (0.5 mM final concentration) and 0.1 units/ml glutathione reductase (10 units/ml), all in 0.2 M potassium phosphate buffer, pH 7.0, at 25 °C. Data were acquired using a SF-2004 stopped-flow instrument (KinTek Corp.). At each GH concentration, the first-order rate constant for the disappearance of NADPH fluorescence was determined by nonlinear least squares fitting to a monoeponential decrease followed by a slower change in absorbance with time due to a slow oxidation of NADPH. This linear correction was significant only at concentrations of GH below 0.2 mM, and, even then, the correction was small compared with the total change in fluorescence. To monitor the competence of the trapping reaction for GSSG, 3 μM GSSG was reduced by NADPH and glutathione reductase at the same concentrations used to follow Grx1 reduction. The rate constant for the disappearance of GSSG (0.85 s⁻¹) was at least 4 times the maximum observed rate constant for Grx1 reduction. The rate constants for Grx1 reduction as a function of GH...
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provide are not important to peptide oxidase activity. However,
PDI (Table I) and the potential substrate interactions they may
induced. Typical data are shown in Fig. 1, and the results for a
more extensive series of catalysts are summarized in Table I.
With this peptide substrate, native PDI and Grx1 are both
monitored. Grx1C14S has no significant effect on the peptide oxi-
dation through a monothiol mechanism that requires only the
nucleophilic cysteine (Fig. 1 and Table I).

RESULTS

Catalysis of Peptide Oxidation—Protein substrates are com-
plex, with multiple disulfides, both native and nonnative. To
observe the intrinsic reactivity of the various active sites on a
well defined substrate, and to determine whether protein sub-
strates are unique substrates for PDI and/or Grx1, the cata-
lized oxidation of a small peptide (NRCSQGSCWN) was exam-
ined. Typical data are shown in Fig. 1, and the results for a
more extensive series of catalysts are summarized in Table I.
With this peptide substrate, native PDI and Grx1 are both
efficient oxidases. PDI requires both active site cysteines to
catalyze peptide oxidation, consistent with a dithiol mechanism
of disulfide formation as with protein substrates. The individ-
ual catalytic domains of PDI are also effective oxidases, com-
parable with the full-length molecule. The other domains of
PDI (Table I) and the potential substrate interactions they may
provide are not important to peptide oxidase activity. However,
Grx1 is still a superior oxidase (5-fold based on active site
concentrations) when compared with PDI. A CXXS mutation in
Grx1(Grx1C14S) has no significant effect on the peptide oxi-
dation activity, suggesting that Grx1 catalyzes the peptide oxida-
tion through a monothiol mechanism that requires only the
nucleophilic cysteine (Fig. 1 and Table I).

Catalysis of Protein Oxidation—During the catalysis of the
 oxidative refolding of reduced RNase, the initial phase of disul-
fide formation rarely produces native RNAse (32). Conse-
sequently, there is a significant lag time before the appearance of
the enzymatically active, native RNAse. This lag is associated
with the formation of RNAse disulfides that must be broken
and reformed to produce native RNAse, and it can be used as an
indicator of the rate of protein oxidation (8). As can be seen in
Fig. 2, the addition of the catalyst of oxidative folding, PDI,
significantly decreases the lag compared with the uncatalyzed
reaction as well as catalyzing the isomerization of incorrect
disulfides leading to native RNAse formation. A small amount
of Grx1, in addition to the PDI that is present, greatly
decreases the lag (accelerates the oxidation) without substan-
tially contributing to the subsequent isomerase activity, con-
sistent with the results reported by Lundström-Ljung and
Holmgren (33). PDI is a 600-fold better oxidant than Grx1 ther-
modynamically (9), but enzymatically, the active site of
Grx1 is a 30-fold better oxidase than PDI in a redox buffer
consisting of 1 mM GSH and 0.2 mM GSSG (Table II).

During the oxidation of protein substrates, PDI catalyzes
direct transfer of its active site disulfide into the substrate;
both PDI active site cysteines are essential for effective catal-
ysis of protein oxidation. PDI mutants with only the nucleo-
philic (N-terminal) cysteine (CGHS) are not active oxidases and
do not decrease the lag during the oxidative refolding of reduced
RNAse (Table II). However, mutation of the active site of Grx1
to remove the C-terminal cysteine has no significant effect on
the ability of Grx1 to decrease the lag in RNAse refolding. The
observation that wild type Grx1 and Grx1C14S plus 1 μM PDI; E,
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straight dotted line on the time axis is the lag time.
The initial velocity was estimated from the lag time observed in the oxidative folding of reduced RNase as described under “Materials and Methods.” Errors represent the S.D. The reactions were performed in the optimum glutathione redox buffer for RNase folding, 1 mM GSH, and 0.2 mM GSSG. The activity is reported on a molar basis of catalyst and not corrected for the number of active sites/molecule.

| Protein                        | Protein oxidase activity (µmol/min/µmol protein) |
|--------------------------------|--------------------------------------------------|
| Wild type Grx                  | 47 ± 6                                           |
| Grx1C14S                       | 72 ± 12                                          |
| Wild type PDI                  | 1.6 ± 0.1                                        |
| Mutant PDI (N<sub>GcRHS</sub>C<sub>CGHS</sub>) | 0.3 ± 0.02                                     |
| Mutant PDI(N<sub>GcRHS</sub>C<sub>CGHS</sub>)<sup>a</sup> | 0.05 ± 0.01                                    |
| Mutant PDI(N<sub>GcRHS</sub>C<sub>CGHS</sub>)<sup>b</sup> | 0.07 ± 0.01                                    |
| Grx-b-b'-a’(SGHS)-c            | 43 ± 3                                           |

<sup>a</sup> Data from Ref. 8.

### Catalysis of the reduction of scrambled RNase by PDI, Grx1, and mutants of PDI and Grx1 with single cysteines at the active site

Experiments were performed at pH 8.0, 25 °C in 0.1 M Tris-HCl. The reduction of scrambled RNase (25 μM) by 3 mM GSH was followed by coupling the formation of GSSG to the oxidation of NADPH (0.15 mM) through glutathione reductase (1 unit/ml). The background reduction of scrambled RNase by GSH has been subtracted. The error limits are shown as the S.D.

| Protein                        | Reductase activity (µmol/min/µmol protein) |
|--------------------------------|-------------------------------------------|
| Wild type Grx                  | 390 ± 25                                    |
| Grx1C14S                       | 25 ± 3                                      |
| Wild type PDI                  | 13.0 ± 1.3                                  |
| Mutant PDI (N<sub>GcRHS</sub>C<sub>CGHS</sub>) | 8.0 ± 0.5                                   |
| Grx-b-b'-a’(SGHS)-c            | 110 ± 3                                     |

Given that the active site thermodynamics suggest that Grx1 is a 600-fold better reductant (redox potential 83 mV more negative) than PDI, the 15-fold difference observed in reductase activities (Table III) is surprisingly low. Darby et al. showed that the individual domains of PDI (a or a') by themselves are very poor reductases (2–3% of wild type PDI activity) (22). Thus, the multidomain structure of PDI enhances the reductase activity considerably (30–50-fold); the catalytic domains are very poor reductases in the absence of the catalytic-substrate interactions that are available from its multiple domains. If substrate interactions contribute 30–50-fold to catalysis of reduction, and Grx1 is a 15-fold better reductant than PDI, we estimate that Grx1 is a 450–750-fold better reductant than the catalytic domains of PDI, in line with the 600-fold (83 mV) difference in reduction potentials.

The effect of creating a multidomain structure with a Grx1 catalytic domain was replaced by Grx1 to create a Grx1-PDI chimera, Grx-b-b'-a'(SGHS)-c. The two cysteines of the C-terminal catalytic domain of PDI were also mutated to Ser so that the activity of Grx1 in the context of the rest of the PDI molecule might be observed. As with PDI, the addition of the protein interaction domains of PDI had no significant effect on the ability of Grx1 to function as an oxidant (Table II). Whereas we cannot be sure that the orientation of the catalytic site in the chimeric protein would allow Grx1 to take advantage of a peptide/protein binding site from the b-b'-a' domains of PDI, the ability of Grx1 and PDI to serve as oxidases relies almost exclusively on the high reactivity of an active site disulfide to introduce disulfides into protein substrates. In the case of Grx1, this active site disulfide is most likely a disulfide with glutathione, whereas for PDI, it represents an intramolecular disulfide between the two active site cysteines.

**Catalysis of Reduction**—Because Grx1 is such a superior oxidase compared with PDI, we were also interested in directly comparing the ability of the two proteins to catalyze reduction. In line with its very negative reduction potential, Grx1 is a more effective reductase than PDI (16-fold on a molar basis) in catalyzing the reduction of the protein disulfides in scrambled RNase. However, Grx1 now requires both active site cysteines to be an effective reductant. Mutation of the more C-terminal cysteine in the Grx1 active site decreases the reductase activity by 15-fold, indicating that both PDI, both active site cysteines make a significant contribution to catalyzing protein disulfide reduction (Table III). Bushweller et al. (35) have found that Grx1 reduction of ribonucleotide reductase requires both active site cysteines, consistent with our observation that catalysis of protein disulfide reduction by Grx1, in general, needs both active site cysteines.

**Fig. 3.** Time course of the reduction of protein-SG mixed disulfides (SG-RNase) catalyzed by Grx1 and PDI. The reaction mixture contained 100 mM potassium phosphate buffer, pH 7.0, 1 mM GSH, 100 μg/ml bovine serum albumin, 100 μM NADPH, 0.14 units/ml yeast glutathione reductase, and 5 μM SG-RNase. The velocity of reduction was monitored by following the reduction of the product GSSG by NADPH at 340 nM.

**TABLE II**

| Protein                        | Protein oxidase activity (µmol/min/µmol protein) |
|--------------------------------|--------------------------------------------------|
| Wild type Grx                  | 47 ± 6                                           |
| Grx1C14S                       | 72 ± 12                                          |
| Wild type PDI                  | 1.6 ± 0.1                                        |
| Mutant PDI (N<sub>GcRHS</sub>C<sub>CGHS</sub>) | 0.3 ± 0.02                                     |
| Mutant PDI(N<sub>GcRHS</sub>C<sub>CGHS</sub>)<sup>a</sup> | 0.05 ± 0.01                                    |
| Mutant PDI(N<sub>GcRHS</sub>C<sub>CGHS</sub>)<sup>b</sup> | 0.07 ± 0.01                                    |
| Grx-b-b'-a’(SGHS)-c            | 43 ± 3                                           |

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| Wild type Grx                  | 390 ± 25                                    |
| Grx1C14S                       | 25 ± 3                                      |
| Wild type PDI                  | 13.0 ± 1.3                                  |
| Mutant PDI (N<sub>GcRHS</sub>C<sub>CGHS</sub>) | 8.0 ± 0.5                                   |
| Grx-b-b'-a’(SGHS)-c            | 110 ± 3                                     |

**TABLE IV**

| Protein                        | Reductase activity (µmol/min/µmol protein) |
|--------------------------------|-------------------------------------------|
| Wild type Grx                  | 690 ± 150                                  |
| Grx1C14S                       | 243 ± 54                                   |
| Wild type PDI                  | 3.8 ± 1.1                                  |
| PDI-a                          | 3.6 ± 1.0                                  |
| Mutant PDI-a (CGHS)            | —<sup>a</sup>                             |
| Mutant PDI-a’ (CGHS)           | —<sup>a</sup>                             |

<sup>a</sup> —, the observed activity was equivalent to the background in the absence of Grx1 or PDI.
additional substrate-catalyst interactions that may be available from the noncatalytic domains of PDI, whereas the same interactions do enhance the reductase activity of the intrinsically poor reductant, PDI.

Lundström-Ljungström et al. (26), found that the glutaredoxins, including Grx1, are good reductases for protein-glutathione mixed disulfides. Comparing Grx1 and PDI for their ability to catalyze reduction of the mixed disulfides of denatured RNase and glutathione (SG-RNase), Grx1 is 200-fold more active against this substrate than a single catalytic domain of PDI (Fig. 3, Table IV). For this substrate, mutating the second active site cysteine to serine decreases the reductase activity of Grx1 by 3-fold. This indicates that there may be some contribution of a two-cysteine reduction mechanism for protein-SG mixed disulfides but that a single-cysteine dominates the reduction mechanism. The PDI catalytic domain (a domain; Table IV), on the other hand, loses its ability to reduce protein-SG mixed disulfides when the more C-terminal cysteine is mutated to serine, suggesting that both active site cysteines are needed, even for this substrate. Thus, for protein-SG mixed disulfides, the specificity of Grx1 for glutathione (35) provides an alternative mechanism of protein reduction that involves only one active site cysteine.

Reduction of the Grx1 Active Site—During catalysis of oxidation, the active site of Grx1 is reduced by the protein substrate and then recycled through oxidation by GSSG (Fig. 4). To observe the reactivity of the Grx1 disulfide and the Grx1-SG mixed disulfide, rapid reaction kinetics were employed. The oxidation of glutathione to GSSG by the oxidized active site of Grx1 requires two consecutive reactions, the formation of a Grx1-SG mixed disulfide and the subsequent reaction of the mixed disulfide with GSH (Scheme 1). These reactions must occur during the catalysis of oxidation and reduction by Grx1 or PDI in order to recycle the catalyst for another round of reduction (or oxidation). To determine the fundamental rate constants for the reaction of Grx1 with glutathione, oxidized Grx1 was rapidly mixed with various concentrations of GSH in a stopped-flow fluorescence spectrophotometer. The GSSG formed as a product of the reaction was trapped by the glutathione reductase-catalyzed oxidation of NADPH to NADP⁺ (31). Conditions were chosen to ensure that there would be sufficient NADPH to reduce all of the GSSG formed (5 μM NADPH and 3 μM Grx1) and that the trapping of GSSG was faster than the observed reactions (Fig. 5).

The observed pseudo-first-order rate constants for Grx1 reduction were determined at various GSH concentrations. At low concentrations of GSH, the reduction of Grx1 is clearly second-order in GSH concentration (Fig. 5). This second-order dependence requires that the reaction of GSH with the active site disulfide is at equilibrium \( k_2 [GSH] \) (Scheme 1), that the Grx-active site disulfide is not fully converted to the mixed disulfide, and that the rate-limiting step is the reaction of the Grx1-SG mixed disulfide with GSH \( (k_3) \). For PDI, at GSH
concentrations of about 0.3 mM, there is a change in the rate-determining step to a reaction that is first-order in GSH, as the reaction of the mixed disulfide of PDI with GSH becomes rate-limiting (31). In this case, the reaction changes from second-order to first-order in GSH concentration. For Grx1, a fit of the data to the entire kinetic model, including the potential for a change in rate-limiting step (Equation 1), is not statistically better than a simple second-order dependence (Equation 2). Thus, at the GSH concentrations that can be used without the rate of Grx1 reduction becoming faster than the trapping reaction, there is no observable change in rate-determining step for Grx1 reduction. However, this places a lower limit on the GSH concentration where this change in rate-limiting step (Equation 1), is not statistically limited by our ability to set only a lower limit on $k_1$. In addition, Grx1 and PDI reduction and oxidation by GSH and GSSG are sufficiently fast that the rate-limiting half-reaction during catalyzed protein and peptide oxidation and reduction must be the reaction of Grx1 and PDI with the peptide or protein substrates rather than the recycling of the active site redox state through reaction with GSH or GSSG. Consequently, the relative rates of peptide and protein oxidation and reduction by Grx1 and PDI reflect reactions of the catalysts with the peptide or protein substrates.

### DISCUSSION

For most members of the thioredoxin family, the kinetic reactivities of the dithiol active sites generally parallel the thermodynamics of disulfide formation within the active site. The least stable disulfides are the best oxidases, whereas the least stable dithiols are the best reductases. Grx1 is an exception. As expected from its redox potential, Grx1 is a better reductase than an individual catalytic domain of PDI (30–200-fold; Tables III and IV), but surprisingly, Grx1 is also a better oxidase than PDI (Tables I and II). One potential explanation for the superiority of Grx1 as an oxidase might lie in the proportion of the active site that is in the oxidized state under the conditions where its oxidase activity is measured. In the redox buffers used to measure the oxidase activity (Tables I and II), only a fraction of the PDI is expected to be in the oxidized state (~10%), whereas Grx1 is expected to be mostly oxidized (~99%). In addition, the proportions of the Grx1 active site that are present as the intramolecular disulfide and the Grx1-SG mixed disulfide can be calculated from the micro-

### Table V

| Constant  | Units | Grx1 | PDI $^{a}$ | Grx1/PDI $^{a}$ |
|-----------|-------|------|------------|-----------------|
| $k_{GHG}^{b}$ | mm$^{-2}$ s$^{-1}$ | 2.3 ± 0.3 | 0.14–0.43 | >1.4 |
| $k_1$ | mm$^{-1}$ s$^{-1}$ | >0.6 | 10–30 | >0.75 |
| $k_2$ | s$^{-1}$ | >40 | 10–30 | >0.75 |
| $K_{cat}^{d}$ | mm | 68 | 69 | 1 |
| $k_3/k_3^{a}$ | mm | >0.25 | 0.2–0.5 | >1 |
| $k_4$ | mm$^{-1}$ s$^{-1}$ | 160 | 60 | 2.7 |
| $k_5$ | mm$^{-1}$ s$^{-1}$ | 930 | 0.6 | 1600 |
| $K_{con}$ | mm | 6 | 0.01 | 600 |
| $E_{c}^{d}$ | mm | 404 | 0.7 | 580 |
| $E_{c}^{d}^{*}$ | mV | -228 | -145 |          |

$^{a}$ Taken from Ref 30.
$^{b}$ Observed second order rate constant for reduction of Grx1 at low GSH concentrations as shown in Scheme I.
$^{c}$ As defined in Scheme 1.
$^{d}$ Equilibrium constant for the formation of the Grx1 active site disulfide and GSH from the Grx1-SG mixed disulfide. Values are taken from Ref. 43.
$^{e}$ Equilibrium constant for the formation of the Grx1-SG mixed disulfide from reduced Grx1 and GSSG. Values are taken from Ref. 43.
$^{f}$ Equilibrium constant for the overall oxidation of Grx1 by glutathione disulfide. Values are taken from Ref. 43.
$^{g}$ Standard redox potential for the reduction of the active site disulfide using an $E_{c}^{d}$ for the Grx redox couple of 240 mV. Values are taken from Ref. 43.

**FIG. 5. Reduction of Grx by GSH.** Grx1 (3 mM) was reduced by GSH at pH 7.0, 0.2 M potassium phosphate, 25 °C using 5 μM NADPH and 10 units/ml glutathione reductase to trap the GSSG produced. A, time course for the change in fluorescence with time after mixing in a stopped-flow instrument (KinTek Corp). B, rate constants for Grx1 reduction as a function of the GSH concentration. The points are the averages and S.D. of replicate points (n = 2–4). The curve is drawn according to a nonlinear least squares fit to a simple second-order reaction ($k = k_{obs}[GSH]^2$).
scopic equilibrium constants (\(K_{\text{mix}}\) and \(K_{\text{tria}}\); Table V). At equilibrium with the redox buffer (1 mM GSH, 0.2 mM GSSG), Grx-SG would represent 1.4% of the total Grx1, whereas the intramolecular disulfide would represent 97%. Although a minor species at equilibrium, the high reactivity of the Grx1-SG mixed disulfide suggests that it can be a kinetically competent oxidant. For example, it reacts with GSH – 400-fold faster than the active site disulfide of PDI. The Grx1-SG mixed disulfide is the most reactive glutathione mixed disulfide described to date, and its increased thermodynamic stability allows it to accumulate to a significant extent in a redox buffer.

In contrast to Grx1, PDI requires both active site cysteines to accomplish effective disulfide formation. The fact that the velocity of substrate oxidation is less than 10% of a wild type active site (depending on the substrate) when the PDI active site has only the nucelophilic cysteine suggests that disulfide formation by PDI is dominated by a direct transfer of a disulfide to the substrate (Fig. 4A) and that glutathione involvement is not required. This is further supported by in vivo data from a genetic analysis in yeast that has defined a core pathway for protein disulfide bond formation in the ER, whereby PDI transfers oxidizing equivalents derived from Ero1p to secretory proteins by direct thiol-disulfide exchange reaction. In addition, depletion of glutathione from the ER has no effect on disulfide bond formation in secretory proteins (36, 37). By contrast, the second active site cysteine in Grx1 contributes minimally to catalysis. Grx1 and the Grx1C14S mutant, have comparable activity in shortening the lag phase of RNase folding and introducing a disulfide bond into a peptide (Tables I and II). It has been previously shown that the synergistic effects of Grx1 together with PDI in refolding of RNase appear exclusively in the presence of glutathione (33). Thus, Grx1-catalyzed oxidation generally occurs by mono-thiol mechanisms (Fig. 4B), where glutathione involvement is necessary and only one active site cysteine is formally required.

The high oxidase activity of Grx1-SG suggests that in addition to its usual role as a cytoplasmic reducing agent, Grx1 may also participate in catalyzing protein oxidation during oxidative stress. The high reactivity of the disulfide of Grx1-SG may be due to the abnormally low pK\(_a\) of the N-terminal Cys residue in Grx1 and the resulting ability to serve as a good leaving group when attacked by a protein thiol (17, 38–40).

The oxidase activity of PDI does not utilize protein-protein interactions that might be available from the multidomain structure of PDI. A single catalytic domain is just as good an oxidase as wild type PDI. By contrast, PDI-catalyzed reduction does require the multidomain structure; the individual catalytic domains are ineffective reductases. Protein reduction is facilitated by interactions between the substrate and the other domains of PDI, substantially increasing the ability of PDI to serve as a protein reductant. The function of the additional substrate interactions may be to affect local unfolding of the substrate, or the enhanced binding interaction may decrease the dissociation rate of the substrate, making reduction more likely. Which mechanism dominates in the reduction of protein substrates by PDI is not clear. However, adding the protein-interaction domains of PDI to Grx1 does not enhance its oxidase or reductase activities, suggesting that the extraordinary reactivity of the Grx1 active site and the flexibility of providing a Grx1-SG mixed disulfide is responsible for enhancing its abnormal oxidase activity.

In summary, Grx1 and PDI are unusual members of the thioredoxin family. Grx1 provides mechanisms for protein oxidation and reduction that are not available to the other family members, mechanisms that involve a single-cysteine mechanism and the high reactivity of a Grx-SG mixed disulfide. This alternative mechanism compensates for the very negative reduction potential of the Grx1 active site and suggests that Grx1, despite its highly reducing nature, may also catalyze significant protein oxidation. The formation of protein-SG mixed disulfides by Grx1 through a monothiol mechanism may play an important role in protecting against more drastic, irreversible modifications of protein thiols, particularly when the redox state of the cytoplasm becomes more oxidizing, as under conditions of oxidative stress. Several monothiol glutaredoxins have been identified in yeast (yGrx3, yGrx4, and yGrx5) (41). The lethality of yGrx5 mutations suggests that monothiol glutaredoxins are very specific for their substrates, and their functions cannot be replaced by their dithiol counterparts.

The biological activities of yGrx3 and yGrx4 are not known, but yGrx5 is involved in the maturation of Fe-S cluster-containing proteins in the mitochondria (21) and plays a central role in defense against protein oxidative damage (41). PDI is unusual because it is a more effective reductant than expected based on its redox potential. In this case, the efficiency of reduction is increased by involving PDI-substrate interactions in facilitating substrate reduction, an activity that is required to affect disulfide isomerization during oxidative protein folding (42).

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