Insights into Deglutathionylation Reactions

DIFFERENT INTERMEDIATES IN THE GLUTAREDOXIN AND PROTEIN DISULFIDE ISOMERASE CATALYZED REACTIONS ARE DEFINED BY THE γ-LINKAGE PRESENT IN GLUTATHIONE

Received for publication, June 12, 2006, and in revised form, August 28, 2006. Published, JBC Papers in Press, September 5, 2006, DOI 10.1074/jbc.M605602200

Mirva J. Peltoniemi§, Anna-Riikka Karala‡, Jaana K. Jurvansuu§, Vuokko L. Kinnula§, and Lloyd W. Ruddock*§1

From the *Biocenter Oulu and Department of Biochemistry, University of Oulu, 90014 Oulu and the +Department of Medicine, Division of Pulmonary Diseases, University of Helsinki and Helsinki University Hospital and Biomedicum Helsinki, 00029 Helsinki, Finland

Glutaredoxins are small proteins with a conserved active site (-CXX(C/S)-) and thioredoxin fold. These thiol disulfide oxidoreductases catalyze disulfide reductions, preferring GSH-mixed disulfides as substrates. We have developed a new real-time fluorescence-based method for measuring the deglutathionylation activity of glutaredoxins using a glutathionylated peptide as a substrate. Mass spectrometric analysis showed that the only intermediate in the reaction is the glutaredoxin-GSH mixed disulfide. This specificity was solely dependent on the unusual recognition site (1–4), that belong to the thioredoxin superfamily (5, 6). Glutaredoxins use the reducing power of GSH to recognize the substrate via the glutathione moiety. In addition, wild-type glutaredoxin showed a sigmoidal dependence on GSH concentrations, the activity being significantly decreased at low GSH concentrations. Thus, under oxidative stress conditions, where the ratio of GSH/GSSG is decreased, the activity of glutaredoxin is dramatically reduced, and it will only have significant deglutathionylation activity once the oxidative stress has been removed. Different members of the protein disulfide isomerases (PDI) family showed lower activity levels when compared with glutaredoxins; however, their deglutathionylation activities were comparable with their oxidase activities. Furthermore, in contrast to the glutaredoxin-GSH mixed disulfide intermediate, the only intermediate in the PDI-catalyzed reaction was PDI peptide mixed disulfide.

Glutaredoxins are small thiol disulfide oxidoreductases, with a conserved active site sequence -CXXC- or -CXXS- and a GSH recognition site (1–4), that belong to the thioredoxin superfamily (5, 6). Glutaredoxins use the reducing power of GSH to catalyze disulfide reductions (2). They preferentially catalyze reductions of GSH-mixed disulfides but have also been suggested to function as general protein disulfide reductants (7). Multiple glutaredoxins are known in different organisms (8–12), but the role of these proteins in cells and in different cell organelles is still unclear. Glutaredoxins are thought to have a primary role in defense against oxidative stress (13–16), but they have been suggested to participate in other functions, such as cellular differentiation (17), redox regulation of signal transduction (14, 18) and prevention of apoptosis (15).

GSH is the primary cellular low molecular weight reductant, and accordingly, there are high levels of GSH in most cells, the cellular levels ranging from 3.5 to 6.6 mM in Escherichia coli (19) and from 1 to 8 mM in mammalian cells (20). Glutathione may also be found in oxidative cellular compartments such as the endoplasmic reticulum as a disulfide linked dipeptide, GSSG, or as protein-GSH mixed disulfides (21, 22). Protein-GSH mixed disulfides have also been observed to accumulate in other cellular compartments, e.g. the cytoplasm, during oxidative stress (23). The primary function of glutaredoxins is thought to be the reduction of protein-GSH mixed disulfides, liberating the native functional protein (24).

Glutaredoxins exist in monothiol (CXXS) and dithiol (CXXC) active site variants. The N-terminal active site cysteine is essential and sufficient for the reduction of GSH-mixed disulfides (25, 26). The monothiol mechanism has been suggested to proceed via a pathway where the thiolute of Grx2 initiates a nucleophilic attack on the mixed disulfide between protein thiol and GSH, leading to a formation of a new disulfide between Grx and GSH and the release of a protein substrate in the reduced form (Fig. 1, reaction 1) (24, 27–30). The mixed disulfide between Grx and GSH can be reduced by GSH through a nucleophilic attack on the disulfide to form GSSG and reduced Grx as the final products (Fig. 1, reaction 2) (25, 27, 28). The dithiol mechanism proceeds via the same pathway except that there is partitioning at the Grx-GSH mixed disulfide state to form oxidized Grx (Fig. 1, reaction 3). This can be retrieved to the functional pathway by reduction by GSH (Fig. 1, reaction 4) (31).

Grx activity is often measured using an artificial nonspecific substrate β-hydroxyethyl disulfide (HED) in a spectrophotometric coupled assay (1). Less frequently, substrates such as cysteine-glutathione mixed disulfide (31) or glutathionylated protein substrates are used (28, 29). Here we report the develop-
Deglutathionylation Activity of Glutaredoxins

PROTEIN EXPRESSION AND PURIFICATION

All constructs used in the experiments were cloned into an expression vector generated previously (32), which incorporates an N-terminal His tag to the cloned gene. E. coli Grx1 was cloned from E. coli strain XL1-Blue, and yeast Grx1 was cloned from Saccharomyces cerevisiae strain W303. Site-directed mutagenesis was performed according to the instructions of the QuikChange™ kit (Stratagene, La Jolla, CA). All plasmids were checked for correctness by sequencing. Proteins were expressed in E. coli strains BL21 (DE3) pLysS or Rosetta-gami and purified by immobilized metal affinity chromatography and ion exchange chromatography as described for the a domain of PDI (33). Pure fractions, as determined by Coomassie Brilliant Blue-stained SDS-PAGE, were combined and buffer-exchanged into 20 mM sodium phosphate buffer, pH 7.3, and stored frozen. The concentration of each protein was determined spectrophotometrically using a calculated molar absorption coefficient. All purified proteins were analyzed for authenticity by matrix-assisted laser desorption/ionization-time of flight mass spectrometry.

ASSAY FOR DETERMINING THE DEGLUTATHIONYLATION ACTIVITY

The glutathionylated substrate peptide SQLWC(glutathione)LSN was incubated with ECG, QCG, or paraECG mixed disulfides were purified by reverse phase-high pressure liquid chromatography using SOURCE 5RPC ST 4.6/150 column (Amersham Biosciences, Uppsala, Sweden). The peptides were eluted from the column with a linear gradient from buffer A (0.1% trifluoroacetic acid) to 100% buffer B (90% acetonitrile, 0.1% trifluoroacetic acid) over 10 column volumes. The peptides were dried by speed vacuum and resuspended into 20 mM phosphate buffer, pH 7.3.

ANALYSIS OF REACTION INTERMEDIATES BY MASS SPECTROMETRY—E. coli Grx1 wild type and C145S mutant and PDI a C39S mutant were used in trapping experiments. The protein was reduced with 1 mM dithiothreitol for 30 min at room temperature. Excess of dithiothreitol was removed by gel filtration (NAP™ 10 columns, Amersham Biosciences), and the sample was fur-
ther concentrated with Biomax Ultrafree centrifugal filter device (Millipore, Bedford, MA). The reduced protein (40 μM) was reacted with either substrate peptide (50 μM) or buffer alone in a total volume of 100 μl in McIlvaine buffer at pH 7.0. The reaction was quenched with 50 mM N-ethylmaleimide or 1.1 M iodoacetamide (both from Sigma). The excess of N-ethylmaleimide/iodoacetamide was removed with pepClean™ C-18 spin columns (Pierce) according to the manufacturer’s instructions. Proteins were eluted with 50% acetonitrile and CH₃COOH to a final concentration of 0.1% was added to samples. Molecular masses were measured with an electrospray ionization mass spectrometer (Micromass LCT, Manchester, UK) using positive ionization. Additional time-dependent trapping experiments were carried out using an RQF3 quenched-flow apparatus (KinTek, Austin, TX). The reduced protein (80 μM) was reacted with either substrate peptide (100 μM) or buffer alone in a total volume of 30 μl in McIlvaine buffer at pH 7.0. After the desired reaction time (0.01–1 s), the reaction was quenched with 0.5 M HCl, to ensure that the pH of the quenched product remained below pH 2.0 and was treated as previously.

Stopped-flow Measurements—Stopped-flow experiments were performed with a SF2004 stopped-flow apparatus (KinTek) with 20 μM E. coli C14S Grx1 and 0.14 mg/ml (357 μM) Ellman’s reagent in McIlvaine buffer at the desired pH (4.5–7.5). The absorbance at 412 nm was measured for 0.2 s after mixing the two reagents, and the pseudo first-order rate constants were calculated using KinTek StopFlow v9.06 software.

RESULTS

Determination of the Deglutathionylation Activity—To directly monitor the deglutathionylation activity of Grx, a glutathionylated substrate peptide was designed. The criteria for the design were that the peptide should contain a single thiolated cysteine residue with an adjacent fluorescent group. The peptide would also need to be small (for ease of synthesis) and water-soluble and contain no residues with charged side chains to eliminate any pH dependence of reaction rates from effects on the peptide. A similar strategy has previously been utilized to generate peptide substrates to monitor disulfide oxidation (34). The peptide synthesized here, SQLWC(glutathione)LSN, had an emission maximum at 356 nm (Fig. 2A), consistent with an aqueous exposed tryptophan. Upon removal of the glutathione, i.e. the reduction of the cysteine moiety, a 38% increase in total fluorescence was observed with no shift in the emission maximum. This change is consistent with quenching of the fluorescence of tryptophan residues by adjacent disulfide bonds (35). Thus, deglutathionylation of the peptide could be measured directly in real time by monitoring the change in fluorescence at 356 nm in the presence of reducing agents such as GSH. At pH 7.0 in McIlvaine buffer, the non-catalyzed reaction was very slow, just 3% of the enzyme-catalyzed reaction with 20 nM E. coli Grx1 and 1 mM GSH (Fig. 2B). Since one of the products of the reaction, GSSG, may reglutathionylate the deglutathionylated peptide substrate, glutathione reductase and NADPH were added to the reaction to remove the GSSG formed.

Analysis of the Reaction Kinetics—To analyze the kinetics as a function of enzyme, substrate, and GSH concentrations, the initial rates of reaction were determined from each time course and plotted against the concentration of the varying substance. As expected, E. coli Grx1 showed a linear dependence of the initial rate of reaction on the enzyme concentration (Fig. 3A). In addition, the initial rate of reaction increased with increasing substrate concentrations and fitted well to the Michaelis-Menten equation with $K_m = 7.8 \mu M$ and $k_{cat} = 4.4 \text{s}^{-1} ([\text{GSH}] = 1 \text{mM}; \text{Fig. 3B})$. In contrast, although an increase in the initial rate with increasing GSH concentration was observed, with a plateau at the highest GSH concentrations measured, the plot could not be fitted to the Michaelis-Menten equation and instead showed a sigmoidal shape ($[\text{substrate peptide}] = 5 \text{μM}$; Fig. 3C).

Analysis of Grx1C14S Mutant-catalyzed Deglutathionylation Reaction—Often, a sigmoidal shape for the dependence of the enzyme-catalyzed rate on the substrate concentration implies cooperativity of substrate binding; however, this is unlikely for glutaredoxin since it is a small monomeric enzyme with a single glutathione binding site (31, 36). Instead, the sigmoidal shape for the GSH dependence of E. coli Grx1 activity probably represents the net effect of the partitioning of the Grx-GSH mixed disulfide intermediate between the formation of reduced or oxidized Grx and the re-reduction of oxidized Grx1 by GSH (Fig. 1). To test this hypothesis, the C-terminal active site C14S mutant of E. coli Grx1 was made since this mutant cannot proceed to the oxidized state, and thus, there should be no partitioning. Under standard conditions

FIGURE 2. Fluorescence analysis of peptide deglutathionylation. A, typical emission spectra for glutathionylated (---) and deglutathionylated (-----) peptide (5 μM) at pH 7.0. B, representative time-dependent fluorescence profiles during the deglutathionylation of the substrate peptide catalyzed by 20 nM E. coli Grx1 (black) and the non-catalyzed reaction (gray). Concentrations were as follows: McIlvaine buffer, pH 7.0, [GSH] = 1 mM, [substrate peptide] = 5 μM.
Deglutathionylation Activity of Glutaredoxins

![Graphs showing reaction kinetics](image)

Deglutathionylation Activity of Glutaredoxins

([GSH] = 1 mM, [substrate] = 5 μM), the initial rate of the C14S mutant-catalyzed reaction was only 23% of that catalyzed by the wild-type enzyme. Similar to wild type, the C14S mutant showed a linear dependence of the initial rate of reaction on the enzyme concentration (Fig. 4A). Furthermore, the data with increasing substrate concentrations fitted well to the Michaelis-Menten equation (Fig. 4B).
GSSG, the amount of GSSG in the GSH stock was calculated to NADPH and glutathione reductase-catalyzed reduction of glutathione reductase. Using changes in the absorbance of in the presence of varying amounts of GSSG and in the absence of glutathionylation reactions were performed with Grx1 and substrate peptide in the absence of GSH. However, analysis of the reaction products indicated only the presence of oxidized Grx1 (mass = 10637 Da), indicating that the nucleophilic attack by the C-terminal active site Cys residue on the Grx-GSH mixed disulfide was significantly faster than the nucleophilic attack by Grx1 on the glutathionylated substrate (Fig. 1). Since the C14S Grx1 mutant cannot undergo the same partitioning reaction, similar equilibrium and quenched-flow reactions were undertaken with this mutant. The results indicated that no Grx-peptide mixed disulfide (mass = 11572 Da) was observed, with only the Grx-GSH (mass = 10928 Da) mixed disulfide being formed. Under the test conditions used ([Grx1] = 80 μM, [substrate] = 100 μM), the half-time for this reaction was around 100 ms.

Effects of Oxidized Glutathione—Since Grx forms mixed disulfides with GSH and not with peptide and since the Km for the C14S for GSH (48 μM) and glutathionylated-substrate (44 μM) are similar, it is likely that oxidized glutathione (GSSG) will act as a competitive substrate (i.e. glutathionylated glutathione) for the deglutathionylation reaction catalyzed by Grx. However, since the product of the reaction using GSSG as a substrate is GSSG, the net effect of this is that GSSG would act like a competitive inhibitor of the peptide deglutathionylation reaction. To test this, the deglutathionylation reactions were performed in the presence of varying amounts of GSSG and in the absence of glutathione reductase. Using changes in the absorbance of NADPH and glutathione reductase-catalyzed reduction of GSSG, the amount of GSSG in the GSH stock was calculated to be 0.05%, i.e. 5 μM when 1 mM GSH was used. The initial rate of both the wild-type Grx1 (20 nM)- and the C14S mutant (100 nM)-catalyzed peptide deglutathionylation reaction decreased with increasing concentrations of GSSG (Fig. 5). Using the Km values for the peptide substrate calculated above, the competitive inhibition constant, Ki, for GSSG was calculated to be 5.6 μM for wild type and 50 μM for the C14S mutant.

pH Dependence of the Deglutathionylation Reaction—To further examine the catalyzed deglutathionylation pathway, the initial rate of reaction was examined as a function of pH. At pH 4.0 and below, denaturation of Grx1 was observed (data not shown). Over the pH range 4.5–7.5, the initial rate of reaction increased with increasing pH. When corrected for the pH dependence of the non-catalyzed reaction, the Grx1-catalyzed reaction showed a single pKa-dependent event with a pKa = 6.47 ± 0.04 for the wild-type enzyme-catalyzed reaction and a pKa = 5.90 ± 0.07 for the C14S-catalyzed reaction (Fig. 6). These values are significantly higher than the pKa values previously reported for the N-terminal active site cysteine (3.8–5.0) (38–40). Thus, the pH dependence of activity most likely does not simply represent the nucleophilicity of the N-terminal active site cysteine. To confirm this, measurements for the pH dependence of the reaction of the C14S Grx1 with Ellman’s reagent were done by stopped-flow. These data fitted to a single pKa-dependent event with a pKa = 4.86 ± 0.07 for the N-terminal cysteine (data not shown).

Determination of Glutathiolation Activity—To monitor the glutathiolation activity of Grx, dehydrothiolated substrate peptide was added to GSSG in the presence and absence of enzyme. Upon addition of a glutathione moiety, a decrease of 69 arbitrary units in total fluorescence was observed with no shift in emission maximum, consistent with the glutathiolation results (increase of 70 arbitrary units in total fluorescence). This reaction was pseudo first-order, with a rate constant of 0.002 s⁻¹ at pH 7.0 ([peptide] = 5 μM, [GSSG] = 5 mM;

FIGURE 5. Competitive inhibitory effect of GSSG on glutaredoxin-catalyzed deglutathionylation. The variation of the initial rate of reaction with GSSG concentration during enzyme-catalyzed peptide deglutathionylation (McIlvaine buffer, pH 7.0, [GSH] = 1 mM, [substrate peptide] = 5 μM, [Grx1] = 20 nM or [C14S Grx1] = 100 nM) is shown. Initial rates are expressed as mean ± S.D. The line of best fit is to competitive inhibition of the Michaelis-Menten equation, using the value of Km determined previously. A, Grx1. B, C14S Grx1.

 Deglutathionylation Activity of Glutaredoxins

---

![Deglutathionylation Activity of Glutaredoxins](image-url)
Deglutathionylation Activity of Glutaredoxins

**FIGURE 6. pH dependence of enzyme-catalyzed deglutathionylation.** The variation of the initial rate of reaction as a function of pH (McIlvaine buffer, pH 7.0, [GSH] = 1 mM, [substrate peptide] = 5 μM, [enzyme] = 20 nM) is shown. Initial rates are expressed as mean ± S.D. with the non-catalyzed rate at each pH deducted. The lines of best fit are to single pK_a dependent events. ——, the Grx1-catalyzed reaction; — — —, the C14S Grx1-catalyzed reaction; ○, wild type; ○, C145 mutant.

**FIGURE 7. Fluorescence analysis of peptide glutathionylation.** Representative time-dependent fluorescence profiles during the glutathionylation of the substrate peptide catalyzed by 200 nM E. coli C145 Grx1 (black) and the non-catalyzed reaction (gray) are shown. Concentrations were as follows: McIlvaine buffer, pH 7.0, [GSSG] = 5 mM, [substrate peptide] = 5 μM.

The addition of *E. coli* Grx1 at levels comparable with those used in the deglutathionylation assay (20 nM) or at 10-fold higher levels resulted in no observed difference when compared with the non-catalyzed rate (data not shown). Similarly, the addition of the C14S Grx1 mutant at 200 nM resulted in only very minor changes in rate when compared with the non-catalyzed rate (Fig. 7). A lower concentration of GSSG (1 mM) did not make this catalytic effect more evident. These results are consistent with partitioning to the oxidized state for the wild-type enzyme and the binding specificity for glutathione when compared with peptide.

**Deglutathionylation Activity of Different Enzymes**—In addition to the glutaredoxins, there is a range of enzymes that may have deglutathionylation activity in various cellular compartments. To screen for such activity, the deglutathionylation activity of different members of the thioredoxin superfamily was measured under standard conditions (pH 7.0, [GSH] = 1 mM, [substrate] = 5 μM). The results indicate that all members of the thioredoxin superfamily tested showed detectable activity above background when the concentration of enzyme was increased to 200 nM from 20 nM (Table 1). Although yeast Grx1 and *E. coli* Grx1 show comparable activity, the different members of the human PDI family members showed considerably lower deglutathionylation activity than glutaredoxins ranging from ~1% (PDIP) to 22% (ERp46) of the *E. coli* Grx1 wild type-catalyzed reaction. Since Grx is thought to have a distinct GSH binding site, whereas the PDI family does not, it is possible that this difference in activity represents different reaction mechanisms. To test this, an analysis of the intermediate in the PDI-catalyzed deglutathionylation reaction was undertaken with the C39S mutant of the first catalytic domain of human PDI reacting with the glutathionylated peptide substrate. The mass spectrometry results revealed only the presence of PDI a domain-peptide mixed disulfides (mass = 15274 Da) and no PDI a domain-GSH mixed disulfide intermediates (mass = 14631 Da).

**Specificity of the Intermediate**—It has previously been reported that human Grx shows specificity for forming a mixed disulfide with glutathione using a cysteine-glutathione mixed disulfide and cysteinylglycine-glutathione mixed disulfide substrates (28, 31, 41). Here we report the exclusive formation of a Grx-GSH mixed disulfide and PDI-peptide mixed disulfide during the deglutathionylation of a homogenous glutathionylated peptide substrate. Although the specificity may arise from salt bridges formed between the glutathionyl moiety and Grx, as seen in the structures (25, 31, 42), the unusual γ-glutamyl structure of GSH may also significantly impact on the specificity. To test this, we made a mixed disulfide between glutamyl-cysteinylglycine and the substrate peptide, this compound being the equivalent of the GSH-peptide substrate used in the deglutathionylation assay except that the glutamic acid-cysteine linkage was a normal peptide bond rather than a γ-linkage. When mass spectrometric analysis was undertaken of the intermediate reaction products in the enzyme-catalyzed reduction of this compound, the results indicated only the presence of Grx-peptide (mass = 11572 Da) or PDI a domain-peptide (mass = 15274 Da) mixed disulfide intermediates. No Grx-glutamylcysteinylglycine intermediates were detected, implying that the specificity of Grx for glutathione is absolutely dependent on the γ-linkage. The same result was obtained when using peptide-QCG and -paraECG mixed disulfides as substrates.

**DISCUSSION**

The majority of previous assays for glutaredoxin activity have been based on either indirect measurements in coupled reac-

**TABLE 1**

| Enzyme              | Turnover | Relative activity |
|---------------------|----------|-------------------|
| E. coli Grx1        | 86       | 100               |
| E. coli C14S Grx1   | 27       | 32                |
| Yeast Grx1          | 92       | 108               |
| Human PDI           | 4        | 5                 |
| Human PD1a          | 5        | 6                 |
| Yeast PD1a          | ~1       | ~1                |
| Human Erp57         | 9        | 10                |
| Human Erp57a        | 5        | 6                 |
| Human PdIp          | ~1       | ~1                |
| Human P5            | 10       | 11                |
| Human Erp18         | 2        | 2                 |
| Human Erp46         | 19       | 22                |
| Human Erp72         | 8        | 9                 |

The glutaredoxins were tested at 20 nM, and other enzymes were tested at 200 nM.

lower deglutathionylation activity than glutaredoxins ranging from ~1% (PDIP) to 22% (ERp46) of the *E. coli* Grx1 wild type-catalyzed reaction. Since Grx is thought to have a distinct GSH binding site, whereas the PDI family does not, it is possible that this difference in activity represents different reaction mechanisms. To test this, an analysis of the intermediate in the PDI-catalyzed deglutathionylation reaction was undertaken with the C39S mutant of the first catalytic domain of human PDI reacting with the glutathionylated peptide substrate. The mass spectrometry results revealed only the presence of PDI a domain-peptide mixed disulfides (mass = 15274 Da) and no PDI a domain-GSH mixed disulfide intermediates (mass = 14631 Da).

Specificity of the Intermediate—It has previously been reported that human Grx shows specificity for forming a mixed disulfide with glutathione using a cysteine-glutathione mixed disulfide and cysteinylglycine-glutathione mixed disulfide substrates (28, 31, 41). Here we report the exclusive formation of a Grx-GSH mixed disulfide and PDI-peptide mixed disulfide during the deglutathionylation of a homogenous glutathionylated peptide substrate. Although the specificity may arise from salt bridges formed between the glutathionyl moiety and Grx, as seen in the structures (25, 31, 42), the unusual γ-glutamyl structure of GSH may also significantly impact on the specificity. To test this, we made a mixed disulfide between glutamyl-cysteinylglycine and the substrate peptide, this compound being the equivalent of the GSH-peptide substrate used in the deglutathionylation assay except that the glutamic acid-cysteine linkage was a normal peptide bond rather than a γ-linkage. When mass spectrometric analysis was undertaken of the intermediate reaction products in the enzyme-catalyzed reduction of this compound, the results indicated only the presence of Grx-peptide (mass = 11572 Da) or PDI a domain-peptide (mass = 15274 Da) mixed disulfide intermediates. No Grx-glutamylcysteinylglycine intermediates were detected, implying that the specificity of Grx for glutathione is absolutely dependent on the γ-linkage. The same result was obtained when using peptide-QCG and -paraECG mixed disulfides as substrates.

**DISCUSSION**

The majority of previous assays for glutaredoxin activity have been based on either indirect measurements in coupled reac-
Deglutathionylation Activity of Glutaredoxins

Deglutathionylation activity in vitro is comparable with the catalyzed rate of deglutathionylation under standard conditions, thus confirming that glutathionylation activities of a range of thioredoxin superfamily members are low. Deglutathionylation activities of a range of thioredoxin superfamily members were also tested, most notably the activities of various human PDI family members. These endoplasmic reticulum-resident enzymes are involved in native disulfide bond formation in vivo, an activity that may require deglutathionylation. Direct in vitro studies on PDI family member deglutathionylation are limited (29, 43), but additional evidence for such an activity comes from in vitro refolding studies since PDI is able to form native disulfide bonds in fully glutathionylated protein substrates (for example, see Ref. 44), and the catalyzed folding pathway for reduced protein substrates in a glutathione buffer never results in the significant accumulation of glutathionylated protein (for example, see Ref. 45). Although the deglutathionylation activities of the PDI family members tested are low when compared with that of glutaredoxin (ranging from 1–22%), they are probably physiologically relevant since the catalyzed rate of deglutathionylation under standard conditions is comparable with the catalyzed rate of peptide oxidation under standard conditions (34, 46). However, further studies...
need to be undertaken on the deglutathionylation activity of these enzymes, not least because the reaction pathway for deglutathionylation reaction catalyzed by human PDI differs very significantly from that catalyzed by glutaredoxin as the PDI-peptide mixed disulfide is the exclusive intermediate in the reaction.

Finally, by comparing the intermediates in the Grx-catalyzed reduction of GSH-peptide and glutamylcysteinylglycine-peptide mixed disulfides, the exquisite sensitivity of the specificity of Grx substrate binding to the γ-linkage was demonstrated. Although Grx has previously been reported to show specificity for GSH using cysteine-glutathione and cysteinylglycine-glutathione mixed disulfides (28, 30), this is, to our knowledge, the first demonstration of the importance of the highly unusual γ-linkage found in GSH on Grx specificity. The structural features of Grx that dictate this specificity remain to be elucidated.

Acknowledgments—We thank Peter Klappa, Robert Freedman, and John Mieyal for useful discussions.

REFERENCES

1. Holmgren, A. (1979) J. Biol. Chem. 254, 3664–3671
2. Holmgren, A. (1979) J. Biol. Chem. 254, 3672–3676
3. Hög, O. J., Jönvall, H., Holmgren, A., Carlquist, M., and Persson, M. (1983) Eur. J. Biochem. 136, 223–232
4. Nordstrand, K., Sandström, A., Äslund, F., Holmgren, A., Otting, G., and Berndt, K. D. (2000) J. Mol. Biol. 303, 423–432
5. Xia, T.-H., Bushweller, J. H., Miranda-Vizuete, A., Billeter, M., Björnberg, O., Holmgren, A., and Wüthrich, K. (1992) Protein Sci. 1, 310–321
6. Martin, J. L. (1995) Structure (Lond.) 3, 245–250
7. Holmgren, A. (1989) J. Biol. Chem. 264, 13963–13966
8. Åslund, F., Ehn, B., Miranda-Vizuete, A., Pueyo, C., and Holmgren, A. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 9813–9817
9. Gvakharia, B. O., Hanson, E., Koonin, E. K., and Mathews, C. K. (1996) J. Biol. Chem. 271, 15307–15310
10. Rodríguez-Manzaneque, M. T., Ros, J., Cabisco, E., Sorribas, A., and Herrero, E. (1999) Mol. Cell. Biol. 19, 8180–8190
11. Lundberg, M., Johansson, C., Herrero, E. (1999) Structure (Lond.) 7, 289–300
12. Takashima, Y., Hirota, K., Nakamura, H., Nakamura, T., Akiyama, K., Cheng, F. S., Maeda, M., and Yodoi, J. (1999) Immunol. Lett. 68, 397–401
13. Vlaminck, R., Starke, D. W., Mieyal, J. J., and Gronostajski, R. M. (1998) J. Biol. Chem. 273, 392–397
14. Apontoweil, P., and Berends, W. (1975) Biochim. Biophys. Acta 399, 1–9
15. Rodríguez-Manzaneque, M. T., Ros, J., Cabiscol, E., Sorribas, A., and Herrero, E. (1999) Free Radic. Biol. Med. 27, 922–935
16. Bass, R., Ruddock, L. W., Collinson, E. J., and Grant, C. M. (2003) J. Biol. Chem. 278, 5257–5262
17. Takashima, Y., Hirota, K., Nakamura, H., Nakamura, T., Akiyama, K., Cheng, F. S., Maeda, M., and Yodoi, J. (1999) Immunol. Lett. 68, 397–401
18. Bandyopadhyay, S., Starke, D. W., Mieyal, J. J., and Gronostajski, R. M. (1998) J. Biol. Chem. 273, 392–397
19. Apontoweil, P., and Berends, W. (1975) Biochim. Biophys. Acta 399, 1–9
20. Griffith, O. W. (1999) Free Radic. Biol. Med. 27, 922–935
21. Bass, R., Ruddock, L. W., Collinson, E. J., and Grant, C. M. (2004) J. Biol. Chem. 279, 5257–5262
22. Starke, D. W., Chock, P. B., and Mieyal, J. J. (2005) Antioxid. Redox. Signal. 7, 348–366
23. Shetlon, M. D., Chock, P. B., and Mieyal, J. J. (2006) Antioxid. Redox. Signal. 8, 283–291
24. Ljung, J., Johansson, M., and Holmgren, A. (1991) Biochemistry 30, 9288–9293
25. Lundström-Ljung, J., Vlamis-Gardikas, A., Åslund, F., and Holmgren, A. (1999) FEBS Lett. 443, 85–88
26. Gravina, S. A., and Mieyal, J. J. (1993) Biochemistry 32, 3368–3376
27. Yang, Y., Jiao, S., and Mieyal, J. J. (1997) Biochemistry 36, 3199–3206
28. Ruddock, L. W., and Mieyal, J. J. (1998) Biochemistry 37, 17145–17156
29. Alanen, H. I., Salo, K. E. H., Pirneskoski, A., and Ruddock, L. W. (2000) Antioxid. Redox. Signal. 2, 1183–1198
30. Lee, Y. J. (2002) Biochemistry 41, 5736–5742
31. Lappi, A.-K., Lensink, M. F., Alanen, H. I., Salo, K. E. H., Lobell, M., Juffer, A. H., and Ruddock, L. W. (2004) J. Biol. Chem. 279, 30374–30380
32. Mieyal, J. J., and Rodríguez-Manzaneque, M. T. (1999) Biochemistry 38, 3928–3933
33. Lappi, A.-K., Lensink, M. F., Alanen, H. I., Salo, K. E. H., Pirneskoski, A., and Ruddock, L. W. (2003) Biochemistry 42, 46566–46575
34. Ruddock, L. W., Hirst, R. T., and Freedman, R. B. (1996) Biochemistry 35, 1001–1005
35. Neves-Petersen, M. T., Grycynski, Z., Lakowicz, J., Fojan, P., Pedersen, S., Petersen, E., and Petersen, S. (2002) Protein Sci. 11, 588–600
36. Nordstrand, K., Åslund, F., Holmgren, A., Otting, G., and Berndt, K. D. (1999) J. Biol. Chem. 264, 423–432
37. Lundberg, M., Johansson, C., Herrero, E. (1999) Structure (Lond.) 7, 289–300
38. Follope, N., and Millo, L. (2004) Eur. J. Biochem. 271, 463–468
39. Follope, N., Sagemark, J., Nordstrand, K., Berndt, K. D., and Nilsson, I. (2001) J. Biol. Chem. 276, 52629–52635
40. Gladyshev, V. N., Liu, A., Novoselov, S. V., Krysan, K., Sun, Q.-A., Kryukov, V. M., Kryukov, G. V., and Lou, M. F. (2001) J. Biol. Chem. 276, 30374–30380
41. Collinson, E. J., and Grant, C. M. (2003) J. Biol. Chem. 278, 22492–22497
42. Starke, D. W., Chock, P. B., and Mieyal, J. J. (2003) J. Biol. Chem. 278, 14607–14613
43. Murata, H., Ibara, Y., Nakamura, H., Yodoi, J., Sumikawa, K., and Kondo, T. (2003) J. Biol. Chem. 278, 50226–50233
44. Song, J. J., Rhee, J. G., Suntharalingam, M., Walsh, S. A., Spitz, D. R., and Lee, Y. J. (2002) J. Biol. Chem. 277, 46566–46575
45. Ruoppolo, M., and Freedman, R. B. (1995) Biochemistry 34, 9380–9388
46. Ruoppolo, M., Freedman, R. B., Pucci, P., and Marino, G. (1996) Biochemistry 35, 13636–13646
47. Alanen, H. I., Salo, K. E. H., Pirneskoski, A., and Ruddock, L. W. (2006) Antioxid. Redox. Signal. 8, 283–291