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Insights into Deglutathionylation Reactions

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

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Glutaredoxins are small proteins with a conserved active site (-C*XX*(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 γ-linkage present in glutathione. The deglutathionylation activity of both wild-type *Escherichia coli* glutaredoxin and the C14S mutant was competitively inhibited by oxidized glutathione, with K_i values similar to the K_m values for the glutathionylated peptide substrate, implying that glutaredoxin primarily recognizes 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 -C*XX*C- or -C*XX*S- 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 (C*XX*S) and dithiol (C*XX*C) 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 thiolate of Grx[2] 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 devel-

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[2] The abbreviations used are: Grx, glutaredoxin; HED, β-hydroxyethyl disulfide; PDI, protein disulfide isomerases.

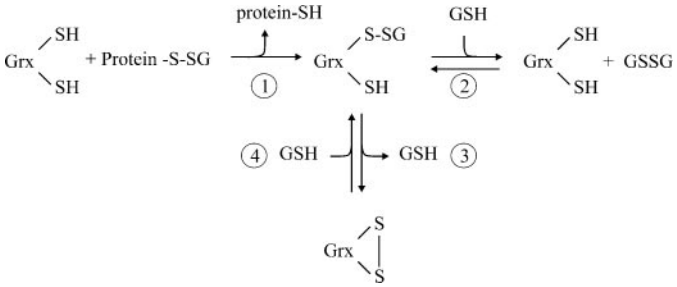

FIGURE 1. **Schematic representation of the proposed reaction scheme for deglutathionylation catalyzed by glutaredoxins.** The initial reaction is a nucleophilic attack by the thiolate of the N-terminal active site cysteine of Grx on the mixed disulfide between protein thiol and GSH, leading to a formation of a new disulfide between Grx and GSH and the release of a protein substrate in the reduced form (*reaction 1*). The mixed disulfide between Grx and GSH is then reduced by GSH through a nucleophilic attack on the disulfide to form GSSG and reduced Grx as the final products (*reaction 2*). For the dithiol (C*XX*C) glutaredoxin variants, there is the possibility of the partitioning at the Grx-GSH mixed disulfide state to form oxidized Grx (*reaction 3*). This can be retrieved to the functional pathway by reduction by GSH (*reaction 4*). The assay developed here used a glutathionylated peptide substrate, but otherwise, the reaction scheme is as shown.

opment of a new real-time method for measuring the deglutathionylation activity of glutaredoxins. The assay is based on a homogeneous glutathionylated peptide and measures the actual formation of deglutathionylated product rather than indirectly the consumption of NADPH as in the traditional HED assay. This method offers new insights into the mechanism of action of the glutaredoxins and allows the glutaredoxin-like activity of other thioredoxin superfamily members, such as protein disulfide isomerases (PDI), to be determined. We also demonstrate that the reaction intermediate in the deglutathionylation reaction is exclusively Grx-GSH mixed, disulfide confirming a previous report (31), and that it is dependent on the unusual γ-linkage present in GSH.

EXPERIMENTAL PROCEDURES

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 (Strategene, 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 ordered from The Biomolecular Science Facility, Department of Biosciences, University of Kent (Canterbury, Kent, UK). Fluorescence measurements were performed with a PerkinElmer Life Sciences LS50B spectrometer using a 315-μl cuvette. Assays were carried out in McIlvaine buffer (0.2 M disodium hydrogen phosphate, 0.1 M citric acid) at pH 4.5–7.5, including appropriate amounts of GSH (0–4 mM), NADPH (50 μM), glutathione reductase (0 or 20 nM) (all from Sigma), EDTA (1 mM), substrate peptide (0–20 μM), and the enzyme of interest (0–200 nM). To prevent loss of Grx due to adsorption on the walls of plastic tubes and the cuvette, dilutions of Grx and glutathione reductase were made into solutions containing bovine serum albumin, the final concentration of bovine serum albumin in the assay being 1 μg/ml. All measurements were done at 25 °C, excitation 280 nm, emission 356 nm, and slit widths 5 nm.

Analysis of Fluorescence Data—The fluorescence data were analyzed with Igor Pro 3.14 (Wavemetrics Inc., Lake Oswego, OR). Initial rates of reaction were determined from linear fits over 20 units of change in fluorescence signal or other applicable linear part of the curve and correlating it to the total change in fluorescence observed during the reaction. The first few data points were ignored due to extra noise being observed during the first few seconds. The corresponding non-catalyzed background reaction rates (*i.e.* in the absence of Grx) were subtracted from the catalyzed rates. The change in fluorescence during the deglutathionylation reaction was proportional to the substrate concentration and otherwise independent of the reaction conditions except when glutathione reductase was omitted. In the absence of glutathione reductase, the overall change in fluorescence was up to 25% smaller, presumably due to the presence of an equilibrium between the deglutathionylation and glutathionylation reactions.

Assay for Determining the Glutathionylation Activity—The reduced substrate peptide SQLWCLSN was ordered from The Biomolecular Science Facility, Department of Biosciences, University of Kent. Fluorescence measurements were performed in McIlvaine buffer (0.2 M disodium hydrogen phosphate, 0.1 M citric acid) at pH 7.0, including GSSG (5 mM), bovine serum albumin (1 μg/ml), EDTA (1 mM), reduced substrate peptide (5 μM), and the enzyme of interest (0–200 nM). Measurements were done at 25 °C, excitation 280 nm, emission 356 nm, and slit widths 5 nm.

Synthesis of Alternative Substrate—The reduced substrate peptide (100 μM) was incubated with ECG, QCG, or paraECG (1 mM) in 0.1 M sodium phosphate buffer at pH 8 for 1 h. Peptide-ECG, -QCG, and -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 C14S 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 CH_3COOH 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 glutathionylated 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. 2*A*), 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. 2*B*). 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. 3*A*). 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 μM and k_{cat} = 4.4 s^{-1} ([GSH] = 1 mM; Fig. 3*B*). 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 ([substrate peptide] = 5 μM; Fig. 3*C*).

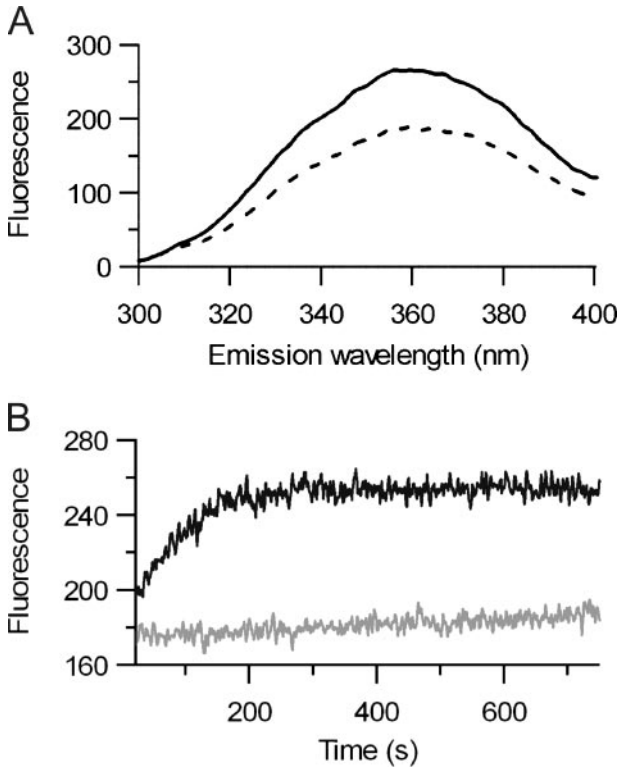

FIGURE 2. **Fluorescence analysis of peptide deglutathionylation.** *A*, typical emission spectra for glutathionylated (– – –) and deglutathionylated (------) peptide (5 μM) at pH 7. 0. *B*, representative time-dependent fluorescence profiles during the deglutathionylation of the substrate peptide catalyzed 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.

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

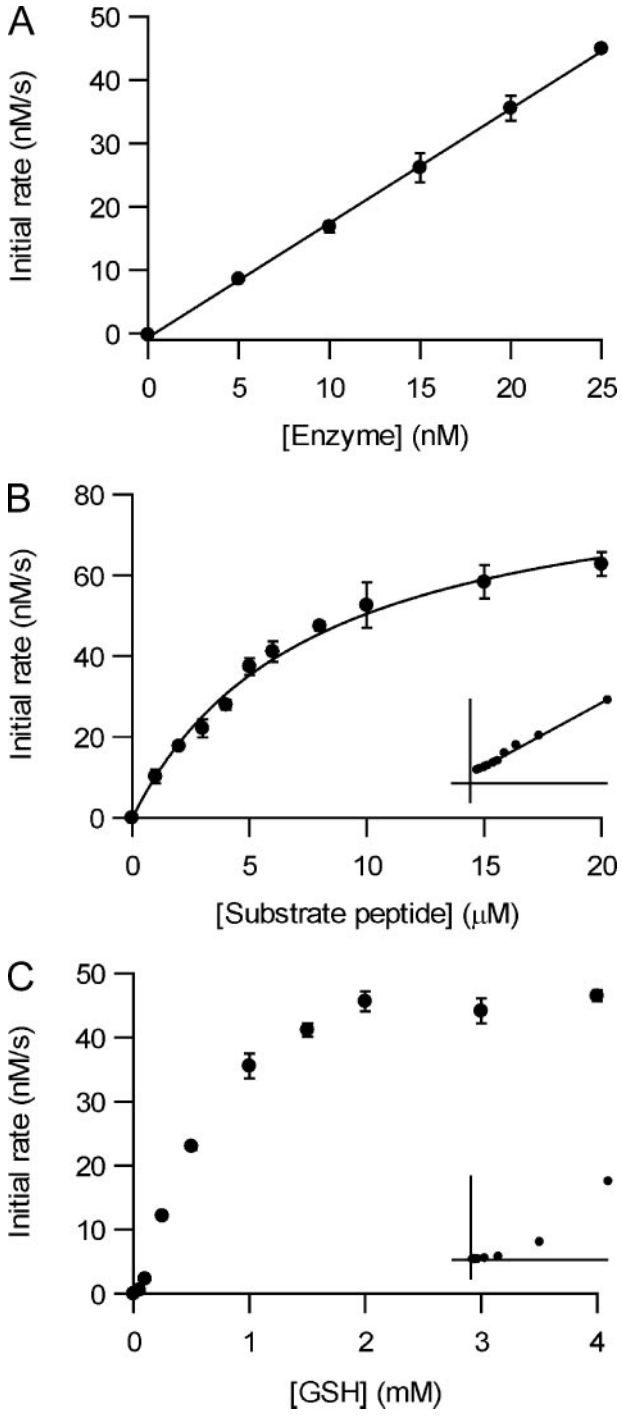

FIGURE 3. **Analysis of the reaction kinetics of glutaredoxin-catalyzed deglutathionylation.** *A*, linear dependence of the initial rate of peptide deglutathionylation on Grx1 concentration (McIlvaine buffer, pH 7.0, [GSH] = 1 mM, [substrate peptide] = 5 μM). Initial rates are expressed as mean ± S.D. *B*, variation of the initial rate of reaction with substrate concentration during peptide deglutathionylation catalyzed by 20 nM Grx1 (McIlvaine buffer, pH 7.0, [GSH] = 1 mM). Initial rates are expressed as mean ± S.D. The line of best fit is to the Michaelis-Menten equation, and the *inset* shows a linear fit to a Lineweaver-Burk plot. *C*, variation of the initial rate of reaction with GSH concentration during peptide deglutathionylation catalyzed by 20 nM Grx1 (McIlvaine buffer, pH 7.0, [substrate peptide] = 5 μM). Initial rates are expressed as mean ± S.D. The *inset* shows a Lineweaver-Burk plot.

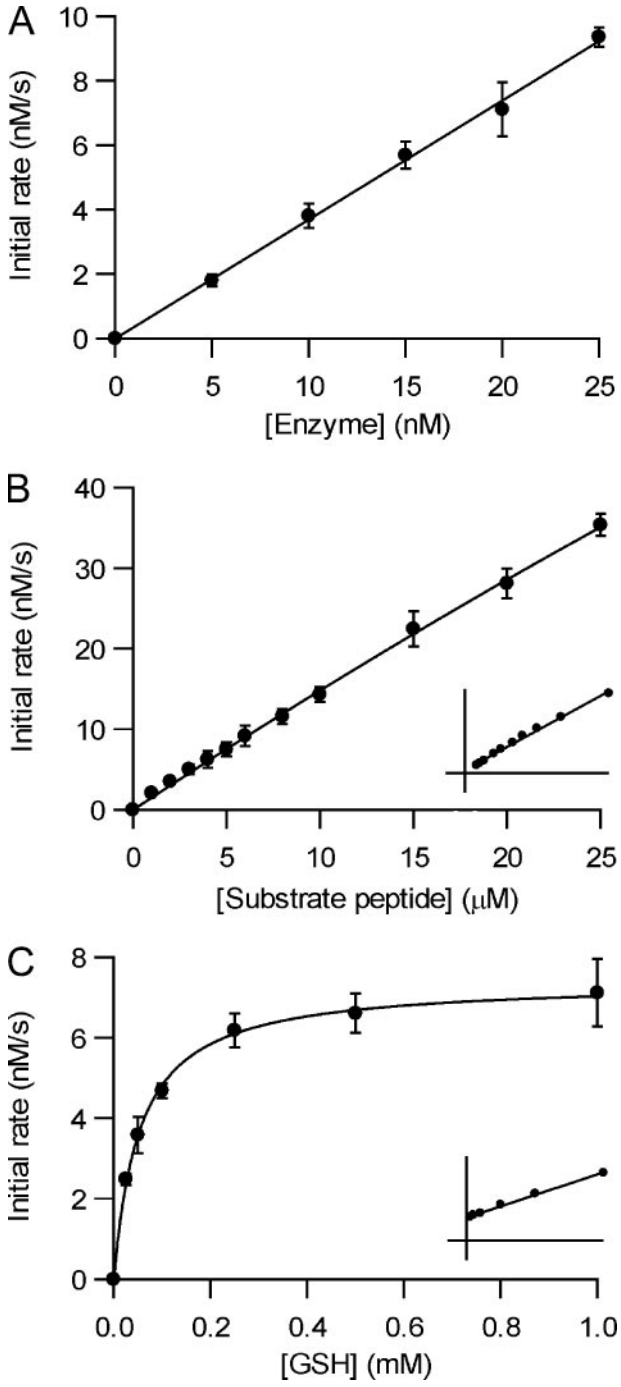

FIGURE 4. **Analysis of the reaction kinetics of C14S glutaredoxin-catalyzed deglutathionylation.** *A*, linear dependence of the initial rate of peptide deglutathionylation on C14S Grx1 concentration (McIlvaine buffer, pH 7.0, [GSH] = 1 mM, [substrate peptide] = 5 μM). Initial rates are expressed as mean ± S.D. *B*, variation of the initial rate of reaction with substrate concentration during peptide deglutathionylation catalyzed by 20 nM C14S Grx1 (McIlvaine buffer, pH 7.0, [GSH] = 1 mM). Initial rates are expressed as mean ± S.D. The line of best fit is to the Michaelis-Menten equation, and the *inset* shows a linear fit to a Lineweaver-Burk plot. *C*, variation of the initial rate of reaction with GSH concentration during peptide deglutathionylation catalyzed by 20 nM C14S Grx1 (McIlvaine buffer, pH 7.0, [substrate peptide] = 5 μM). Initial rates are expressed as mean ± S.D. The line of best fit is to the Michaelis-Menten equation, and the *inset* shows a linear fit to a Lineweaver-Burk plot.

([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. 4*A*). Furthermore, the data with increasing substrate concentrations fitted well to the Michae-

lis-Menten equation with K_m = 44 μM and k_{cat} = 4.0 s^{-1} ([GSH] = 1 mM; Fig. 4*B*), although no plateau was observed due to the narrow range of possible substrate concentrations that could be used. In contrast to wild type, the increase observed in the initial rate with increasing GSH concentrations could be fitted to the Michaelis-Menten equation, giving K_m = 48 μM and k_{cat} = 0.36 s^{-1} ([substrate peptide] = 5 μM; Fig. 4*C*). A lower mid-point for glutathione dependence for the mutant (here K_m = 48 μM) when compared with the wild-type enzyme (here mid-point ~0.5 mM) has previously been reported for human Grx and assigned to the effects of the partitioning reaction to the oxidized state for the wild-type enzyme (31).

Intermediates in the Deglutathionylation Reaction—It is presumed (31, 37) that the major intermediate in the deglutathionylation reaction catalyzed by Grx is the Grx-GSH mixed disulfide. To try to confirm this, experiments for trapping the reaction intermediate in equilibrium as well as quenched-flow reactions were undertaken 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 K_m 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 K_m values for the peptide substrate calculated above, the competitive inhibition constant, K_i, 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

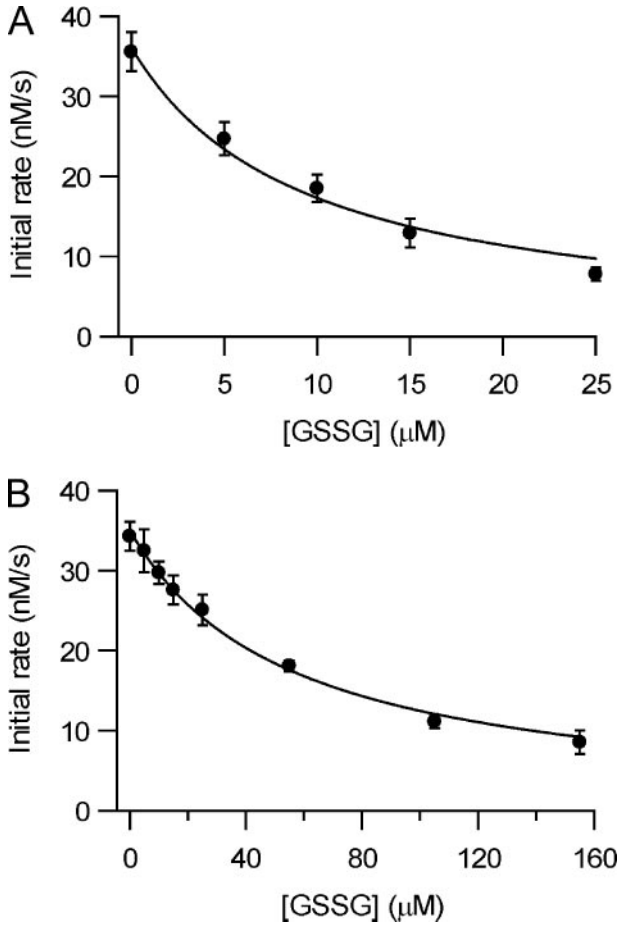

FIGURE 5. **Competitive inhibitory effect of GSSG on glutaredoxin-catalyzed deglutathionylation.** The variation of the initial rate of reaction with GSSG concentration during enzyme-catalyzed peptide deglutathionylation (McIlvaine buffer, pH 7.0, [GSH] = 1 mM, [substrate peptide] = 5 μM, [Grx1] = 20 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 K_m determined previously. *A*, Grx1. *B*, C14S Grx1.

dependence of the non-catalyzed reaction, the Grx1-catalyzed reaction showed a single pK_a-dependent event with a pK_a = 6.47 ± 0.04 for the wild-type enzyme-catalyzed reaction and a pK_a = 5.90 ± 0.07 for the C14S-catalyzed reaction (Fig. 6). These values are significantly higher than the pK_a 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 pK_a-dependent event with a pK_a = 4.86 ± 0.07 for the N-terminal cysteine (data not shown).

Determination of Glutathionylation Activity—To monitor the glutathionylation activity of Grx, deglutathionylated 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 deglutathionylation 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;

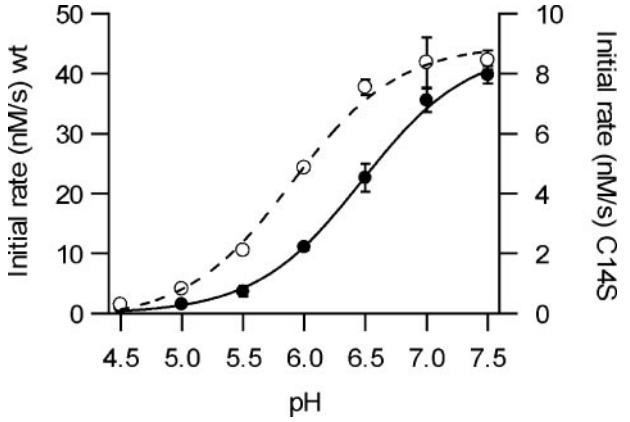

FIGURE 6. **pH dependence of enzyme-catalyzed deglutathionylation.** The variation of the initial rate of reaction as a function of pH (McIlvaine buffer, pH 7.0, [GSH] = 1 mM, [substrate peptide] = 5 μM, [enzyme] = 20 nM) is shown. Initial rates are expressed as mean ± S.D. with the non-catalyzed rate 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; ○, C14S mutant.

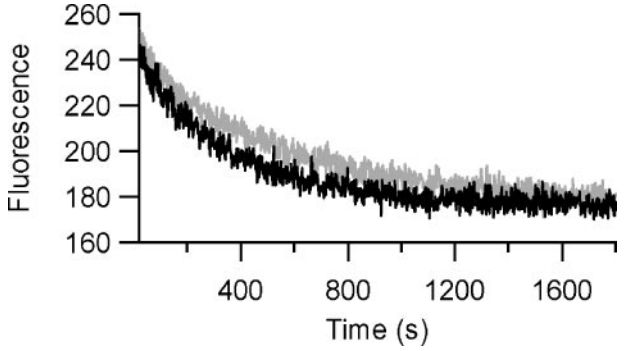

FIGURE 7. **Fluorescence analysis of peptide glutathionylation.** Representative time-dependent fluorescence profiles during the glutathionylation of the substrate peptide catalyzed by 200 nM *E. coli* C14S Grx1 (*black*) and the non-catalyzed reaction (gray) are shown. Concentrations were as follows: McIlvaine buffer, pH 7.0, [GSSG] = 5 mM, [substrate peptide] = 5 μM.

Fig. 7). 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

TABLE 1

Deglutathionylation activity of different enzymes under standard conditions (McIlvaine buffer pH 7.0, [GSH] = 1 mM, [substrate peptide] = 5 μM)

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

Enzyme	Turnover	Relative activity
	min^{-1}	%
E. coli Grx1	86	100
E. coli C14S Grx1	27	32
Yeast Grx1	92	108
Human PDI	4	5
Human PDIa	5	6
Yeast PDIa	~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

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 glutamylcysteinylglycine 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-

tions, usually on non-physiological substrates, *e.g.* the HED assay (1), or discrete interval measurements on substrates that may not be homogeneous, *e.g.* deglutathionylation of radiolabeled bovine serum albumin-, papain-, or hemoglobin-GSH mixed disulfides (28, 30). The assay introduced here directly measures in real time the deglutathionylation of a homogenous peptide substrate and thus allows for the sensitive elucidation of reaction parameters. The limitations of the assay are based on the fluorescence detection method, making it unsuitable for activity determination in crude lysates and giving a fairly narrow range of permissible substrate concentrations due to the inner filter effect.

The assay allowed us to determine apparent K_m and k_{cat} values for wild-type *E. coli* Grx1 and the C14S mutant of this enzyme, with the exception of the K_m value for GSH for the wild-type enzyme (see below). Although the C14S mutant showed only 23% of the wild-type enzyme activity under standard assay conditions, this was found to represent a decrease in K_m for the substrate (and probably GSH) rather than a decrease in k_{cat}. Thus, consistent with previous results (25, 26), the mechanism of action of deglutathionylation of the glutathionylated peptide substrate by glutaredoxins requires only the N-terminal active site thiol. Since the reduction in K_m for the substrate probably represents the effects of making a mutation in a wild-type enzyme rather than reflecting intrinsic differences between one-cysteine and two-cysteine active site glutaredoxins, the following question arises: why have the second active site cysteine?

The answer to this question is probably linked to three other observations from our assay: (*a*) that the only intermediate seen in the reaction is the Grx1-GSH mixed disulfide, *i.e.* that no Grx1-peptide mixed disulfide was observed; (*b*) that the GSH dependence of wild-type Grx1 is sigmoidal due to partitioning of the Grx-GSH mixed disulfide to oxidized Grx1 (Fig. 5); and (*c*) that the K_m values for the C14S mutant for the substrate (44 μM) and for GSH (48 μM) and the K_i value for GSSG (50 μM) were effectively equivalent, as were the K_m value for substrate (7.8 μM) and K_i value for GSSG (5.6 μM) for the wild-type enzyme. These observations have two implications.

Firstly, the specificity of glutaredoxin for glutathionylated substrates comes from the glutathione moiety (although probably with a factor dependent on the accessibility of this moiety in glutathionylated proteins (30)). This allows glutaredoxins to potentially act equally well on all glutathionylated substrates, resolving the issue of the lack of specificity of the glutathionylation reaction under oxidative stress. In addition, this implies that glutaredoxins are likely to be very poor general protein disulfide reductases and, consistent with our observations, that glutaredoxins are likely to be poor catalysts of glutathionylation reactions.

Secondly, the combination of these observations implies that glutaredoxins do not, as currently thought, work under conditions of oxidative stress. Studying the *in vivo* activity of glutaredoxins under such conditions is extremely difficult; however, these conditions can be mimicked in the *in vitro* assay. In terms of the intracellular glutathione pool, oxidative stress conditions can be defined as either conditions in which the concentration of GSH is significantly reduced or conditions in which the concentration of GSSG is increased. Since wild-type Grx1 shows a sigmoidal dependence on GSH concentrations, its activity is very significantly decreased at low GSH concentrations (due to partitioning to the oxidized state, Fig. 1, *reaction 3*) and consistent with a lower K_m for GSH of the mutant Grx when compared with the wild-type enzyme (31). Likewise Grx1 is potently inhibited by GSSG. Thus, under conditions that mimic oxidative stress, the activity of Grx1 is very markedly reduced, and it will only be able to have significant deglutathionylation activity once the oxidative stress has been removed, *i.e.* that it is an enzyme that repairs the effects of damage after the event rather than limiting the effects during the event. Given that glutaredoxin is an enzyme that relies on a highly reactive active site cysteine residue that will itself be very sensitive to oxidative stress, these two protective mechanisms during oxidative stress, forming a less reactive mixed disulfide by reacting with GSSG and forming a stable intramolecular disulfide between the two active site cysteines, ensure that glutaredoxin is less sensitive to oxidative damage until the oxidative stress is removed. Thus, the need for a second active site cysteine residue in most of the glutaredoxins comes about. Its effects are not to modulate the deglutathionylation activity of the enzyme, but rather, the effects of the C-terminal active site cysteine residue are to protect the reactive N-terminal active site cysteine residue by forming a less reactive disulfide, a reaction that is readily reversed once GSH levels rise, *i.e.* once oxidative stress is removed, generating an active enzyme.

These results show the complexity of enzyme systems. What at first sight appears to be a relatively simple reaction to catalyze shows two substrate ping-pong kinetics (30), with partitioning to an inactive form from the covalent intermediate formed with substrate one (the Grx-GSH mixed disulfide), product inhibition (by GSSG), and substrate inhibition (by GSH at concentrations greater than 20 mM, data not shown). Although these complexities have not been previously appreciated, they all combine to provide the enzyme with the ideal properties required for its physiological role.

In addition to developing the deglutathionylation assay and investigating the mechanisms of action of glutaredoxins, the 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.

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REFERENCES

1. Holmgren, A. (1979) *J. Biol. Chem.* **254,** 3664–3671
2. Holmgren, A. (1979) *J. Biol. Chem.* **254,** 3672–3678
3. Höög, J. O., Jörnvall, H., Holmgren, A., Carlquist, M., and Persson, M. (1983) *Eur. J. Biochem.* **136,** 223–232
4. Nordstrand, K., Sandström, A., Åslund, F., Holmgren, A., Otting, G., and Berndt, K. D. (2000) *J. Mol. Biol.* **303,** 423–432
5. Xia, T.-H., Bushweller, J. H., Sodano, P., 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., Cabiscol, E., Sorribas, A., and Herrero, E. (1999) *Mol. Cell Biol.* **19,** 8180–8190
11. Lundberg, M., Johansson, C., Chandra, J., Enoksson, M., Jacobsson, G., Ljung, J., Johansson, M., and Holmgren, A. (2001) *J. Biol. Chem.* **276,** 26269–26275
12. 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
13. Collinson, E. J., and Grant, C. M. (2003) *J. Biol. Chem.* **278,** 22492–22497
14. Starke, D. W., Chock, P. B., and Mieyal, J. J. (2003) *J. Biol. Chem.* **278,** 14607–14613
15. Murata, H., Ihara, Y., Nakamura, H., Yodoi, J., Sumikawa, K., and Kondo, T. (2003) *J. Biol. Chem.* **278,** 50226–50233
16. 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
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., Klappa, P., and Freedman, R. B. (2004) *J. Biol. Chem.* **279,** 5257–5262
22. Hwang, C., Sinskey, A. J., and Lodish, H. F. (1992) *Science* **257,** 1496–1502
23. Cotgreave, I. A., and Gerdes, R. G. (1998) *Biochem. Biophys. Res. Commun.* **242,** 1–9
24. Shelton, M. D., Chock, P. B., and Mieyal, J. J. (2005) *Antioxid. Redox. Signal.* **7,** 348–366
25. Bushweller, J. H., Åslund, F., Wüthrich, K., and Holmgren, A. (1992) *Biochemistry* **31,** 9288–9293
26. Yang, y., and Wells, W. W. (1991) *J. Biol. Chem.* **266,** 12759–12765
27. Fernandes, A. P., and Holmgren, A. (2004) *Antioxid. Redox. Signal.* **6,** 63–74
28. Srinivasan, U., Mieyal, P. A., and Mieyal, J. J. (1997) *Biochemistry* **36,** 3199–3206
29. Lundström-Ljung, J., Vlamis-Gardikas, A., Åslund, F., and Holmgren, A. (1999) *FEBS Lett.* **443,** 85–88
30. Gravina, S. A., and Mieyal, J. J. (1993) *Biochemistry* **32,** 3368–3376
31. Yang, Y., Jao, S., Nanduri, S., Starke, D. W., Mieyal, J. J., and Qin, J. (1998) *Biochemistry* **37,** 17145–17156
32. Alanen, H. I., Salo, K. E., Pekkala, M., Siekkinen, H. M., Pirneskoski, A., and Ruddock, L. W. (2003) *Antioxid. Redox. Signal.* **5,** 367–374
33. Lappi, A.-K., Lensink, M. F., Alanen, H. I., Salo, K. E. H., Lobell, M., Juffer, A. H., and Ruddock, L. W. (2004) *J. Mol. Biol.* **335,** 283–295
34. Ruddock, L. W., Hirst, T. R., and Freedman, R. B. (1996) *Biochem. J.* **315,** 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. Mol. Biol.* **286,** 541–552
37. Bushweller, J. H., Billeter, M., Holmgren, A., and Wüthrich K. (1994) *J. Mol. Biol.* **235,** 1585–1597
38. Foloppe, N., and Nilsson, L. (2004) *Structure* (*Lond.*) **12,** 289–300
39. Foloppe, N., Sagemark, J., Nordstrand, K., Berndt, K. D., and Nilsson, L. (2001) *J. Mol. Biol.* **310,** 449–470
40. Sun, C., Berardi, M. J., and Bushweller, J. H. (1998) *J. Mol. Biol.* **280,** 687–701
41. Rabenstein, D. L., and Millis, K. K. (1995) *Biochim. Biophys. Acta* **1249,** 29–36
42. Jao, S. C., English Ospina, S. M., Berdis, A. J., Starke, D. W., Post, C. B., and Mieyal, J. J. (2006) *Biochemistry* **45,** 4785–4796
43. Xiao, R., Lundström-Ljung, J., Holmgren, A., and Gilbert, H. F. (2005) *J. Biol. Chem.* **280,** 21099–21106
44. Ruoppolo, M., and Freedman, R. B. (1995) *Biochemistry* **34,** 9380–9398
45. Ruoppolo, M., Freedman, R. B., Pucci, P., and Marino, G. (1996) *Biochemistry* **35,** 13636–16346
46. Alanen, H. I., Salo, K. E. H., Pirneskoski, A., and Ruddock, L. W. (2006) *Antioxid. Redox. Signal.* **8,** 283–291

Insights into Deglutathionylation Reactions: DIFFERENT INTERMEDIATES IN THE GLUTAREDOXIN AND PROTEIN DISULFIDE ISOMERASE CATALYZED REACTIONS ARE DEFINED BY THE γ-LINKAGE PRESENT IN GLUTATHIONE

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