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Original Research Communication

The C-Terminal Active Site Cysteine of *Escherichia coli* Glutaredoxin 1 Determines the Glutathione Specificity of the Second Step of Peptide Deglutathionylation

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Abstract

Glutaredoxins are oxidoreductases specialized in reducing glutathione-protein mixed disulfides. In the first step of deglutathionylation, glutaredoxins form a mixed disulfide with glutathione, releasing reduced peptide. The specificity of this reaction is based on the unusual amide linkage formed between the γ-carboxylate of the N-terminal glutamic acid and the α-amino group of the cysteine present in glutathione. In the second step of deglutathionylation, glutathione reduces the glutaredoxin-glutathione mixed disulfide. Here we show that the specificity of this second reaction for *Escherichia coli* Grx1, but not for human or yeast Grx1, also is based on the unusual γ-linkage present in glutathione. Mutating Tyr13, Thr58, and/or Asp74 to alanine in *E. coli* Grx1 results in the glutaredoxin-peptide mixed disulfide being thermodynamically favored over the glutaredoxin-glutathione mixed disulfide in the first step of the reaction. An increased propensity to form glutaredoxin-protein mixed disulfides was observed *in vivo* for these same mutants. Furthermore, we demonstrate that all mutations studied in Cys14, the C-terminal active site cysteine, abolish the specificity of *E. coli* Grx1 for glutathione over the corresponding tripeptide Glu-Cys-Gly, which has a normal peptide bond linking Glu-Cys instead of the γ-linkage present in glutathione, in the second step of deglutathionylation. *Antioxid. Redox Signal.* 11, 1819–1828.

Introduction

Classic glutaredoxins (Grx) are small proteins with a conserved active site -CPYC- and a glutathione recognition site (2, 3, 8, 11). They belong to the thioredoxin fold superfamily based on their three-dimensional structures (10, 17, 19, 21). Glutaredoxins catalyze the reduction of protein disulfides or GSH-protein mixed disulfides, generally preferring the latter as substrates (4). The reduction of protein disulfides is thought to proceed *via* the dithiol pathway by using both active-site cysteines, whereas the monothiol reaction mechanism used for reducing glutathionylated substrates requires only the N-terminal active-site cysteine (22–24). The first intermediate in the monothiol reaction is a mixed disulfide formed between Grx and the GSH moiety released from the substrate (7, 14, 18, 22). The specificity of this first step (reaction 1 in Fig. 1) is dependent on the unusual amide linkage formed from the side chain or γ-carboxylate of the N-terminal glutamic acid and the α-amino group of the cysteine that is present in GSH (16, 22). Another molecule of GSH is needed for the re-reduction of the Grx-GSH mixed disulfide back to the active dithiol form of Grx. The oxidized glutathione formed in the reaction is in turn reduced by glutathione reductase by using NADPH as hydrogen donor (see Fig. 1). Alternatively the Grx-GSH mixed disulfide can kinetically partition to the inactive intramolecular disulfide or oxidized form of Grx. Glutaredoxin returns to the Grx-GSH mixed disulfide state after nucleophilic attack by GSH.

Although other enzymes are capable of reducing GSH-protein mixed disulfides, Grx is thought to be the specialist in these reactions. Structural studies on glutaredoxins, with the C-terminal active-site cysteine mutated to serine, in mixed disulfide with GSH, implicated a number of conserved residues in the interaction of Grx with the GSH moiety. These

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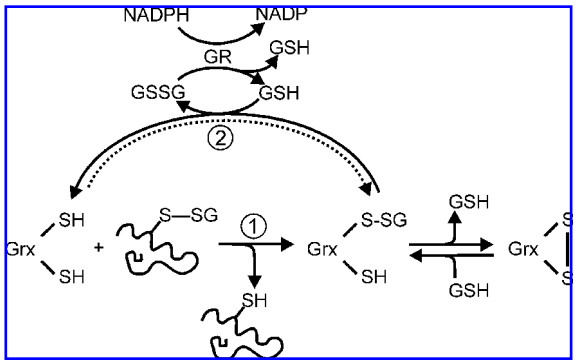

FIG. 1. The deglutathionylation reaction catalyzed by glutaredoxin. The monothiol pathway is used in the reduction of GSH-mixed disulfides. The initial reaction (deglutathionylation reaction 1) is nucleophilic attack by the N-terminal active-site cysteine of glutaredoxin on the protein or peptide GSH-mixed disulfide, with the formation of the Grx-GSH mixed disulfide. From this state, kinetic partitioning occurs. Glutathionylated Grx can undergo nucleophilic attack by a molecule of GSH to form GSSG and regenerate the reduced state of Grx (deglutathionylation reaction 2), or the Grx-GSH mixed disulfide can undergo nucleophilic attack by the C-terminal active-site cysteine to release GSH and the oxidized form of Grx. At high GSH concentrations, the intermolecular reaction is kinetically faster, and the reaction pathway depends only on the N-terminal active-site cysteine of Grx; hence, it is known as the monothiol pathway. At low GSH concentrations, the intramolecular reaction is kinetically faster, and the oxidized form of Grx is formed. Oxidized Grx is returned to the Grx-GSH mixed-disulfide state by the nucleophilic attack of GSH on the active-site disulfide bond. This kinetic partitioning gives rise to the sigmoidal dependence of the catalyzed reaction on GSH concentration. The oxidized form of Grx is also an obligatory intermediate in the dithiol mechanism for the reduction of protein disulfide bonds. The GSSG generated in the deglutathionylation reaction is reduced to GSH by glutathione reductase (GR) by using NADPH as reducing power.

include Tyr13, Thr58, Val59, Tyr72, Thr73, and Asp74 in *Escherichia coli* Grx1 (3) and residues in analogous positions in human Grx1 (22).

In this study we used site-directed mutagenesis, activity measurements, Western blot analysis, and mass spectrometric studies on reaction intermediates to study the effects of selected conserved residues on the glutathione specificity of *E. coli* Grx1. We show that the specificity of the second step of deglutathionylation (reaction 2 in Fig. 1) also depends on the γ-linkage present in GSH. We further show that although Tyr13, Thr58, and Asp74 contribute to the thermodynamics of glutathione specificity, mutations in these residues do not alter the specificity of the initial reaction, with the formation of the Grx-GSH mixed disulfide rather than the alternative Grx-peptide mixed disulfide being kinetically favored for all mutants. Unexpectedly, mutations in Cys14, the C-terminal active-site cysteine, have a very marked effect on the specificity of the second step in peptide deglutathionylation, removing the requirement for the γ-linkage in the second substrate.

To examine the extent to which the results obtained for *E. coli* Grx1 can be generalized across the glutaredoxin family, we also examined the specificity of the first and second steps of deglutathionylation as catalyzed by human Grx1 and *Saccharomyces cerevisiae* Grx1. Both of these glutaredoxins showed a specificity for the γ-amide linkage being present rather than a normal peptide bond in the first step of deglutathionylation, as per *E. coli* Grx1. However, in marked contrast to *E. coli* Grx1, human Grx1 showed no specificity for the γ-linked glutamyl in GSH over an α-amide–linked glutamyl group for the second step of the deglutathionylation reaction, even though the presence of the glutamyl moiety was previously shown to be important (6, 18); *S. cerevisiae* Grx1 favored the use of a tripeptide that had a normal peptide bond over GSH in the second step of deglutathionylation. These results have significant implications for the study of glutaredoxin structure–function relations.

Materials and Methods

Protein expression and purification

Constructs used in the experiments were cloned into an expression vector, which incorporates either an N-terminal His-tag, for purification, or an Myc-tag, to facilitate Western blot analysis, to the cloned gene. *Escherichia coli* Grx1 was cloned from *E. coli* strain XL1-Blue; *S. cerevisiae* Grx1, from strain W303; and human Grx1 was cloned from a liver cDNA library (Clontech, Mountain View, CA). The **a** domain of human PDI was cloned previously (13). Site-directed mutagenesis was performed according to 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 BL21 (DE3) pLysS RARE or Rosetta-gami, and His-tagged proteins were purified by immobilized metal affinity chromatography and ion-exchange chromatography, as described previously (13), with the exception that for human Grx1, a monoS cation-exchange column was used with a 20 m*M* phosphate buffer, pH 6.5, in place of the final monoQ anion exchange column with 20 m*M* phosphate buffer, pH 7.3. Pure protein fractions, as determined by Coomassie brilliant blue-stained SDS-PAGE, were combined and buffer exchanged into 20 m*M* sodium phosphate buffer, pH 7.3, and stored frozen.

Measuring deglutathionylation activity

The deglutathionylation activity of the wild-type and mutant glutaredoxins was measured as described previously (16). In brief, assays were carried out in McIlvaine buffer (0.2 *M* disodium hydrogen phosphate/0.1 *M* citric acid) at pH 7.0, including GSH (1 m*M* or varying) or ECG (1 m*M* or varying), bovine serum albumin (BSA) (1 μg/ml), EDTA (1 m*M*), substrate peptide (5 μ*M*) [SQLWC(glutathione)LSN or SQLWC (ECG)LSN; University of Kent, Department of Biosciences, Canterbury, Kent, UK], and the enzyme of interest (0–500 n*M*). Fluorescence measurements were performed with a Perkin-Elmer LS50B spectrometer at 25°C; excitation, 280 nm; emission, 356 nm; and slit widths, 5 nm. Statistical analysis of the results from the enzymatic analysis was performed by using a two-tailed *t* test for independent samples.

Analysis of reaction intermediates with mass spectrometry

Mutants of *E. coli* Grx1 in the C14S background were used in trapping experiments, as described previously (16). In brief,

the reduced protein of interest (40 μM) was reacted with either substrate peptide (50 μM) or buffer alone in McIlvaine buffer at pH 7.0 for the desired time. The reaction was quenched with 50 m*M* NEM (*N*-ethylmaleimide) or 1.1 *M* IAA (iodoacetamide) (both from Sigma-Aldrich, St. Louis, MO), and the excess of NEM/IAA was removed with pepClean C-18 spin columns (Pierce, Rockford, IL) according to manufacturer's instructions. The results were the same and thus independent of the blocking reagent used. Molecular masses were measured with an electrospray ionization mass spectrometer (ESI-MS; Micromass LCT, Manchester, UK) by using positive ionization. Additional time-dependent trapping experiments were carried out by using a RQF3 quenched-flow apparatus (KinTek, Austin, TX) with HCl as a quencher. $\Delta\Delta G$ values for the formation of the Grx-GSH *versus* Grx-peptide mixed disulfide adducts were calculated by using the equation

$$\Delta\Delta G = -RT \ln(\text{proportion Grx-peptide}/\text{proportion Grx-GSH})$$

where T is the temperature in Kelvin, and R is the gas constant 8.3145 Jmol/K.

Western blot analysis

The level of expression of myc-tagged Grx proteins and their association *via* the formation of mixed disulfide bonds with other proteins in *E. coli* lysates were assessed according to Western blot analysis with anti-myc antibodies. The *E. coli* lysates were centrifuged, and the supernatants were mixed with either reducing or nonreducing electrophoresis sample buffer. Proteins were separated with SDS-PAGE and transferred to PVDF membrane (Immobilon-P Transfer Membrane; Millipore Corporation, Bedford, MA). The blotted membranes were incubated with antibody against Myc-tag (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), followed by treatment with an appropriate horseradish peroxidase–conjugated secondary antibody. The proteins were detected by using the enhanced chemiluminescence (ECL) system (Amersham Pharmacia Biotech AB, Uppsala, Sweden), and the luminol excitation was imaged on x-ray film.

Biophysical analysis

Far UV circular dichroism spectra were recorded on a Jasco J600 spectrophotometer. All scans were collected at 25°C as an average of eight scans, by using a cell with a path length of 0.1 cm, scan speed of 20 nm/min, a spectral band width of 1.0 nm, and a time constant of 0.5 s. The maximal HT voltage was <750 V.

Results

Both steps of the deglutathionylation reaction exhibit γ-linkage specificity

To catalyze the efficient deglutathionylation of peptides and proteins, glutaredoxins must be able to form a mixed disulfide selectively with GSH as the first intermediate in the reaction and then show selectivity toward GSH in the second step of the reaction. We and others showed that the specificity of the first step is dependent on the unusual γ-linkage present in glutathione (16, 22). Although it is known that human Grx1 exhibits specificity for GSH in the second reaction compared with Cys or the dipeptide Cys-Gly (18), it is not known whether this specificity is due to the absence of the glutamic acid *per se* or due to the absence of the spatial array of noncovalent interactions associated with the unusual γ-amide linkage of the glutamyl moiety in GSH. To test this, we examined the ability of wild-type *E. coli* Grx1 to catalyze the deglutathionylation of a model peptide under standard conditions (16) by using (a) a model peptide substrate in a mixed disulfide with either GSH or ECG as the first reactant; and (b) GSH or the corresponding tripeptide ECG that has a normal peptide bond instead of the γ-linkage as the second reactant in the system. For all four combinations, the noncatalyzed rates were slow and statistically indistinguishable. With the peptide-ECG substrate in place of the peptide-GSH substrate, we found a decrease in wild-type Grx1 activity by 99% (Fig. 2A). This is consistent with our previous results on the requirement of the γ-linkage for the specificity of the first step in the deglutathionylation reaction (16). Similarly, when GSH was replaced with ECG as the second reactant by using peptide-GSH as the substrate, the activity of wild-type Grx1 decreased by >99% (Fig. 2A). No significant activity over the background rate could be observed for Grx by using ECG as the second reactant with peptide-ECG as the substrate. These results suggest that the γ-linkage is important for the specificity of the second step in the deglutathionylation reaction catalyzed by *E. coli* Grx1 as well as for the first step.

When the GSH concentration dependence of the reaction catalyzed by wild-type enzyme was analyzed, a sigmoidal curve was observed with a midpoint $\sim$0.5 m*M* (Fig. 2B), consistent with our previous results (16) and with the idea of kinetic partitioning from the Grx-GSH mixed disulfide state (see Fig. 1). This prevents the determination of an apparent K_m value of Grx for GSH at this concentration of peptide-GSH substrate. The ECG concentration dependence of the reaction catalyzed by the wild-type enzyme was also consistent with a sigmoidal curve; however, a plateau was not observed by the highest concentration that could be tested (Fig. 2C). Because the V_{max} might be expected to be similar, with either GSH or ECG for the deglutathionylation of the GSH-peptide substrate, these results imply that the midpoint for the ECG reaction is >12 m*M*. Hence, consistent with the results shown in Fig. 2A, the wild-type Grx apparently shows a strong dependence on the γ-linkage of GSH for the specificity of the second reaction of deglutathionylation. This is consistent with previously published results for human Grx1 by using Cys or the dipeptide Cys-Gly (18), but our results demonstrate explicitly the dependence on the γ-linkage over the tripeptide ECG, which has a normal peptide bond linking Glu-Cys.

Effect of selected conserved residues on GSH specificity of Grx in vitro

Structural studies on human and *E. coli* glutaredoxins in mixed disulfide with GSH have implicated a number of conserved residues in the interaction of Grx with the GSH moiety. These include Tyr13, Thr58, Val59, Tyr72, Thr73, and Asp74 in *E. coli* Grx1 (3). To investigate the role of selected conserved residues on the GSH specificity of *E. coli* Grx1, the mutations Y13A, T58A, Y72A, and D74A were made, either singly or in combination. The Y72A mutants were not solubly expressed and were not tested further. Five of the solubly expressed

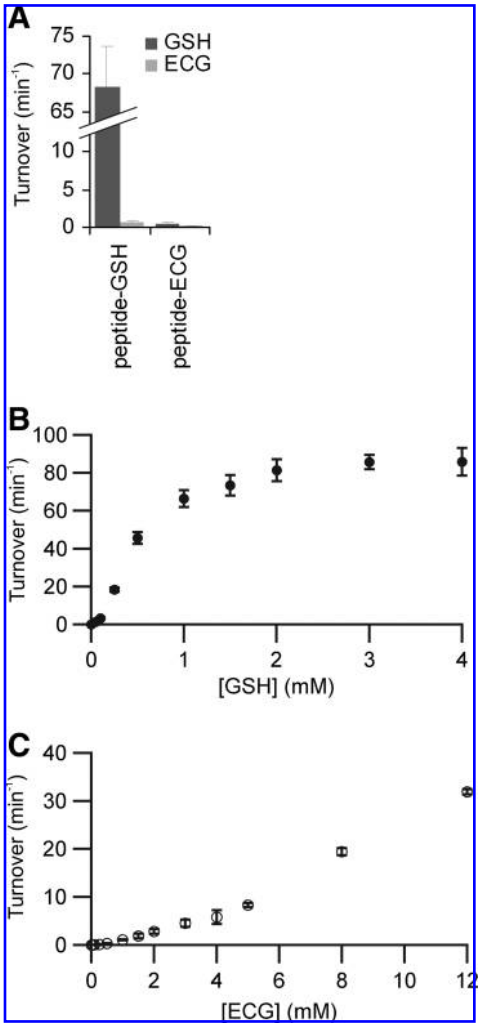

FIG. 2. γ-Linkage specificity of both steps of the deglutathionylation reaction catalyzed by wild-type Grx1. (**A**) The deglutathionylation reactivity of *E. coli* Grx1 wild type was measured by using peptide-GSH or peptide-ECG as a substrate and GSH or ECG as the second reactant [McIlvaine buffer, pH 7.0; (substrate peptide) = 5 μM; (GSH or ECG) = 1 mM]; see also Table 3. (**B**) GSH and (**C**) ECG dependence of the reaction catalyzed by wild-type enzyme was determined [McIlvaine, pH 7.0, (substrate peptide) = 5 μM]. Measurements were done with varying amounts of enzyme, depending on the level of activity, and the initial rates were normalized to turn over accordingly. The noncatalyzed rates were subtracted. Turnover is expressed as mean ± SD, $n \geq 3$.

mutants along with the wild-type control were then purified to homogeneity. When tested for their ability to catalyze the deglutathionylation of a model peptide under standard conditions (16), all of the mutants showed a lower activity than the wild-type enzyme (Table 1). The Y13A mutation had the greatest effect for a single point mutation. The effects of all of the mutations appear to be additive with each other, with the Y13A mutation in combination with other mutations leading to almost inactive enzyme. To confirm that the effects of the mutations were not due to gross structural changes, all of the mutants were analyzed with circular dichroism, and all showed spectra that were essentially identical to the wild-type spectra (data not shown). This implies that all of the mutants have the same degree of regular secondary structure (α-helices and β-sheets) and that the differences in activity were not due to gross structural changes.

Table 1. Effect of Selected Conserved Residues on Activity

Construct	*Turnover/min*	*Activity (% of wt)*
Wild-type	68 ± 5	100
Y13A	2.8 ± 0.2	4
T58A	8.8 ± 0.5	13
D74A	23 ± 1	34
Y13A T58A	0.1 ± 0.1	<1
Y13A D74A	0.7 ± 0.2	1
C14S	18 ± 1	27
C14A	14 ± 5	20
Y13A C14S	2.4 ± 0.3	3
C14S T58A	1.7 ± 0.1	3
C14S D74A	4.2 ± 0.3	6
Y13A C14S T58A	0.04 ± 0.2	<1
Y13A C14S D74A	0.25 ± 0.24	<1

The activities were measured by using either 20 or 100 nM enzyme [McIlvaine buffer, pH 7.0, (GSH) = 1 mM; (substrate peptide–GSH) = 5 μM; $n \geq 3$] and normalized accordingly. The noncatalyzed rates were subtracted.

The reduction in activity observed for the mutants, combined with the previous implications for these residues to be involved in interaction with glutathione, are consistent with the mutations having an effect on the K_m values for either the GSH-peptide in the first step and/or GSH in the second step of deglutathionylation. However, in the wild-type active-site background, it is not possible to determine the K_m of GSH for the mutants, as they would all be expected to show sigmoidal dependence because of kinetic partitioning (see earlier). To avoid this problem, the mutants were all made in the C14S Grx1 background, and the proteins were purified to homogeneity. The C14S mutant showed a reduced activity compared with the wild-type enzyme, but again, mutations in Y13, T58, and D74 decreased the activity still further, consistent with the data observed for these mutants in the wild-type background (see Table 1 for activity data under standard conditions). However, it was subsequently found that the C14S mutation affects the specificity of the second step of the deglutathionylation reaction (see later), and hence, the specific effects of the Y13, T58, and D74 mutations on the K_m values of glutaredoxin for GSH could not be determined.

Although the Y13, T58, and D74 mutations may have an effect on the K_m values, it also is possible that they change the nature of the intermediate formed in the first reaction from Grx-GSH mixed disulfide to the Grx-peptide mixed disulfide. Determination of kinetic intermediates under catalytic conditions is extremely challenging. To circumvent this problem, an equilibrium measurement was made to examine the preference of the mutants to form mixed disulfides with either GSH or peptide by coincubating the mutants with a slight excess of the activity assay peptide-GSH substrate. For all mutants, equilibrium was reached within 10 min at RT. Whereas the C14S control showed a clear preference for forming Grx-GSH mixed disulfides, the mutants showed an increasing preference to form Grx-peptide mixed disulfides (see Table 2). Because this is an equilibrium measurement,

Table 2. ΔΔG Measurements for Relative Binding Affinity

Construct	% *Grx-GSH*	% *Grx-peptide*	$\Delta\Delta G \pm SD$ *(kJ per mol)*
C14S	66 ± 5	34 ± 5	1.7 ± 0.5
C14A	62 ± 4	38 ± 4	1.2 ± 0.4
Y13A C14S	41 ± 2	59 ± 2	−0.9 ± 0.2
C14S T58A	31 ± 2	69 ± 2	−2.0 ± 0.2
C14S D74A	45 ± 4	55 ± 4	−0.5 ± 0.4
Y13A C14S T58A	36 ± 3	64 ± 3	−1.4 ± 0.2
Y13A C14S D74A	30 ± 2	70 ± 2	−2.1 ± 0.3

The relative amount of Grx-GSH mixed disulfide and Grx-peptide mixed disulfide was determined at equilibrium by mass spectrometry after quenching with NEM or IAA. The relative amounts of each mixed disulfide were converted to ΔΔG for relative mixed disulfide adduct formation with *E. coli* Grx1 with GSH *versus* peptide [McIlvaine buffer, pH 7.0, (substrate peptide-GSH) = 50 μM, (enzyme) = 40 μM; $n \geq 3$]. Note that initial product for each mutant is the Grx-GSH mixed disulfide with the subsequent formation of Grx-peptide mixed disulfides.

these can be converted into ΔΔG measurements for relative mixed-disulfide adduct formation. The C14S mutant preferred to form a mixed disulfide with GSH by 1.7 kJ/mol, whereas the Y13A, C14S, and D74A mutant preferred to form a mixed disulfide with peptide by 2.1 kJ/mol, showing an overall net difference of 3.9 kJ/mol (see Table 2). Although the effects were not large, the Y13A, T58A, and D74A mutations all clearly had an effect on the relative adduct formation of *E. coli* Grx1 with GSH *versus* peptide, with some of the effects being additive. These results are consistent with the decrease in activity of the mutants (Table 1) and with their apparent role in interacting with the GSH moiety in the mixed disulfide Grx-GSH structure, although it should be noted that cross-comparisons between kinetic and thermodynamic properties should be made with great caution, as they are not interchangeable.

Unexpectedly, 34% Grx-peptide mixed disulfide was observed at equilibrium incubation (>10-min incubation) with the C14S mutant. This result is inconsistent with the previous exquisite specificity observed for the first reaction by Grx (16) at time points up to 1 s. To resolve this apparent contradiction, the reaction specificity of the initial nucleophilic attack of the mutant glutaredoxins on the glutathionylated-peptide was studied in more detail at the subsecond timescale. Quenched flow reactions at 50 ms, 0.1 s, 0.5 s, and 1 s revealed that the reaction of the C14S mutant with the peptide-GSH was significantly faster than the reaction of the Y13A C14S or Y13A C14S D74A mutants. However, for all three mutants, the first step in the reaction was always the formation of the Grx-GSH mixed disulfide, with the subsequent appearance of Grx-peptide mixed disulfides. The implies that although thermodynamically, the Grx-peptide may be the most favored mixed-disulfide form for some of the mutants, the kinetically favored form for the initial product of the first step of the deglutathionylation reaction is the Grx-GSH mixed disulfide for all of the mutants. It is unclear from these results what causes this kinetic partitioning, but perhaps the peptide makes a significantly better leaving group than GSH. However, the initial formation of only the Grx-GSH mixed disulfide and the slow kinetics for the subsequent formation of the Grx-peptide mixed disulfide are consistent with the ability of glutaredoxins to catalyze deglutathionylation reactions in which the formation of the Grx-peptide is limited by GSH being the favored second substrate both by specific binding by the enzyme and by mass action, because GSH is usually in large excess in such reactions.

Formation of Grx-protein mixed disulfides in E. coli

Because mutations in the residues implicated in interacting with glutathione gave rise to altered thermodynamics of forming the glutathione-Grx and peptide-Grx mixed disulfides *in vitro*, the effects of these mutations on the formation of glutaredoxin mixed-disulfide adducts *in vivo* was tested. *Escherichia coli* lysates were prepared from strains expressing Myc-tagged *E. coli* Grx1 mutants. All of the experiments were done with the C14S background, as per the *in vitro* equilibrium measurements, so that stable mixed disulfides could be formed. Coomassie brilliant blue–stained reducing SDS-PAGE (Fig. 3A) revealed that all of the proteins were expressed to comparable levels. Western blot analysis was then performed by using an anti-myc antibody to detect the *E. coli* Grx1. Under reducing conditions, a single band was observed running at the position of the *E. coli* Grx1 monomer (Fig. 3B), with the exception of one additional higher-molecular-weight band that was not reducible in the Y13A C14S and Y13A C14S

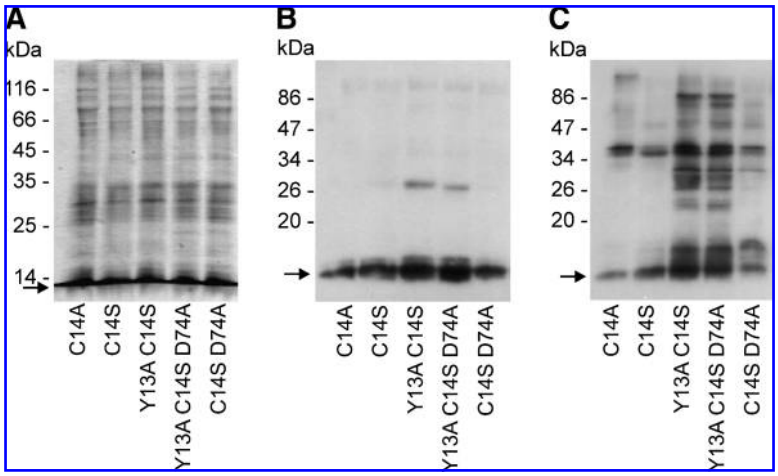

FIG. 3. Grx-mixed disulfides formed *in vivo* and *in vitro*. (**A**) Coomassie brilliant blue–stained nonreducing SDS-PAGE of *E. coli* cell lysates expressing selected Myc-tagged Grx1 constructs. (**B, C**) Representative data from Western blot analysis of Grx-mixed disulfides formed *in vivo* in *E. coli* cell lysates expressing selected Myc-tagged Grx1 constructs separated on SDS-PAGE under reducing (**B**) or nonreducing (**C**) conditions. The blotted membranes were treated with antibody against Myc-tag 1:2,000 and with anti-rabbit secondary antibody, 1:10,000. The position of the *E. coli* Grx1 monomer is marked by an *arrow* in each panel.

D74A mutant lysates. In contrast to this, under nonreducing conditions, many higher-molecular-weight bands were observed in the lysates expressing *E. coli* Grx1 Y13A C14S, C14S D74A, and Y13A C14S D74A mutants, most of which were not observed with the C14S or C14A controls (Fig. 3C). These higher-molecular-weight bands represent the formation of *E. coli* Grx1 mutant mixed disulfides with *E. coli* proteins. Because they were not observed for the C14S or C14A controls, these results imply that the formation of mixed disulfides for the Y13A C14S and Y13A C14S D74A mutants is much broader than that of the controls. Although the detailed mechanisms for the formation of these mixed disulfides *in vivo* cannot be determined, this result is consistent with the *in vitro* equilibrium measurements for the formation of Grx mixed disulfides with GSH or peptide.

Effect of selected conserved residues on deglutathionylation activity in vitro

Because the *in vitro* equilibrium analysis and the *in vivo* analysis indicated that the mutations in *E. coli* Grx1 allowed the formation of mixed disulfides with peptides and proteins other than GSH, the effects of these mutations on the specificity of glutaredoxin toward substrates were further examined *in vitro*.

First, selected mutants were tested for their specificity in the first step of deglutathionylation by comparing their ability to reduce the peptide-GSH and peptide-ECG substrates with GSH. Whereas the activity of wild-type *E. coli* Grx1 shows a very strong dependence on the γ- rather than α-amide linkage present in GSH in the mixed-disulfide peptide substrate (Fig. 2A), the activity of the catalytic **a** domain of human protein disulfide isomerase (PDI) does not (Table 3). This was expected, as Grx1 forms a mixed disulfide with GSH as the first step in the reaction, whereas the **a** domain of PDI recognizes and forms a mixed disulfide with the peptide and not with glutathione (16). The activities of all of the mutants were lower with the peptide-ECG substrate compared with the peptide-GSH substrate, but these effects for the Y13A and D74A mutants were less than that observed for the wild-type enzyme. Hence, with peptide-GSH as the substrate, the Y13A and D74A mutants showed only 4% and 34%, respectively, of the activity of the wild-type enzyme, whereas they both showed around 108% of the activity of wild-type enzyme with the peptide-ECG substrate (Table 3).

To complete the catalytic cycle, Grx must show specificity for GSH over peptide in the second step in the reaction (*i.e.*, the conversion of the Grx-mixed disulfide to generate reduced Grx and GSSG). Because the mass-spectrometric analysis implied that the mutant glutaredoxins tested kinetically retained the glutathione specificity for the first reaction, but an altered equilibrium pattern for mixed-disulfide bond formation, we decided to investigate the specificity of the second reaction. To examine this, the peptide deglutathionylation reaction was performed by using either GSH or the corresponding tripeptide ECG as the second reactant. When GSH was replaced with ECG, the activity of the wild-type *E. coli* Grx1 decreased by >99% (Fig. 2A and Table 3). Similarly, the activity of the Y13A, T58A, and D74A mutations showed a strong dependence on GSH *versus* ECG; although the effect was less marked for the Y13A mutation than for the wild-type enzyme, it was still statistically significant (Table 3). These results imply that none of these residues in isolation is responsible for the γ-linkage specificity of the second reaction. Combinations of these mutations could not be studied because of their low basal activity.

Cys14 affects the specificity of the second step in the deglutathionylation reaction

Because the *in vitro* equilibrium measurements and the *in vivo* formation of Grx-protein mixed disulfides had to be

Table 3. Catalytic Turnover per Minute for Wild-type and Mutant Proteins with Different Substrates

	Peptide-GSH		*Peptide-ECG*	
Construct	*+GSH*	*+ECG*	*+GSH*	*+ECG*
E. coli Grx1	68 ± 5	0.65 ± 0.19	0.55 ± 0.11	0.12 ± 0.04
E. coli Grx1 C14S	18 ± 1	18 ± 2	0.27 ± 0.02	0.05 ± 0.02
E. coli Grx1 Y13A	2.8 ± 0.2	0.71 ± 0.29	0.60 ± 0.07	0.06 ± 0.02
E. coli Grx1 T58A	8.8 ± 0.5	0.31 ± 0.36	ND	ND
E. coli Grx1 D74A	23 ± 1	0.47 ± 0.33	0.59 ± 0.08	ND
E. coli Grx1 Y13A T58A	0.1 ± 0.1	-0.05 ± 0.15	ND	ND
E. coli Grx1 Y13A D74A	0.7 ± 0.2	0.07 ± 0.15	0.27 ± 0.03	0.02 ± 0.04
Human Grx1	138 ± 7	141 ± 3	1.5 ± 0.1	3.0 ± 0.2
Human Grx 1 C26S	145 ± 1	135 ± 2	ND	ND
S. cerevisiae Grx1	54 ± 1	123 ± 10	0.60 ± 0.03	3.1 ± 0.1
S. cerevisiae Grx1 C30S	75 ± 3	139 ± 1	ND	ND
Human PDI **a** domain	4.8 ± 1.0	2.3 ± 0.4	3.4 ± 0.1	2.4 ± 0.4

The activities were measured by using peptide-GSH or peptide-ECG mixed disulfides as the first substrate and GSH or ECG as the second [McIlvaine buffer, pH 7.0 (substrate peptide-GSH or –ECG) = 5 μM; (GSH or ECG) = 1 mM] with varying amounts of enzyme ranging upward from 20 nM, depending on the level of activity of the mutant. The initial rates were normalized to turnover accordingly after the noncatalyzed rates were subtracted. All of the constructs tested show significant differences between the use of peptide-GSH and peptide-ECG ($p < 0.005$, except for *E. coli* Grx1 Y13A D74A double mutant: $p = 0.0509$). All of the *E. coli* Grx1 constructs, except for the C14S and the Y13A T58A double mutant, which showed activity that was statistically indistinguishable from the noncatalyzed rate, showed a significant difference between the use of GSH and ECG as the second substrate ($p < 0.005$; except for Y13A D74A double mutant, $p < 0.05$). Turnover is expressed as the mean $\pm$ standard deviation, $n \geq 3$.

ND, not determined.

performed in the C14S background, the specificity of Y13A, T58A, and D74A mutants for the second step in the reaction were also examined in this background. Consistent with our previous observations (16), the *E. coli Grx1* C14S mutant showed only one fourth of the activity of the wild-type enzyme with peptide-GSH and GSH as substrates (Table 1). However, in contrast to the strong specificity of the wild-type enzyme, the C14S mutation showed no difference in catalytic efficiency when either GSH or ECG was used as the second reactant. Furthermore, the Y13A, T58A, and D74A mutants in the C14S background also showed no specificity for GSH or ECG in the second reaction (Fig. 4A). These results imply that C14 has a major role in the γ-linkage specificity of the second reaction.

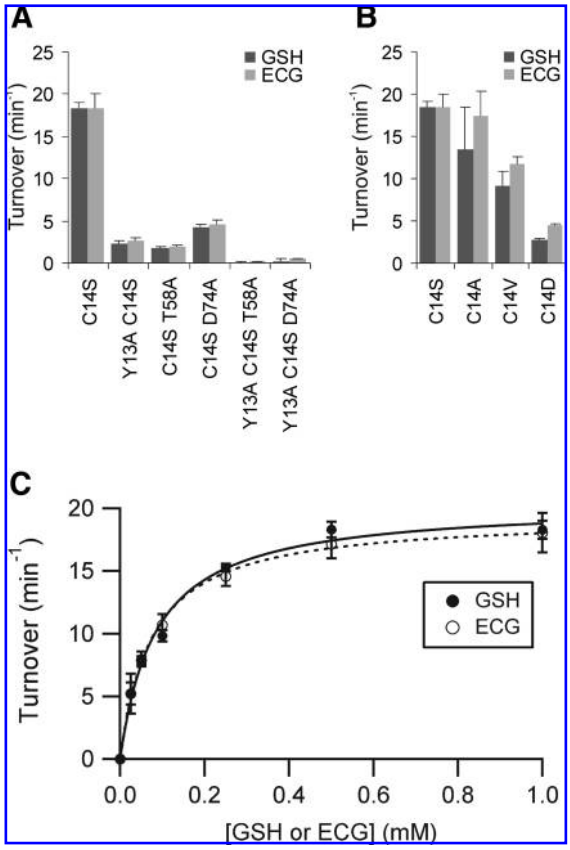

FIG. 4. Effect of conserved residues on the specificity of second step in the deglutathionylation reaction. (**A, B**) The deglutathionylation reactivity of selected *E. coli* Grx1 mutants was measured by using either GSH or ECG as the second reactant [McIlvaine buffer, pH 7.0, (substrate peptide-GSH) = 5 μM; (GSH or ECG) = 1 mM]. Measurements were done with varying amounts of enzyme, depending on the level of activity of the mutant, and the initial rates were normalized to turn over accordingly. The noncatalyzed rates were subtracted. Turnover is expressed as mean ± SD, $n \geq 2$. No significant differences were fund for these constructs between the use of GSH and ECG as the second substrate, except for C14D, which has a higher turnover with ECG ($p < 0.05$). (**C**) The deglutathionylation reactivity of C14S Grx1 was determined with varying concentrations of GSH or ECG as a reductant [McIlvaine buffer, pH 7.0, (substrate peptide) = 5 μM; (enzyme) = 100 nM]. The noncatalyzed rates were subtracted. Turnover is expressed as mean ± SD, $n \geq 2$.

Because the serine in the C14S mutant has the potential to form a stable hydrogen bond, which could affect the local structure, a series of other mutants were made in C14 of *E. coli* Grx1. The C14M and C14F mutants were insolubly expressed, but the C14A, C14V, and C14D mutants were expressed and purified to homogeneity. Although the C14A mutant was prone to aggregation, making quantification more difficult, none of the four mutations in C14 showed specificity for GSH over ECG, and indeed, the C14D mutant showed a nearly twofold increase in activity with ECG over GSH (Fig. 4B).

Further to study this effect on specificity, the GSH and ECG dependence of the deglutathionylation reaction for the wild-type *E. coli* Grx1 and C14S mutants was examined. When the GSH or ECG dependence of the reaction catalyzed by the wild-type enzyme was analyzed, a sigmoidal curve was observed for both (Fig. 2B and C), with the wild-type enzyme showing a strong preference for GSH over ECG as the second reactant. In contrast, for the C14S-catalyzed reaction, which lacks the potential partitioning reaction to the inactive intramolecular disulfide state, the curves could be fitted to Michaelis–Menton equation. This gave similar apparent K_m and k_{cat} values for the C14S mutant for both GSH and ECG (Fig. 4C) ($K_m = 85 \pm 12\ \mu M$; $k_{cat} = 0.34$ per second; and $K_m = 75 \pm 4\ \mu M$; $k_{cat} = 0.32$ per second for GSH and ECG, respectively; when [peptide-GSH] = 5 μM). These results imply that, unlike the wild-type enzyme, the C14S mutant shows no specificity for GSH or ECG as the second reactant.

Specificity for the γ-linkage of other glutaredoxins

Because *E. coli* Grx1 appears to show specificity for the γ-linkage in glutathione in both the first and second steps of deglutathionylation, with the latter being dependent on the C-terminal active-site cysteine residue, we decided to investigate whether the results could be applied to other glutaredoxin family members. We chose human Grx1 and *S. cerevisiae* Grx1, as other well-studied two-thiol glutaredoxin family members. Whereas the overall sequence identity between the three proteins is low at *circa* 20%, all of the residues previously mutated and studied in *E. coli* Grx1 (*i.e.*, Y13, C14, T58, and D74) are conserved between the three. Human Grx1 and *S. cerevisiae* Grx1 as well as their C-terminal active site mutants, C26S and C30S, respectively, were cloned, expressed, purified, and tested in the deglutathionylation assay. With peptide-GSH and GSH as substrates under standard assay conditions, the catalytic turnover of human Grx1 was *circa* twofold greater than that of *E. coli* Grx1, whereas *S. cerevisiae* Grx1 showed a slightly lower catalytic turnover (Table 3). All three proteins showed a *circa* 99% decrease in catalytic turnover when the peptide-ECG mixed disulfide was used in place of the peptide-GSH mixed disulfide, implying that all three have a substrate specificity for the first step of deglutathionylation that depends strongly on the γ-linkage present in glutathione. In contrast, the catalytic **a** domain of human protein disulfide isomerase showed only a slight reduction in catalytic turnover (Table 3).

In sharp contrast to these results, and to the results obtained for *E. coli* Grx1, when the specificity for the second step in the deglutathionylation reaction was examined, human Grx1 showed no difference in catalytic turnover when GSH or ECG was used, and *S. cerevisiae* showed a *circa* twofold greater catalytic turnover when using ECG than with GSH (Table 3).

Similar trends were observed with the C-terminal active-site cysteine mutants of human Grx1 and *S. cerevisiae* Grx1 as with the wild-type proteins, implying that, unlike *E. coli* Grx1, the C-terminal active-site cysteine residue does not appear to play a role in determining the specificity of the second step of the deglutathionylation reaction.

Discussion

The role of glutaredoxins in reducing GSH-mixed disulfides requires the specific recognition of potential substrates. The specificity of glutaredoxins toward glutathionylated peptide and protein substrates is thought to depend on the unusual γ-linkage present in GSH (18, 22). Previous structural studies using C-terminal active-site Cys to Ser mutants implicated several conserved residues in the potential GSH interaction site of glutaredoxins (3, 22). These include Tyr13, Thr58, and Asp74 in *E. coli* Grx1 assessed in this study.

Asp74 is thought to form an electrostatic attractive force with the N-terminus of GSH. The N-terminal of GSH would be in a significantly different position if a normal peptide bond were present between the Glu and Cys of the tripeptide GSH, and hence, this interaction depends on the presence of the γ-linkage. Another very significant difference would be the position of the free carboxyl group at the γ-carbon of Glu instead of attached to the α-carbon; however, Grx does not appear to have a positively charged residue close to the glutathione binding site to make an attractive electrostatic interaction with this group. The mutation D74A results in a shift in the equilibrium of mixed disulfides formed by Grx (Table 2), significantly decreases the activity of Grx (Table 1), and results in the formation of mixed disulfides with proteins in an *E. coli* lysate. All of these are consistent with a role for Asp74 in substrate interaction.

The side chains of Tyr13 and Thr58 help form the binding groove for GSH in the mixed disulfide structures of *E. coli* and human Grx (3, 22). The conserved tyrosine (Tyr13) is found between the active-site cysteines in most of the Grx family members, although in some glutaredoxins (pig, vaccinia virus, tomato, rice, and monothiol glutaredoxins), another aromatic residue, phenylalanine, is found in this position. Interestingly, human omega class glutathione S-transferase has the sequence -CPFA- in an analogous position to the active site of glutaredoxins, and furthermore has been shown to have Grx-like deglutathionylation activity and ability to bind GSH (1). The conserved Thr58 is located close to the *cis*-proline that is found in all thioredoxin-superfamily members that bind proteins or peptides and that is directly implicated in interactions with substrates (3, 21, 22). Our observations on the capability of Y13A and T58A mutants to form mixed disulfides with peptide and proteins (Table 2 and Fig. 3), significantly reduce the activity of the enzyme (Table 1), and are in line with their role in defining the GSH interaction site and hence the specificity of Grx. The effects of the mutations in Tyr13, Thr58, and Asp74 were additive, suggesting that all contribute to the GSH interaction site.

To complete efficiently the catalytic cycle for deglutathionylation glutaredoxins should show specificity for GSH as the second reactant. Previously it was shown that human Grx1 exhibits a specificity for GSH and the γ-glutamylcysteine dipeptide over Cys or the Cys-Gly dipeptide (18). Here we demonstrate for the first time that the specificity of *E. coli* Grx1 for GSH depends on the γ-linkage present in GSH, as the comparable tripeptide ECG having a normal peptide bond results in the wild-type enzyme exhibiting $<1\%$ of the activity it shows when GSH is the second reactant (Fig. 2A). Similarly, the Y13A, T58A, and D74A mutants all showed specificity for GSH over ECG (Table 3). Whereas the Y13A and D74A mutants showed a marked decrease in activity with ECG rather than GSH, this effect was not so strong as for the wild-type enzyme, and both mutants showed a higher activity than the wild-type enzyme with ECG.

In contrast to the wild-type enzyme or the Y13A, T58A, or D74A mutants, the C14S, C14A, and C14M mutants showed no specificity for GSH over ECG in the second reaction (Fig. 4), and the C14D shows a higher turnover with ECG than with GSH. Hence, although the wild-type enzyme requires the γ-linkage of GSH for it to act as a substrate in the second reaction, the Cys14 mutants do not. This finding is significant on several levels. First, C-terminal active-site mutants of Grx have been used in structural studies on Grx-GSH mixed disulfides to define the GSH interaction site in Grx (3, 22) and have been used by us, and others, as a mimic of the intermediate state in the deglutathionylation reaction (2, 16, 22). These findings that the Cys14 mutants of *E. coli* Grx1 have an altered specificity for the second reaction suggest that these mutants, although providing important information on the interaction of this protein with GSH, cannot be used as the starting point for defining the determinates of the specificity of the second reaction of deglutathionylation. However, it is unclear how general these findings are for the glutaredoxin family (see later). Second, C-terminal active-site mutants of other thioredoxin superfamily members have been used establish function and mechanisms of action [for examples, see (12, 20, 25)] and to determine their *in vivo* substrate specificity [for example, ERp57; (9)]. If the C-terminal active-site Cys mutants of these enzymes similarly change the substrate specificity, then caution must be used in the interpretation of these results in determining the natural substrate specificity of the enzymes.

Our observations on the requirement for the γ-linkage in substrates and the role of Y13 and T58 for ligand binding by *E. coli* Grx1 are consistent with a recent elegant study on *E. coli* Grx3 substrate specificity (5). By using ligand-induced stability measurements, Elgán and Berndt (5) showed that for *E. coli* Grx3, interactions with the γ-glutamyl moiety of GSH contributed 2.13 kcal/mol toward substrate binding, when compared with binding to a Cys-Gly dipeptide. In contrast, the addition of an α-amide–linked glutamyl moiety to Cys-Gly (to form ECG) changed the substrate binding affinity by only 0.67 kcal/mol. The ethylene linker of the γ-Glu makes contacts with Y13 and T51 of Grx3 (equivalent to Y13 and T58 in *E. coli* Grx1) and, by using a variety of ligands, the contribution from the interactions of the ethylene linker were found to be 1.41 kcal/mol. Hence, these interactions contribute 70% of the net total from the addition of the γ-glutamyl moiety, suggesting that the positioning of the charged groups in the γ-amide–linked c.f. α-amide–linked glutamyl moiety play a relatively minor role in determining the specificity of *E. coli* Grx3. Although the full ligand analysis was not performed for *E. coli* Grx1, the same method showed a difference in substrate-binding affinity of 2.14 kcal/mol between GSH and ECG for this enzyme, larger than the 1.46 kcal/mol difference in substrate-binding affinity for *E. coli* Grx3 (5).

It was previously reported that different glutaredoxins show different effects when the C-terminal active-site cysteine is mutated. For example, *E. coli* Grx1 C14S has reduced activity in both the 2-hydroxyethyl disulfide assay (2, 15) and in our peptide deglutathionylation assay [(16) and results presented here]. Similarly, *E. coli* Grx3 C14S (15) and *S. cerevisiae* Grx2 C30S (4) have reduced activities compared with the wild-type enzymes. In contrast, *S. cerevisiae* Grx1 C30S [(4) and results presented here] and human Grx2 C40S (6) show increased activities compared with the wild-type enzymes. Unexpectedly, unlike *E. coli* Grx1, human Grx1 showed no specificity for GSH over ECG as the second substrate in the reaction, and *S. cerevisiae* Grx1 showed increased turnover with ECG (Table 3). Furthermore, mutation of the C-terminal cysteine of both enzymes had minimal effect on the catalytic turnover with ECG as the second substrate. It is unclear why these three glutaredoxins show such a marked difference in specificity for the second step in deglutathionylation, whereas all have a strong specificity for the γ-linkage in glutathione in the first step of deglutathionylation. Clearly differences must exist in the structures of the Grx-GSH mixed-disulfide intermediates.

However, these differences cannot easily be studied, because the intermediate is extremely transient, and stabilizing the intermediate (*e.g.*, by making the C-terminal active-site cysteine mutant) changes the specificity of *E. coli* Grx1. It should be noted that Grx1 from *E. coli*, human, and *S. cerevisiae* share very low sequence identity. In particular, the sequence around the active site, except for the CPCY motif, is very poorly conserved in comparison with conservation in other thioredoxin-superfamily families (*e.g.*, the active site of PDI-family members) (13). In addition, a recent study on the distinct biochemical properties of *S. cerevisiae* Grx1 and Grx2 implicated Ser/Ala 23 and Gln/Glu 52 as being at least partially responsible for the differences (4). These residues are not conserved between these two proteins or between them and *E. coli* or human Grx1. Given that other significant differences can be found between glutaredoxins [*e.g.*, the reduction potential of *E. coli* Grx1 and Grx3 are 35 mV different (26)], it is clear that generalizations regarding the functional properties of the glutaredoxin family should be made with caution.

Overall, this study demonstrates the role of conserved residues in the proximity of proposed GSH binding site to the GSH-binding specificity of *E. coli* Grx1. Opening the binding groove and removing charged residues enabled *E. coli* Grx1 more readily to form mixed disulfides with other molecules besides GSH. Furthermore, the C-terminal active site cysteine of *E. coli* Grx1 was shown to be required for the γ-linkage specificity of the second step of deglutathionylation, whereas human Grx1 showed no specificity toward a requirement for the γ-linkage over a normal peptide bond, and *S. cerevisiae* Grx1 had a higher turnover with ECG than with GSH (Table 3). These observations have significant implications for studies on Grx structure–function relations.

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Abbreviations

ECG, glutamylcysteinyl glycine; Grx, glutaredoxin; PDI, protein disulfide isomerase.

Author Disclosure Statement

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