Glutathione-Thiol Radical Scavenging and Transferase Properties of Human Glutaredoxin (Thioltransferase)

POTENTIAL ROLE IN REDOX SIGNAL TRANSDUCTION*

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David W. Starke‡, P. Boon Chock‡, and John J. Mieyal††

From the ‡Department of Pharmacology, School of Medicine, Case Western Reserve University, Cleveland, Ohio 44106-4965 and §Biochemistry Section NHLBI, National Institutes of Health, Bethesda, Maryland 20892-8012

Glutaredoxin (GRx, thioltransferase) is implicated in cellular redox regulation, and it is known for specific and efficient catalysis of reduction of protein-S-S-glutathione-mixed disulfides (protein-SSG) because of its remarkably low thiol pKₐ (~3.5) and its ability to stabilize a catalytic S-glutathionyl intermediate (GRx-SSG). These unique properties suggested that GRx might also react with glutathione-thiol radicals (GS·) and stabilize a disulfide anion radical intermediate (GRx-SSG·), thereby facilitating the conversion of GS·-GSSG or transfer of GS· to form protein-SSG. We found that GRx catalyzes GSSG formation in the presence of GS-thiol radical generating systems (Fe⁺³/ADP/H₂O₂ + GSH or horseradish peroxidase/H₂O₂ + GSH). Catalysis is dependent on O₂ and results in concomitant superoxide formation, and it is distinguished from glutathione peroxidase-like activity. With the horseradish peroxidase system and [³⁵S]GSH, GRx enhanced the rate of GS·-radiolabel incorporation into GAPDH. GRx also enhanced the rate of S-glutathionylation of glyceraldehyde-3-phosphate dehydrogenase with GSSG or S-nitrosothioglutathione, but these glutathionyl donors were much less efficient. Both actin and protein-tyrosine phosphatase-1B were superior substrates for GRX-facilitated S-glutathionylation with GS-radical. These studies characterize GRX as a versatile catalyst, facilitating GS-radical scavenging and S-glutathionylation of redox signal mediators, consistent with a critical role in cellular regulation.

Human glutaredoxin (thioltransferase) (GRX, EC 1.8.4.2) is known for its unique properties of specific and efficient catalysis of glutathionylation of protein-S-S-glutathione-mixed disulfides (protein-SSG). These catalytic properties of glutaredoxin have identified the enzyme for prominent roles in homeostasis of protein sulfhydryl groups both in a protective mode under overt oxidative stress associated with aging and various disease states including cardiovascular and neurodegenerative diseases, diabetes, AIDS, and cancer (2, 5, 6) and in a regulatory mode whereby reversible glutathionylation represents a mechanism of redox-activated signal transduction (7–11). These physiological roles are supported further by the documentation that glutaredoxin accounts for essentially all of cellular protein-SSG glutathionylase activity in mammalian cells (4, 12, 13) and its inactivation by cadmium is correlated with inhibition of intracellular glutathionylase activity (4, 11).

Although reversible formation of protein-SSG is a prevalent form of protein sulfhydryl modification, mechanisms of protein-SSG formation are not resolved. Unless intracellular GSSG concentrations reach unusually high levels, GSSG is unlikely to be the mediator of protein-SSG formation based on typical redox potentials for cysteine residues (14, 15). Consequently, glutathione-thiol radical and S-nitrosothioglutathione (GS-NO) have been considered as potential alternative mediators (5, 6, 10). In this context, we considered the unusually low pKₐ (~3.5) of the active site cysteine of glutaredoxin (16, 17) and the selective stabilization of the glutathionyl moiety in the glutaredoxin-SSG catalytic intermediate (1–3) along with the known ability of thiyl radicals to be stabilized by the formation of disulfide anion radicals (18, 19). Accordingly, we reasoned that glutaredoxin might react preferentially with the glutathione-thiol radical to form a glutaredoxin-S-S-glutathione-disulfide anion radical (GRX-SSG·) and that this enzyme intermediate could facilitate transfer of the GS-radical either to form GSSG or protein-SSG adducts. To test this hypothesis, two GS-radical generating systems were used, i.e. Fe(II)-ADP/H₂O₂ + GSH (20, 21) and HRP/H₂O₂ + GSH (22). Here we report that glutaredoxin catalyzes the formation of GSSG in the GS-radical generating systems. The catalysis of GSSG formation is dependent on molecular oxygen, and it is distinguished from glutathione peroxidase-like activity. Moreover, glutaredoxin enhanced the rate of S-glutathionylation of GAPDH in the presence of GS-radicals. This model reaction mimics the intracellular formation of GAPDH-SSG under oxidative conditions where GSSG content is not substantially changed (23–25). Glutaredoxin also enhanced the rate of formation of GAPDH-SSG when GSSG or GS-NO were used as the glutathionyl donors, but these reactions were much less efficient than the GS-radical transfer reaction. In comparison to GAPDH, both actin and PTP1B were found to be superior substrates for GRX-facilitated S-glutathionylation under GS-radical transfer.

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† Supported in part by NIA, National Institutes of Health Program Project Grant AG15885 and a Veterans Affairs merit review grant. To whom correspondence should be addressed: Dept. of Pharmacology, School of Medicine, Case Western Reserve University, Cleveland, OH 44106-4965. Tel.: 216-368-3383; Fax: 216-368-3395; E-mail: jjm5@po.cwru.edu.

‡ The abbreviations used are: GRX, glutaredoxin (thioltransferase); BSA-CM, S-carboxymethyl bovine serum albumin; GS-NO, S-nitroso-glutathione; ct c, cytochrome c; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GSH, glutathione (reduced form); GSSG, glutathione disulfide (oxidized form); HRP, horseradish peroxidase; NADPH, D-nicotinamide adenine dinucleotide phosphate; protein-SSG, protein-S-S-glutathione mixed disulfides; PTP1B, protein tyrosine phosphatase 1B.
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EXPERIMENTAL PROCEDURES

Materials—Water was initially purified by reverse osmosis. It was further purified by a Millipore MilliQ system. Finally, it was treated with 5 g of Chelex (Bio-Rad)/liter to remove potentially contaminating metals. Glucose, sodium nitrite, sodium dibasic phosphate, and potassium monobasic phosphate were from Fisher Scientific. ADP, glutathione reductase from yeast, glutathione peroxidase from bovine erythrocytes, glucose, trichloroacetic acid, and mono-carboxymethyl-BSA (BSA-CM) were from Sigma. NADPH, GAPDH, horseradish peroxidase, acetylated cytochrome c, and glucose oxidase were from Roche Molecular Biochemicals. [35S]Glutathione was from ICN Radiochemicals. Glutaredoxin was isolated and purified as described previously (3). Sodium-potassium phosphate buffer (0.1 M) was used in all of the assays, diluted from 1 M stock solution that was prepared by adding 1 M potassium monobasic phosphate to 1 M sodium dibasic phosphate until the measured pH was 7.5. Glutaredoxin catalyzed formation of GSSG—Stock solutions of Fe(II)-ADP complex were made by mixing equal volumes of FeCl2 (20 mM) and ADP (100 mM) solutions at least 1 h before beginning the experiments. Complete reaction mixtures contained 0.1 M sodium-potassium phosphate (pH 7.5), various amounts of glutaredoxin, 0.2 mM NADPH, 0.5 mM GSH, 2 units/ml GSSG reductase, and premixed 0.5 mM FeCl2, 2.5 mM ADP, and 0.05 mM H2O2. Reactions were initiated with the Fe(II)-ADP and H2O2 mixture. Controls omitting glutaredoxin and/or Fe(II)-ADP and H2O2 were performed in parallel. NADPH oxidation (equivalent to GSSG formation) was monitored at 340 nm using an extinction coefficient of ε340 = 6.2 mmol cm⁻¹. Factors correcting for path length in the plate reader were calculated and used to express rates as nmol GSSG formed per minute. Glutaredoxin enzyme was added as indicated in the figure legends, and time courses of product formation and dependence on enzyme concentration were determined. Oxygen Depletion Experiments—Experiments were conducted as described above with the exception that all of the solutions were bubbled for 15 min with an N2 lance. In some experiments, glucose (0.1 mM) and glucose oxidase (300 units/ml) were added to ensure complete oxygen depletion. Experimental results with N2 purging in the absence or presence of the glucose oxidase system were indistinguishable.

Concurrent Detection of Superoxide Formation—Reactions were conducted as described above with the exception that partially acetylated cytochrome c (30–90 μM) was added to the initial mixture. Rates of GSSG formation were determined as above. Changes in absorbance at 550 nm were recorded in a plate reader, and rates of cytochrome c oxidation were calculated using ε550 = 18.5 mmol cm⁻¹. Glutathionyl Radical-mediated S-Glutathionylation of GAPDH—Glutathionyl radicals were generated by horseradish peroxidase utilizing H2O2 and GSH adapted from the studies of Harman and Mason et al. (26). The assays measured the incorporation of [35S]glutathione into GAPDH. Reaction mixtures contained 0.1 mM sodium-potassium phosphate, pH 7.5, 0.2 mg/ml HRP, 0.5 mM FeCl2, 2.5 mM ADP, and 0.05 mM H2O2. Reactions were initiated with Fe(II)-ADP and H2O2 mixture. Controls omitting glutaredoxin and/or Fe(II)-ADP and H2O2 were performed in parallel. NADPH oxidation (equivalent to GSSG formation) was monitored at 340 nm using an extinction coefficient of ε340 = 6.2 mmol cm⁻¹. Factors correcting for path length in the plate reader were calculated and used to express rates as nmol GSSG formed per minute. Glutaredoxin enzyme was added as indicated in the figure legends, and time courses of product formation and dependence on enzyme concentration were determined.

RESULTS

Glutaredoxin Catalysis of GS-Radical Dependent GSSG Formation—Production of reactive oxygen species in cells is broadly implicated in signal transduction mechanisms as well as pathophysiological changes. However, characterization of the proximal mediators of these events is still unclear and under active investigation. To examine the participation of glutathionyl radical and the potential roles of glutaredoxin in regulating these events, we used the well known system comprised of the Fe(II)-ADP complex and H2O2 (20, 21, 29) to produce hydroxyl radicals in the absence or presence of GSH and glutaredoxin. Hydroxyl radical production in the absence of GSH was documented by the conversion of salicylate to 2,3-dihydroxybenzoate as reported previously (30), and the radical transfer reaction of hydroxyl radicals with GSH to produce glutathionyl thyl radicals (18) was confirmed by documenting GSH concentration-dependent inhibition of 2,3-dihydroxybenzoate formation in the Fe(II)-ADP/H2O2 system with salicylate (data not shown). Although glutathionyl radical can form the disulfide anion radical (GSSG⁺) and react with oxygen to form superoxide and GSSG (31, 32), this overall reaction is disfavored at neutral pH (33) (See “Discussion,” Scheme 1). Upon the addition of glutaredoxin, the formation of GSSG is accelerated over a time course that is linear for at least 5 min, consistent with a catalytic role for the enzyme. Accordingly, the reaction is enzyme concentration dependent (Fig. 1), and it depends on the native enzyme for activity, i.e. boiled enzyme is inactive (data not shown). The accelerated formation of GSSG is distinguished from a glutathione peroxidase-like activity of glutaredoxin, because glutaredoxin had no effect on the rate of formation of GSSG.

confirmed in separate model preparations with unlabeled GSH that this reaction gives stoichiometric conversion based on the extinction coefficient of GSSG at 338 nm (ε338 = 980 M⁻¹ cm⁻¹) (28).

Glutathionylation of GAPDH by Various Oxidants—GS-radical, GSSG, and GSSG were compared as glutathionyl donors for their relative ability to support S-glutathionylation of GAPDH in the absence or presence of glutaredoxin. Reaction mixtures contained 0.1 mM sodium-potassium phosphate, pH 7.5, 2 mg/ml BSA-CM, and 28 μM GSSG, and one of the GS-donors was added to initiate the reaction as follows: [35S]GSSG (~1 nCi/μmol; 0.05 or 0.5 mM) or [35S]GS-NO (~1 nCi/μmol; 0.05 or 0.5 mM) or GS-thiyl radical (~0.5 nCi/μmol; estimated as ≤0.01 mM). The concentration of GS-radicals probably represents an overestimate based on amount of GSSG accumulated (GSSG reductase assay) in 15 min in the HRP GS-radical generating system. The reactions were quenched with trichloroacetic acid, and [35S]GAPDH-SG was quantified according to the specific radioactivity of the respective glutathionyl donor as described above.
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**Glutaredoxin Catalysis of GSSG Formation from GS-Radicals**

To determine whether the glutaredoxin-catalyzed reaction involves the participation of molecular oxygen, we studied the effect of depleting oxygen. Fig. 2 documents that the enzyme-catalyzed reaction requires O₂ for efficient turnover and suggests coincident formation of superoxide (Fig. 3). Estimation of the stoichiometry of GSSG formation from GS-radicals was varied as shown. Either glutathione peroxidase (0.002 units/ml, triangles) or glutaredoxin (1 unit/ml, rectangles) was added as potential catalysts.

Glutaredoxin Acceleration of GS-Radical-mediated Protein-SSG Formation—A significant consequence of oxidative stress in cells is the accumulation of protein-SSG-mixed disulfides, possibly representing a protective mechanism (2, 5). S-Glutathionylation of specific proteins has now been demonstrated also upon activation of signaling cascades in cell culture as well and probably represents a mode of regulation (9, 11). Although many key proteins with specific cysteine residues that are subject to reversible glutathionylation have been characterized (2, 10), the mechanism(s) of formation of protein-SSG is unclear. One such protein whose glutathionylation has been studied in various contexts is glyceraldehyde-3-phosphate dehydrogenase. This abundant cytosolic protein has been observed to accumulate as GAPDH-SSG in cells after oxidative insults even though the GSSG concentration did not change substantially (23–25, 34), suggesting that a form of the glutathione moiety other than GSSG was the proximal mediator of glutathionylation. Therefore, we tested the relative effectiveness of GS-radical to serve as the precursor of protein-SSG formation and examined whether glutaredoxin would facilitate the reaction. In this case, the GS-radical generating system was horseradish peroxidase, utilizing H₂O₂ as oxidant and GSH as reductant. This system allowed us to minimize direct reaction of glutaredoxin with GAPDH (35), because we could use a concentration of H₂O₂ with GAPDH 10-fold lower than that for the Fe(II)-ADP/H₂O₂ system (described above) and still generate a significant steady-state concentration of GS-radical over the reaction time. Therefore, S-glutathionylation of GAPDH could be studied under conditions more closely related to a cellular signaling environment, i.e. low H₂O₂. Using this HRP GS-radical generating system, glutaredoxin did accelerate the rate of [³⁵S]GAPDH-SSG formation in a time-dependent and concentration-dependent manner (Fig. 4, inset). No enhancement of GAPDH-SSG formation occurred when the glutaredoxin enzyme was boiled before it...
was added or when either H₂O₂ or HRP was omitted from the reaction mixture (data not shown). Therefore, we conclude that the enhancement of GAPDH-SSG formation requires native glutaredoxin and it is dependent on GS-radical formation.

**Effect of Oxygen Deprivation on GAPDH-SSG Formation by GS-Radical in the Absence or Presence of Glutaredoxin**—Fig. 5 shows that removal of oxygen does not affect net GAPDH-SSG formation from GS-radicals in the presence of GRx (hatched bars), in contrast to the effect on GSSG formation (Fig. 2). However, in the absence of GRx, less GAPDH-SSG is formed under anaerobic conditions. Thus, the enhancement of GAPDH glutathionylation by GRx is greater in the absence of O₂. This distinction suggests an oxygen-independent rate-limiting step for the GRx-mediated glutathionylation of proteins (see “Discussion,” Scheme 4).

**Acceleration of Protein-SSG Formation by Glutaredoxin: Alternative GS Donors**—Besides GSSG and the glutathione-thiol radical, considerable attention has been focused on GS-NO as a potential mediator of protein-SSG formation (10, 36). Therefore, we tested the relative ability of GSSG, GS-radical, and GS-NO to mediate GAPDH-SSG formation in the absence and presence of glutaredoxin (Table I). Although both GSSG and GS-NO could substitute for the GS-radical-generating system and glutaredoxin accelerated GAPDH-SSG formation, the relative efficiency of these reactions was poor. Comparing the values in the right-most column of Table I, the GRx-mediated reactions for GS-NO and GSSG at 50 μM (⩾5× the concentration of GS-radical (<10 μM)) yielded less than half of the amount of GAPDH-SSG formed in the GS-radical reaction. Only at a very high concentration (500 μM) were GS-NO and GSSG as good or better donors for GRx-mediated GAPDH-glutathionylation as GS-radical at <10 μM.

**Glutathionylation of Actin and PTP1B**—Changes in the intracellular S-glutathionylation status of PTP1B and actin in response to a physiological redox stimulus have been reported recently; however, the glutathionylation mechanism has yet to be resolved. Therefore, we also examined the capability of glutaredoxin to mediate GS-radical-dependent formation of PTP1B-SSG and actin-SSG relative to GAPDH-SSG (Fig. 6). Both actin and PTP1B were much more readily glutathionylated by glutaredoxin-mediated GS-radical transfer compared with GAPDH. The *upper unshaded* portions of the bars at the right in each set in Fig. 6 represent the GRx-mediated protein-SSG formation, showing that actin and PTP1B are glutathionylated by the GRx and GS-radical system >12 times and >15 times more, respectively, than GAPDH.

**DISCUSSION**

**Glutaredoxin Catalysis of Radical Scavenging**—A generalized scheme has been proposed (31, 32, 37) that depicts the central role of GSH in mediating the dissipation of radicals within cells. The scheme involves intermediate formation of the GS-thyl radical and the GSSG-disulfide-anion radical. Ultimately, the radical is dissipated by reaction with molecular oxygen to yield the superoxide anion radical, which is converted finally to H₂O and O₂ by the combined actions of super-

![Graph 1](image1.png)

**Fig. 4.** Time course of glutaredoxin-mediated GAPDH-glutathionylation with the GS-radical generating system and dependent on glutaredoxin concentration (inset). Reaction mixtures contained 0.1 mM sodium-potassium phosphate, pH 7.5, 2 mg/ml BSA-CM, 28 μM GAPDH, 0.5 mM [³⁵S]GSH (~1 nCi/nmol), 0.2 mg/ml HRP, and 8.6 μM GRx. Reactions were initiated with 0.05 mM H₂O₂ and allowed to proceed for up to 15 min. Aliquots (0.02 ml) were withdrawn periodically (as shown) and added to an equal volume of 20% trichloroacetic acid to precipitate the protein. The radioactivity associated with the precipitated, washed, and resolubilized GAPDH was measured. Inset, conditions were the same as described above with the exception that the concentration of GRx was varied as shown (in a total volume of 0.02 ml), and all of the reactions were allowed to proceed for 15 min.

![Graph 2](image2.png)

**Fig. 5.** Effect of oxygen deprivation on GAPDH-SSG formation from GS-radicals in the absence and presence of glutaredoxin. Reactions were conducted as in Fig. 4 with the exception that for experiments labeled Anaerobic, all solutions were pre-bubbled with an N₂ lance for 15 min on ice. Glutaredoxin concentration was 0.1 μM. The hatched portion of the right bar in each case represents the net gain in [³⁵S]GAPDH-SSG because of the presence of glutaredoxin.

**Table I**

<table>
<thead>
<tr>
<th>GS donor</th>
<th>minus GRx</th>
<th>plus GRx</th>
<th>GRx-mediated</th>
</tr>
</thead>
<tbody>
<tr>
<td>GS-radical (&lt;10 μM)</td>
<td>0.7 ± 0.1</td>
<td>1.9 ± 0.2</td>
<td>1.2 ± 0.3</td>
</tr>
<tr>
<td>GS-NO (50 μM)</td>
<td>0.1 ± 0.1</td>
<td>0.4 ± 0.1</td>
<td>0.3 ± 0.2</td>
</tr>
<tr>
<td>GS-NO (500 μM)</td>
<td>1.0 ± 0.4</td>
<td>3.0 ± 0.6</td>
<td>2.0 ± 1.0</td>
</tr>
<tr>
<td>GSSG (50 μM)</td>
<td>0.2 ± 0.0</td>
<td>0.7 ± 0.2</td>
<td>0.5 ± 0.2</td>
</tr>
<tr>
<td>GSSG (500 μM)</td>
<td>1.1 ± 0.2</td>
<td>3.9 ± 0.3</td>
<td>2.8 ± 0.5</td>
</tr>
</tbody>
</table>
FIG. 6. Relative glutaredoxin-mediated S-glutathionylation of GAPDH, actin, and PTP1B. GAPDH, actin, and PTP1B were compared as glutathionyl recipients in the GS-radical generating system in the absence and presence of glutaredoxin. Reaction mixtures contained 0.1 M sodium-potassium phosphate, pH 7.5, 2 mg/ml BSA-CM (as co-precipitant), and GAPDH or actin or PTP1B, each at 28 μM, in the presence of the HRP GS-radical generating system as described under “Experimental Procedures.” Reactions were initiated with H$_2$O$_2$ and allowed to proceed for 15 min. Proteins then were precipitated with trichloroacetic acid, and the radioactivity associated with the respective GLUTATHIONYL-CONTAMINATED PLASMA PROTEINS was measured. The left-most solid black bar represents the overall glutaredoxin controls, and the right bar in each case represents the plus glutaredoxin results. The upper shaded bar in each case represent the minus glutaredoxin controls, and the right-hand bars in each case represents the plus glutaredoxin results. The upper shaded bar in each case represent the minus glutaredoxin controls, and the right-hand bars in each case represents the plus glutaredoxin results.

GLUTATHIONYLATION REACTIONS 1–4

1. R + GSH → RH + GS
2. GSH → GS$^+$ + H$^+$
3. GS + GS$^+$ ⇌ GSSG
4. GSSG$^+$ + O$_2$ → GSSG + O$_2^-$ . . . . . . . . . . . coupled to SOD, GPXase

Even though the formation of the glutathione-disulfide anion radical (Reaction 3) is inefficient because of a relatively low second order rate constant and the fact that GSH is predominantly in the protonated form at physiological pH (Reaction 2), the overall reaction leading to formation of superoxide is facilitated by the favorable second order rate constant for Reaction 4 (33). In this study, we made the remarkable discovery that the slow step in this reaction sequence (Reaction 3) could be supplanted by an enzyme-catalyzed reaction involving glutaredoxin (thioltransferase). The extent of acceleration of GSSG formation by GRx demonstrates catalytic turnover of the enzyme. From Fig. 1, the initial linear region of dependence of GSSG formation rate on GRx concentration gives a turnover of ~7 min$^{-1}$ (0.1 s$^{-1}$). At a typical cellular concentration of GRx (i.e., 1 μM) (29), 10 μM GS-radical would be converted to GSSG in 1.5 min. In fact, local dissipation of the GS-radical could occur much more rapidly, because its reaction with GRx (Scheme 2, step 1) would occur much faster than the overall reaction. It seems likely that the properties of glutaredoxin that impart this catalytic activity for scavenging the GS-radical would be related to those that are responsible for its efficient catalysis of protein-SSG deglutathionylation, namely exquisite selectivity for the glutathionyl moiety (1, 3), and unusually low $pK_a$ of the active site cysteine thiol ($pK_a$ of C-22-SH = 3.5) (16, 17). These properties would favor stabilization of a GRx-SSG$^-$ disulfide anion radical intermediate as depicted in Scheme 1.

To distinguish whether the putative glutaredoxin-glutathione disulfide anion radical intermediate might be turned over directly by reacting with a second GS-radical or involve participation of molecular oxygen (Scheme 2), we studied the effect of depleting oxygen. The overall data for GRx catalysis of GS-radical scavenging are consistent with the solid-line portion of Scheme 2, which is analogous to the reaction scheme we established for GRx catalysis of reduction of glutathionyl-mixed disulfides (Scheme 3) (2). Catalytic formation of GSSG is dependent on conditions that generate the glutathione-thiyl radical, and catalysis is abolished by the removal of molecular oxygen. With O$_2$ present, concomitant formation of superoxide along with the GSSG was shown by reduction of cytochrome c. Less than stoichiometric reduction of cyt c and GSSG formation was observed, probably because of competition between cytochrome c and Fe(III)-ADP (and GSH) for reaction with the superoxide. Accordingly, increasing the concentration of cytochrome c led to increased rates of reduction (Fig. 3).

Thus, Scheme 2 depicts a novel mechanism for scavenging radicals within cells where GSH is present in abundance and serves as the proximal acceptor, forming the GS-radical. Then glutaredoxin captures the GS-radical as the stabilized GRx-SSG$^-$ disulfide anion radical intermediate that can react readily with molecular oxygen, resulting in net conversion of the original radical to superoxide anion radical. The superoxide radical would then be dissipated by the coupled actions of superoxide dismutase and catalase and/or glutathione peroxidase or the thioredoxin peroxidase system to regenerate molecular O$_2$ and water as the final products of the scavenging cascade. Thus, more deleterious radical species (e.g., hydroxy radicals) would be eliminated by GSH coupled to the synergistic catalytic actions of glutaredoxin and superoxide dismutase. An analogous oxygen-dependent effect of thioredoxin on turnover of phenoxy-radicals was reported previously (38).

Glutaredoxin-mediated Protein S-Glutathionylation—There is a growing interest in reversible S-glutathionylation as a modulatory mechanism of signal transduction (6, 7, 10, 39). Although much circumstantial evidence favors this mechanism, there are many unanswered questions. Perhaps the most prominent question is whether there are enzymatic mecha-
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nisms for controlled formation of specific protein-SSG adducts as signaling intermediates. This study provides data pertinent to this consideration and offers an explanation for the presence of intracellular protein-SSG adducts under conditions where GSSG does not accumulate sufficiently to support simple thiol-disulfide exchange consistent with thermodynamic equilibria. Instead, glutathione-thiyl radical has been suggested as an alternative GS donor (5, 10) and, consistent with this hypothesis, intracellular GS-thiyl radicals have been trapped and identified by electron spin resonance spectroscopy (40, 41).

As shown in Table I, GRx-mediated formation of GAPDH-SSG was most efficient when GS-radical was the donor of the GS moiety, GS-radical \(\rightarrow\) GSSG \(\rightarrow\) GS-NO. Although the mechanisms by which GRx facilitates GAPDH-SSG formation with any of these GS donors are unknown, the comparison among GS donors indicates that a reaction involving radical intermediates is favored and that direct production of GS-radical (by the HRP system) is more efficient than homolytic cleavage of GS-NO either in the absence or presence of glutaredoxin.

Based on current data, Scheme 4 is a plausible working model for how GRx may facilitate GS-radical-mediated protein-SSG formation; however, additional studies are necessary to delineate the mechanism. Under the particular conditions used for the HRP-generating system, the formation of GS-radical probably is rate limiting for the overall reaction. Accordingly, glutaredoxin would react very quickly with the GS-radicals to form the GRx-SSG anion radical intermediate (step 1). This intermediate then could abstract a hydrogen atom from the target protein-SH group (step 2) to form protein-S' thiyl radical and GSH (step 2). Completion of the reaction would involve a radical recombination reaction (step 3), forming the protein-SSG and recycling the glutaredoxin enzyme. The net reaction would involve two GS-radicals for each protein-SSG product formed.

Alternatively, the target protein-S' thiolate might react directly with the GRx-radical intermediate to form protein-SG\(^{\delta}\) (step 2') and then transfer the radical to molecular oxygen to give protein-SSG and superoxide (step 3'). This alternative sequence, however, would not be favorable unless the target protein had properties analogous to glutaredoxin (low \(pK_a\) for the SH moiety and stabilization site for the glutathionyl moiety).

Using GAPDH as a model, the GRx-dependent rate of formation of protein-SSG was enhanced under anaerobic conditions (Fig. 5, upper shaded portion of right-hand bar). This effect is consistent with a higher steady-state amount of the GRx-SSG\(^{\delta}\) intermediate available for reaction with the target protein-thiol moiety when the competing reaction with O\(_2\) is eliminated.

As net accumulation of protein-SSG would also be affected by GRx catalysis of deglutathionylation. To confirm this interpretation with the GAPDH model, reaction mixtures containing glutathionylated GAPDH were concentrated and utilized as substrates in a typical deglutathionylation assay for GRx in the absence of the GS-radical generating system. These samples were deglutathionylated (release of radiolabel from \([\text{35S}]\text{GAPDH}-\text{SSG}\) in a time-dependent manner at rates proportional to the GRx concentration (data not shown). Therefore, as the concentration of protein-SSG accumulated in the complete reaction mixture, its rate of deglutathionylation would increase until a steady-state was reached. In this regard, we also investigated the relative reactivities of actin and PTP1B as targets of S-glutathionylation by the glutaredoxin-mediated GS-radical system (Fig. 6), because both of these proteins have recently been reported to have their intracellular activities modulated by S-glutathionylation (9, 11). Since considerable quantities of GAPDH-SSG, actin-SSG, and PTP1B-SSG accumulated, this suggests that the GRx-mediated S-glutathionylation of these proteins under the limited GS-radical-generating conditions of our experiments was efficient enough to overcome the competing deglutathionylation reaction in each case. Thus, the HRP-GSH-H\(_2\)O\(_2\) system as adjusted for the current experiments may appropriately simulate intracellular conditions where a continuous production of a low level of GS-radicals may occur, e.g. when a redox signaling pathway is activated.

Under equivalent conditions, PTP1B was glutathionylated by GRx most extensively, i.e. PTP1B-SSG \(>\) actin-SSG \(>\) GAPDH-SSG. Although the reactivity of PTP1B and GAPDH with the GRx-SSG anion radical intermediate could be ascribed to their low \(pK_a\) thiolates, the same interpretation cannot be applied to actin whose most accessible cysteine residue has a normal \(pK_a\) (11). As noted above, the relative accumulation of each of the S-glutathionylated proteins reflects their relative efficiencies as substrates for the two different reactions catalyzed by glutaredoxin, GS-radical-dependent S-glutathionylation versus GSH-dependent deglutathionylation.

It remains an open question how protein S-glutathionylation actually takes place intracellularly and whether it is in fact
enzyme-mediated; however, the possibility that glutaredoxin (or other enzyme(s) with comparable properties) catalyzes the formation of specific protein-SSG adducts via transfer of the glutathione-thiyl radical is supported by the current studies that provide proof of principle. The additional catalytic properties of glutaredoxin revealed by the current studies characterize it as a versatile enzyme important for a variety of cell functions, reinforcing the concept that glutaredoxin plays a vital role in sulfhydryl homeostasis and redox signal transduction.

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