

Irreversible Inactivation of Glutaredoxins 1 and 2 by Peroxynitrite

Byungki Jang and Sanghwa Han*

Department of Biochemistry, Kangwon National University, Chunchon 200-701, Korea. *E-mail: hansh@kangwon.ac.kr

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Formation of a mixed disulfide between a protein thiol and glutathione, *i.e.* S-glutathionylation (or S-glutathiolation), is considered a potential regulatory mechanism especially under oxidative stress.¹⁻³ Most enzymes with an active site cysteine lose activity upon S-glutathionylation and regain activity when reduced by thiols. While the cellular mechanism of S-glutathionylation remains unresolved, deglutathionylation is known to be catalyzed by glutaredoxins.³

Glutaredoxins 1 and 2 are disulfide-dithiol oxidoreductases localized in cytosol and mitochondrial matrix, respectively.⁴ They catalyze the transfer of a glutathione moiety from an S-glutathionylated protein to glutathione. Glutaredoxin 1 (Grx1) contains a conserved CPYC sequence with the N-terminal cysteine having a very low pK_a . A similar CSYC motif is found in Grx2. As expected, down-regulation of a glutaredoxin results in extensive S-glutathionylation of cytosolic proteins.⁵

Interactions of glutaredoxins with nitric oxide and its congeners have not been studied extensively. To our knowledge, the only report is by Aykac-Toker *et al.*⁶ who showed that rat liver cytosolic fractions lose glutaredoxin activity when treated with peroxynitrite. Considering the potential role of S-glutathionylation in signaling, inactivation of glutaredoxins by reactive nitrogen species deserves detailed study.

We report here that peroxynitrite among the nitric oxide congeners is a very effective inactivator of purified Grx1 and Grx2. Inactivated glutaredoxins are not reactivated by glutathione. Glutathione, however, protects glutaredoxins from peroxynitrite-induced inactivation.

Results and Discussion

We first examine the reactions of purified glutaredoxins with various reactive nitrogen species. Grx1 or Grx2 (5 μM) was treated with a reactive nitrogen species (500 μM) for 30 min at 25 °C and the thioltransferase activity was measured by the HED assay as described in Experimental Section. Bolus addition of 500 μM peroxynitrite to 5 μM Grx1 inactivated the enzyme by ~90% (see Table 1). Morpholinonydronimine (SIN-1), which simultaneously generates NO and superoxide, was as effective as peroxynitrite. Decomposed peroxynitrite was not effective (not shown). BF_4NO , an NO^+ donor that can S-nitrosylate thiols, did not inactivate Grx1. GSNO, which can S-nitrosylate and/or S-glutathionylate proteins depending on the target,⁷ inactivated Grx1

Table 1. Inactivation of glutaredoxins by reactive nitrogen species. Glutaredoxins (5 μM) were incubated with a reactive nitrogen species (500 μM) for 30 min and the activity was measured by the HED assay as described in Experimental Section. Turnover number (TN) was calculated by dividing the rate of NADPH oxidation by [Grx]. Values are mean \pm SD, $n = 4$

reactive nitrogen species	TN of glutaredoxin-1	TN of glutaredoxin-2
control	25.9 \pm 1.9	4.05 \pm 0.04
peroxynitrite	2.8 \pm 0.5	2.28 \pm 0.07
SIN-1	3.3 \pm 0.1	3.61 \pm 0.18
BF_4NO	25.6 \pm 1.8	4.23 \pm 0.07
GSNO	23.1 \pm 2.0	3.79 \pm 0.07
DEANO	20.5 \pm 2.1	4.14 \pm 0.14

marginally. DEANO, a NO donor, also inactivated Grx1 only slightly. Peroxynitrite inactivated Grx2 by ~45%, which is much smaller than the value for Grx1. Interestingly SIN-1, which was a stronger inactivator for Grx1 than peroxynitrite, inactivated Grx2 only by ~10%. The other reactive nitrogen species were almost ineffective.

Shown in Figure 1 is the degree of Grx1 inactivation as a function of peroxynitrite (or SIN-1) concentration. At a Grx1 concentration of 5 μM , the concentrations of peroxynitrite and SIN-1 for 50% inactivation (IC_{50}) were 50 μM and 25 μM , respectively. Grx1 treated with different concentrations of peroxynitrite were subjected to SDS-PAGE and protein bands were visualized by a Western blot using anti-Grx1 antibody (Fig. 1b). Under non-reducing condition, the band at ~12 kD of native Grx1 disappeared as the peroxynitrite concentration increased with concomitant increase in the intensity of multimeric bands. This is in parallel with decrease in the Grx1 activity. In the presence of mercaptoethanol, the bands corresponding to multimeric forms disappeared and collapsed into a single band at 12 kD, implying that the multimers were intermolecular disulfides produced upon oxidation of cysteine residues. At higher peroxynitrite concentrations, a weak dimeric band was visible presumably due to formation of an intermolecular dityrosine cross-link, which can not be reduced by thiols. The samples treated with SIN-1 exhibited a similar pattern (Fig. 1c). When superoxide dismutase was included during the treatment of SIN-1, neither inhibition nor multimeric bands were observed (not shown) suggesting that the effect of SIN-1 was due to peroxynitrite that was produced by the reaction of NO and superoxide.

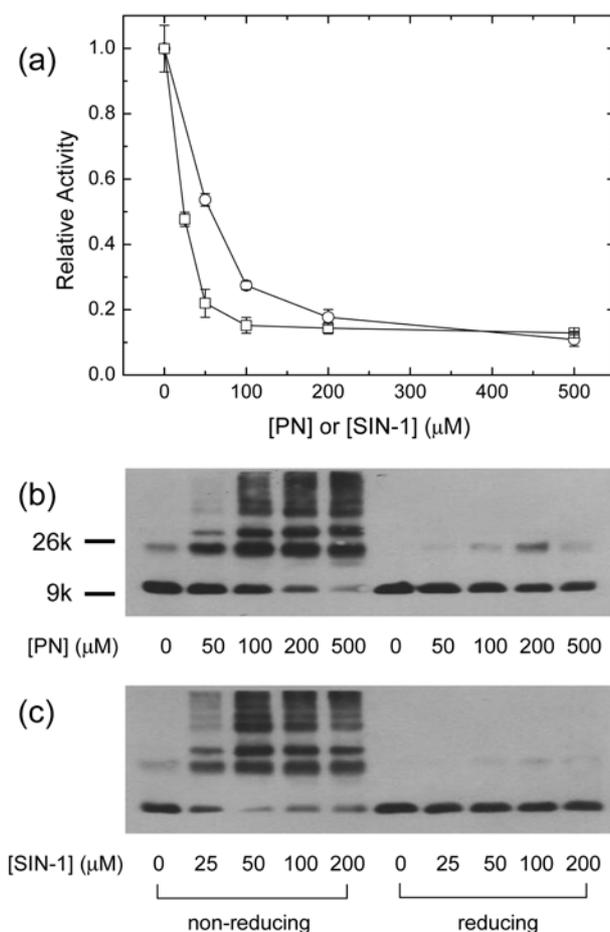


Figure 1. Inactivation of Grx1 by peroxynitrite and SIN-1. Grx1 was reacted with various concentrations of peroxynitrite (PN) or SIN-1. Decrease in thioltransferase activity of Grx1 as a function of [PN] or [SIN-1] is shown in (a) and the corresponding Western blots with anti-Grx1 antibody are presented in (b) and (c). Mercaptoethanol was excluded in non-reducing gels. "C" denotes the untreated sample. See Experimental Section for detail. Values are mean \pm SD, $n = 4$.

Figure 2 shows the effect of glutathione, an abundant intracellular reductant, on peroxynitrite-induced inactivation of Grx1. Glutathione at 500 μ M was enough to almost completely suppress the peroxynitrite-induced inactivation if glutathione was present prior to addition of peroxynitrite (Fig. 2a, open circles). SDS-PAGE under non-reducing condition (Fig. 2b) also indicated that glutathione protected the enzyme against peroxynitrite. The result indicates that peroxynitrite rapidly reacted with glutathione before attacking the thiolate group in the active site of Grx1. However, when glutathione was added after the peroxynitrite treatment, the enzyme activity was not recovered at all (Fig. 2a, filled circles). Corresponding SDS-PAGE under non-reducing condition (Fig. 2c) also showed that most of the multimeric bands were reduced by glutathione but the intensity of the \sim 12 kD band (and the activity of Grx1) was not recovered to the level of the control.

Figure 3 shows the effect of GSH on peroxynitrite-induced inactivation of Grx2. When GSH was present before peroxy-

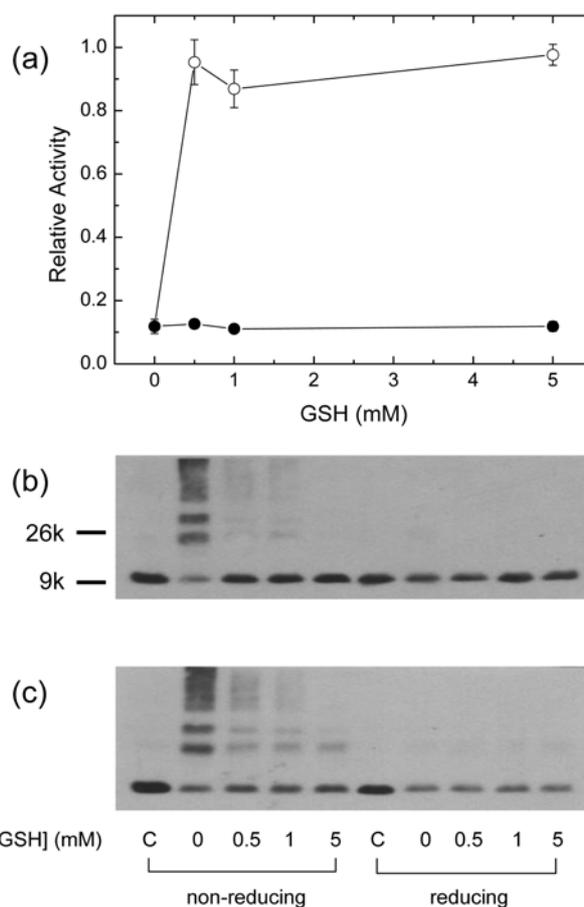


Figure 2. Effects of glutathione on peroxynitrite-induced inactivation of Grx1. GSH was added before (open circles) or after (closed circles) the treatment of Grx1 with peroxynitrite. Changes in thioltransferase activity of Grx1 as a function of [GSH] is shown in (a). Western blots of samples corresponding to open and closed circles in (a) are presented in (b) and (c), respectively. Values are mean \pm SD, $n = 4$.

nitrite addition, the enzyme was fully protected from peroxynitrite (Fig. 3a). However, GSH was not able to reactivate the enzyme that was pre-treated with peroxynitrite. These results are similar to those for Grx1. SDS-PAGE under reducing conditions showed that peroxynitrite produced a dimeric form of Grx2, which was not reduced by thiols (GSH and mercaptoethanol). This means that the dimerization was not due to formation of an intermolecular disulfide bond. A dityrosine bond could be formed between two Grx2 molecules as frequently encountered in the reactions of proteins and peroxynitrite.⁸ Under non-reducing conditions SDS-PAGE showed faint bands for monomeric and dimeric forms produced upon peroxynitrite treatment (lane 2). This may be due to weakened reactivity of oxidized Grx2 against the antibody. When GSH was present prior to peroxynitrite addition, these bands became strong (lane 4).

We next examined peroxynitrite-induced inactivation of intracellular Grxs. When RAW 274.7 cells were treated with 500 mM peroxynitrite, no indication of Grx inactivation was observed presumably due to abundant intracellular GSH, which protects Grxs from peroxynitrite attack (Fig. 4). When

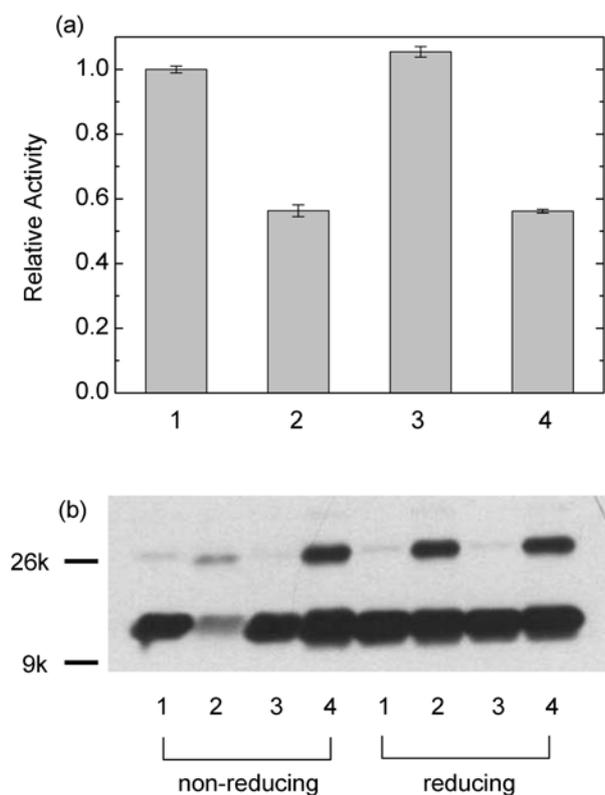


Figure 3. Effects of glutathione on peroxynitrite-induced inactivation of Grx2. (a) Changes in Grx2 activity. 1, untreated; 2, treated with 500 μ M peroxynitrite in the absence of GSH; 3, 1 mM GSH was present before peroxynitrite treatment; 4, 1 mM GSH was added after peroxynitrite treatment. Values are mean \pm SD, $n = 4$. (b) Western blots of samples corresponding to those in (a).

GSH was depleted by BSO prior to addition of peroxynitrite, the total Grx activity decreased by \sim 30%. GSH depletion alone without peroxynitrite treatment did not affect the Grx activity. Our result agrees with Aykac-Toker *et al.*⁶ although cell lysates instead of cells were treated with peroxynitrite in their work.

Peroxyntirite inactivates enzymes by modifying amino acid residues⁹ or releasing active site metal ions.¹⁰ But the major targets of peroxynitrite in proteins are tyrosine and cysteine. Peroxynitrite reacts with tyrosine to produce 3-nitrotyrosine and dityrosine.^{11,12} These modifications are often harmless unless the modified tyrosine is located in the active site. Formation of dityrosine was marginal, however, under the experimental conditions used in this study. On the other hand, many enzymes contain a cysteine residue in the active site and these enzymes are often inactivated by peroxynitrite. The modifications of cysteine residues by peroxynitrite include intermolecular disulfide formation,¹³ oxidation to sulfenic acid,¹⁴ S-nitrosylation,^{15,16} and S-glutathionylation in the presence of GSH.¹⁵ High reactivity of thiols toward peroxynitrite, however, makes GSH a good cellular protector against the peroxynitrite attack. Considering that intracellular GSH is maintained at a high concentration, it is conceivable that a cysteine residue in proteins remains unmodified until glutathione is depleted by continu-

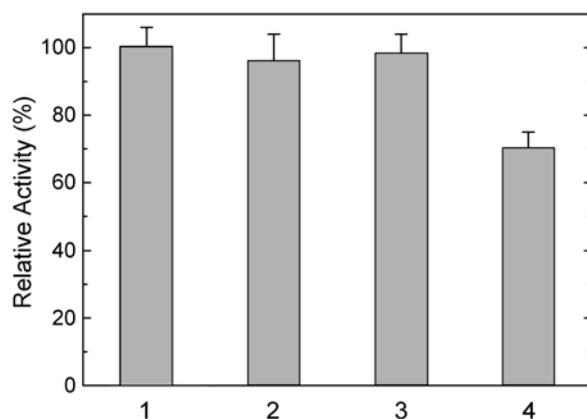


Figure 4. Effect of peroxynitrite on intracellular glutaredoxins. RAW 264.7 cells were treated with 500 μ M peroxynitrite and the Grx activity was measured by the HED assay. 1, control; 2, treated with BSO to deplete GSH; 3, treated with peroxynitrite only; and 4, treated with peroxynitrite after GSH depletion. Values are mean \pm SD, $n = 3$.

ous generation of peroxynitrite or by sustained oxidative stress. Inactivation of Grx may be responsible for the S-glutathionylated proteins observed under nitrosative stress.

Other reactive nitrogen species can also modify cysteine residues in proteins. NO is an S-nitrosylating species when oxidized to N_2O_3 . A nitrosyl donor such as GSNO can S-nitrosylate and/or S-glutathionylate cysteine residues.⁷ These reactions are known to be facilitated when the cysteine residue is ionized. The active site cysteines in Grx1 and Grx2 are expected to be easily modified because they have a low pK_a . In our study, however, both Grx1 and Grx2 were resistant to DEANO (a NO donor) and GSNO (a NO⁺ donor). One can argue that protein-SNO was reduced back to protein-SH during the assay since GSH was present in the assay system. It is known, however, protein-SNO can only be reduced by ascorbate not by GSH.¹⁷ So it appears that Grxs did not react with DEANO or GSNO.

In conclusion, Grx1 was extremely prone to inactivation by peroxynitrite and SIN-1. Grx2 was also inactivated by peroxynitrite but not by SIN-1. Once inactivated, both Grx1 and Grx2 were not reactivated by GSH. GSH scavenged peroxynitrite efficiently so that Grxs were protected from peroxynitrite attack unless GSH was depleted.

Experimental Section

Materials. Recombinant human glutaredoxins 1 and 2 and their antibodies were obtained from Labfrontier (Seoul, Korea). Glutaredoxin solutions were dialyzed against a phosphate buffer to remove 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (HEPES), which otherwise reacts with peroxynitrite. Peroxynitrite was prepared by reacting isoamyl nitrite with hydrogen peroxide and stored in an alkaline solution.¹⁸ All other chemicals were from Sigma-Aldrich Korea (Yongin, Korea) except diethylamine nitric oxide (DEANO) which was from Cayman Chemical (MI, USA).

Solutions were treated with Chelex 100 to remove trace transition metal ions and DTPA (100 μM) was included in the reaction mixtures to provide a metal-free environment.

Reaction with Reactive Nitrogen Species. Glutaredoxin (5 μM) in 0.1 M potassium phosphate buffer (pH 7.4) was rapidly mixed with reactive nitrogen species and incubated for 30 min at 25 °C. All solutions of reactive nitrogen species were prepared freshly before use. For a control experiment with decomposed peroxyxynitrite, alkaline peroxyxynitrite was diluted in a phosphate buffer (pH 7.4) and incubated for 1 h.

Activity Measurements of Glutaredoxins. Thioltransferase activity of glutaredoxins was measured by the hydroxyethyl disulfide (HED) assay. HED (700 μM), glutathione (1 mM) and NADPH (115 μM) were dissolved in 0.1 M phosphate buffer containing bovine serum albumin (0.1 mg/mL). Reaction was initiated by adding 20 nM Grx1 (or inactivated Grx1). Due to low activity, Grx2 was added at a concentration of 100 nM. Thioltransferase activity of Grxs was measured by monitoring the oxidation of NADPH, which can be followed by decrease in the absorbance at 340 nm.

Electrophoresis and Western Analyses. Samples of Grx1 and Grx2 were electrophoresed on a 13.5% polyacrylamide gel with sodium dodecylsulfate and mercaptoethanol. For a non-reducing condition, mercaptoethanol was excluded. Protein bands were transferred to a PVDF membrane and probed with anti-Grx1 and anti-Grx2 antibodies.

Experiment with Cells. RAW 264.7 cells were grown in DMEM. For GSH depletion, cells were treated with buthionine sulfoximine (BSO) for 24 h. Both control and BSO-treated cells were washed with 0.1 M potassium phosphate buffer before incubating with 500 μM peroxyxynitrite for 30 min. Cells were washed with PBS and lysed on ice for 30

min. Lysate was centrifuged at 16,000 g for 10 min and an aliquot (200 μg protein) of the supernatant was used for the HED assay.

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