Glutathione peroxidase (GPx) was inactivated by S-nitroso-N-acetyl-L-penicillamine (SNAP), a nitric oxide donor (Asahi, M., Fujii, J., Suzuki, K., Seo, H. G., Kuzuya, T., Horii, M., Tada, M., Fujii, S., and Taniguchi, N. (1995) J. Biol. Chem. 270, 21035–21039). The structural basis of the inactivation was studied. We also show that 3-morpholinosydnonimine \(N\)-ethylcarbamide, a peroxynitrite precursor, as well as synthetic peroxynitrite also inactivated bovine GPx. The degree of incorporation of a precursor, as well as synthetic peroxynitrite also inactivated bovine GPx decreased after pretreatment with SNAP as evidenced by mass spectrometry. To identify the modification site of this enzyme by SNAP, both SNAP-pretreated and untreated GPxs were reacted with \(n\)-octyldithionitrobenzoic acid and digested with lysylendopeptidase, and the resulting peptides were subjected to mass spectrometry. This technique identified a bridge between two peptides, one of which contains Sec\(^{45}\) at the catalytic center and Cys\(^{74}\), and the other contains Cys\(^{91}\). Although there are two possible combinations, selenocysteine 45 (Sec\(^{45}\)) and Cys\(^{91}\) or Cys\(^{74}\) and Cys\(^{91}\), the tertiary structure of GPx indicates that a cross-link between Sec\(^{45}\) and Cys\(^{91}\) is more feasible. This is consistent with the experimental evidence that SNAP specifically inactivates GPx, in which Sec\(^{45}\) forms the catalytic center. Thus, we conclude that SNAP mainly oxidized Sec\(^{45}\) to form a selenenyl sulfide (Se-S) with a free thiol, leading to the inactivation of the enzyme. These data suggest that nitric oxide and its derivatives directly inactivate GPx in a specific manner via the production of a selenenyl sulfide, resulting in an increase in intracellular peroxides that are responsible for cellular damage.

Nitrile oxide (NO)\(^1\) has been implicated in a number of physiological processes such as vasoconstriction, inhibition of platelet aggregation, and neurotransmission (1). The half-life of NO is very short, however, in vivo because it easily reacts with molecular oxygen and other reactive oxygen species as well as free thiols in, for example, glutathione and proteins to form nitrosothiols. Nitrosothiols functionally mimic NO and are also thought to play an important role in vivo (2, 3). Recent studies of glyceraldehyde-3-phosphate dehydrogenase (4, 5), protein kinase C (6), low molecular weight phosphotyrosine protein phosphatase (7), and NADPH oxidase (8) indicate that NO interacts with thiol residues on these enzymes and as a result influences cellular function (9).

The interaction of NO and superoxide produces a potent oxidant, peroxynitrite (ONOO\(^{-}\)) (10). The rate of formation of peroxynitrite is approximately 30-fold faster than the reaction of NO with oxyhemoglobin and 3-fold faster than the reaction of superoxide with superoxide dismutase (11). Stimulated macrophage (12), neutrophils (13), and endothelial cells (14) have been shown to generate peroxynitrite in vivo. Recent data have provided evidence for the in vivo formation of peroxynitrite in human atherosclerotic coronary vessels (15), in human acute lung injury (16), and in chronic inflammation (17). The produced peroxynitrite induces protein modifications by a mechanism that is different from that for reactive oxygen species or NO in that it rapidly reacts with tryptophan and tyrosine as well as cysteine and induces the formation of protein carbonyl groups (18). The oxidative modification of proteins, including enzymes by peroxynitrite, causes protein fragmentation and thus alters enzyme activity and in addition induces many injury processes associated with oxidative biological damage (19, 20).

Glutathione peroxidase (GPx), a potent anti-oxidative enzyme, scavenges various peroxides. Overexpression of this enzyme was observed to suppress reactive oxygen-induced apoptosis in a variety of cells, suggesting that the inhibition of this enzyme is closely related to apoptotic cell death (21, 22). GPx contains a rare amino acid, selenocysteine, which is essential for peroxidase activity (23, 24). This selenocysteine residue resembles a cysteine residue in terms of chemical properties but has a higher reactivity (25, 26). In prior studies in this laboratory, we have examined the effects of oxidants, on the inactivation of GPx and recently have shown (27) that treatment of GPx with S-nitroso-N-acetyl-D,L-penicillamine (SNAP), a nitric oxide precursor, results in its inactivation. SNAP appears to inactivate GPx in a complex manner involving two discrete steps. The first step appears to be reversible, and during this step dithiothreitol can recover its activity, whereas the second step is irreversible.

Because peroxynitrite is a much more potent oxidant than NO (28, 29), it is possible that GPx is also inactivated by peroxynitrite. This paper reports a further characterization of the mechanism of inactivation of GPx by NO and a determina-
tion of the specific site of GPx, which is oxidized and results in inactivation. Experiments were also carried out using SIN-1, a precursor of peroxynitrite, to investigate the nature of this oxidant on the inactivation of the enzyme.

**EXPERIMENTAL PROCEDURES**

**Materials**—Bovine intracellular GPx was purchased from Toyobo Co. Ltd. ODNB was obtained from Fluka. SNAP and DTT were purchased from Wako Pure Chemical Co. Ltd. SIN-1 was obtained from Dojin. Lysylendopeptidase and 5(6)-carboxyfluorescein-N-hydroxysuccinimide ester (FLUOS) were obtained from Boehringer Mannheim.

**Preparation of Peroxynitrite—** Peroxynitrite was prepared by the method of Beckman et al. (30) with minor modifications. Briefly, 50 mM sodium nitrate was added to an equivalent amount of hydrogen peroxide in 1 N HCl, and the pH of the solution was then immediately adjusted to 10 by the addition of 1.2 N sodium hydroxide. Concentrations of peroxynitrite were determined by its absorption at 302 nm (1,670 mol⁻¹ cm⁻¹).

**Modification of Free Thiols and Selenol—** Free thiols and selenol were modified by ODNB as described by Faulstich et al. (31). ODNB is a reagent that reacts with free thiols and selenols in protein more rapidly than Ellman’s reagent.

**Preparation of Reduced GPx and Its Modification by NO and ODNB—** To convert all the purified GPx into its active form, the GPx in 0.1 M potassium phosphate buffer (pH 7.4) was reduced with DTT. After spontaneous oxidation by air, the sample solution was flushed with N₂ to protect GPx from excess ODNB for 30 min at room temperature. To protect GPx from spontaneous oxidation by air, the sample solution was flushed with N₂ during the reaction. GPx was treated with and without SNAP, a donor of NO⁻ and NO₂⁻ as well as NO (32) in the same buffer as the above at 37 °C for 1 h and then reacted with a 20-fold excess ODNB for 30 min at room temperature. To protect GPx from spontaneous oxidation by air, the sample solution was flushed with N₂ during the reaction. GPx was treated with and without SNAP and then modified with ODNB, designated as NO-GPx-ODNB and GPx-ODNB, respectively, and was used for further experiments.

**Reversed Phase HPLC Analysis—** GPx molecules and peptides derived from GPx by digestion with lysylendopeptidase were separated by RP-HPLC (Waters) at a flow rate of 1.0 ml using a 150 × 4.6-mm I.D. Develosil 300 C4-HG-5 column and a 150 × 4.6-mm I.D. Develosil ODS-HG-5 column (Nomura chemical, Aichi, Japan), respectively. A gradient system formed between solvent A (0.1% trifluoroacetic acid in water) and solvent B (0.1% trifluoroacetic acid in acetonitrile) was used.

**Mass Spectrometry—** Positive ion MALDI-TOP-MS was performed using a Voyager-TOF or Voyager-Elite time-of-flight (TOF) mass spectrometer equipped with a delayed-extraction system (PerSeptive Biosystems, Flemington, MA) as described previously (33). Solutions (1 µl) containing the GPx molecules fractionated by RP-HPLC or the peptides derived from GPx by digestion with lysylendopeptidase were separated by MALDI-TOF-MS. The molecular masses of fractionated peptide fragments were examined by MALDI-TOF-MS, and the amino acid sequences were assigned using the data obtained.

**SDS-PAGE—** Proteins were separated on 15% SDS-PAGE according to Laemmli (35) in the presence or the absence of 5% 2-mercaptoethanol.

**GPx Activity Determination—** GPx activity was determined according to the method of Lawrence and Burk (36). One unit was defined as the amount of enzyme required to oxidize 0.5 µmol of NADPH (corresponding to 1 µmol of reduced glutathione) per min.

**RESULTS**

**Inactivation of GPx by SIN-1 and Peroxynitrite—** We have previously shown that GPx is inactivated by SNAP treatment more specifically than glyceraldehyde-3-phosphate dehydrogenase in vitro (27). In this study, we examined the inactivation of GPx by peroxynitrite, which is a more potent oxidant than NO, and the reversibility of the inactivation. When we examined the effects of SIN-1, a precursor of peroxynitrite that generates both NO and superoxide (39), we found that GPx was inactivated by SIN-1 in a dose-dependent manner (Fig. 1a). To determine the actual molecule that attacks GPx, we examined the effects of manganese-superoxide dismutase on inactivation of GPx by SIN-1 and SNAP. a, purified GPx (1 mg/ml) was incubated with varying concentrations of SIN-1 at 37 °C for 1 h. Enzyme activity was determined after dilution with assay buffer. b, purified GPx (1 mg/ml) was incubated with 1 mM SIN-1 or SNAP in the presence or the absence of 100 units/ml manganese-superoxide dismutase at 37 °C for 1 h. Enzyme activities were measured in triplicate. The means ± S.D. are shown. CTR; control; SOD; superoxide dismutase.

**Purification of Manganese-superoxide Dismutase—** Manganese-superoxide dismutase was purified by chromatography on DEAE-cellulose, hydroxyapatite, and butyl-Toyopearl as described previously (37).

**Peptide Electrophoresis—** To identify the peptides modified with SNAP, the lysylendopeptidase digests were first incubated with the fluorescent probe, FLUOS, to amplify the signal derived from peptide bands (38) and then subjected to 20% SDS-PAGE. The bands were detected by a fluorescein image analyzer (FluoImager SI, Molecular Dynamics).

**Statistical Analysis—** The paired Student’s t test was used to compare the significance of the differences between data.

**TABLE 1.** Effect of manganese-superoxide dismutase on inactivation of GPx by SIN-1 and SNAP. a, purified GPx (1 mg/ml) was incubated with varying concentrations of SIN-1 at 37 °C for 1 h. Enzyme activity was determined after dilution with assay buffer. b, purified GPx (1 mg/ml) was incubated with 1 mM SIN-1 or SNAP in the presence or the absence of 100 units/ml manganese-superoxide dismutase at 37 °C for 1 h. Enzyme activities were measured in triplicate. The means ± S.D. are shown. CTR; control; SOD; superoxide dismutase.

**FIG. 1.** a, inactivation of GPx by SIN-1. b, effect of manganese-superoxide dismutase on inactivation of GPx by SIN-1 and SNAP. a, purified GPx (1 mg/ml) was incubated with varying concentrations of SIN-1 at 37 °C for 1 h. Enzyme activity was determined after dilution with assay buffer. b, purified GPx (1 mg/ml) was incubated with 1 mM SIN-1 or SNAP in the presence or the absence of 100 units/ml manganese-superoxide dismutase at 37 °C for 1 h. Enzyme activities were measured in triplicate. The means ± S.D. are shown. CTR; control; SOD; superoxide dismutase.
did not induce the inactivation.

Characterization of GPx Treated with SNAP—The facts that the activity of SNAP-inhibited GPx can be partially recovered by brief incubation with DTT and that this reversibility is diminished after prolonged incubation with SNAP (27) suggest that the mechanism by which GPx is inactivated is a multistep process. Because nitroso-GPx cannot be detected by NMR (27), the intermediate may not be as stable as that found for the case of serum albumin (2). Because NO is known to be an oxidant, it may mediate oxidation, possibly of cysteine side chains or selenocysteine residues. We then incubated GPx with or without 1 mM SNAP and further reacted the samples with ODNB, a sulfhydryl reagent, to quantitate the free SH or SeH residues in GPx. GPx treated with the above reagents was eluted from the C-4 column in the order of intact GPx > NO-GPx-ODNB > GPx-ODNB (Fig. 3), which suggests differences in the amount of ODNB attached to GPx, because this reagent gives molecular species of increasing hydrophobicity. These same molecular species also displayed slightly different electrophoretic mobilities on SDS-PAGE under nonreducing conditions (Fig. 4). In addition, NO-GPx-ODNB (Fig. 4, lanes 7 and 8) gave two bands, along with a broad smear in the high molecular weight region. Under reducing conditions, this complex mixture reverted to a single band, corresponding to 22 kDa (Fig. 4). The result implies that modification of GPx by SNAP occurs in part at some specific ODNB-reactive site, that is, at free thiol or selenol groups in the GPx.

To determine the number of ODNB molecules incorporated into each GPx, the fractions obtained by RP-HPLC (Fig. 3) were subjected to MALDI-TOF-MS. Intact GPx gave a relatively broad molecular ion peak, the center of which corresponds to \( m/z \) 21,575 as \( MH^+ \), which is close to the value (21,586) calculated from the amino acid sequence (5–198) of GPx (see Fig. 6b) (41). The broadening of the molecular ion peak can be attributed to the heterogeneity of the N-terminal portion of GPx, which was verified by N-terminal sequence analysis of intact GPx (data not shown) and should have caused a slightly larger error in mass than expected. The NO-GPx-ODNB and GPx-ODNB samples showed molecular ion peaks at \( m/z \) 22,004 and 22246, respectively, which are larger than the observed mass (21,575) of the intact GPx by 429 and 671. The increased values are consistent with the incorporation of three and five \( n \)-octane-1-thiol, respectively, derived from ODNB into GPx treated with and without SNAP. These results strongly suggest that two thiol or selenol residues were modified by SNAP treatment, preventing them from reacting with ODNB.

Identification of Modified Residues—To identify the modification site in GPx treated with SNAP, GPx-ODNB and NO-GPx-ODNB were digested with lysylendopeptidase and then fractionated by RP-HPLC using a C-18 column. Eight peptides were produced from GPx-ODNB by lysylendopeptidase digestion (see Fig. 6c). The observed molecular weights obtained by ESI-MS of these fractions allowed the complete identification of bovine GPx (see Fig. 6b). It should be noted that peak 1, which contains the N-terminal peptide fragment gave several molecular ion signals in ESI-MS (data not shown), all of which were
Collectively, these data indicate that Sec45 or Cys74 in peptide 2 and Cys91 in peptide 3 were cross-linked via Se-S or S-S bridges in NO-GPx-ODNB in a specific manner.

On the other hand, the recoveries of peptides 2 and 3 in the digest of NO-GPx-ODNB were decreased, relative to the remainder of the peptides that were obtained with similar intensities to those observed for GPx-ODNB (Fig. 6a). This suggests that cross-linking through disulfide or selenenyl sulfide occurs between Sec45 or Cys74 in peptide 2 and Cys91 in peptide 3 by SNAP treatment, although a cross-linked peptide has not been eluted from the reverse-phase column under the present conditions.

To examine the presence of high molecular weight cross-linked peptides in the digest, it was directly subjected to SDS-PAGE modified for the separation of relatively low molecular weight proteins, which allows separations ranging from 3 to 10 kDa (see “Experimental Procedures”) (Fig. 7). Although the lysylendopeptidase digest of GPx-ODNB showed two bands of approximately 5 kDa, which may correspond to the two large fragments (peptides 2 and 8, Fig. 6b) of GPx (Fig. 7, lane 2), the digest of NO-GPx-ODNB gave an additional electrophoretic band at a position between bovine insulin (5.7 kDa) and horse cytochrome c (12.2 kDa) (Fig. 7, lanes 3 and 4), which could correspond to a cross-linked fragment that was not eluted from the reverse-phase column.

To further identify the cross-linked fragment in NO-GPx-ODNB, the digest was subjected to MALDI-TOF-MS. The molecular ion signals observed for the digests of GPx-ODNB and NO-GPx-ODNB could be correlated to the amino acid sequence. The N-terminal peptides gave a series of ions, which correspond to the amino acid sequence truncated at the N terminus. The result agreed with the observation of the relatively broad signals in MALDI-TOF-MS of intact GPx (Fig. 5). With respect to the elution of the high molecular weight peptide around 7 kDa for NO-GPx-ODNB in SDS-PAGE (Fig. 7, lanes 3 and 4), MALDI-TOF-MS indicated a molecular weight of m/z 6839.9, which was not observed in the spectrum of GPx-ODNB (Fig. 8). This value is consistent with the sum of peptides 2 and 3. Collectively, these data indicate that Sec45 or Cys74 in peptide 2 and Cys91 in peptide 3 were cross-linked via Se-S or S-S bridges in NO-GPx-ODNB in a specific manner.

**DISCUSSION**

We previously reported the inactivation of purified bovine GPx by SNAP and cellular GPx by endogenously produced NO via induction of NOS II (27). In this study, we found that GPx was also inactivated by both SIN-1 and peroxynitrite, a more potent oxidant than NO (28, 29). It is noteworthy in this respect that recent reports also demonstrate that aconitase, an enzyme in Krebs cycle and well known as a target of NO, was inactivated by peroxynitrite more strongly than NO (42, 43). Aconitase has an iron-sulfur cluster at the active center, and the inactivation by peroxynitrite is caused by binding to ferrous iron. However, the inactivation mechanism of GPx would be expected to be different from that of aconitase. Because GPx has an essential selenocysteine residue in its active center, which would form a transient selenenyl sulfide with substrate reduced glutathione during the catalytic cycle, nitrososelenocysteine or selenenyl sulfide formation of the residue would lead to inactivation. Although SNAP-mediated inactivation involves reversible and irreversible steps (27), peroxynitrite mediated only irreversible inactivation (Fig. 2), suggesting that the oxidation of the essential thiol or selenol is a cause of the inactivation. Whereas S-nitrosothiols and SNAP act as NO$^+$,
NO, and NO donors under physiological conditions (29). NO\(^+\) would be predicted to be the actual effector molecule that inhibits GPx because NO\(^+\) binds strongly to thiol groups (44, 45). That the essential selenocysteine residue in the active center of GPx binds NO\(^+\) and is then oxidized to form a selenenyl sulfide is a reasonable hypothesis. Peroxynitrite would mediate only selenenyl sulfide formation by its strong oxidant activity. Although there is a report showing that NO does not inactivate GPx (46), the authentic NO and NO donor used in that study would provide NO\(^\cdot\) but not NO\(^+\). This might lead to a different conclusion because NO\(^+\) has a much higher reactivity with respect to thiol and selenol.

Mass spectrometry proved to be a useful technique for the identification of the residues involved in the inactivation of GPx by SNAP. The data indicate that selenocysteine residue (Sec\(^{45}\)) or cysteine residue (Cys\(^{74}\)) are the residues that are oxidized. Despite of the difference in chemical characteristics between Se-S and S-S bonds, any method to definitely distinguish them has not been reported. However, because a selenol is more easily oxidized than a thiol, Sec\(^{45}\) may well represent the specific oxidative site. The formation of a smear on electrophoresis as shown in Fig. 4 also indicates an additional nonspecific oxidative site. Based on selenol reactivity, Sec\(^{45}\) could also be involved in these nonspecific bridges. Destruction of the catalytic center via the formation of a selenenyl sulfide may cause irreversible inactivation of this enzyme. The specific cross-link between Sec\(^{45}\) and Cys\(^{91}\) observed in the present study could be well explained by the evidence that the atomic distance between Se of Sec\(^{45}\) and S of Cys\(^{91}\) in the tertiary structure is 134.2 nm, which is in sufficiently close proximity to allow the formation of a selenenyl sulfide (47). Only a few reports that demonstrate NO-mediated modification directly by identifying specific sites have appeared (7).

Inactivation induced by NO under anaerobic conditions would be expected to proceed via a different pathway. Reaction of NO with the free thiol group of human serum albumin yields a sulfenic acid (R-S-OH) and nitrous oxide (N\(_2\)O) under anaerobic conditions (48). It is hypothesized that human serum albumin with a sulfenic acid (R-S-OH) reacts with reduced glutathione or Cys to form mixed disulfides in plasma. Sulfenic acid is rather stable in human serum albumin because it is derived from the only free thiol group in the molecule. However, GPx has one SeH and 4 SH residues, which would accelerate oxidative linkage between the residues, especially between Sec\(^{45}\) and Cys\(^{91}\). The following schemes may explain the oxidative linkage between the residues under physiological conditions (E; GPx).

\[
\text{E} \xrightarrow{\text{SH}} \text{SeH} + 2 \cdot \text{NO} \rightarrow \text{E} \xrightarrow{\text{SH}} \text{SeOH} + \text{N}_2\text{O} \quad (\text{Eq. 1})
\]

\[
\text{E} \xrightarrow{\text{SeH}} \text{SeOH} \rightarrow \text{E} \xrightarrow{\text{S}} \text{S} + \text{H}_2\text{O} \quad (\text{Eq. 2})
\]

\[
\text{E} \xrightarrow{\text{SH}} \text{SeH} + 2 \cdot \text{NO} \rightarrow \text{E} \xrightarrow{\text{S}} \text{S} + \text{N}_2\text{O} + \text{H}_2\text{O} \quad (\text{Eq. 3})
\]

Thus, for the case of GPx, the selenenic acid residue would easily react with a thiol in the molecule to form a selenenyl sulfide, at which point GPx undergoes irreversible inactivation. Although this work was carried out under anaerobic conditions, similar cross-linking would occur in GPx in vivo where oxygen pressure is very low.

Recent reports demonstrated that oxidative stress is one of the direct causes of apoptotic cell death and that GPx, as well as bcl2, a proto-oncogene that blocks apoptotic cell death in multiple contexts, can prevent apoptosis (21). Reactive oxygen...
species also participate in many cellular events, including signal transduction by growth factors. Hence, the destruction of balance between oxidants and antioxidants by reactive nitrogen species such as NO and peroxynitrite through inhibition of GPx could easily affect cellular homeostasis.

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