S-Nitrosoglutathione Is Cleaved by the Thioredoxin System with Liberation of Glutathione and Redox Regulating Nitric Oxide*

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In activated human neutrophils a burst of nitric oxide (NO) converts intracellular GSH to S-nitrosoglutathione (GSNO) which is subsequently cleaved to restore GSH by an unknown mechanism. We discovered that GSNO is an NADPH oxidizing substrate for human or calf thymus thioredoxin reductase (TR) with an apparent $K_m$ value of 60 $\mu$M and a $K_{cat}$ of $0.6 \times s^{-1}$. Addition of human thioredoxin (Trx) stimulated the initial NADPH oxidation rate severalfold but was accompanied by progressive inactivation of TR. Escherichia coli TR lacked activity with GSNO, but with E. coli Trx present, GSNO was oxidized without inhibition of the enzyme. Chemically reduced E. coli Trx-(SH)$_2$ was oxidized to Trx-S$_2$ by GSNO with a rate constant of 760 $m^{-1}s^{-1}$ (7-fold faster than by GSSG) as measured by tryptophan fluorescence. Analysis of this reaction in the presence of metmyoglobin revealed quantitative formation of metmyoglobin indicative of NO$^\cdot$ release. Analysis of GSNO reduction demonstrated that oxidation of NADPH produced a stoichiometric amount of free GSH. These results demonstrate a homolytic cleavage mechanism of GSNO, giving rise to GSH and NO$. $ GSNO efficiently inhibited the protein disulfide reductase activity of the complete human or calf thymus thioredoxin systems. Our results demonstrate enzymatic cleavage of GSNO by TR or Trx and suggest novel mechanisms for redox signaling.

In physiological systems NO$^\cdot$ has many functions in redox signaling (1–4). Among targets of NO, protein thials are specifically S-nitrosylated (5–7). Thus, nitrosylation of a critical Cys-residue in p21$^{ras}$ activates this G protein (8). In cells glutathione readily reacts with activated NO intermediates, forming the stable adduct S-nitrosoglutathione (GSNO) as shown in activated human neutrophils (9, 10). The observed time-dependent recovery of GSH in the cells (10, 11) could not be accounted for by spontaneous degradation of GSNO. The mechanism of cleavage is thus unknown.

Thioredoxin is a 12-kDa protein with a redox active disulfide in the conserved active site sequence -Cys-Gly-Pro-Cys- located on a protrusion in its three-dimensional structure (12). Reduced thioredoxin (Trx-(SH)$_2$) operates together with thioredoxin reductase and NADPH (the thioredoxin system) as a general protein disulfide reductase (13, 14). NADPH will reduce an active site disulfide in thioredoxin reductase (TR) which then reduces oxidized thioredoxin (Trx-S$_2$) to Trx-(SH)$_2$ which is reoxidized in a direct reaction with a disulfide (Reactions 1–3).

NADPH + $H^+$ + TR-S$_2$ $\rightarrow$ NADP$^+$ + TR-(SH)$_2$  
REACTION 1

TR-(SH)$_2$ + Trx-S$_2$ $\rightarrow$ TR-S$_2$ + Trx-(SH)$_2$  
REACTION 2

Trx-(SH)$_2$ + protein-S$_2$ $\rightarrow$ Trx-S$_2$ + protein-(SH)$_2$  
REACTION 3

Since the disulfides of insulin efficiently oxidize Trx-(SH)$_2$ (Reaction 3 (15)), this protein is used as a classical substrate to test the activity of the Trx system (15). The direct reaction between Trx-(SH)$_2$ and insulin ($K_m > 5 \times 10^2 \text{m}^{-1} s^{-1}$) is more than 10$^4$ times faster than the equivalent reaction with the well known dithiothreitol (DTT) molecule (13). Mammalian thioredoxin reductases (M, 116,000) show an intriguing substrate specificity, reducing not only thioredoxins from many species but also 5,5$'$-dithiobis(2-nitrobenzoic acid) (DTNB), selenodiglutathione, vitamin K, or alloxan (13–15). In contrast, the well characterized Escherichia coli TR (M, 70,000) is highly specific for the homologous Trx and some related prokaryotic thioredoxins (13–15).

The aim of the present study was to investigate whether TR or Trx could catalyze the reduction of GSNO. Our results showed that oxidation of NADPH by GSNO in the presence of TR and Trx released GSH and nitric oxide. Incubations with GSNO also inhibited the activity of the mammalian thioredoxin system.

MATERIALS AND METHODS

GSNO was synthesized by reaction of GSH and acidified sodium nitrite as described elsewhere (16) and was of better than 97% purity by HPLC analysis (17). Upon synthesis GSNO was stored in the dark at 20 °C. The GSNO concentration was determined using a millimolar extinction coefficient of 0.92 at 335 nm (16). TR was purified to homogeneity from human placenta and calf thymus essentially as described previously (15). Human Trx was a pure recombinant preparation (18) and was reduced prior to use by incubation with DTT followed by a desalting step on a NAP-5 Sephadex G-25 column (18). Homologous preparations of E. coli TR and E. coli Trx were prepared as described elsewhere (15). Horse heart oxymyoglobin (Sigma) was prepared by dithionite reduction of myoglobin in 100 mM potassium phosphate, pH 7.4, followed by desalting on a NAP-5 Sephadex G-25 (Pharmacia Biotech Inc.) column equilibrated with the same buffer. This oxymyoglobin solution was stored at −20 °C until use (5). Metmyoglobin was prepared by oxidizing horse heart myoglobin in 100 mM potassium phosphate, pH 7.0, with a 5% molar excess of potassium ferricyanide in the same buffer. This was followed by a desalting step on a NAP-5 Sephadex G-25
Reactivity of Nitrosothiols with Thioredoxin System

column equilibrated with the same buffer (5).

Spectrophotometric Measurements—All kinetic experiments were performed in 50 mM Tris-Cl, 1 mM EDTA, pH 7.5, containing 400 μM NADPH at 25°C. The oxidation of NADPH was followed at 340 nm in semimicro Quartz cuvettes (final volume of the reaction was 0.5 ml) using a Shimadzu UV2100 or a Zeiss PMQIII spectrophotometer and calculated with a millimolar extinction coefficient of 6.2. Since GSNO absorbs light at 340 nm, the reference cuvette usually contained GSNO at the same concentration as the sample cuvette to correct for the absorption of this compound. Fresh stock solutions of GSNO were prepared in H2O and kept on ice between experiments. During the time and under our experimental conditions spontaneous degradation of GSNO was negligible with a measured half-life of more than 10 h. Details of enzyme activity determinations using insulin reduction experiments have been described previously (13, 15).

Anaerobic Experiments—The method previously described was used, with a modification of solution of GSNO and measurements in special cuvettes covered by rubber septa (19).

Myoglobin Assay—The myoglobin assay was utilized to determine whether NO or NO· species were released (5) in the reaction of Trx-(SH)2 with GSNO. NO· release was followed by monitoring the conversion of oxymyoglobin to metmyoglobin spectrophotometrically (20, 21). Spectra from 520 to 640 nm were recorded at specified time intervals. Decreases in absorbance at 542 and 580 nm, accompanied by increases at 632 were considered indicative of metmyoglobin formation and NO·

Decreases in absorbance at 542 and 580 nm, accompanied by increases at 632 were considered indicative of metmyoglobin formation and NO· release. The assay was performed in 100 mM potassium phosphate, 1 mM EDTA, pH 7.4, by mixing equimolar amounts (100 μM) of Trx-(SH)2 and GSNO in the presence of 40 μM oxymyoglobin. NO· release was monitored spectrophotometrically by following the reduction of oxymyoglobin to metmyoglobin in the presence of anaerobic conditions (20, 21). Spectra from 520 to 640 nm were recorded at specified time intervals. Increases in absorbance at 542 and 580 nm, accompanied by decreases at 640 nm were considered indicative of the reduction of Fe(II) myoglobin to Fe(II) myoglobin and NO· release. Assay solutions contained 40 μM metmyoglobin to which equimolar amounts (100 μM) of Trx-(SH)2 and GSNO were added in 100 mM potassium phosphate, 1 mM EDTA, pH 7.4. The extinction coefficient for oxymyoglobin at pH 7.0 is 13.9 mM−1 cm−1 at 542 nm and 14.4 mM−1 cm−1 at 580 nm (21), while for GSNO it is 0.015 mM−1 cm−1 at 545 nm (16) and its contribution to the absorbance at these wavelengths was disregarded. The measurements were performed using a Shimadzu UV2100 spectrophotometer.

Fluorescence Measurements—Protein fluorescence of E. coli Trx was measured with a thermostated Spec FluoroMax spectrophotofluorimeter at 25°C. Trx-S5 was reduced by a 100-fold molar excess of DTT at 37°C for 15 min followed by careful removal of DTT on a 3 ml column of NAP-5 Sephadex G-25, which was equilibrated with N2-treated 50 mM Tris-Cl, 1 mM EDTA, pH 7.5. Excitation of tryptophan fluorescence in Trx was done at 280 nm and emission spectra from 300 to 400 were recorded (22). For spectral recordings at different times the samples contained 1.0 μM Trx-(SH)2 in 50 mM Tris-Cl, 1 mM EDTA, pH 7.5 to which 10 μM GSNO was added. Reaction rates were obtained by following the decrease of fluorescence at the emission wavelength of 350 nm after mixing 1.0 μM GSNO and 1.0 μM Trx-(SH)2. In other reactions the rate using GSNO (10 μM) in place of GSNO was determined.

Determination of the Reaction Stoichiometry—A reaction between GSNO and E. coli TR and Trx was performed in 50 mM Tris-Cl, 1 mM EDTA, pH 7.5, containing 400 μM NADPH in a final volume of 750 μl. To both the sample and the reference cuvette was added 200 μM GSNO. The reaction was started by adding to the sample cuvette E. coli TR and Trx to final concentrations of 0.1 and 5.0 μM, respectively. The oxidation of NADPH was started at 340 nm for 20 min. Aliquots (10 μl) were taken from both the sample and the reference cuvette, every 15 min and analyzed by HPLC separation (17) as described below. The net free thiol content in the sample after a 45-min reaction was determined at 412 nm by addition of 1 ml of 1 M DTNB in 6 mM guanidine HCl (15, 23).

The reaction stoichiometry using homogeneous human placenta TR was also analyzed in 1.00 ml of 50 mM Tris-Cl, 1 mM EDTA, pH 7.5, containing 200 μM NADPH. To both the sample and the reference cuvette was added 50 μM GSNO. The reaction was started by adding human placenta TR (0.1 μM) to the sample cuvette and oxidation of NADPH was followed at 340 nm during 20 min. Aliquots (100 μl) were taken from both the sample and the reference cuvettes at 5, 10, and 20 min and the free thiol content was determined at 412 nm after addition of 5 volumes of 1 mM DTNB (15, 23), and GSNO was determined by HPLC analysis.

HPLC Analysis of GSNO and Reaction Products—Aliquots (50 μl) to be analyzed were immediately treated by centrifugal filtration for 5 min at 4°C to remove protein (Ultrafree-MC, Millipore), and the filtrates were analyzed by HPLC separation (17). Chromatographic conditions were as follows: stationary phase, C18 column, 250 × 4.6 mm; particle size, 5.0 μm (Supelco, Sweden); and mobile phase, isocratic elution at 1 ml/min with 80% methanol, 80% NAP-PO4 containing 1 mM 1-octanesulfonic acid, pH 2.4. Detection was at 220 nm. The retention times of authentic GSNO and nitrite were 6.4 ± 0.5 and 7.6 ± 0.5 min, respectively. Standards were run of GSNO, GSSG, and sodium nitrite.

RESULTS

GSNO Is a Substrate for Mammalian Thioredoxin Reductase—When NADPH and calf thymus TR (0.1 μM) were mixed with different concentrations of GSNO (10–200 μM) the absorbance at 340 nm decreased, demonstrating a time-dependent oxidation of NADPH. The reactions were enzyme-dependent and linear for extended periods of time. When the initial rates were plotted against the concentrations of GSNO (Fig. 1A), the apparent K_m value for GSNO in the reaction was 60 μM and the apparent V_max was 3.5 μM min⁻¹ equivalent to a K_cat of 0.6 × 1 s⁻¹. The K_m/K_cat calculated for the reaction was 10⁵ M⁻¹ s⁻¹ which is about 2% of the corresponding K_m/K_cat (10⁷ M⁻¹ s⁻¹) for Trx-S2, the natural substrate for the calf thymus enzyme (15). Human placenta TR showed the same reactivity as the calf thymus enzyme. Glutathione reductase from yeast or rat liver showed no activity with GSNO (data not shown) in agreement with the result of others (17) and the preparation of TR used was devoid of such activity. Determination of the reaction stoichiometry at different time points showed that the oxidation of 1 mol of NADPH was followed by the formation of 1.0 ± 0.1 mol of free SH groups as determined by DTNB (15, 23), indicating release of GSH.

Effects of Addition of Human Thioredoxin to Thioredoxin Reductase—With calf thymus TR (0.1 μM), the addition of human TRx (5.0 μM) strongly increased the initial reaction rates with lower concentrations of GSNO indicating that the reduced forms of both TR and Trx were oxidized by GSNO (Fig. 1B). However, with Trx present, linear reactions were observed only for progressively shorter time periods dependent on increasing GSNO concentrations. This is shown by the fact that the plot of the rates calculated between the first and second min actually decreased at high (>50 μM GSNO) concentrations (Fig. 1B). This time-dependent inhibition of the activity made comparisons of K_m values difficult, but as seen from Fig. 1B the apparent K_m value for GSNO with Trx present may be in the range of 10 μM, apparently lower than that seen with only TR.

Reactivity of GSNO with E. coli Thioredoxin Reductase and Thioredoxin—As seen from Fig. 1C a concentration-dependent oxidation of NADPH was obtained with GSNO without any major signs of inhibition at higher concentrations. The apparent K_m value for GSNO in this reaction was 100 μM. The direct activity of E. coli TR with NADPH and GSNO was very low (Fig. 1C), and the reaction was thus dependent on Trx. This is in agreement with the known high specificity of the E. coli enzyme (13–15). Repeated experiments showed that oxidation of 1 mol of NADPH by 1 mol of GSNO was accompanied by formation of 1.1 mol of free SH groups as determined by DTNB in 6 mM guanidine-Cl (15), indicating release of GSH.

Anaerobic Enzyme Assays—The reactivity of GSNO with the mammalian and the E. coli thioredoxin system was also examined in the absence of oxygen. Thus, anaerobic measurements using the E. coli thioredoxin system and GSNO showed decreased reaction rates as compared to aerobic conditions (Table I). Also the reactivity of the mammalian thioredoxin system with GSNO was significantly lower (Table I). With the E. coli TR system admission of air resulted in increased reaction rates after a short lag phase. Under both aerobic and anaerobic conditions the oxidation of 1 mol of NADPH resulted in the generation of 1 mol of free SH groups, as analyzed by DTNB.

HPLC Analysis of the Reaction Products—Characterization...
of products from the reactions of GSNO with the Trx systems
was achieved by HPLC analysis (Fig. 2). Aliquots were taken
from the reaction mixture at defined time points, and elution
profiles were compared with known standards. This proved
that consumption of GSNO lead to formation of the products
GSH and nitrite (NO2
2
); the loss of GSNO was the same as
calculated from the oxidation of NADPH independently deter-
mained at 340 nm.

Oxidation of Trx-(SH)2 by GSNO—The direct reaction be-
tween GSNO and chemically reduced Trx-(SH)2 was also de-
termined. E. coli Trx-(SH)2 containsonly twosulfhydrylgroups
which are located in the active site and has a 3-fold higher
tryptophan fluorescence emission compared with the oxidized
form (22). Mixing GSNO and Trx-(SH)2 resulted in a time-de-
pendent fluorescence decrease demonstrating oxidation to
Trx-S2 (Fig. 3A). From analysis of the rate using equimolar or
excess of GSNO, the reaction was shown to follow apparent
second order kinetics, with a value for K
2
 of 760 M
2
 s
2
1.
The corresponding reaction with GSSG, one of the potential prod-
cuts of GSNO cleavage, was also measured (Fig. 3B). GSSG
showed a slower reaction (100 M
2
 s
2
1), in agreement with
previous spectrophotometric measurements showing that
GSSG is a relatively inert substrate for Trx-(SH)2 (13). The results thus demonstrated that GSNO was around 7-fold more
efficient than GSSG as an oxidant of Trx-(SH)2.

Myoglobin Assay (NO
z
/NO
2
)—When oxymyoglobin (Fe(II))
was incubated with Trx-(SH)2 and GSNO a time-dependent
complete conversion to metmyoglobin (Fe(III)) was observed,
indicating release of NO
z
 (Fig. 4A). In the same time frame
there was no significant interaction between oxymyoglobin and
either Trx-(SH)2 or GSSG in separate incubations (data not
shown). There was little if any conversion of metmyoglobin to
Fe(II)-nitrosylmyoglobin when incubated with Trx-(SH)2 and

\begin{table}
\centering
\begin{tabular}{|c|c|c|c|c|}
\hline
GSNO & Mammalian Trx system & \multicolumn{2}{|c|}{E. coli Trx system} \\
& Anaerobic & Aerobic & Anaerobic & Aerobic \\
\hline
\hline
10 & 0.012 & 0.012 & 0.002 & 0.003 \\
40 & 0.009 & 0.014 & 0.007 & 0.012 \\
100 & 0.002 & 0.010 & 0.010 & 0.025 \\
\hline
\end{tabular}
\caption{Comparison of influence of oxygen on the rate of GSNO reduction}
\end{table}
GSNO (Fig. 4B), thus showing that NO\(^{-}\) was not released. Parallel fluorescence measurements showed that oxidation of Trx-(SH)\(_2\), (1.0 \(\mu\)M) in \(\mathrm{N}_2\)-treated 50 mM Tris-Cl 1 mM EDTA, pH 7.5, at 25 \(^\circ\)C was determined from 300 to 400 nm, using excitation at 280 nm. The reaction was started by addition of GSNO (10 \(\mu\)M) and the fluorescence spectrum was measured at defined time points. B, comparison of the oxidation rate of E. coli Trx-(SH)\(_2\) with GSNO and GSSG. The reactions were followed as the decrease in fluorescence emission at 350 nm using excitation at 280 nm. The reaction was started by mixing 1.0 \(\mu\)M Trx-(SH)\(_2\) with either 1 \(\mu\)M of GSNO or GSSG in a final volume of 1.0 ml.

**DISCUSSION**

Our results demonstrate that GSNO is reduced by either human or calf thymus thioredoxin reductase or also by thioredoxin. That GSNO is an oxidizing substrate for Trx-(SH)\(_2\) was directly shown by spectrophotometric measurements in the presence of E. coli TR which had negligible activity by itself or by direct recording of the well known intrinsic tryptophan fluorescence change characteristic of formation of the disulfide in Trx-S\(_2\) (15, 22). E. coli Trx-(SH)\(_2\), with known high resolution structure in solution (24), has only the sulfur of Cys 32 exposed in a hydrophobic active site surface, whereas the thiol group of Cys-35 is buried. The Cys-32 thiol acts as a potent nucleophile (12). The reaction of Trx-(SH)\(_2\) with GSNO is proposed to operate by Reactions (4–7). The products of this direct reaction, Trx-S\(_2\) and NO\(^{+}\), have been determined experimentally. Superoxide radical (O\(_2^{-}\)) has been qualitatively detected by the \(\mathbf{l}\)-
epinephrine method (25) where the formation of the product, adrenochrome, was followed as the change of absorbance at 480 nm (data not shown). The mechanism shown in Reactions 6 and 7 has been proposed for phenoxyl radical-catalyzed oxidation of E. coli Trx (26). Reaction 8 summarizes our experimental results and is consistent with a mechanism of homolytic cleavage catalyzed by thioredoxin (Reaction 8).

$$\text{Trx-(SH)}_2 + \text{GSNO} + \text{O}_2 \rightarrow \text{Trx-S}_2 + \text{GSH} + \text{NO}^+ + \text{O}_2^-$$  

**REACTION 8**

Trx-S2 will be generated in the presence of TR and NADPH (Reactions 1 and 2) and thus one mol of NADPH will be consumed per mol of GSNO reduced. The fact that Reaction 8 is oxygen-dependent can explain the observed faster reaction rates of the mammalian and the E. coli thioredoxin system with physiological concentrations of GSNO in the presence of oxygen as compared to anaerobic conditions.

Thioredoxin reductase from mammalian cells directly reduced GSNO. This enzyme also presumably contains a dithiol in the active site after reduction by NADPH (Reactions 1 and 2) which will be reoxidized by GSNO. In particular, it should be noted that human or calf thymus TR have no activity with GSSG as a substrate so this rules out any mechanism with GSSG as substrate in the enzyme-catalyzed reaction. However, since the amount of the enzyme available is the limiting factor we have not yet been able to further pursue the elucidation of the mechanism for the enzyme with GSNO. Although Trx-(SH)2 can reduce GSSG, a homolytic cleavage mechanism involving GSSG is unlikely and we demonstrated that GSNO itself oxidized Trx-(SH)2 7-fold faster than GSSG.

As seen from Reaction 8, the product of GSNO reduction by Trx-(SH)2 is NO. The NO/rhemp is an unstable species which reacts with oxygen (27, 28) yielding nitrile (Reactions 9 and 10). NO can also react with superoxide radical (29) giving rise to peroxynitrite (Reaction 11), which can decompose to toxic hydroxyl radical or ultimately to nitrate (Reaction 12).
strate, GSNO was shown to inhibit the mammalian thioredoxin system, through a novel mechanism which may regulate activity. Both low molecular weight S-nitrosothiols, like GSNO or S-nitrosocysteine, and S-nitrosoproteins have been suggested to store NO and drastically prolong the half-life in vivo (4, 30). The observed ability of the mammalian thioredoxin system to cleave the S-nitrosothiol bond in GSNO indicates that other cellular nitrosothiols, such as nitrosylated proteins, are good substrates. Trx and TR are expressed in variable amounts in different cells and also do not always coexist (31). This in combination with a relation to cell membranes may be relevant for the release and metabolism of nitrogen oxides in specific tissues. Further studies of the reactivity of the thioredoxin system with S-nitrosylated proteins like p21 ras, the mechanisms of the reaction and its in vivo effects are in progress.

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