Selenodiglutathione Is a Highly Efficient Oxidant of Reduced Thioredoxin and a Substrate for Mammalian Thioredoxin Reductase*

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Selenium compounds like selenite (SeO₄²⁻) may form a covalent adduct with glutathione (GSH) in the form of selenodiglutathione (GS-Se-SG), which is assumed to be important in the metabolism of selenium. We have isolated GS-Se-SG and studied its reactions with NADPH and thioredoxin reductase from calf thymus or with thioredoxin reductase and thioredoxin from *Escherichia coli*. Incubation of 0.1 μM calf thymus thioredoxin reductase or 0.1 μM thioredoxin reductase and 1 μM thioredoxin from *E. coli* with 5, 10, or 20 μM GS-Se-SG resulted in a fast initial reaction, followed by a large and continued oxidation of NADPH. However, anaerobic incubation of 0.1 μM calf thymus thioredoxin reductase and 20 μM GS-Se-SG resulted only in oxidation of a stoichiometric amount of NADPH; admission of oxygen started continuous NADPH oxidation. Contrary to the mammalian enzyme, GS-Se-SG was not a substrate for thioredoxin reductase from *E. coli*. The rate of the oxygen-dependent reaction between calf thymus thioredoxin reductase and GS-Se-SG was increased 2-fold in the presence of 4 mM GSH, indicating that HSe⁻ was the reactive intermediate. Glutathione reductase from rat liver reduced GS-Se-SG with a very slow continued oxidation of NADPH, and the presence of the enzyme did not affect the oxygen-dependent nonstoichiometric oxidation of NADPH by GS-Se-SG and thioredoxin reductase. Fluorescence spectroscopy showed GS-Se-SG to be a very efficient oxidant of reduced thioredoxin from *E. coli* and kinetically superior to insulin disulfides. Thioredoxin-dependent reduction of CDP to dCDP by ribonucleotide reductase was effectively inhibited by GS-Se-SG.

Selenium is an essential trace element for higher eukaryotes. The best established function is its presence in the detoxifying enzyme glutathione peroxidase as a selenocysteine residue in the active site (Rotruck et al., 1973). Several selenium compounds have been shown to have inhibitory effects on mammalian cell growth, particularly tumor cells (Shamberger, 1985; Medina and Oborn, 1984); to a limited extent, this property has even been used in animal antitumor therapy (Weisberger and Suhrland, 1956). Furthermore, selenium is an essential trace nutrient for growth of cells in synthetic media containing a low concentration of serum or under serum-free conditions (McKeohan et al., 1976). The physiological and antiproliferative mechanisms of action of selenium compounds are largely unknown. One suggested antiproliferative mechanism is an interaction with reduced glutathione (GSH) (Batist et al., 1986).

Selenite and reduced glutathione react according to Painter (1941):

\[ 2H^+ + 4\text{GSH} + \text{SeO}_4^{2-} \rightarrow \text{GS-SG} + \text{GS-Se-SG} + 3\text{H}_2\text{O} \]

Bordet and Milner (1941) have suggested that GS-Se-SG is a substrate for the thioredoxin reductase, resulting in reduction to GS-Se⁻ and GSH and oxidation of a stoichiometric amount of NADPH. Poirier and Milner (1984) have suggested that GS-Se-SG is one of the most effective selenium compounds in inhibiting the growth of neoplastic cells; Vernier et al. (1979) have also suggested that GS-Se-SG is a strong inhibitor of protein synthesis in Swiss 3T3 fibroblasts. However, proof for this effect is lacking, and the mechanism remains unknown.

Thioredoxin is a small (12 kDa) ubiquitous protein with a redox-active dithiol/disulfide in the active site. It operates in the thioredoxin system as a general protein disulfide reductase (Holmgren, 1988).

\[
\begin{align*}
\text{Trx-S}_{\text{SH}} + \text{NADPH} + H^+ \xrightarrow{\text{TR}} \text{Trx-(SH)}_2 + \text{NAPDH}^+ \\
\text{Trx-(SH)}_2 + \text{protein-S}_{\text{SH}} \rightarrow \text{Trx-S}_{\text{SH}} + \text{protein-(SH)}_2
\end{align*}
\]

One function of thioredoxin involves acting with thioredoxin reductase as an NADPH-dependent hydrogen donor for ribonucleotide reductase (Thelander and Reichard, 1979). This enzyme produces the precursors for DNA replication. In addition, thioredoxin can regulate the activity of a system via thiol redox control (Holmgren, 1985, 1989). This process involves changes in the activity of an enzyme or receptor via dithiol/disulfide interchange reactions.

Selenite was recently found to be a substrate for the thioredoxin system, causing nonstoichiometric oxidation of NADPH. The purpose of our work was to determine whether a product of selenium metabolism (GS-Se-SG) is a substrate for the thioredoxin system. GSSG is not a substrate for thioredoxin reductase and is a poor disulfide substrate for reduced thioredoxin (Holmgren, 1979b, Holmgren, 1985). However, contrary to expectation, insertion of a selenium atom in GSSG made this compound a highly reactive substrate for the thioredoxin system, capable of redox cycling in the presence of oxygen. Thus, interactions among GS-Se-SG, thioredoxin, and thiol-disulfide interchange reactions may be involved in selenium's effects on cell growth and proliferation.

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1 The abbreviations used are: GS-Se-SG, selenodiglutathione; Trx-S₂, oxidized thioredoxin; Trx-(SH)₂, reduced thioredoxin; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HPLC, high-pressure liquid chromatography.

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triodoxin, and triiodoxin reductase suggest mechanistic explanations for some of the physiological actions of selenium compounds on cell proliferation.

**MATERIALS AND METHODS**

Selenium dioxide was from Fluka AG Chemische Fabrik. Dithiothreitol, GSH, and GSSG were from Sigma. NADPH was from Boehringer Mannheim, and bovine insulin was from Nordisk Insulin A/S (Gentofte, Denmark). The C18 column used to separate GS-Se-SG from GSSG was a 30-μm Versapack (10 μm) with an inner diameter of 4.1 mm, from Altitech Associates, Inc. [3H]CDP was from Amersham Corp., and other components of the ribonucleotide reductase assay were as described previously (Holmgren, 1979a).

**Enzyme Preparations**—Thioredoxin from Escherichia coli was a homogenous preparation; the concentration was determined at 280 nm using a molar extinction coefficient of 13,700 (Holmgren and Reichard, 1967). Thioredoxin reductase from E. coli was from IMCO Corp. (Stockholm, Sweden). Thioredoxin reductase from calf thymus was purified to homogeneity essentially as described for the rat liver enzyme (Luthman and Holmgren, 1982). Ribonucleotide reductase from E. coli was a kind gift from Prof. B.-M. Sjöberg (Stockholm University). Glutathione reductase was a pure preparation from rat liver (Carlberg and Mannervik, 1975). The activity of the enzyme was determined as described by Worthington and Rosemeyer (1974). One unit of glutathione reductase is equal to 1 μmol of GSSG reduced per min, and 1.0 unit/ml is taken as 40 μmol enzyme.

**Preparation of Selenodiglutathione**—Selenium dioxide (SeO₂) was dissolved in water (SeO₂ + H₂O → SeO₂⁻ + 2H⁺) to 0.1 M and 10 μl was mixed with 60 μl of 0.1 M HCl. Finally, 40 μl of 0.1 M GSH was added to the reaction mixture. After incubation in room temperature for 1 min, the reaction products (GSSG and GS-Se-SG) were separated by HPLC (Nakagawa et al., 1988) on a C18 column in water adjusted to pH 2 with HCl; the flow rate was 2 ml/min. The separation of GS-Se-SG and GSSG was followed at 263 nm, and the concentration of GS-Se-SG was determined using a millimolar extinction coefficient of 1.87 (Ganther, 1971); the accuracy of this was checked by amino acid analysis (data not shown).

**Enzyme Assays**—The reactions between selenodiglutathione and triiodoxin reductase from calf thymus, glutathione reductase from rat liver, or triiodoxin plus triiodoxin reductase from E. coli were performed in 50 mM Tris-HCl (pH 7.5), 1 mM EDTA, generally containing 200 μM NADPH. When 10 nM triiodoxin reductase was used, 0.1% bovine serum albumin was included in the reaction mixture. The oxidation of NADPH was followed at 340 nm in semimicro quartz cuvettes (final volume of the reaction mixture was 500 μl) using a Zeiss PMQ3 spectrophotometer and determined with a millimolar extinction coefficient of 1.87 (Ganther, 1971); the accuracy of this was checked by amino acid analysis (data not shown).

**Anaerobic Enzyme Assays**—The anaerobic reactions between triiodoxin reductase from calf thymus and GS-Se-SG were performed in special cuvettes covered with rubber septa. To the cuvettes was added 50 mM Tris-HCl (pH 7.5), 1 mM EDTA containing 200 μM NADPH plus triiodoxin reductase (to a final volume of 500 μl). The content of the cuvettes and GS-Se-SG in a separate tube were bubbled with argon through a needle penetrating the rubber septum. Through another needle, air was evacuated. To remove any contaminating oxygen, the argon was washed by pretreatment with solutions of 2 and 0.2% sodium dithionite in 0.1 M NaOH. After the argon treatment, the reaction was started by injection of GS-Se-SG into the cuvette and of an equal volume of buffer in the reference cuvette by Hamilton syringes.

**Kinetics of Reaction Between Reduced Thioredoxin and GS-Se-SG, GSSG, or Insulin Determined by Fluorescence Spectroscopy**—Thioredoxin from E. coli (300 nmol) was reduced by a 100-fold molar excess of dithiothreitol at 37 °C for 10 min and desalted on a 9-ml PD-10 column (Pharmacia LKB Biotechnology Inc.) equilibrated with N2-treated 50 mM Tris-HCl (pH 7.5), 1 mM EDTA. The oxidation of Trx-(SH)₂ by GS-Se-SG, GSSG, or insulin was followed as a decrease in fluorescence at an emission wavelength of 350 nm with excitation at 280 nm; fluorescence was measured on a Shimadzu RF-501PC fluorescence spectrophotometer. To each cuvette was added 50 mM Tris-HCl (pH 7.5), 1 mM EDTA containing 200 nM Trx-(SH)₂ plus 200 nM GS-Se-SG, GSSG, or insulin (final volume of the reaction mixture was 1.0 ml). The reaction was started by addition of GS-Se-SG or the disulfide compound to the cuvette.

**Inhibition of Triiodoxin-dependent Ribonucleotide Reductase Activity by GS-Se-SG**—A reaction mixture was made with 45 μl of 25 mM [3H]CDP, 30 μl of 0.1 M ATP, 120 μl of 0.2 M MgCl₂, 60 μl of 20 mg/ml bovine serum albumin, 75 μl of 1.0 M HEPES (pH 7.6), 45 μl of 40 mg/ml NADPH, and 37.5 μl of water. To each tube were added 40 μl of reaction mixture and triiodoxin and triiodoxin reductase from E. coli to final concentrations of 500 and 50 nM, respectively, and 57 μg of ribonucleotide reductase from E. coli, all in a final volume of 120 μl. GS-Se-SG was also added to a final concentration 0–100 μM. Since the stock solution of GS-Se-SG was acidic (pH 2), the pH was increased to 7.0 by addition of 2 M sodium acetate immediately prior to use. After 5 min of incubation at 37 °C, the reactions were stopped by addition of 1 ml of 1 M perchloric acid. The nucleotides were hydrolyzed by heating, and dCMP was separated from CMP by chromatography on a Dowex 50 column as described by Thelander and Reichard (1979) and Thelander et al. (1978).

**RESULTS**

**Preparation of Selenodiglutathione**—Ganther (1971) prepared GS-Se-SG by mixing SeO₂⁻ and GSH in a ratio of 1:4 and separating GSSG and GS-Se-SG using Dowex 50 in the presence of a metal ion. We found that reverse-phase separation with HPLC (Nakagawa et al., 1988) gave better results. The reaction mixture made by mixing SeO₂⁻ and GSH was eluted in three major well-separated peaks (Fig. 1); the third peak was pure GS-Se-SG. Control experiments showed that GSSG and any unreacted SeO₂⁻ eluted well before the GS-Se-SG peak within the first minutes after injection. A sample of SeO₂⁻ injected separately was detected by assay with calf thymus triiodoxin reductase. Also, GSSG in the expected amount, generated in a complete Painter (1941) reaction, eluted as one single peak identical in area and position to the second peak from the reaction mixture. Thus, a pure preparation of GS-Se-SG was obtained.

**Oxidation of Reduced Thioredoxin**—E. coli Trx-(SH)₂ is known to decrease its tryptophan fluorescence emission 3-fold upon oxidation to Trx-S²⁻ (Holmgren, 1972). Addition of 200 nM GS-Se-SG to 200 nM Trx-(SH)₂ resulted in a fast oxidation of thioredoxin; after 25 s, all thioredoxin was oxidized (Fig. 2). The reaction showed an increasing velocity. The reaction between Trx-(SH)₂ and insulin was slower and followed an apparent second-order reaction (Krause et al.,
fig. 2. kinetics of oxidation of Trx-(SH)2 by GS-Se-SG and disulfide compounds. The fluorescence of Trx-(SH)2 and Trx-S2- was measured at an emission wavelength of 350 nm with excitation at 280 nm. The reaction was started by rapid mixing of 200 nM GS- Se-SG (C), 200 nM GSSG (O), or 200 nM insulin (■) with 200 nM E. coli Trx-(SH)2, after which the fluorescence was recorded. The concentra-tions of Trx-(SH)2 at various times were calculated from the relative height of reduced and oxidized thioredoxin fluorescence.

fig. 3. GS-Se-SG-dependent oxidation of NADPH catalyzed by calf thymus thioredoxin reductase. The reaction was performed in 50 mM Tris-HCl (pH 7.5), 1 mM EDTA containing 200 μM NADPH with 100 nM thioredoxin reductase plus 5 μM (C) and 10 μM (■) GS-Se-SG or with 10 nM thioredoxin reductase plus 10 μM GS-Se-SG (O). The reference cuvette contained all components except GS-Se-SG. The arrow indicates addition of GS-Se-SG to the sample cuvette.

1991). In contrast, GSSG was a very poor oxidant of Trx-(SH)2 (Fig. 2), in agreement with previous results (Holmgren, 1979b).

reaction between GS-Se-SG and Mammalian Thioredoxin Reductase—Incubation of 100 nM thioredoxin reductase from calf thymus and 5 or 10 μM GS-Se-SG resulted in a fast initial oxidation of a stoichiometric amount NADPH, followed by a continued oxidation (Fig. 3). After 60 min, the presence of 5 μM GS-Se-SG resulted in oxidation of 100 μM NADPH or a ratio of GS-Se-SG to NADPH of 1:20 (data not shown). A plot of the rate (ΔA340 × min⁻¹) of the continuous reaction against increasing initial concentrations of GS-Se-SG (5–20 μM) was sigmoidal in shape (data not shown). Incubation of a low concentration of thioredoxin reductase (10 nM) with 10 μM GS-Se-SG resulted in a slow but continuous oxidation of NADPH (Fig. 3).

The continuous reaction was oxygen-dependent (Fig. 4) since anaerobic incubation resulted only in the fast stoichiometric oxidation. When air was bubbled through the cuvette, the continuous reaction started with a slight lag phase (Fig. 4). Addition of 4 mM GSH (a physiological concentration) to the reaction mixture resulted in a 2-fold increased reaction rate (Fig. 5), indicating that the presence of GSH facilitates the formation of active GS-Se-SG metabolites. This is physiologically important since the concentration of GSH is high in virtually all tissues.

Interaction among Mammalian Thioredoxin Reductase, Glutathione Reductase, and GS-Se-SG—When 10 μM GS-Se-SG was added to 1 unit/ml (40 nM) rat liver glutathione reductase, there was a stoichiometric fast oxidation of 10 μM NADPH (Fig. 6). However, from this point, any further reaction was very slow; after 15 min, slightly less than 20 μM NADPH was oxidized. Thus, the reaction between mammalian thioredoxin reductase and GS-Se-SG was superior in oxidizing NADPH (Figs. 3 and 4). To investigate if the presence of glutathione reductase influenced the reaction between thioredoxin reductase and GS-Se-SG, we added 10 μM GS-Se-SG and 100 nM thioredoxin reductase to 40 nM glutathione reductase (Fig. 6). Contrary to expectation, the presence of glutathione reductase did not influence the continuous oxidation of NADPH by thioredoxin reductase and GS-Se-SG. This is important since glutathione reductase and thioredoxin reductase are both present in mammalian cells.

reaction between Thioredoxin Reductase Plus Thioredoxin
from E. coli and GS-Se-SG—Contrary to mammalian thioredoxin reductase, GS-Se-SG was not a substrate for E. coli thioredoxin reductase (data not shown). However, after addition of E. coli thioredoxin, there was a fast stoichiometric oxidation, followed by a continuous oxidation of NADPH. Thus, mixing 100 nM thioredoxin reductase, 1 μM thioredoxin, and 10 μM GS-Se-SG resulted in oxidation of 40 μM NADPH in 15 min (Fig. 7).

Inhibition of CDP Reduction—Selenodiglutathione was an inhibitor of CDP reduction by ribonucleotide reductase. GS-Se-SG at 100 μM completely inhibited formation of dCDP by ribonucleotide reductase with E. coli thioredoxin reductase plus thioredoxin as a hydrogen donor (Fig. 8). The most obvious mechanism of this inhibition is oxidation of reduced thioredoxin by GS-Se-SG. However, we have not excluded a direct effect of GS-Se-SG on the redox-active SH groups in the active site of ribonucleotide reductase.

DISCUSSION

This paper reports novel interactions between the thioredoxin system and selenodiglutathione. The result of these reactions was a large consumption of NADPH and oxidation of reduced thioredoxin. GSSG is no substrate for thioredoxin reductase or a very poor substrate for the thioredoxin system (Holmgren, 1978b). However, introduction of a selenium atom into the molecule made it highly reactive.

Ganther (1971) has shown that GS-Se-SG is a substrate for NADPH and glutathione reductase from yeast. In this reaction, the stoichiometry is slightly higher than 1 mol of NADPH oxidized per mol of GS-Se-SG, as also seen with our experiments using the rat liver enzyme. Our results show that the reaction between calf thymus thioredoxin reductase and GS-Se-SG was much more efficient in oxidizing NADPH. In 15 min, 10 μM GS-Se-SG oxidized 60 μM NADPH; and after this time, there was no sign of decreased reaction rate. Because of the efficient decomposition of GS-Se-SG by glutathione reductase (Ganther, 1971), an influence of the reaction between thioredoxin reductase and GS-Se-SG by glutathione reductase could be expected. However, the presence of 1 unit/ml (40 nM) glutathione reductase neither decreased nor inhibited the reaction with thioredoxin reductase. Considering that both glutathione reductase and thioredoxin reductase are present in mammalian cells, this observation is important for the relevance of the reaction between GS-Se-SG and thioredoxin reductase in cells.

The following reaction sequences have been used to describe the decomposition of GS-Se-SG by glutathione reductase (Shamberger, 1985).

\[
\text{GS-Se-SG} + \text{NADPH} \rightarrow \text{GS-Se}^- + \text{GSH} + \text{NADP}^+ \quad (3)
\]

\[
\text{GS-Se}^- + \text{H}_2\text{O} \rightarrow \text{Se}^0 + \text{GSH} + \text{OH}^- \quad (4)
\]

\[
\text{GS-Se}^- + \text{NADPH} + \text{H}_2\text{O} \rightarrow \text{HSe}^- + \text{GSH} + \text{NADP}^+ + \text{OH}^- \quad (5)
\]

\[
\text{GS-Se}^- + \text{GSH} \rightarrow \text{HSe}^- + \text{GS} \quad (6)
\]

\[
\text{HSe}^- + (\text{O}) \rightarrow \text{Se}^0 + \text{OH}^- \quad (7)
\]

\[
\text{HSe}^- + (\text{O}) + 2\text{GSH} \rightarrow \text{HSe}^- + \text{GS} \quad (8)
\]

This sequence could serve as a model also for thioredoxin reductase from calf thymus or thioredoxin reductase plus thioredoxin from E. coli. The fast stoichiometric oxidation is probably due to heterolytic cleavage of GS-Se-SG (Reaction 3) by thioredoxin reductase or, in the E. coli system, thioredoxin reductase plus thioredoxin. We have shown that the continued part of the reaction is oxygen-dependent. This oxygen dependence could be explained by the following reac-
preventing the reduction of thioredoxin. As an example, we expressed and secreted by activated human lymphocytes (Takatsu, cultured human cells. Recently, thioredoxin was found to be reduced to >90% in a physiological system by thioredoxin plus thioredoxin reductase and selenium compounds in a physiological system. This suggests important physiological regulatory mechanisms for protein disulfide formation and thiol redox control. Holmgren, 1985, 1989). We have studied the oxidation of reduced E. coli thioredoxin because Trp-28 gives this protein useful fluorescence properties (Holmgren, 1972; Krause and Holmgren, 1991). Thioredoxin from mammalian cells has the same apparent specific activity in disulfide reductions and a conserved active site (Holmgren, 1979c, Krause, and Holmgren, 1991). Thioredoxin and insulin disulfides described previously (Gilbert, 1990), suggesting that the thioredoxin system plays an important role in the physiological actions of selenium compounds.

We are now studying interactions between human thioredoxin plus thioredoxin reductase and selenium compounds in cultured human cells. Recently, thioredoxin was found to be expressed and secreted by activated human lymphocytes (Takatsu, 1990). There are also new data suggesting important roles for thioredoxin in the growth process of virus-infected lymphocytes (Wakasugi et al., 1990). Changes in the ratio of Trx(SH)2 to Trx-S-SG as a result of reactions between thioredoxin and selenium compounds could therefore be a physiological regulatory mechanism in mammalian cells.

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REFERENCES

Carlgren, J., and Mannervik, B. (1975) J. Biol. Chem. 250, 5475-5480
Gantner, H. E. (1971) Biochemistry 10, 4089-4098
Holmgren, A. (1979a) J. Biol. Chem. 254, 3672-3678
Holmgren, A. (1979b) J. Biol. Chem. 254, 9113-9119
Holmgren, A. (1979c) J. Biol. Chem. 254, 9627-9632
Painter, E. P. (1941) Chem. Rev. 28, 179-213

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