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Selective Inactivation of Glutaredoxin by Sporidesmin and Other Epidithiopiperazinediones*

Usha Srinivasan[†,‡], **Aveenash Bala**[φ,‡], **Shu-chuan Jao**[†], **David W. Starke**[†], **T. William Jordan**[φ], and **John J. Mieyal**[†]

[†]Department of Pharmacology, School of Medicine, Case Western Reserve University, Cleveland, Ohio 44106-4965 [φ]School of Biological Sciences and Centre for Biodiscovery, Victoria University of Wellington, New Zealand

Abstract

Glutaredoxin (thioltransferase) is a thiol-disulfide oxidoreductase that displays efficient and specific catalysis of protein-SSG deglutathionylation and is thereby implicated in homeostatic regulation of the thiol-disulfide status of cellular proteins. Sporidesmin is an epidithiopiperazine-2,5-dione (ETP) fungal toxin that disrupts cellular functions likely via oxidative alteration of cysteine residues on key proteins. In the current study sporidesmin inactivated human glutaredoxin in a time- and concentration-dependent manner. Under comparable conditions other thiol-disulfide oxidoreductase enzymes, glutathione reductase, thioredoxin, and thioredoxin reductase, were unaffected by sporidesmin. Inactivation of glutaredoxin required the reduced (dithiol) form of the enzyme, the oxidized (intramolecular disulfide) form of sporidesmin, and molecular oxygen. The inactivated glutaredoxin could be reactivated by dithiothreitol only in the presence of urea, followed by removal of the denaturant, indicating that inactivation of the enzyme involves a conformationally inaccessible disulfide bond(s). Various cysteine-to-serine mutants of glutaredoxin were resistant to inactivation by sporidesmin, suggesting that the inactivation reaction specifically involves at least two of the five cysteine residues in human glutaredoxin. The relative ability of various epidithiopiperazine-2,5-diones to inactivate glutaredoxin indicated that at least one phenyl substituent was required in addition to the epidithiodioxopiperazine moiety for inhibitory activity. Mass spectrometry of the modified protein is consistent with formation of intermolecular disulfides, containing one adducted toxin per glutaredoxin but with elimination of two sulfur atoms from the detected product. We suggest that the initial reaction is between the toxin sulfurs and cysteine 22 in the glutaredoxin active site. This study implicates selective modification of sulfhydryls of target proteins in some of the cytotoxic effects of the ETP fungal toxins and their synthetic analogs.

Keywords

gliotoxin; glutaredoxin; mixed disulfides; sporidesmin; thioltransferase

Glutaredoxin (GRx, also known as thioltransferase) is a member of the thiol-disulfide oxidoreductase enzyme family, which also includes thioredoxin and their corresponding reductase enzymes GSSG reductase and thioredoxin reductase, respectively. Mammalian

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Address for correspondence and reprint requests: John J. Mieyal, Ph.D., Department of Pharmacology Case Western Reserve University School of Medicine Cleveland, OH 44106-4965 Phone: (216) 368-3383 Fax: (216) 368-3395 jjm5@cwru.edu.
‡According to their equal contribution, Usha Srinivasan and Aveenash Bala are considered co-first authors.

GRx1 is a 12 kDa cytosolic protein that has been characterized *in vitro* as a specific catalyst for the reduction of protein-glutathionyl mixed disulfides (protein-SSG) (1–6). The reaction catalyzed by GRx1 is also selective for GSH as the reducing substrate (4). This thiol-disulfide interchange reaction is likely crucial for maintaining intracellular thiol status (2–7) under a variety of physiological and pathophysiological processes like aging, cardiovascular and neurodegenerative diseases, AIDS, and cancer chemotherapy where sulfhydryl redox balance is challenged and there is a tendency to accumulate protein-SSG (7–11). Likewise the intracellular redox balance can be shifted and protein-thiols modified by exogenous disulfides including toxins like sporidesmin (12).

Sporidesmin is a natural product that belongs to the class of epidithiopiperazine-2,5-dione (ETP) fungal toxins. Sporidesmin and its analog gliotoxin are among the best characterized ETPs that contain a strained disulfide bridge across the piperazinedione ring (Chart 1). Sporidesmin and other ETPs exhibit a variety of biological activities, including antibacterial, antiviral, immunosuppressive and antineoplastic actions (12, 13). The mode of action of the ETP compounds is unknown but multiple effects have been reported including altered calcium flux (14), interference with NFκB action (15), inhibition of farnesyl and geranylgeranyl transferases (16) and formation of mixed disulfides between the ETP moiety and cysteine residues on vital proteins, as reported for creatine kinase (17). Also implicated is the ability of the ETPs to undergo redox cycling in the presence of oxygen, thereby generating reactive oxygen species (18). Thus thiol-disulfide exchange reactions are central to the potential mechanisms of ETP toxicity. Since the relative toxicity of the ETPs like sporidesmin is dependent on the redox status of the dithiopiperazinedione moiety, we examined the interaction of sporidesmin with GSH and with the various thiol disulfide oxidoreductases that might mediate its reduction in cells. Here we report sporidesmin in the presence of GSH is catalytically reduced by GRx1. However, in the absence of GSH sporidesmin inactivates GRx1 in a concentration-, time-, and oxygen-dependent fashion that appears to involve disulfide formation with specific cysteine residues on the enzyme. We observed similar inactivation of GRx1 by gliotoxin and other ETPs. In contrast to GRx1, GSSG reductase, thioredoxin, and thioredoxin reductase are not inactivated by sporidesmin under analogous conditions. These results implicate modification of sulfhydryl residues in selective target proteins in some of the cytotoxic effects of the ETPs.

METHODS

Gliotoxin was purchased from Sigma Chemical Company (St Louis, MO). Sporidesmin (sporidesmin A) and sporidesmin D (bis-thiomethyl sporidesmin) were a gift from AgResearch (Hamilton, New Zealand). Synthetic ETPs (1,4-diethyl ETP, 1,4-dibenzyl ETP and 1-benzyl,4-methyl ETP) were a gift from Dr Christina Chai (Australian National University, Canberra, Australia).

Human recombinant GRx1 (40-60 U mg^{-1}) expressed in *E. coli* was isolated as previously described (19). Cysteine to serine mutants of the human enzyme were also prepared by previously described methods (5), including the previously used triple (C7S, C78S, C82S) and quadruple (C7S, C25S, C78S, C82S) mutants. Additionally, a C25S mutant was prepared using the PCR primer 5′ CCT GGA GTA CG 3′. The structures of the mutants were confirmed by nucleotide sequencing.

Human GRx1 activity was routinely measured using cysteine-SSG (cys-SSG) as substrate and coupling GSSG formation to NADPH oxidation by GSSG-reductase (5). Assays were carried out at 30 °C in 0.1 M phosphate buffer pH 7.5, containing GRx1, yeast GSSG reductase (2 U ml^{-1}), 0.1 mM cys-SSG, 0.2 mM NADPH, and 0.5 mM GSH. The substrate activity of sporidesmin was measured analogously in assays that contained sporidesmin in

place of cys-SSG. Sporidesmin was added dissolved in ethanol. The final concentration of ethanol (2–5% v/v) did not affect GRx1 activity.

Yeast GSSG reductase (0.125 μM) was assayed according to GSSG-dependent loss of NADPH absorbance at 340 nm (20). *E.coli* thioredoxin (0.2 mg ml^{-1}) and thioredoxin reductase (0.1 mg ml^{-1}) were assayed spectrophotometrically by coupling reduction of thioredoxin-disulfide to oxidation of NADPH (ΔA340 nm) by thioredoxin reductase, as adapted from previous procedures (21, 30). Cys-SSG was used as the initiating disulfide substrate to produce thioredoxin-disulfide, and thioredoxin and thioredoxin reductase concentrations were adjusted so that one or the other was limiting for the assays of their respective activities. Bovine thioredoxin was assayed by a turbidometric assay using insulin as the substrate and DTT as the reductant for the oxidized thioredoxin (22).

Enzyme inactivation was measured by incubating GRx1 or other enzymes with sporidesmin in 0.1 M phosphate buffer, pH 7.5 at 30 °C followed by 100-fold dilution of the reaction mixture and measurement of enzyme activity using standard conditions.

One-dimensional polyacrylamide gel electrophoresis and Western blotting of GRx1 were carried out as previously described using a rabbit polyclonal antibody to recombinant human GRx1 (23).

Removal of Metal Ions from Assay System

Chelex-100 (0.4 meq ml^{-1}) was used to treat the assay buffer (0.1 M potassium phosphate, pH 7.5, 5% ethanol). This buffer was used to reconstitute GRx1 (30 μM) and to make sporidesmin stock solutions (10 mM). 3 μM GRx1 was incubated with 1 mM sporidesmin at 30 °C and GRx1 activity was determined as described above.

Analysis of ETP Analogs

2 μM GRx1 was treated with the indicated ETP analog at 1 mM final concentration. The diethyl, dibenzyl and methylbenzyl ETPs were dissolved in DMSO to a final concentration of 100 mM and diluted 1:100 into the GRx1 reaction mixture containing 0.1 M potassium phosphate, pH 7.5, 10% ethanol. The analogs were incubated with GRx1 for 20 min at 30 °C. The control samples for the synthetic ETPs were treated with 1% DMSO. GRx1 activity was then assessed as described above.

Mass spectrometry

MALDI spectra of GRx1 and the reaction products with ETPs were obtained using a PerSeptive DE PRO mass spectrometer in positive ion mode with accelerating voltage 25,000, grid voltage 88%, 0.1% guide wire voltage and delay time 300 nsec. Samples were prepared in sinapinic acid matrix in 0.5 % TFA:acetonitrile (1:1) (24). Masses were calibrated using close external standards *E. coli* thioredoxin (m/z 11,674.48) and equine apomyoglobulin (m/z 16,952.56) for GRx1. Native GRx1, sporidesmin and GRx1-sporidesmin adducts were also examined using ESI mass spectrometry in a PerSeptive Mariner ESI-TOF mass spectrometer in positive ion mode, with the sample in acetonitrile: 0.1% acetic acid (1:1) infused directly into the instrument.

RESULTS

Sporidesmin is both a substrate and inactivator of GRx1

Initial experiments showed that sporidesmin was both a substrate and inactivator of GRx1. Inactivation was measured using cys-SSG as the typical substrate of GRx1, after preincubation with sporidesmin (see below). In this assay GRx1 activity is determined by

coupling formation of product GSSG to oxidation of NADPH by GSSG reductase (*see* Figure 11, step 7). Independent assay of GSSG reductase using 1 mM GSSG substrate directly indicated that sporidesmin did not modify the reductase activity (preincubation of reductase with 2 mM sporidesmin at 30 °C for 20 min). Also sporidesmin (0.1 mM) was not a substrate for GSSG reductase, as expected. The following experiments therefore represent the effects of sporidesmin on GRx1.

Sporidesmin is a substrate for GRx1 in the presence of GSH

This activity was measured in assays that contained GRx1, sporidesmin, GSH, NADPH and GSSG reductase. The presence of GSH prevented inactivation of GRx1 under these conditions. Enzyme activity required all components and was not detected in the absence of any one of the assay components NADPH, GSH, or GSSG reductase. Spontaneous reaction of sporidesmin with GSH to form GSSG occurred in assays from which GRx1 was omitted. Spontaneous reaction rates were typically less than 10% of total activity at standard assay conditions (40 μM sporidesmin, 0.5 mM GSH) and were subtracted from total reaction rates to give rates of GRx1 catalyzed reactions. Sporidesmin D, in which the disulfide is modified by methylation to give a bis-thiomethyl derivative, was not a substrate for GRx1. Gliotoxin and synthetic ETP compounds 1,4-diethyl, 1,4-dibenzyl and 1-benzyl,4-methyl ETP were also substrates for GRx1 (data not shown).

Figure 1 shows the two-substrate kinetics for GSH and sporidesmin. The apparent Km for sporidesmin was approximately 50 μM under standard assay conditions for GRx1 with 0.5 mM GSH. Enzyme activity was routinely assayed under non-saturating conditions because of large spontaneous reaction rates at higher sporidesmin and GSH concentrations. The rate of enzyme-catalyzed reaction (formation of GSSG/oxidation of NADPH) with 40 μM sporidesmin plus 0.5 mM GSH was comparable to that for the prototype disulfide substrate cys-SSG at 100 μM (standard assay conditions).

The spontaneous reaction of sporidesmin with GSH suggested possible formation of a sporidesmin-SSG mixed disulfide that may serve as the actual substrate for GRx1 (1). The products of spontaneous reaction of the two substrates were therefore examined by ESI mass spectrometry. Protonated ions at m/z 474 and 308 were obtained for solutions of 0.1 mM sporidesmin and 0.5 mM GSH respectively. Reaction of sporidesmin with GSH gave predominant ions for GSH and sporidesmin plus an ion at m/z 781 corresponding to the formation of the mixed disulfide sporidesmin-SSG. Approximately 20-40% conversion of sporidesmin to the mixed disulfide was detected after 2 min incubation at 20°C prior to mass spectrometry. This result is consistent with the typical mechanism of GRx1 catalysis where the enzyme catalyzes reduction of glutathione-containing mixed disulfides (see Discussion).

Inactivation of GRx1

Sporidesmin inactivated GRx1 in a time and concentration-dependent manner (Figure 2). Inactivation followed pseudo first order kinetics and was analyzed according to a modified form of the Kitz-Wilson relationship (25, 26). This method was simplified, as described previously (26), by using a single time of incubation of GRx1 with each concentration of sporidesmin rather than generating a complete kinetic curve to obtain $t_{1/2}$ for each amount of toxin. It was confirmed separately that first order kinetics were obtained throughout the concentration range during the 5 min inactivation period. K_I and k_{inact} were determined by nonlinear regression analysis of the relationship between sporidesmin concentration and enzyme activity remaining at time *t*, according to the following expression $\ln (E_o/E_t) / t = \underline{\text{kinact [I]}/(K_I + [I])}$, where E_o and E_t represent concentrations of active GRx1 at time 0 and 5 min, [I] is the concentration of inactivator (sporidesmin), K_I is the concentration of inactivator that gives half maximal inactivation, and k_{inact} is the net rate constant of

inactivation. This expression can be rearranged to give a linear double reciprocal format (t / ln (E_o / E_t) vs. 1/[I], which fits the data shown in Figure 3.

Selectivity of sporidesmin-mediated inactivation

To examine the selectivity of sporidesmin inactivation it was tested as an inactivator of other TDOR enzymes, all of which in their reduced forms contain a dithiol motif at their respective active sites. Besides GRx1, yeast GSSG reductase, *E. coli* thioredoxin and thioredoxin reductase, and bovine thioredoxin reductase were examined. Only GRx1 was inactivated by 1 mM sporidesmin (Figure 4).

ETP structural requirements for inactivation of GRx1

Sporidesmin, gliotoxin and the synthetic compounds 1,4-dibenzyl ETP and 1-benzyl, 4-methyl ETP all inactivated GRx1 (Figure 5). However, 1,4-diethyl ETP did not inactivate GRx1. These results indicate a structural requirement for an aromatic (or extensive hydrophobic) residue adjacent to the disulfide-bridged ETP ring. In addition, S,S-dimethyl-sporidesmin (sporidesmin D), in which the cyclic disulfide is precluded by methylation of the sulfurs, did not inactivate GRx1, implicating the intramolecular disulfide moiety of the toxin in the inactivation mechanism.

Requirements for the sporidesmin-mediated inactivation of GRx1

Inactivation of GRx1 by sporidesmin required reduced GRx1, and molecular oxygen. Cys-SSG and hydroxyethyl disulfide convert the active site cysteine dithiol motif of GRx1 to an intramolecular disulfide (26) and should therefore prevent inactivation if sporidesmin interacts with either the cys22 or the cys25 thiols. Incubation of 200 μM GRx1 with 1 mM hydroxyethyl disulfide or cys-SSG for 5 min, prior to 100-200 fold dilution and incubation with 1 mM sporidesmin for 10 min at 30 °C, completely protected against inactivation of GRx1, as measured by the standard spectrophotometric assay. Preincubation of sporidesmin with GSH for 10 min before dilution into the solution containing GRx1 also prevented sporidesmin inactivation of the enzyme, indicating that the disulfide form of sporidesmin was required for the inactivation reaction, as indicated also by the inactivity of the S,S-dimethyl analog (Figure 5). Addition of 0.5 mM GSH to GRx1 prior to addition of sporidesmin also completely protected GRx1 against inactivation.

Sporidesmin (1 mM) inactivated GRx1 by greater than 80% in the presence of oxygen, but purging the reaction mixture with argon led to a marked decrease in the inactivation of GRx1 by sporidesmin. The requirement for molecular oxygen was demonstrated by incubating 30 μM GRx1 with or without 1 mM sporidesmin for 15 min in pH 7.5 phosphate buffer at 30 °C. When buffer that had been purged with argon for 3 h was used, 84 ± 7% of GRx1 activity was retained after incubation with sporidesmin. In contrast, only 18 ± 2% activity was retained when normal oxygen saturated buffer was used. Since oxygen involvement in inactivation is often associated with free radical oxygen species whose formation is known to be metal catalyzed, we examined the effect of metal ions on sporidesmin inactivation of GRx1. Removal of metal ions by treatment of all solvents with chelex resin did not alter the inactivation of GRx1 by sporidesmin, indicating that that metal ion-catalyzed formation of reactive oxygen species did not play a significant role in the inactivation mechanism.

Evidence for formation of a mixed disulfide between sporidesmin and GRx1

The possibility of formation of a mixed disulfide of sporidesmin with GRx1 cysteine residues was investigated in a number of experiments. Initially we investigated whether disulfide bond breakage by DTT could be used to reactivate sporidesmin-treated GRx1.

DTT alone did not reactivate the sporidesmin-modified enzyme, nor did treatment with urea in the absence of DTT. However, treatment of inactivated GRx1 with 50 mM DTT plus 8 M urea resulted in recovery of GRx1 activity after dialysis to remove urea and unreacted sporidesmin (Figure 6). This experiment indicated that inactivation of GRx1 by sporidesmin may proceed via formation of a disulfide bond that is conformationally shielded from reduction by DTT unless the enzyme is denatured to allow access to the disulfide bond (see Discussion).

Since our results indicated that sporidesmin inactivation of GRx1 involved disulfide formation we investigated whether there was intermolecular disulfide bond formation resulting in GRx1 dimers or higher molecular weight oligomers. Western blotting with anti-GRx1 antibody (23) after electrophoresis of samples on non-reducing SDS-PAGE revealed only the monomeric species for 18 μM GRx1 incubated alone or with 1 mM sporidesmin (data not shown). This result eliminated formation of GRx1 dimers or higher molecular weight aggregates as the mechanism of inactivation, and supported either formation of a GRx1-S-S-sporidesmin adduct or intramolecular disulfide formation between GRx1 cysteines, neither of which could be reversed by DTT alone.

Use of cysteine mutants of GRx1 to probe the sporidesmin-enzyme reaction

Cysteine to serine mutants of GRx1 were used to test the involvement of specific cysteine residues with sporidesmin. Human GRx1 contains two cysteines (C22, C25) at the active site and three additional cysteines at positions 7, 78 and 82. Cys22 is essential for enzyme activity. A C25S mutant GRx1 and triple (C7S, C78S, C82S) and quadruple (C7S, C25S, C78S, C82S) mutants were resistant to inactivation when incubated with 1 mM sporidesmin. All mutants retained greater than 90% activity after incubation at pH 7.5 and 30 °C for 20 min (Figure 7). Wildtype GRx1 showed greater than 80% loss of activity under the same conditions. These results indicate roles for cys25 and at least one of the non active site cysteines in the inactivation mechanism (see Discussion).

Mass spectrometry of sporidesmin-inactivated GRx1

Mass spectrometry of GRx1 and its reaction products was used to investigate the formation of a possible GRx1-ETP adduct. The reaction of sporidesmin (monoisotopic mass 473.05) with GRx1 (average mass 11,644.55) was expected to give rise to a mixed disulfide adduct at mass 12,117 that would be observed as an ion at m/z 12,118. MALDI spectra of GRx1 contained a major ion at 11,645 +/-2 m/z as expected, subject to the expected +/− 2 Da mass accuracy in this mass range (Figure 8A). Reaction of the enzyme with sporidesmin or gliotoxin (monoisotopic mass 326.04) was carried out using GRx1 (6 μg) incubated in 10 μl of MilliQ water for 20 min at 30 °C either alone or in the presence of 8 μg of sporidesmin, or 5 μg gliotoxin, in 1 μl of ethanol. Unexpectedly reaction of GRx1 with sporidesmin gave a prominent product ion at m/z 12,054 with no trace of the expected adduct ion m/z 12,118 (Figure 8B). The GRx-sporidesmin reaction product was therefore 64 Da less than expected and we hypothesize that this represents elimination of two sulfur atoms from the adduct. The simplest explanation for the 12,054 m/z value is that 2 sulfur atoms are lost from the adduct in the mass spectrometer. Alternately, the m/z of 12,054 could represent loss of two sulfurs from GRx1 or sporidesmin, or one sulfur each from the GRx1 and sporidesmin moieties, being eliminated as sulfinic acid during the reaction of GRx1 and sporidesmin before mass spectrometry.

Sporidesmin alone showed prominent ions at m/z 474 and 410 (Figure 8C) using MALDI or ESI mass spectrometry, but the low mass region of the spectrum following reaction with GRx1 contained major ions at 479, 459, 443 and 410 (Figure 8D). These ions were consistent with the presence of sporidesmin (calculated m/z 474.0555), sporidesmin minus

two sulfur atoms (calculated m/z 410.1113). Following reaction with GRx1, the prominent m/z values are consistent with modified sporidesmin in which a sulfur atom was replaced by hydrogen (calculated m/z 443.0912), and that species was further changed by the remaining sulfur being oxidized to a sulfenic acid by addition of a single oxygen atom (calculated m/z 459.0862). The identity of the m/z 479 ion is unknown. These results support the possibility of alterations of sporidesmin by elimination of sulfur and oxygenation of sulfur during reaction with GRx1.

The reaction of gliotoxin with GRx1 also gave an adduct mass 64 Da less than expected but adduct ions were not detected when gliotoxin or sporidesmin were reacted with the C7S, C25S, C78S, C82S quadruple mutant of GRx1 or when native GRx1 was reacted with sporidesmin D in which the toxin sulfurs are methylated. These results are consistent with direct involvement of GRx1 and ETP sulfurs in the reaction mechanism. Further evidence for involvement of GRx1 sulfhydryls was therefore sought by alkylation of native GRx1 and the GRx1-sporidesmin reaction product with iodoacetamide, which can be expected to add 57 Da to each free cys residue in each case. As predicted, reaction of 0.1 M iodoacetamide with GRx1 at 40 ° C for 1 h gave a major ion at m/z 11,930 +/− 3 Da (addition of 5x57 = 285 + 11,645 Da) in the MALDI spectrum consistent with carboxamidomethylation of each of the five GRx1 thiols in the native protein (Figure 9A). When the product of reaction of GRx1 with sporidesmin was subsequently alkylated with iodoacetamide an ion at m/z 11,702 +/− 2 was consistent with the presence of sporidesmin-free GRx1 with a single carboxamidomethylation (11,645 + 57 Da), along with an ion at m/z 12,054 corresponding to the GRx1-sporidesmin adduct that was present prior to alkylation. Unexpectedly, ions corresponding to carboxamidomethylation of the GRx1-sporidesmin adduct (*i.e.*, m/z > 12,054) were not detected (Figure 9B). These results suggest that reactivity of all GRx1 cys residues was lost in the adduct possibly due to conformational inaccessibility, elimination of sulfur, steric hindrance by the adducted toxin, or other modification including sulfur oxygenation. There also appeared to be release of toxin-free GRx1 alkylated on a single cysteine (ion at m/z 11,702).

We sought further mass characterization of the adduct after reacting it with DTT. When the product of reaction of GRx1 with sporidesmin was incubated with 35 mM DTT for 1 h at room temperature there was partial conversion of the m/z 12,054 species to ions at m/z 11,613 +/− 2 (Figure 10A) and 443 (Figure 10B) that may represent release of GRx1 and sporidesmin each less one sulfur atom, as would be expected if two sulfurs had been eliminated from the adduct, one each from GRx1 and sporidesmin. Greater than 90% conversion of adduct to these products was achieved after overnight reaction with DTT. This observation of the separate m/z 11,613 and 443 species did not require denaturation of the adduct with urea prior to DTT treatment. This differed from reconstitution of enzyme activity which required denaturation of the adduct with urea prior to reduction indicating that conformational modification of the enzyme may have occurred during formation of the adduct. In control experiments DTT did not alter the mass of native GRx1, but when sporidesmin was reacted with 35 mM DTT it was converted to an m/z 476 species, probably representing reduction of the disulfide bridge to give sporidesmin dithiol.

DISCUSSION

Sporidesmin is a substrate for GRx1

We have previously shown that GRx1 is specific for glutathionyl containing substrates (1, 2). It catalyzes the thiol-disulfide interchange reaction most efficiently in a monothiol fashion involving formation of a glutathionyl mixed disulfide intermediate on the Cys-22 residue of the enzyme, whereas formation of the Cys22-S-S-Cys-25 intramolecular disulfide detracts from the catalytic efficiency (16). Non-GSH-containing disulfides acted as

substrates for GRx1 only when the second substrate was GSH, and this could be attributed to the pre-formation of glutathionyl-mixed disulfide substrate in a non-enzymatic reaction between the original disulfide and GSH. Similarly, sporidesmin is a substrate for GRx1 when the second substrate is GSH. Consistent with the usual interpretation we did observe a new peak in the mass spectral analysis of mixtures of GSH and sporidesmin that corresponded to the sporidesmin-SSG mixed disulfide. However, this mass spectral species was in relatively low abundance when GRx 1 was present, consistent with catalysis of its breakdown by the enzyme. There was insufficient sporidesmin-SSG to detect by HPLC analyses of complete reaction mixtures containing GRx1. Thus, the data support the typical catalytic mechanism and specificity of GRx1 (depicted by steps 4,5,7 of Figure 11), whereby non-enzymatic reaction of sporidesmin with GSH leads to formation of sporidesmin-SSG which reacts further with GSH only very slowly in the absence of catalysis by the GRx1 enzyme (1,2,4,5). This scheme is supported by the sequential pattern observed for the two-substrate kinetics analysis of the GRx1-catalyzed reaction involving sporidesmin and GSH as substrates (Figure 1). However, an alternative mechanism is conceivable. Namely, GRx1 may react directly with sporidesmin to form a GRx1-S-S-sporidesmin intermediate (step 1, Figure 11). GSH could then turn over this intermediate by releasing reduced sporidesmin and forming GRx1-SSG which would enter the normal catalytic cycle for GRx1 (steps 6, 7, Figure 11). Also shown is the coupling reaction with GSSG reductase at step 7. Regardless of the particular mechanism, the fact that GRx1 catalyzes the reduction of sporidesmin may be relevant in cellular systems. In cases where sporidesmin mediates its toxicity by redox cycling, catalysis of its reduction may enhance toxicity. In cases where the mechanism of toxicity involves the disulfide form of sporidesmin, then GRx1-mediated reduction may be a detoxifying reaction.

Mechanistic model of GRx1 inactivation by sporidesmin

As shown, inhibition of GRx1 required the presence of reduced GRx1 (cysteine-thiols) and oxidized sporidesmin (bridged disulfide). This implicates disulfide exchange as essential for inactivation. Accordingly, the S,S-dimethyl analogue of sporidesmin did not inactivate GRx1, and pretreatment of GRx1 with hydroxyethyl disulfide or cys-SSG prevented inactivation of GRx1. Mass spectrometry was used primarily to distinguish whether inactivation of GRx1 by sporidesmin was due to a covalent sporidesmin-GRx1 adduct or an internal disulfide-modified form of GRx1 with release of modified sporidesmin. Mass spectral analysis demonstrated formation of an adduct consistent with a mixed disulfide between one molecule of GRx1 and one molecule of sporidesmin (or gliotoxin). The detected products were 64 Da less than expected, suggesting elimination of two sulfur atoms in the mass spectrometer or possibly as part of the reaction mechanism. GRx1 was inactivated by the toxins but activity was recovered when the inactivated enzyme was denatured in urea and reduced with DTT which would be expected to break mixed disulfide bonds between the GRx1 and ETP sulfurs. Although restoration of enzyme activity required denaturation with urea, prior to reduction and renaturation, DTT treatment in the absence of urea followed by analysis in the mass spectrometer showed separate m/z species corresponding to separate, modified forms of GRx1 and toxin, each apparently missing one sulfur atom. Overall these results may be rationalized according to the schemes shown in Figures 11 and 12 (see below). The mass analysis after DTT treatment alone, which showed separate GRx1 and sporidesmin species, differed from reconstitution of GRx1 enzyme activity which required denaturation of the adduct with urea prior to reduction, indicating that conformational modification of the enzyme likely occurred during formation of the adduct, shielding one of the GRx1-sporidesmin intermolecular disulfide bonds from DTT (see Figure 11). This shielded disulfide might correspond to the one that is lost due to desulfuration in the mass spectrometer (Figure 12), thus providing an explanation for the paradoxical effect of DTT alone when coupled to analysis by mass spectrometry.

GRx1 has five cysteines at amino acid positions 7, 22, 25, 78, and 82. Active site cys22 is the most reactive because of its unusually low pKa = 3.5 (26). Mutation of cys22 results in complete loss of catalytic activity, as does modification of this residue by electrophilic reagents like N-ethyl maleimide or iodoacetamide. Therefore it is reasonable to expect that initial attack of GRx1 on sporidesmin involves the cys22 thiolate. Site directed replacement of all other cysteines with serine gives a mutant GRx1 (quadruple mutant) that is fully catalytically active (5); however, this mutant was resistant to sporidesmin- inactivation, suggesting that cysteines on GRx1 other than cys22 are involved in the inactivation process. Cys25 is implicated in the inactivation mechanism, because the singular C25S mutant was also resistant to sporidesmin inactivation. The inactivation cannot be explained, however, by exclusive reactions with cys22 and cys25, because the triple mutant (C7S, C78S, C82S) which retains only these two cysteines was also resistant to inactivation. Overall the results of the mutant studies (Figure 7) along with the requirements for disulfide exchange and molecular oxygen lead to the hypothetical scheme depicted in Figure 11, whereby inactivation of GRx1 by sporidesmin is proposed to involve stepwise disulfide bond formation of sporidesmin with cys22, then cys25 and cys82 on GRx1. The requirement for O_2 implicates an additional oxidation step forming a second disulfide bond beyond initial adduction of sporidesmin to GRx1 via mixed disulfide formation. Based on the higher reactivity of cys22 we suggest initial adduction of sporidesmin occurs on cys22 (step 1, Figure 11). In the absence of GSH the neighboring cys25, that is within disulfide bonding distance from cys22 attacks the C22-S-S-sporidesmin adduct forming a reversible C25-S-S-sporidesmin adduct (step 2, Figure 11). The irreversible inactivation reaction is completed by oxygen-dependent formation of a second disulfide bond between cys82 and sporidesmin (step 3, Figure 11). This interpretation is supported by the proximity of the cys82 residue to cys25 according to the 3-dimensional NMR structure of human GRx1 (5, 27). According to the hypothetical scheme (Figure 11), GRx1 is irreversibly inactivated even though the active site cys22 is not blocked. This could be explained in two different ways. Inactivation of GRx1 may sufficiently change the active site topology that the cys22 is no longer extending in solution or the reactivity (pKa) of cys22 has been altered dramatically due to conformational changes in GRx1.

One of the remaining challenges is explanation of the apparent loss of two sulfur atoms from the adduct. The cumulative mass data suggest elimination of one sulfur atom from sporidesmin and one from GRx1. Figure 12 presents a plausible mechanism whereby a sporidesmin-GRx1 adduct could expel two sulfurs as S_2, yet persist as a disulfide-linked bimolecular adduct that would be reducible by DTT. Although there are other explanations for the loss of 64 Da (*e.g.*, loss of four oxygen atoms or loss of a sulfur and two oxygens) we suggest that loss of two sulfurs is most compatible with the experimental evidence.

Initial mass analysis of tryptic peptides of the sporidesmin-GRx1 reaction product (data not shown) were not so definitive to establish the hypothetical scheme in Figure 12, hence additional studies would be necessary to identify the exact nature of the adduct and its two-sulfur depleted by-product. Van Zanden et al. (28) similarly had difficulty in characterizing expected adducts of quercetin with cys47 of glutathione S-transferase P1-1 using electrospary ionization mass spectrometry of tryptic digests of the enzyme inhibited by quercetin. In contrast to the quercetin adduct where there is a carbon-sulfur bond, the ETP-GRx1 adduct is joined by mixed disulfides that likely require conformational stabilization and would be labile in the mass spectrometer as the tryptic peptide adducts.

Is sporidesmin a mechanism based inhibitor of GRx1?

Sporidesmin is both a substrate and an inactivator of GRx1, but not of the other tested thiol-disulfide oxidoreductases, possibly making it a suicide inhibitor of GRx1. The formation of GRx1-S-S-sporidesmin at the cys22 may be the branch point for two parallel reactions to

occur as shown in Figure 11. In the presence of GSH, GRx1-S-S-sporidesmin-SH can form sporidesmin-$(SH)_2$ and GRx1-SSG, which can then be turned over by GSH (steps 6 and 7). However in the absence (or depletion) of GSH, GRx1-S-S-sporidesmin-SH would undergo the sequence of events described above (steps 1, 2, 3). However, even in the absence of GSH there may be some partitioning of the initial sporidesmin-GRx1 complex to give inactivated GRx1 and to produce products of modified sporidesmin, recycling the GRx1 enzyme. Potential evidence for product release from the sporidesmin-GRx1 complex is provided by the apparent complete modification of sporidesmin indicated in Figure 8D where the 474 m/z sporidesmin ion is completely lost after reaction of GRx1 with an approximately 50-fold excess of sporidesmin (0.3 nmol GRx1 + 17 nmol sporidesmin), indicating possible catalytic modification of a sporidesmin sulfur by the enzyme. However quantitative interpretation of this observation is complicated by the observation that sporidesmin alone undergoes desulfuration in the mass spectrometer (Figure 8C).

Structural features necessary for GRx1 inactivation

Besides the requirements for particular cysteine residues on GRx1, the ETP moiety also needs particular substituents to effectively inactivate GRx1. As seen in Figure 5, only the analogs that contained aromatic substituents exhibited significant inhibitory activity against GRx1. Thus, the benzyl ring in sporidesmin appears to be important for inhibition of the enzyme. More likely, both non-covalent and covalent interactions contribute to GRx1 inactivation by sporidesmin, consistent with resistance of the inactivated enzyme to reactivation by DTT unless urea was present.

Analogous inactivation of thiol proteins by gliotoxin

Inactivation of alcohol dehydrogenase by the related fungal toxin gliotoxin is reported to involve two mechanisms (29). One is covalent modification of a specific cysteine residue, identified as either cys281 or cys282 according to analysis of proteolytic fragments of alcohol dehydrogenase treated with radiolabelled gliotoxin. This cysteine is not at the active site of the enzyme, but its modification is associated with enzyme inactivation attributed to conformational changes induced by toxin binding. In this case, reducing agents like DTT and GSH enhanced inactivation rather than reversing inactivation by disulfide reduction. The presence of catalase restored inactivation to the rate observed in the absence of reducing agents. The authors concluded that the enhancement by reducing agents was due to a radical mediated pathway involving metal ion catalysis and reactive oxygen species. In contrast to alcohol dehydrogenase, GSH protected GRx1 against inactivation by sporidesmin. Although inactivation of GRx1 by sporidesmin required molecular oxygen, it was not dependent on metal ions as demonstrated by the lack of effect of chelex treatment. Furthermore, direct addition of Cu^{+1} did not enhance inactivation of GRx1 (data not shown).

Another case of gliotoxin-mediated enzyme inactivation has been reported for creatine kinase (17). Like GRx1 (26,30), creatine kinase is susceptible to inactivation by various sulfhydryl reagents as well as reactive oxygen species (31). The addition of reducing agents prevented gliotoxin-mediated inactivation of creatine kinase and ^{35}S-gliotoxin binding to creatine kinase, presumably by direct reduction of gliotoxin. There are a number of contrasts between the gliotoxin-creatine kinase inactivation and the sporidesmin-GRx1 inactivation. In contrast to GRx1, DTT or GSH in the absence of urea partially restored creatine kinase activity. Instead of an ultimate gliotoxin-creatine kinase mixed disulfide adduct as the basis for inactivation of the enzyme, the gliotoxin adduct is characterized as an intermediate in the formation of intramolecular disulfides on the enzyme. Inactivation of the enzyme is attributed selectively to the disulfide formation that occurs on one of the two subunits of the dimer, i.e. one of the subunits can be fully oxidized without loss of activity and inactivation progresses as the second subunit is oxidized to its C73-C282 disulfide. Thus, both the

inactivation of GRx1 by sporidesmin and the inactivation of creatine kinase by gliotoxin appear to be complex processes involving intermediate stages of disulfide formation.

Sporidesmin as a prototype for design of GRx1 inhibitors

Sporidesmin is neither a high affinity nor highly selective inhibitor of GRx1. The K_I for sporidesmin is 0.15 mM, and it is known to react with several other proteins. Nevertheless the understanding of the mechanism of inactivation of GRx1 by sporidesmin and the ETP analogs reveals specific structural requirements on the enzyme and on the inactivator that lend themselves to refinement of better inhibitors. Selective inhibitors of GRx1 will find utility not only as tools for elucidating the cell biological functions of the enzyme but also for potential therapeutic development for instance in AIDS where our finding that GRx1 may regulate the activity of the HIV-1 protease (32) suggests GRx1 as a therapeutic target.

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Abbreviations

cys-SSG	cysteinyl glutathione disulfide
DTT	dithiothreitol
ETP	epidithiopiperazine–2,5-dione
GRx1	human cytosolic glutaredoxin 1
GSSG	glutathione disulfide

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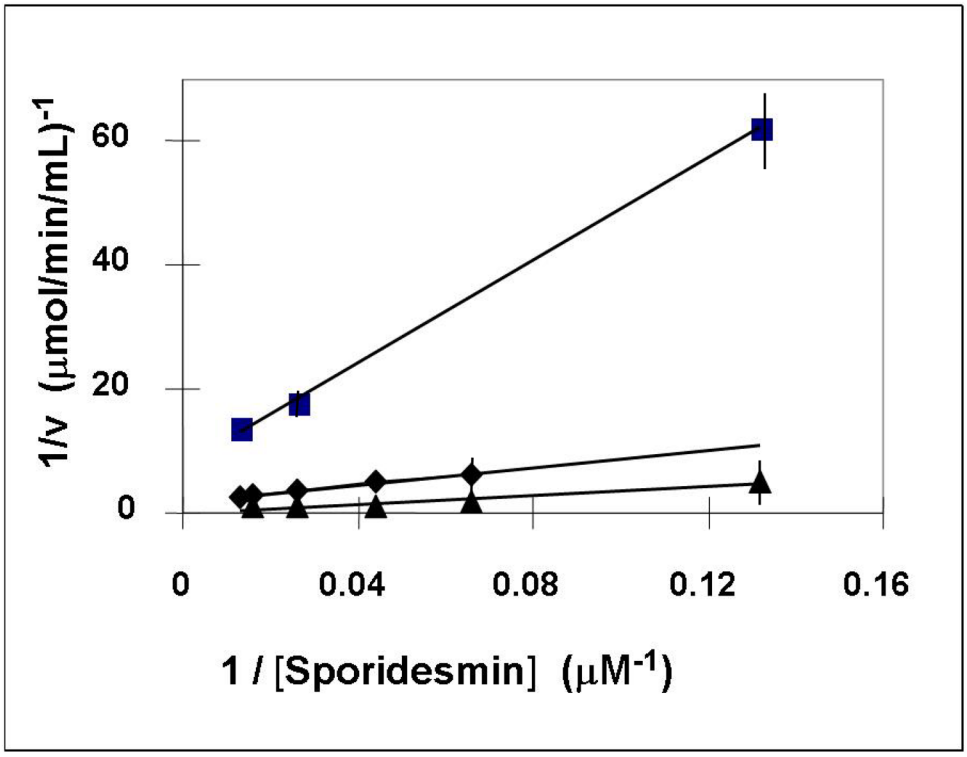

Figure 1. Two substrate kinetics for GSH-dependent GRx1-catalyzed reduction of sporidesmin

Two substrate kinetics for sporidesmin and glutathione with GRx1. Concentrations of GSH and sporidesmin were varied in the presence and absence of GRx1. The two substrate kinetic analysis of sporidesmin (7.6–76 μM) and GSH (0.4 mM, solid square), (2 mM, solid diamond), (5 mM, solid triangle) with GRx1 (0.035 μM) shows an intersecting pattern of lines consistent with a sequential mechanism. Each point represents the mean value, plus and minus the standard error, for at least three determinations. Where error bars are not evident they are within the size of the symbol.

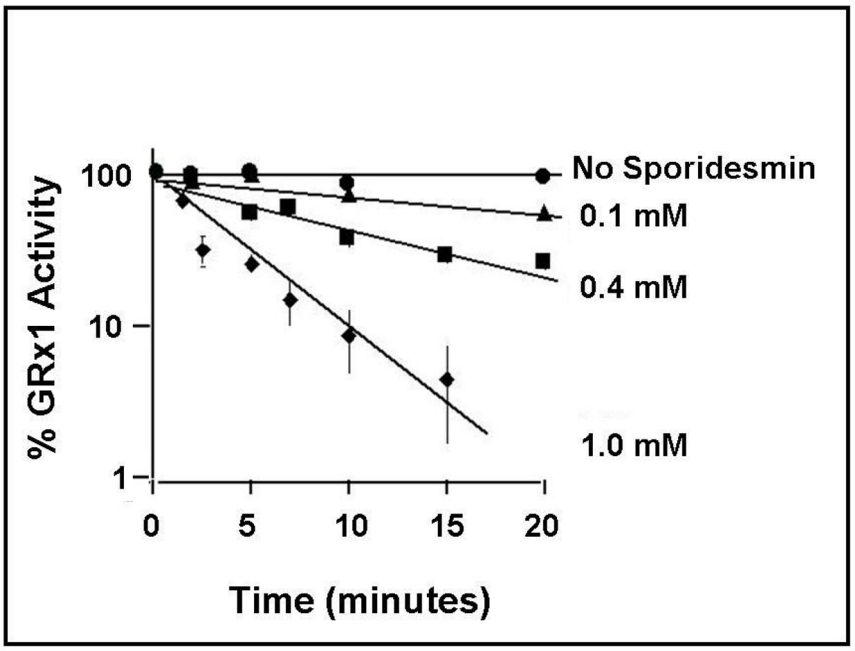

Figure 2. Inactivation of GRx1. Time and concentration dependent loss of GRx1 activity

GRx1 (2-10 μM) was treated with different concentrations (0.1–1 mM) of sporidesmin in 0.1 M K phosphate, pH 7.5, 10 % ethanol. At different times, aliquots were tested for GRx1 activity according to the spectrophotometric assay (GSSG-reductase mediated NADPH oxidation coupled to GSSG formation) using cys-SSG as the prototype substrate. Rates of GSSG formation were also determined with sporidesmin as the disulfide substrate and are designated as non-cys-SSG rates. Since residual sporidesmin is transferred to the assay with the deactivated GRx1, non-cys-SSG rates were subtracted from cys-SSG rates for each time point. Each point represents the mean value, plus and minus the standard error, for at least three determinations. Where error bars are not evident they are within the size of the symbol.

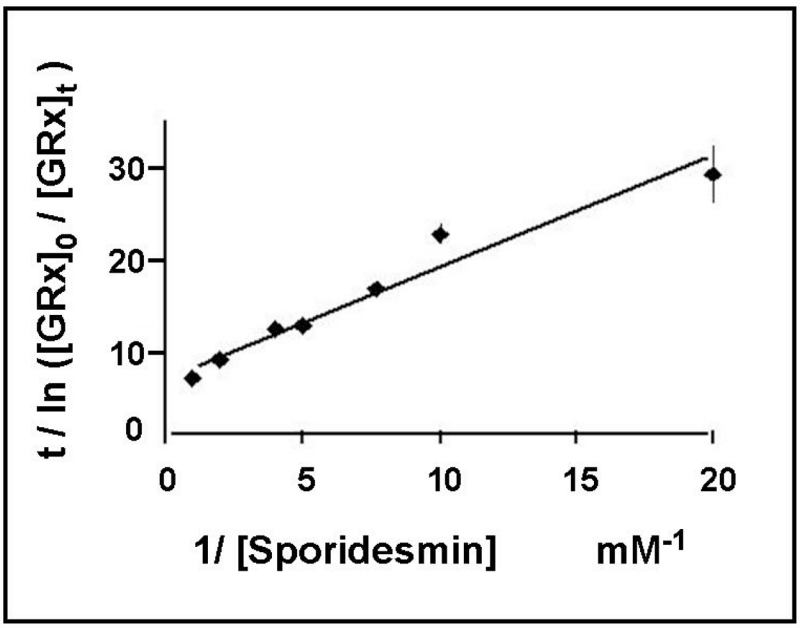

Figure 3. Modified Kitz-Wilson plot for GRx1 inactivation by sporidesmin

GRx1 (0.9 μM) was incubated with different concentrations of sporidesmin (0.05–1 mM) for 5 min at 30 °C, 0.1 M K phosphate, pH 7.5, and residual activities were determined relative to ethanol treated control as described in Figure 2. K_I and k_{inact} were determined according to the relationship: $\ln (E_o/E_t)/ t = k_{inact} [I] / (K_I + [I])$, where E_o and E_t refer to GRx1 concentrations at times 0 and t, [I] refers to sporidesmin concentration, K_I is the concentration of inactivator that gives half the maximal rate of inactivation, and k_{inact} is the net rate constant for inactivation. The K_I of sporidesmin for GRx1 is 0.15 mM and the k_{inact} is 0.72 min^{-1}. Each point represents the mean value, plus and minus the standard error, for at least three determinations. Where error bars are not evident they are within the size of the symbol.

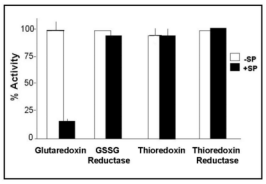

Figure 4. Selective inactivation of GRx1 by sporidesmin

Thiol-disulfide oxidoreductase enzymes were incubated with 1 mM sporidesmin at 30 °C, 0.1 M K phosphate (5% ethanol), pH 7.5 for 20 min. The remaining enzyme activity was expressed as percent of the control activity. Human GRx1 (1.8 μM) activity was determined by GSSG-reductase mediated NADPH oxidation coupled to GSSG formation. Yeast GSSG reductase (0.125 μM) activity was measured by the loss of NADPH absorbance at 340 nm. *E.coli* thioredoxin (0.2 $mg.ml^{-1}$) and thioredoxin reductase (0.1 $mg.ml^{-1}$) were assayed by NADPH oxidation coupled to thioredoxin oxidation using GSSG as the substrate. Mammalian thioredoxin was assayed by a turbidometric assay using insulin as the substrate. Each bar represents the mean value, plus and minus the standard error, for at least three determinations. Where errors are not shown the value represents a single experiment carried out after optimization of conditions.

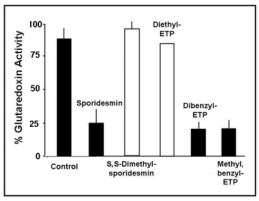

Figure 5. Reaction of ETP analogs with GRx1: Structural requirements for inactivation

GRx1 (2 μM) was treated with different ETP analogues at 1 mM final concentration. The synthetic ETPs were dissolved in dimethyl sulfoxide to a final concentration of 100 mM and diluted 1/100 into the GRx1 –containing solution at 0.1 M K phosphate, pH 7.5, 30 °C. GRx1 was treated with 1 % ethanol or dimethyl sulfoxide as controls for these experiments. After incubation for 20 min, the samples were assayed for GRx1 activity using cys-SSG as the substrate. Each bar represents the mean value, plus and minus the standard error, for at least three determinations. In the case of Diethyl-ETP, no error is shown because this represents a single experiment after optimization of conditions, due to limited supply of this ETP derivative.

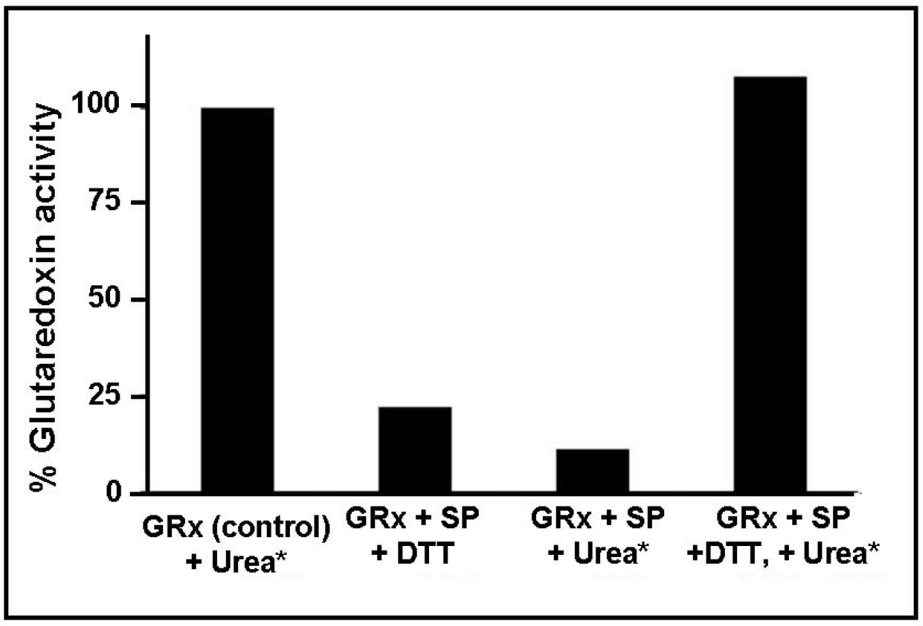

Figure 6. Requirements for reactivation of sporidesmin-inactivated GRx1

GRx1 (100 μM) was treated either with 1 mM sporidesmin or 5 % ethanol at 30 °C, 0.1 M K phosphate, pH 7.5. Enzyme activity was measured after incubation for 15 min. The sporidesmin-treated sample was approximately 90% inactivated. The control was treated with 8 M urea plus 50 mM DTT. The inactivated GRx1 was divided into two aliquots and treated either with either 8 M urea or 50 mM DTT and 8 M urea. The samples (including urea plus DTT treated control) were each loaded on a gel filtration column and separated from excess small molecules. The GRx1 protein was pooled and analyzed for activity and protein content. The results shown in the figure are for a single experiment, after conditions were optimized. The recovery of protein after size exclusion chromatography was 55–60% for all three samples. GRx1 inactivated with sporidesmin and exposed to urea alone or DTT alone showed no reactivation, while treatment with sporidesmin then urea plus DTT gave 60% recovery of activity, i.e. full recovery of GRx activity when normalized to 55–60% protein recovery.

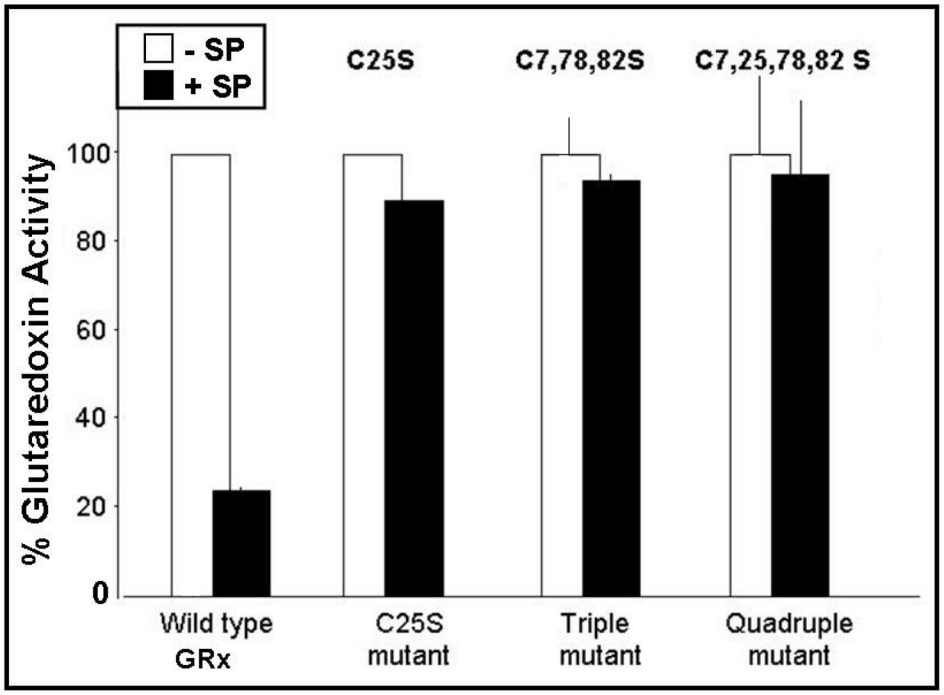

Figure 7. Study of sporidesmin reaction with cysteine-to-serine mutants of GRx1

Site-directed mutagenesis was utilized to produce the cys to ser mutant GRx1 enzymes, as described in Methods. Each enzyme (3–5 μM) was incubated with 1 mM sporidesmin for 20 min in 0.1 M K phosphate, pH 7.5, 10% ethanol at 30 °C. GRx1 activity was then measured using cys-SSG substrate. Each point represents the mean value, plus and minus the standard error, for at least three determinations. Where error bars are not evident they are within the size of the symbol.

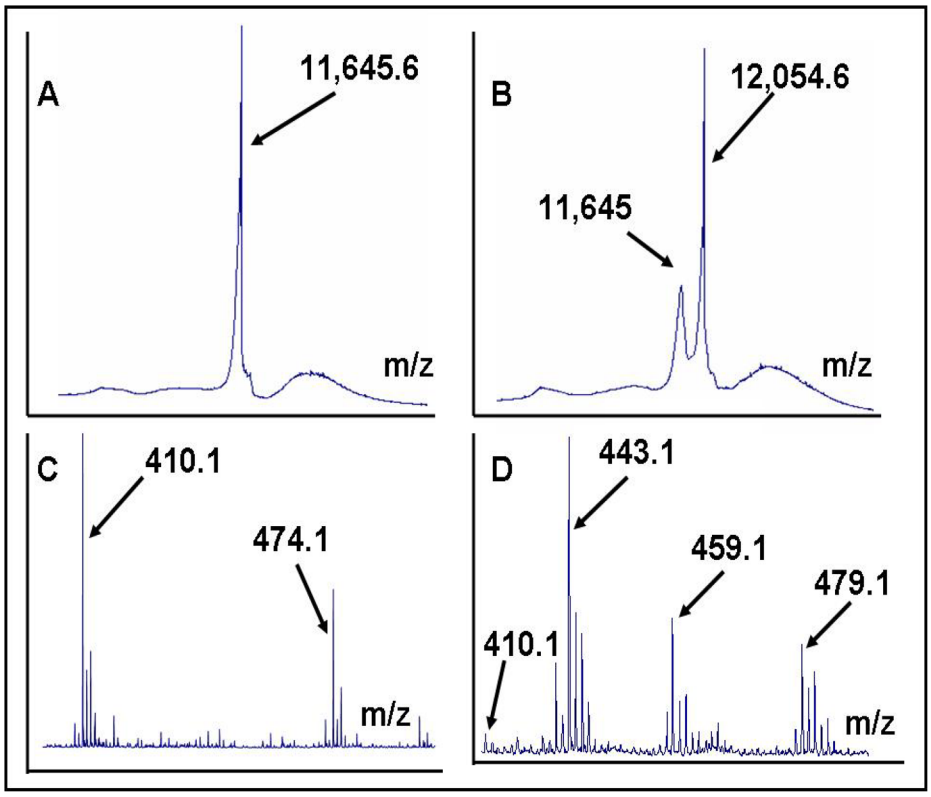

Figure 8. Mass spectrometry of GRx1, sporidesmin and reaction products

MALDI spectra of GRx1 and the reaction product with sporidesmin show ions at m/z 11,645 corresponding to native GRx1 (A), and m/z 12,054 consistent with formation of a mixed disulfide between GRx1 and sporidesmin followed by elimination of two sulfur atoms (B). ESI spectra of sporidesmin (C) and the corresponding low mass regions in the product of reaction with GRx1 (D) contain ions consistent with the presence of sporidesmin (calculated m/z 474.0555), sporidesmin minus two sulfur atoms (calculated mass 410.1113) and in the reaction product ions corresponding to sporidesmin in which a sulfur has been replaced by hydrogen (calculated m/z 443.0912) and the product of monooxygenation of the remaining sulfur atom (calculated m/z 459.0862). The identity of the m/z 479 species is unknown. Low mass regions of the spectra were internally calibrated using the sporidesmin ion.

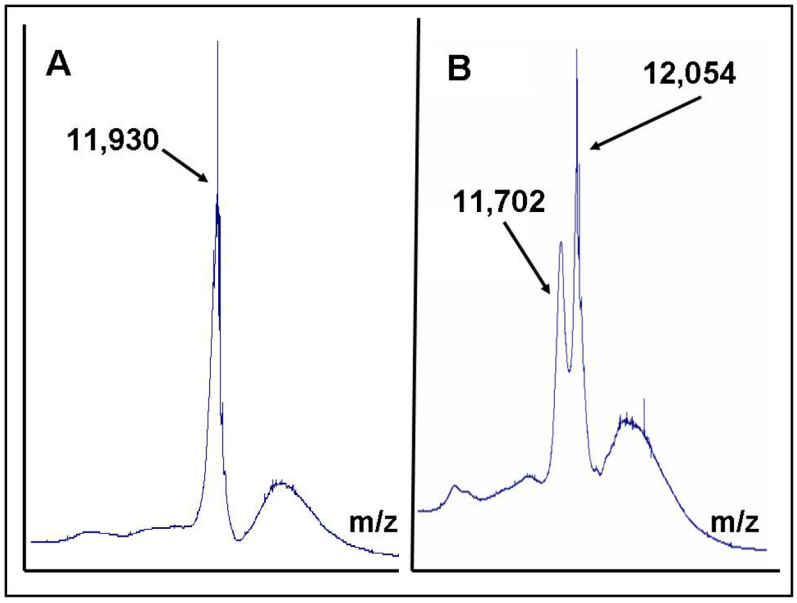

Figure 9. Alkylation of GRx1 cysteines with iodoacetamide (IAM)

MALDI spectra of GRx1 reacted with iodoacetamide (A), and GRx1 first reacted with sporidesmin, then with iodoacetamide (B). IAM-treated GRx1 contained a major ion m/z 11,930 +/− 3 consistent with alkylation of all five cysteines. MALDI analysis of the products of reaction of GRx1 with sporidesmin, then treated with IAM, only showed major ions corresponding to the SP-GRx1 adduct (m/z 12054) and GRx1 modified by carboxamidomethylation of a single cysteine (calculated m/z 11,702).

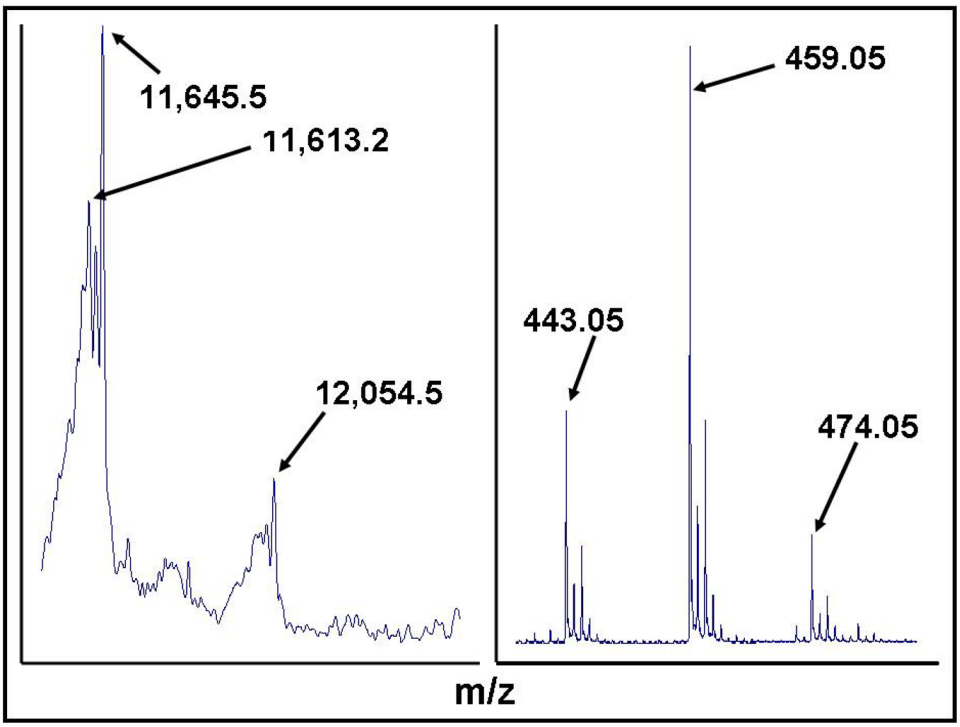

Figure 10. Reaction of GRx1 and adduct with DTT

GRx1 was pretreated with sporidesmin, followed by incubation in the presence of 35 mM DTT for 1 h at room temperature, then subjected to mass spectral analysis. MALDI ions at m/z 11,613 +/− 2 and 443 were consistent with release of GRx1 and sporidesmin, respectively, each less a sulfur atom, as would be expected if two sulfurs had been eliminated from the adduct. Ions consistent with the presence of unreacted GRx1 (11,645) and SP-GRx1 adduct minus 64 Da (12,054), reflecting possible loss of two sulfur atoms, are also evident.

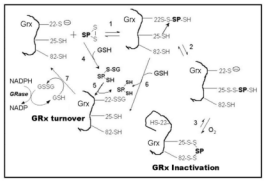

Figure 11. Mechanistic models of sporidesmin as a substrate and inactivator of GRx1

This scheme shows two alternative explanations for sporidesmin as a substrate of GRx1: (a) **steps 4, 5, 7** depict an initial non-enzymatic formation of glutathionyl mixed disulfide of sporidesmin, sporidesmin-S-SG, which is then reduced by GSH in a GRx1 catalyzed reaction to form reduced sporidesmin. This postulated mechanism is consistent with the two-substrate kinetics (Figure 1) and previous reports of the substrate specificity of GRx1 and the mode of substrate activity of non-GSH containing disulfides (1,2,4,5). (b) **Steps 1, 6, 7** depict the direct reaction of sporidesmin with GRx1 to form GRx1 sporidesmin mixed disulfide (GRx1-S-S-sporidesmin). GSH then displaces sporidesmin from this adduct to form GRx1-S-SG which can enter the normal catalytic cycle of GRx1. (c) When GSH is scarce or absent the GRx1-S-S-sporidesmin complex goes on to an irreversibly inactivated form according to **steps 1, 2, 3** involving molecular oxygen.

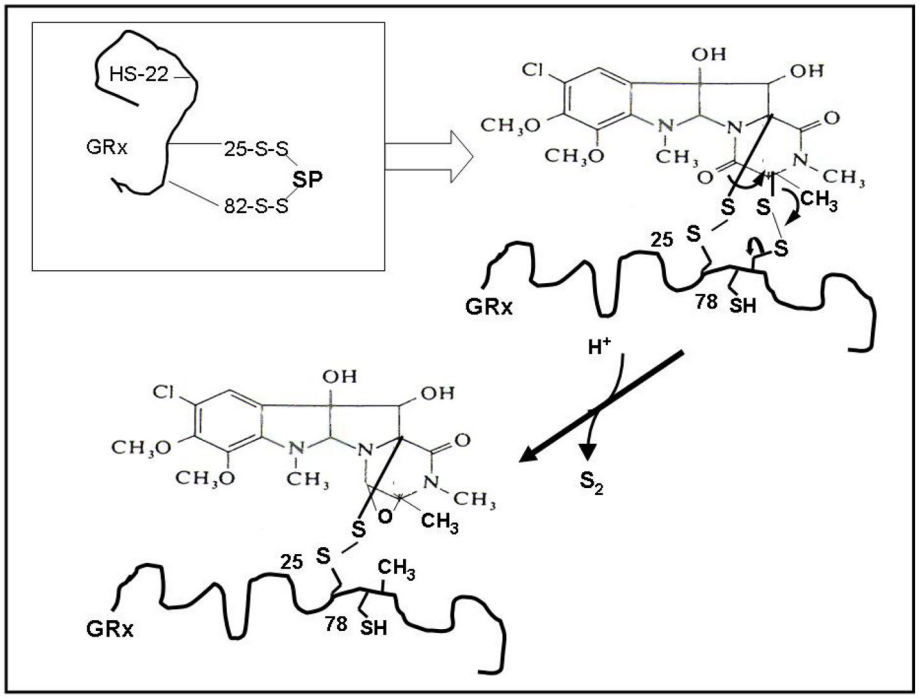

Figure 12. Hypothetical scheme for spontaneous de-sulfuration of the GRx1-sporidesmin adduct

This scheme depicts one plausible mechanism by which the adduct of GRx1 and sporidesmin might spontaneously lose 2 sulfur atom equivalents yet remain intact as a disulfide-bonded bimolecular complex. (As indicated in the text, there are other conceivable ways to lose 64 Da of mass, *e.g.*, loss of four oxygens, or 2 oxygens and 1 sulfur).

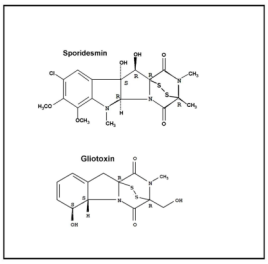

Chart 1. Structures of sporidesmin and gliotoxin

"R" and "S" notations on the molecular structures refer to the configurations at the respective chiral centers.