Glutathione Reductase/Glutathione Is Responsible for Cytotoxic Elemental Sulfur Tolerance via Polysulfide Shuttle in Fungi

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Fungi that can reduce elemental sulfur to sulfide are widely distributed, but the mechanism and physiological significance of the reaction have been poorly characterized. Here, we purified elemental sulfur-reductase (SR) and cloned its gene from the elemental sulfur-reducing fungus *Fusarium oxysporum*. We found that NADPH-glutathione reductase (GR) reduces elemental sulfur via glutathione as an intermediate. A loss-of-function mutant of the SR/GR gene generated less sulfide from elemental sulfur than the wild-type strain. Its growth was hypersensitive to elemental sulfur, and it accumulated higher levels of oxidized glutathione, indicating that the GR/glutathione system confers tolerance to cytotoxic elemental sulfur by reducing it to less harmful sulfide. The SR/GR reduced polysulfide as efficiently as elemental sulfur, which implies that soluble polysulfide shuttles reducing equivalents to exocellular insoluble elemental sulfur and generates sulfide. The ubiquitous distribution of the GR/glutathione system together with our findings that GR-deficient mutants derived from *Saccharomyces cerevisiae* and *Aspergillus nidulans* reduced less sulfur and that their growth was hypersensitive to elemental sulfur indicated a wide distribution of the system among fungi. These results indicate a novel biological function of the GR/glutathione system in elemental sulfur reduction, which is distinguishable from bacterial and archaeal mechanisms of glutathione-independent sulfur reduction.

Glutathione (γ-L-glutamyl-L-cysteinylglycine) is a small peptide occurring in both eukaryotes and prokaryotes. Its physiological function depends on a redox-active cysteine residue that is oxidized by thiolate to disulfide to form oxidized glutathione (GSSG). This redox reaction often couples with mechanisms involving tolerance to cellular oxidative damage. Glutathione peroxidase reduces toxic hydrogen peroxide to water and glutaredoxin reduces protein disulfides; both of these reactions use reduced glutathione (GSH) as a substrate. Oxidized glutathione produced by these reactions is reduced by GSH by NADPH-glutathione reductase (GR) and the cellular GSH:GSSG ratio is maintained. Glutathionylation of small compounds is catalyzed by glutathione S-transferases and characterized as the initial step of detoxifying xenobiotics. Besides these enzymic reactions, GSH reacts with various endogenous and exogenous compounds due to having a chemically active thiolate residue. Because the cellular concentration of glutathione is normally maintained at relatively higher levels (~10 mM in Baker’s yeast), it is also significant as a buffer for cellular redox homeostasis.

Elemental sulfur is ubiquitous in the environment, and its reduction to sulfide is an essential step in the global sulfur cycle. Microorganisms are important players in this process. The sulfur-reducing bacterium *Wolinella succinogenes* produces sulfur reductase (SR), which contains a molybdopterin guanine dinucleotide cofactor and catalyzes the menaquinone-dependent reduction of elemental sulfur. In contrast, whereas SR produced by the thermophilic archaeon *Pyrococcus furiosus* is a flavoprotein that uses NADH as an electron donor, these organisms inhabit anoxic environments and are thought to use sulfur for dissimilation or as a terminal electron acceptor to support anoxic growth. To date, gene cloning and the molecular characterization of SR have been restricted to bacteria and archaea. Eukaryotes and human erythrocytes, as well as *Saccharomyces cerevisiae* and *Fusarium oxysporum* reduce exocellular elemental sulfur to sulfide under hypoxic (low oxygen) conditions. Abe et al. (11) detected NADH- and NADPH-dependent SR activity in cell-free extracts of *F. oxysporum*. However, little is known about the eukaryotic sulfur-reducing mechanism and its physiological role. No SR ortholog of the bacterium and the archaeon has been identified in published genomic nucleotide sequences of these organisms, suggesting that the eukaryotic sulfur-reducing and known mechanisms are distantly related.

Elemental sulfur is almost totally insoluble in water (5 μg liter⁻¹ at 25°C) but sulfur-reducing eukaryotes might transfer reducing equivalents produced by the oxidation of carbon sources to exocellular sulfide. One possible mechanism governing this exocellular electron shuttle is the plasma membrane electron transport (PMET) system found in *S. cerevisiae* and mammalian cells. The best-known PMET in *S. cerevisiae* is cat-

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2 The abbreviations used are: GSSG, oxidized glutathione; CS, colloidal elemental sulfur; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); GR, glutathione reductase; GSH, reduced glutathione; IAA, iodoacetamide; IAM, iodoacetamide; polySR, polysulfide reductase; PS, powdery elemental sulfur; SR, sulfur reductase.
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alyzed by ferric reductase (13). The plasma membrane of mammalian cancer cells contains NADH oxidase (14) and a NADH-dependent PMET in *S. cerevisiae* has been proposed (15), although a direct link between the systems and sulfur reduction has not yet been demonstrated. Another mechanism for shuttling reducing equivalents might depend on soluble redox active molecules that permeate the plasma membrane. Bacterial cells produce sulfur/sulfide- and cysteine/cystine-shuttle mechanisms (16, 17), where sulfide and cysteine, respectively, are exported to the periplasm to reduce electron acceptors, although this type of exocellular electron shuttling mechanism remains obscure in eukaryotes, including fungi.

*F. oxysporum* is a filamentous fungus of the phylum ascomycota that inhabits soil environments. It includes phytopathogenic strains that damage fruits and crops, and thus controlling their proliferation is of considerable agriculture importance. Elemental sulfur has long been used as a fungicide to suppress the growth or germination of strains as well as of other phytopathogenic ascomycotic fungi (18). Therefore to understand the fungal mechanism that responds to and metabolizes elemental sulfur is of ecological and academic importance. Here we investigated the sulfur-reducing mechanism of *F. oxysporum*, and uncovered genetic evidence that the fungus reduces elemental sulfur via the GR/glutathione system. We also demonstrated that the reaction is important for *F. oxysporum* as well as for other fungi to tolerate and survive the oxidative stress imposed by toxic elemental sulfur. This report proposes a novel function of the eukaryotic glutathione system that mediates the NADPH-dependent reduction of exocellular elemental sulfur supported by polysulfide shuttling across the cell membrane.

**EXPERIMENTAL PROCEDURES**

**Strains, Culture, and Media—** *F. oxysporum* JCM11502 generated from the Japanese Collection of Microorganisms was cultured at 120 rpm and 30 °C for 72 h on a rotary shaker in 300 ml of GP medium (30 g liter⁻¹ glycerol, 2 g liter⁻¹ NH₄Cl, 10 mM KH₂PO₄ (pH 7.2), 2 mM MgSO₄, and 1 ml liter⁻¹ trace elements) (19) in 500-ml flasks. The mycelia were harvested by filtration, washed with 9 g liter⁻¹ NaCl, inoculated into 100 ml of MMEA medium (300 ml ethanol, 10 mM NH₄Cl, 10 mM KH₂PO₄, 2 mM MgSO₄, and 1 ml liter⁻¹ trace elements (19) pH 7.2) with or without exogenous elemental sulfur (Wako Pure Chemical Industries, Osaka, Japan) in 500-ml flasks and incubated as described above. Fungal transformants were cultured in the same media containing 50 µg ml⁻¹ hygromycin B.

*Aspergillus nidulans*—FGSC A4 (bia1) (University of Kansas Medical Center) and DGR (bia1, argB2, ΔglrA::argB) (20) were pre-cultured in MMDN medium (10 g liter⁻¹ glucose, 6 g liter⁻¹ NaNO₃, 10 mM potassium phosphate (pH 7.0), 7 mM KCl, 2 mM MgSO₄, 2 ml liter⁻¹ trace elements) (19) at 120 rpm and 30 °C for 24 h. The mycelia were harvested, washed, and inoculated into MMEA medium (100 mM ethanol, 10 mM NH₄Cl, 10 mM KH₂PO₄, 7 mM KCl, 2 mM MgSO₄, and 2 ml liter⁻¹ HUTNER’S trace metals (19), 0.2 µg liter⁻¹ biotin, pH 7.2) with or without elemental sulfur. The flask were incubated at 120 rpm and 30 °C for 24 h. *S. cerevisiae* BY4741 (MATa; his3Δ1; leu2Δ0; met15Δ0 ura3Δ0) and Δglr1Δ (BY4741, Δglr1::kanMX4) (Open Biosystems Products, Huntsville, AL, USA) were pre-cultured in YPD medium (20 g liter⁻¹ tryptone, 10 g liter⁻¹ yeast extract, 20 g liter⁻¹ glucose) at 120 rpm and 30 °C for 12 h. Portions were then transferred to SDU medium (20 g liter⁻¹ glucose, 5 g liter⁻¹ ammonium sulfate, 1.7 g liter⁻¹ yeast nitrogen base without amino acids and ammonium sulfate, and with 20 mg liter⁻¹ each of histidine, leucine, methionine, and uracil) with or without elemental sulfur to an optical density of 0.4, and incubated at 250 rpm and 30 °C for 24 h. To measure sulfide production by the fungi, we purged headspace air with nitrogen gas for 10 min and sealed the flasks with butyl rubber stoppers to prevent sulfide evaporation. *Escherichia coli* was cultured in Luria broth (10 g liter⁻¹ tryptone, 5 g liter⁻¹ yeast extract, 5 g liter⁻¹ NaCl).

**Incubation with Thiolate Reagents—** Pre-cultured cells were washed with 9 g liter⁻¹ NaCl, and suspended in 100 mM potassium phosphate (pH 7.0) containing either 5’t-dithiobis-2-nitrobenzoic acid (DTNB), iodoacetic acid (IAA), or iodoacetamide (IAM) (5 mM each). After an incubation at 25 °C for 2 h, the reaction mixtures were filtered and then collected cells were washed and suspended in 100 mM potassium phosphate (pH 7.0) and 32 mg liter⁻¹ (1 mM eq.) of colloidal sulfur. The reactions were incubated at 25 °C for 15 min, and the amount of evolved sulfide was measured. Thereafter, the cells were collected by filtration, suspended in 1% (w/v) 5-sulfosalicylic acid and sonicated. The suspensions were separated by centrifugation at 10,000 × g for 10 min, and then glutathione in the supernatant was determined as described below.

**Purification of Sulfur Reductase—** *F. oxysporum* JCM11502 was cultured in 3 liters of GP medium at 30 °C for 72 h, collected by filtration, washed with 9 g liter⁻¹ NaCl and transferred to 3 liters of MMEA medium containing 0.64 g (20 mM eq.) of powdered elemental sulfur at 30 °C for 48 h. We routinely obtained 60 g (wet weight) of cells from 9 liters of culture, suspended them in 20 mM Tris-HCl (pH 7.5) containing 10% (w/v) glycerol and 0.3 mM N-tosyl-l-phenylalanine chloromethyl ketone (Sigma), 0.3 mM phenylmethylsulfonyl fluoride, and then homogenized the suspension with aluminum oxide as described (21). The homogenates were centrifuged at 1,500 × g for 10 min, and then the supernatant was separated by centrifugation at 100,000 × g for 60 min. Ammonium sulfate (1.4 M final concentration) was added to the supernatant, and the mixture was applied to a butyl-Sepharose CL-4B (GE Healthcare, Waukesha, WI) column (φ1.6 × 20 cm) equilibrated with buffer A (50 mM Tris-HCl (pH 7.5) containing 10% (w/v) glycerol) containing 1.4 M ammonium sulfate. The column was eluted with a 120 ml of linear ammonium sulfate gradient (1.4 to 1.0 M) in buffer A at a flow rate of 20 ml h⁻¹. Active 3-ml fractions were pooled, dialyzed against buffer A, and applied to a 2’,5’-ADP Sepharose (GE Healthcare) column (φ0.5 × 7 cm) equilibrated with buffer A. Proteins were eluted with buffer A containing 10 mM NADP⁺ at a flow rate of 10 ml h⁻¹. Active 1-ml fractions were combined and stored at −80 °C.

**Enzyme Assays—** Fungal cell-free extracts were prepared as described above except for using 20 mM potassium phosphate (pH 7.2), 10% (w/v) glycerol, 0.3 mM N-tosyl-l-phenylalanine.
choloromethyl ketone, 0.3 mM phenylmethylsulfonyl fluoride. Sulfur reductase activity was assayed in a reaction mixture containing 100 mM potassium phosphate (pH 7.5), 32 mg liter⁻¹ (1 mM eq.) CS, 1 mM GSSG and 1 mM NADPH in 1.5-ml microtubes. The reaction was initiated by adding NADPH, incubated for 5 to 15 min at 25 °C, and then the amounts of produced sulfide were determined. Polysulfide reductase was assayed using the same method by replacing CS with polysulfide (typically 0.1 mM) prepared as described (21). Glutathione reductase was assayed in a reaction mixture containing 100 mM potassium phosphate (pH 7.5), 0.2 mM NADPH, 0.34 mM recombinant human insulin solubilized by the method of Holmgren et al. (22) and 20 μM recombinant thioredoxin (rTrxA) of Aspergillus nidulans (20, 23). The reaction was started by adding rTrxA and the decrease in NADPH was measured at absorbance 340 nm. Catalase and cytochrome c peroxidase were assayed as described (24, 25).

Cloning the Sulfur Reductase Gene (glaR)—Purified protein (6 μg) was resolved by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) on 10% (w/v) polyacrylamide gels as described by Laemmli (26) and then electronically blotted onto polyvinylidene difluoride membranes. The blots were stained with Coomassie Brilliant Blue R-250, and amino-terminal amino acid sequences were determined in excised protein bands using an automated protein sequencer (Model Precise 492, Perkin Elmer, Waltham, MA). Purified SR was digested with trypsin, used for matrix-assisted laser desorption ionization time of flight-mass spectrometry (MALDI-TOF-MS), and peptide mass fingerprints were analyzed using the MASCOT search engine (Matrix Science Ltd., London, UK) as described (27). The template comprised nucleotide fragments corresponding to the gene for SR (glaR) were amplified by PCR using total DNA of F. oxysporum JCM11502 prepared as described by Takasaki et al. (19) and the primers were fogr1 and fogr2 (supplemental Table S1). Nucleotide sequences were determined using primers GRseq 1 to 6 (supplemental Table S1) and an automated DNA sequencer (CEQ2000, Beckman Coulter) according to the manufacturer’s instructions. The nucleotide sequence will appear in the EMBL/DDBJ data base with accession number AB617703.

Gene Disruption of F. oxysporum glaR—A 720-bp DNA fragment encoding the 5’ region of glaR fused with restriction sites was amplified using the primers dgr1 and dgr2. After digestion with SacI and Xbal, the DNA fragment was ligated with pdLD10 (28) that had been pre-spliced with the same restriction enzymes. The 3’-region of glaR was amplified using the primers dgr3 and dgr4, cut with SalI and KpnI and inserted into the same restriction sites of the resulting plasmid to generate pDFOGR1. Protoplasts of F. oxysporum JCM11502 were prepared and transformed as described (29). Total fungal DNA was Southern blotted and analyzed using a DIG DNA labeling and detection kit (Roche, Basel, Switzerland) according to the manufacturer’s instructions. The resulting glaR gene disruptant was designated DGR (disrupted glutathione reductase gene). Fragments of DNA amplified with primers dgr3 and dgr4 served as a hybridization probe. Supplemental Table S1 lists nucleotide sequences of the primers.

Quantitative PCR—Strain JCM11502 was cultured in MMEA medium with or without 0.64 g liter⁻¹ (20 mM eq.) of PS at 30 °C for 12 h, and total RNA was purified using the RNasy plant mini kit (Qiagen, Venlo, The Netherlands) according to the manufacturer’s instructions. First-strand cDNA was synthesized by incubating total RNA (10 μg) in 10 μl of reaction buffer comprising Oligo (dT)₂₀ (Toyobo, Osaka, Japan), 5 × reverse transcriptase buffer and reverse transcriptase M-MLV (200 units) (Takara Bio, Kyoto, Japan) at 42 °C for 90 min. First-strand cDNA (330 ng) synthesized in the reaction was analyzed by quantitative PCR using iQ™ SYBR® Green Supermix (Bio-Rad) and MiniOpticon™ version 3.1 (Bio-Rad) according to the manufacturer’s instructions. The expression of glaR was normalized against that of the actin gene (FOXG_04579.2, Broad Institute, Cambridge, MA). Results are shown as relative expression. The primers were RTfogrF and RTfogrR for glaR, and RTfaoctF and RTfaoctR for the actin gene (supplemental Table S1).

Glutathione Determination—Fungal cells (1 g wet weight) collected by filtration were powdered in liquid nitrogen, suspended in 1% (w/v) 5-sulfosalicylic acid, and incubated on ice for 30 min. After centrifugation at 10,000 g for 10 min, the supernatant was neutralized (pH 6–7) with 1 M potassium phosphate (pH 7.5), and then the GSH and GSSG concentrations were determined as described (30).

Analytical Methods—Sulfide levels in the culture broth were determined using the methylene-blue method (31). Colloidal sulfur was prepared by acidifying sodium thiosulfate with concentrated sulfuric acid as described (32). Polysulfide was prepared as described by Ikeda (21). Protein concentrations were determined using the Protein Assay Reagent (Bio-Rad) according to the manufacturer’s instructions. The molecular weight of native GR was determined by gel filtration through a Superose 6 10/300 GL column (GE Healthcare). Viable cells were determined by spreading appropriately diluted culture broth onto potato-dextrose agar plates, incubated them at 30 °C for 36 h, and then counting the numbers of colonies.

RESULTS

Sulfur Reduction by F. oxysporum—F. oxysporum JCM11502 reduces powdery elemental sulfur (PS) to sulfide (12). Our results indicated that the fungus produced more sulfide from exogenous colloidal elemental sulfur (CS) than from PS (Fig. 1A). Sulfide production linearly increased after an incubation with <5 mM equivalent CS (described as mM eq. hereafter for a more accurate comparison between levels of insoluble elemental sulfur and soluble sulfide) for 24 h (Fig. 1A). At >5 mM eq. CS, sulfide production became saturated at ~2 mM and cell survival severely decreased (to <7%), which agreed with the saturated production of sulfide at >5 mM eq. CS (Fig. 1A). The results obtained using PS were essentially the same although less sulfide was produced and growth was less inhibited com-
pared with CS (Fig. 1, A and B; supplemental Fig. S1). These are probably due to the more hydrophobic and lower dispersal properties of PS, which prevented an efficient reaction between the cells and PS.

We examined the effect of CS and sulfide on growth. Fig. 1 C indicates that *F. oxysporum* generated smaller colonies with a thinner hyphael lawn on agar plates containing 2 mM CS. Adding the same amount of sulfide resulted in a lesser decrease in fungal growth (Fig. 1 D) and a distorted morphology, indicating that CS impairs cell growth more than sulfide. This is consistent with the finding that incubation with sulfide decreased the number of surviving cells, although less effectively than CS (Fig. 1 B). These results indicated that *F. oxysporum* reduced elemental sulfur to less toxic sulfide under these culture conditions.

We examined sulfur reduction by cells incubated with thiolate reagents (Fig. 1 E). Pre-incubating the cells with thiolate-blocking IAA and IAM (5 mM each) almost completely impaired fungal sulfide production from CS, indicating that cellular thiolate residues are involved in the sulfur reduction. By contrast, pre-incubation with 5 mM DTNB had no effect (Fig. 1 E), and 50% of the activity persisted even after incubation with 20 mM DTNB (data not shown). Both IAA and IAM decreased cellular GSH and GSSG, confirming that they could permeate the cell membrane (Fig. 1 F), whereas membrane-impermeable DTNB affected GSH and GSSG concentrations to a lesser extent. These results indicate that intracellular thiolate mediates the sulfur reduction by *F. oxysporum*, which agrees with our findings (described below) that the sulfur reduction mechanism involves glutathione.

**Identification of Fungal SR—**The cell-free extract prepared from *F. oxysporum* JCM11502 cultured under sulfide-producing conditions generated sulfide at a rate of 2.4 nmol min\(^{-1}\) mg\(^{-1}\) in the presence of 5 mM eq. CS and 1 mM NADPH (Fig. 2 A). The reaction with PS substituted for CS also generated...
sulfide, but we used CS for enzyme analysis hereafter since it generated more sulfide, possibly due to its more hydrophilic nature. Adding a filtrate of cell-free extract (<10 kDa) to the dialyzed restored the sulfide-producing activity that was lost by adding dialyzed cell-free extract. Adding 0.1 mM of either GSH or GSSG to the dialyzed enhanced NADPH-dependent sulfide production from CS (Fig. 2A), whereas other physiological or artificial cofactors including benzyl viologen, phenazine methosulfate, FAD, FMN, diithiothreitol, mercaptoethanol, and cysteine did not (data not shown). Sulfide was produced at a rate of 310 μmol min⁻¹ mg⁻¹ in the presence of 5 mM eq. CS and 1 mM GSSG; this was 130-fold faster than that in the absence of GSSG. Hereafter, we refer to this process as GSSG-dependent NADPH-sulfur reductase (SR) activity. We fractionated the cell-free extract by differential centrifugation and recovered >90% of the SR activity from the soluble cytosolic fractions where cytosolic glucose-6-phosphate was concentrated, indicating that most SR resides in fungal cytosol. Minor, but significant activity was detected in particulate fractions containing mitochondria.

We chromatographically purified the SR activity 150-fold with 18% recovery from the fungal cell-free extracts (Table 1). Resolution as a single band (50 kDa) on SDS-PAGE confirmed the homogeneity of the purified preparation (supplemental Fig. S2). The molecular mass of the enzyme calculated from gel-filtration chromatography was 100 kDa, indicating that the purified enzyme was dimeric (data not shown). The rate at which the purified preparation oxidized NADPH in the presence of CS and GSSG was 41 μmol min⁻¹ mg⁻¹, whereas that of NADH oxidation was 0.86 μmol min⁻¹ mg⁻¹, indicating that the enzyme prefers NADPH. Analysis by MALDI-TOF-MS showed that tryptic peptides of purified SR accounted for 90% of the SR activity from the soluble cytosolic fractions where cytosolic glucose-6-phosphate was concentrated, indicating that most SR resides in fungal cytosol. Minor, but significant activity was detected in particulate fractions containing mitochondria.

| Purification procedure | Protein (mg) | Total activity (μmol min⁻¹) | Specific activity (μmol min⁻¹ mg⁻¹) | Yield (%) | Fold |
|-----------------------|-------------|-----------------------------|-----------------------------------|-----------|------|
| Cell-free extract     | 750         | 180 (250)                   | 0.24 (0.37)                       | 100 (100) | 1.0 (1.0) |
| Ultracentrifugation   | 400         | 140 (200)                   | 0.35 (0.51)                       | 78 (75)   | 1.4 (1.4) |
| Butyl-Sepharose       | 22          | 66 (110)                    | 3.0 (4.8)                         | 37 (37)   | 11 (13)   |
| 2′5′-ADP-Sepharose    | 0.74        | 29 (44)                     | 41 (59)                           | 16 (18)   | 150 (160) |

Table 2: Apparent kinetic parameters of purified SR/GR

Enzyme activity was determined by measuring rates of sulfide production except that of GR, which was determined by measuring decreases in absorbance at 340 nm.

![Image of Table 2](image.png)

*Not applicable.

Amino acid sequence, APITKETDYLYL, which was identical to that of purified SR except for the absence of an amino-terminal methionine residue. All of the determined peptide mass fingerprints were found in the calculated masses of the predicted tryptic fragments of the cloned gene, indicating that the isolated gene encoded purified SR. The nucleotide sequence of the open reading frame of the cloned gene was identical to that of BOXG_07937.2 except for 23 nucleotide changes in wobble positions that caused no amino acid changes and two nucleotide substitutions of valine 265 to cysteine. We found a motif typical of the Rossmann-fold superfamily (14GXGXXG), a highly conserved amino acid sequence among the GR involving the catalytic Cys residues (14CVNVGC), a contact site for the isoa-laxozine ring (778DXGXXD) and a binding motif for FAD (1994T(5)X(5)XGXD), which are characteristic among GR (2) (supplemental Fig. S3). The predicted amino acid sequence was similar to those of GR encoded by E. coli gora (50% identity), S. cerevisiae GLR1 (47%), and A. nidulans glrA (74%). These results together with following indicated that the fungal GR functions as SR, and the isolated gene was designated as glrA.

Sulfur Reducing Mechanism by SR/GR in Vitro—We examined the stoichiometry of NADPH consumption and sulfide formation by the purified SR in reactions containing 1 mM NADPH, 0.1 mM GSSG, 1 mM CS, and 5.9 pmol of the purified SR at 25 °C. After a 5-min incubation, 0.12 ± 0.1 μmol of NADPH was consumed and 0.11 ± 0.1 μmol of sulfide was generated. The results are consistent with the stoichiometry of eq. 1 in Fig. 2B. The steady-state kinetics of this reaction indicated that the apparent Kₘ values for CS, GSSG, and NADPH were 1.1 ± 0.2 mM, 63 ± 8 μM, and 21 ± 2 μM, respectively (Table 2). The SR oxidized NADPH in the absence of CS in a GSGG-dependent manner, and the apparent Kₘ values for GSSG and NADPH were comparable (55 ± 5 and 16 ± 2 μM, respectively) to those of the SR activity (Table 2). These values are also similar to GR from other fungi (20, 33), indicating that the purified SR was as active as GR in catalyzing Reaction 1.

NADPH + H⁺ + GSSG → NADP⁺ + 2GSH

**REACTION 1**
Glutathione System Tolerates Elemental Sulfur

The mechanism of the SR reaction is explained as follows. Initially, SR/GR reduces GSSG to GSH using NADPH (Reaction 1). A rapid non-enzymatic reaction generates sulfides from GSH and elemental sulfur under physiological conditions (Reaction 2) (34).

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2\text{GSH} + S^0 \rightarrow GSSG + H_2S
\]

REACTION 2

According to this chemistry, the produced GSH reduces elemental sulfur and is oxidized back to GSSG. The net reaction was consistent with the SR reaction (Fig. 2, eq. 2), and was facilitated by GSSG and/or GSH as the redox mediator. We confirmed GSSG and GSH turnover during the catalysis by detecting more generated sulfide compared with the amount of GSSG added to the reaction.

**Regulation of SR/GR—Expression of the glrA gene was quantified using PCR.** Culture in the presence of 20 mM eq. of elemental sulfur (PS) resulted in the generation of 4.2-fold more glrA transcripts in *F. oxysporum* JCM11502 than that in the absence of PS (Fig. 2C). Intracellular SR activity was consistently higher in the fungus cultured in medium containing PS. The induction ratio of 1.3 was higher than that of intracellular GR activity (1.4-fold; Fig. 2D). These results indicated that elemental sulfur up-regulated SR/GR expression at the transcriptional level, and agreed with the notion that glrA is responsible for producing cellular SR and GR activity under the sulfur-reducing conditions described below.

**SR Confers Tolerance against Sulfur Toxicity**—We constructed a plasmid to double-crossover with the fungal chromosome at the 5′-and 3′-regions of glrA and introduced it into *F. oxysporum* JCM11502 (Fig. 3A). Southern blotting of total DNA from the wild-type strain (WT, JCM11502) and a transformant designated DGR revealed a specific 10-kb EcoRI DNA fragment for glrA in WT, but not in DGR (Fig. 3A). The DGR strain generated a 7.2-kb band, indicating a deletion of the glrA gene. Neither SR nor GR activities was detected in cell-free extracts of DGR (Fig. 2A), indicating that glrA was required to generate these activities. The levels of GSSG were 6–10% of the total intracellular glutathione both in WT and in DGR cultured in the absence of CS (Fig. 3B). Adding CS to the culture medium increased intracellular levels of GSSG both in WT and DGR, and more GSSG accumulated in DGR. These results indicated that glrA is important for the reduction of GSSG to GSH. Fig. 3B also indicated that the total amounts of GSH + GSSG increased 1.8-fold in DGR compared with WT. These findings suggested that GSH synthesis was induced by eliminating GR as in other fungi (20, 35). We examined sulfide evolved by *F. oxysporum* cultured with elemental sulfur. The amounts of sulfide evolved by the DGR strain from CS and PS were 20 and 60%, respectively, of that observed by the WT strain (Fig. 3C), indicating that glrA is required for maximal sulfide production. These results showed that the glrA gene product functions as SR in *F. oxysporum* cells. We examined fungal growth in the presence of GS and sulfide. In the presence of 1 mM sulfide, WT and DGR strains generated similar sizes of colonies to those in the absence of sulfide, whereas DGR formed smaller colonies on plates containing the same concentration of CS (Fig. 3D), indicating that a deletion of SR/GR increased the sensitivity of the fungal growth to CS but less so to sulfide. These results indicate that *F. oxysporum* SR/GR tolerates elemental sulfur by reducing it to less cytotoxic sulfide.

Thioredoxin is a small ubiquitous dithiol protein that is reduced by NADPH-thioredoxin reductase in vivo. We detected this activity in cell-free extracts of *F. oxysporum* (Fig. 4). Adding a recombinant preparation of thioredoxin (TrxA) (20, 23) to the cell-free extract increased NADPH-dependent sulfide production 1.5-fold (Fig. 2A), suggesting that TrxA mediates sulfur reduction by thioredoxin reductase. This TrxA-dependent SR activity might account for sulfide production observed in the DGR strain (Fig. 3C).

**Growth under Other Types of Oxidative Stress**—We examined the growth of *F. oxysporum* strains on agar in the presence of various oxidants. Colonies of WT were decreased by menadione, diamide, and hydrogen peroxide (H₂O₂) (1 mM each) and to a lesser extent by diamide (Fig. 4A). Higher concentrations of menadione, diamide, and H₂O₂ suppressed the growth (data not shown), indicating that these compounds inhibit fungal growth. The growth of DGR was slightly slower under normal conditions as above. Adding the oxidants suppressed the growth of DGR more than that of the WT. The difference in colony sizes between WT and DGR was the most obvious after culture in the presence of diamide (Fig. 4A). These results indicated that glrA contributed to growth in the presence of oxidative stress caused by diamide but less to that in the presence of menadione and H₂O₂. Diamide damages cellular thiolates (36), suggesting that the role of the fungal glutathione system is to
repair such damage. We found that basal levels of intracellular anti-oxidant enzymes such as thioredoxin reductase, catalase and cytochrome c peroxidase of DGR were higher than those of the WT (Fig. 4B). These enzymes helped to sequester cellular \( \text{H}_2\text{O}_2 \) and compensated for the GR deficiency in growth in the presence of \( \text{H}_2\text{O}_2 \) as described for other fungi (20, 35, 37).

We found that the purified SR/GR was active in NADPH-dependent polysulfide reduction (polysulfide reductase (polySR), eq. 2 in Fig. 2B) that was lost in DGR strain (Fig. 2A). Apparent \( K_m \) and \( k_{cat} \) values for CS, GSSG, and NADPH were similar between the SR and the polySR activities (Table 2), indicating that \( F. \text{oxysporum} \) SR also reduces both CS and polysulfide. Fig. 3D shows that the DGR strain formed smaller colonies than WT when plates contained 1 mM polysulfide. The extent of growth inhibition induced by polysulfide was similar to that induced by CS. These results are consistent with the notion that polysulfide generated from elemental sulfur is transported to the cytosol, where it is reduced by GSH to generate sulfide (Fig. 5A). The glutathione system comprising GR and glutathione constitutes the critical mechanism for the reducing reaction (see “Discussion”).

**Evolutionary Conservation of Sulfur Tolerance Mechanism among Fungi**—Because little is known about the distribution of fungi that reduce elemental sulfur to sulfide, we examined this ability in 37 fungal strains. We discovered that 17 strains evolved \( 0.1 \text{ mM sulfide} \) when cultured in the presence of \( 5 \text{ mM} \) eq. PS for 30 h (supplemental Table S2). None of the strains evolved sulfide in the absence of PS, indicating their ability to reduce elemental sulfur to sulfide. These fungi are ascomycetes, basidiomycetes and zygomycetes, indicating a wide distribu-

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**FIGURE 4. Role of GR in oxidative stress responses.** A, effects of menadione (MD), diamide (DA), and hydrogen peroxide (\( \text{H}_2\text{O}_2 \)) (1 mM each) on growth of WT and DGR. Strains were incubated on MMEA for 72 h at 30 °C. B, intracellular activities of thioredoxin reductase (TRR), catalase, and cytochrome c peroxidase (CPX). WT and DGR were incubated in MMEA with (+S) or without (−S) 20 mM eq. PS for 24 h at 30 °C.

**FIGURE 5. Sulfur reduction mediated by SR/GR in \( F. \text{oxysporum} \) and other fungi.** A, model of SR/GR-mediated sulfur reduction. B, sulfide production by WT (filled bars) and GR gene disruptants (unfilled bars) of \( S. \text{cerevisiae} \) and \( A. \text{nidulans} \). \( S. \text{cerevisiae} \) strains BY4741 (WT) and glr1/1 (Δglr1) were used to inoculate medium containing either 20 mM eq. PS or 5 mM eq. CS to optical density of 0.4. The \( A. \text{nidulans} \) WT and DGR1 (ΔglrA) strains (20) (100 mg dry cells) were cultured in MMEA medium containing 20 mM eq. PS or 5 mM eq. CS for 24 h. C, morphology of \( S. \text{cerevisiae} \) colonies on MMEA agar plates with or without sodium sulfide, CS, and polysulfide (polyS) (1 mM each) after incubation for 38 h. D, morphology of \( A. \text{nidulans} \) colonies on MMEA agar plates with or without sodium sulfide, CS, and polysulfide (polyS) (1 mM each) after incubation for 48 h.
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The reduction of elemental sulfur is likely to accompany the transfer of reducing equivalents across the cell membrane because of its low solubility and membrane impermeable properties. One mechanism that can transport them is the PMET, through which intracellular reducing equivalents (herein, those provided from GSH) are transferred to membrane protein thiols that face the outside of the membrane and reduce exocellular compounds. However, this is not true for the fungal sulfur reduction mechanism since high levels of sulfur were produced (elemental sulfur reduction) even after DTNB blocked cell surface thiols (Fig. 1). More likely, the fungus reduces sulfur via a mechanism that shuttles reducing equivalents to exocellular sulfur. One study found that that small amounts of elemental sulfur dissolved in water (5 µg liter⁻¹) provided the substrate for initial sulfide production, and once present, sulfide can spontaneously react with elemental sulfur to generate polysulfide (38, 40) (Reaction 3). Polysulfide is soluble under physiological conditions and transported to the cytosol. A reaction between polysulfide and GSH generates sulfide and polysulfide with the loss of one sulfur atom (Fig. 5A). They are both excreted outside the cells where they react with elemental sulfur. The overall reaction shuts two reducing equivalents to exocellular elemental sulfur in a process that is mediated by polysulfide and/or sulfide. The present finding that GR has polySR activity in the presence of GSSG and that the fungal CS and polysulfide inhibited fungal growth to a similar extent support this notion. Our results demonstrated that the fungus reduces and detoxifies toxic sulfur by a combination of this polysulfide/sulfide shuttle mechanism and the glutathione system. Although our results do not exclude the possibility that some polysulfide progressively reacts with another GSH to generate sulfide after penetrating cells (Fig. 5A), considerable amounts of polysulfide and/or sulfide must be excreted extracellularly because they are required for the continuous import of exocellular elemental sulfur.

We demonstrated that F. oxysporum, A. nidulans, and S. cerevisiae have a GR-dependent mechanism that reduces elemental sulfur. In addition, human erythrocyte cell lysates reduce elemental sulfur after adding GSH, NADPH, or NADH (10) although this mechanism is not understood in detail. Because the glutathione system is conserved among most eukaryotes, our results together with this finding suggest that elemental sulfur reduction is a novel function of the glutathione system that is conserved among eukaryotes. Future studies should clarify this issue. Bacteria and archaea reduce elemental sulfur. W. succinogenes and Acididanus ambivalens produce quinone-dependent SR (7, 41) and P. furiosus as well as Thiobacillus ferroxidans produce NAD(P)H-dependent SR (8, 42). Apparently, glutathione is not required for their activity, indicating that the fungi have adapted independently from bacterial and archaeal mechanisms to reduce elemental sulfur during the course of evolution. Reports indicate that bacterial SR is significant in consuming reducing equivalents for hypoxic fermentative growth. We were unable to address this function of fungal SR (or GR) because adding sub-mM eq. amounts of elemental sulfur causes too much toxicity to measure an effect on fermentative growth. However, the GR requirement for fungal growth on agar plates containing sulfur under aerobic condi-
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