ORIGINAL RESEARCH COMMUNICATION

The Enzymatic and Structural Basis for Inhibition of *Echinococcus granulosus* Thioredoxin Glutathione Reductase by Gold(I)

Gustavo Salinas,^{1,2,*} Wei Gao,^{3-5,*} Yang Wang,^{3,4} Mariana Bonilla,^{2,6} Long Yu,^{3,4} Andrey Novikov,^{3,4} Veridiana G. Virginio,⁷ Henrique B. Ferreira,⁷ Marisol Vieites,⁸ Vadim N. Gladyshev,⁹ Dinorah Gambino,⁸ and Shadong Dai^{3,4}

Abstract

Aims: New drugs are needed to treat flatworm infections that cause severe human diseases such as schistosomiasis. The unique flatworm enzyme thioredoxin glutathione reductase (TGR), structurally different from the human enzyme, is a key drug target. Structural studies of the flatworm *Echinococcus granulosus* TGR, free and complexed with Au^I-MPO, a novel gold inhibitor, together with inhibition assays were performed.

Results: Au^I-MPO is a potent TGR inhibitor that achieves 75% inhibition at a 1:1 TGR:Au ratio and efficiently kills *E. granulosus* *in vitro*. The structures revealed salient insights: (i) unique monomer–monomer interactions, (ii) distinct binding sites for thioredoxin and the glutaredoxin (Grx) domain, (iii) a single glutathione disulfide reduction site in the Grx domain, (iv) rotation of the Grx domain toward the Sec-containing redox active site, and (v) a single gold atom bound to Cys⁵¹⁹ and Cys⁵⁷³ in the Au^I-TGR complex. Structural modeling suggests that these residues are involved in the stabilization of the Sec-containing C-terminus. Consistently, Cys→Ser mutations in these residues decreased TGR activities. Mass spectroscopy confirmed these cysteines are the primary binding site.

Innovation: The identification of a primary site for gold binding and the structural model provide a basis for gold compound optimization through scaffold adjustments.

Conclusions: The structural study revealed that TGR functions are achieved not only through a mobile Sec-containing redox center but also by rotation of the Grx domain and distinct binding sites for Grx domain and thioredoxin. The conserved Cys⁵¹⁹ and Cys⁵⁷³ residues targeted by gold assist catalysis through stabilization of the Sec-containing redox center. *Antioxid. Redox Signal.* 27, 1491–1504.

Keywords: thioredoxin glutathione reductase, gold, selenocysteine, flatworm, *Echinococcus*, glutaredoxin, selenoprotein.

Introduction

THE NEGLECTED DISEASES caused by flatworm (platyhelminth) infections constitute a major cause of human

morbidity and mortality in many developing countries. Major classes of parasitic flatworms are flukes (trematodes) and tapeworms (cestodes). The most prevalent and devastating trematode disease is schistosomiasis, which affects more

¹Worm Biology Lab, Institut Pasteur de Montevideo, Montevideo, Uruguay.

²Cátedra de Inmunología, Facultad de Química, Instituto de Higiene, Universidad de la República, Montevideo, Uruguay.

³Department of Biomedical Research, National Jewish Health, Denver, Colorado.

⁴Department of Immunology and Microbiology, University of Colorado Denver, School of Medicine, Aurora, Colorado.

⁵School of Science, Beijing Forestry University, Beijing, China.

⁶Redox Biology of Trypanosomes, Institut Pasteur de Montevideo, Uruguay.

⁷Laboratório de Genômica Estrutural e Funcional, Centro de Biotecnologia, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil.

⁸Cátedra de Química Inorgánica, Facultad de Química, Universidad de la República, Montevideo, Uruguay.

⁹Brigham and Women's Hospital, Harvard Medical School, Boston, Massachusetts.

*Contributed equally to this work.

Innovation

The crystal structures of *Echinococcus granulosus* thioredoxin glutathione reductase (egTGR), free and in complex with gold, provide insights to understand TGR reaction mechanism and its inhibition, and to improve flatworm TGR-based drug design. A single oxidized glutathione (GSSG) binding site is present in egTGR, and different sites of the thioredoxin reductase domain interact with the Grx domain and thioredoxin. Cys⁵¹⁹ and Cys⁵⁷³ residues constitute a primary TGR site for binding gold and play a key role in positioning the flexible Sec-containing redox center close to N-terminal active site disulfide. Cys⁵¹⁹ and Cys⁵⁷³ sites are the ideal target for designing promising gold prodrug compounds through scaffold adjustments.

than 200 million people. Cestode infections are less prevalent, but include serious human and livestock diseases such as cysticercosis and echinococcosis. The tools available to control flatworms are very limited (26, 27). There is no effective commercial vaccine, and chemotherapy treatments rely on very few drugs; in some cases, there is only a single drug of choice for repeated mass treatment (13). There is concern regarding drug resistance emergence and spread. Research efforts on a rational approach targeting the parasite by new mechanisms may lead to the discovery of drug candidates that may alleviate this pressing need.

The thioredoxin and glutathione systems are vital to several thiol-dependent redox pathways, including deoxyribonucleotide biosynthesis, protein folding, and antioxidant defense, and key to redox regulation, signaling, and homeostasis (25). In contrast to their mammalian hosts and free-living flatworms, parasitic flatworms possess linked thioredoxin–glutathione systems instead of canonical thioredoxin and glutathione systems (52). This unique array of thiol-based redox pathways provides a pharmacological target. The selenoenzyme thioredoxin glutathione reductase (TGR, EC 1.8.1.B1), a natural fusion of thioredoxin reductase (TR) and glutaredoxin (Grx) domains (44), is the single core enzyme for thioredoxin- and glutathione-dependent pathways.

This bottleneck enzyme for flatworm parasites functionally replaces both canonical TR (EC 1.8.1.9) and GR (EC 1.8.1.7) (1, 2, 36, 39), and constitutes a validated drug target. RNAi studies and assays with specific inhibitors have shown that TGR is essential for the trematodes *Schistosoma mansoni*

(31), *Schistosoma japonicum* (42) and the cestode *Echinococcus granulosus* (11). Furthermore, different TGR inhibitors have been shown to partially cure flatworm experimental infection in mice (31, 38, 41, 42).

Gold(I) compounds, such as auranofin (Fig. 1), are potent inhibitors of pyridine nucleotide thiol–disulfide oxide reductases, such as eukaryotic TR, GR, and TGR, and have attracted considerable attention as antitumor and antiparasitic agents (23, 35). Due to the unique thiol-based redox array present in parasitic flatworms, the use of gold-based therapy against flatworm infections has been successfully explored. Auranofin kills cestode and trematode flatworms (11, 31, 33, 42) and is able to cure schistosome infection in a mouse model (31, 42).

The inhibition of *S. mansoni* TGR (smTGR) by auranofin has been studied in detail. Using X-ray crystallography it has been shown that the actual enzyme inhibitor is Au^I, released from auranofin in a reaction that is thought to be catalyzed by TGR (5). This study found that gold can bind at three different TGR sites, including the Cys¹⁵⁴–Cys¹⁵⁹ redox active site and an additional pair of Cys residues (Cys⁵²⁰–Cys⁵⁷⁴). Other Au^I phosphine derivatives have been found to be irreversible inhibitors of human pyridine nucleotide thiol–disulfide reductases, and for human GR it was found that gold was the actual inhibitor with a linear S–Au^I–S coordination at the enzyme CX₄C redox active site (48). However, little is known about structures of medicinally relevant gold compounds.

A gold(I) triphenylphosphine [Au^ICl(PPh₃)₂] complex with pyridine-2-thiol N-oxide (MPO) as coligand, Au₂(MPO)₂(PPh₃)₂ (Au^I–MPO, Fig. 1), has previously been described as a potent antiproliferative agent against trypanosomatids (49).

We report the inhibition of the flatworm parasite *E. granulosus* TGR (egTGR) by Au^I–MPO, Au^ICl(PPh₃)₂, and auranofin (Fig. 1). Au^I–MPO efficiently inhibits egTGR and kills *E. granulosus* protoscoleces. We solved the structure of free egTGR and Au^I–egTGR complex, providing structural and enzymatic insights into egTGR catalysis and inhibition by Au^I.

Results

Overall structure of egTGR

The crystal structure of the full-length form of egTGR, in which the C-terminal penultimate Sec was replaced with Cys, was solved to 2.7 Å resolution. The crystals obtained belong to the space group of P43212. The overall structure of egTGR is presented in Figure 2A. The data collection and statistics are listed in Table 1. egTGR, like mammalian TRs and

FIG. 1. Structures of the compounds assayed as inhibitors of *E. granulosus* TGR. (A) Gold(I) triphenyl phosphine complex with pyridine-2-thiol-N-oxide [Au₂(MPO)₂(PPh₃)₂]; (B) Au(I) triphenyl phosphine [Au^ICl(PPh₃)₂]; (C) auranofin. TGR, thioredoxin glutathione reductase.

smTGR, is a homodimer (4, 22). There is one physiological homodimer in an asymmetric unit.

The overall fold of the TR domains of egTGR is similar to those of smTGR. Figure 2B presents the comparison between smTGR and egTGR structures. Superimposition of all the C α atoms of TR domains with those of smTGR resulted in a root mean square deviation (RMSD) of 0.53 Å. The typical TR domains were clearly identified: the discontinuous FAD-binding domain (residues 106–257, 391–460), the NADPH-binding domain (residues 258–390), and the interface domain (residues 461–492).

The dimer interfaces are similar between egTGR and smTGR, consisting of an interacting domain of one antiparallel five-stranded β -sheet, flanked on each side by two α -helices. In addition, the loop of egTGR between residues 184 and 193 forms an antiparallel β -sheet between two monomers. These interactions are missing in smTGR or human TR1. This implies that egTGR has a more stable dimeric structure due to the extra monomer–monomer interactions. In both free egTGR and egTGR–Au complex structures, the C-terminal tail containing the Sec-containing redox center did not show a clear electron density after residue 592, indicating that this portion of the protein would be mobile, as it has been the case for smTGR and mammalian TRs (3–5, 21, 22).

Although the TR domains of egTGR and smTGR overlaid nicely, their Grx domains (residues 4–105) did not. The Grx of egTGR was found to be rotated 15 degrees toward the C-terminal active site loop (Fig. 2B). This brought the Grx active site 6 Å closer to the Sec-containing C-terminal redox active center. This implies that to accommodate the transfer of electrons from TR to Grx, not only the C-terminal active site loop would undergo a significant conformational change but also the Grx would rotate toward the penultimate Sec⁵⁹⁶ residue, similar to the Grx domain of mammalian TGR (44, 45). The N-terminal Grx domain had a higher B factor than the TR domains (97.5 and 68.5, respectively), suggesting that it is more mobile than the TR domains.

Interactions of the TR domains with Grx domain and Trx

We observed that a β -strand (residues 44–54) and an α -helix (residues 10–19) of the Grx domain interact with two loops of TR domains (residues 131–134 and 455–460, respectively). Recently, a crystal structure of a complex between human TR1 and thioredoxin was determined (21). The egTGR and TR1 homodimers can be superimposed with RMSD of 0.75 Å. Trp¹¹⁴ of TR1 is critical for thioredoxin binding. Although egTGR does not have the Trp¹¹⁴ counterpart as TR1, there is a Phe²¹⁰ at the same site, which should be able to form a similar van der Waals contact network with thioredoxin. The C-terminal active site has a similar distance to both thioredoxin and Grx, around 13 Å. This suggests that the flexible C-terminus needs to make a large conformational change and rotate to access the active sites of both substrates.

For TR1 it has been proposed that Trp⁴⁰⁷, Asn⁴¹⁸, and Asn⁴¹⁹ in the guiding bar suppress motion of the C-terminal arm (21, 22). On thioredoxin binding, there were no major conformational changes in the C-terminal portion interacting with TR guiding bar for controlled electron transfer. Apparently, egTGR has a more flexible C-terminal tail. This may allow

FIG. 2. Crystal structure of egTGR in ribbon representation. (A) Overall structure of egTGR homodimer. The TR domain is depicted in *green*, the Grx domain is depicted in *gold*, and the hypothetical thioredoxin binding model is depicted in *magenta* (based on TR1/thioredoxin complex structure, PDB code 3QFA). FAD is shown as *ball and stick*. (B) Superposition of egTGR (*yellow*) and smTGR monomers (*green*). The structures were overlaid *via* the backbones of the TR1 domains using program Pymol. The TGR C-terminal loop from neighboring monomer is shown in *brown*. (C) Superposition of egTGR (*yellow*) and smTGR Grx domains (*green*). egTGR, *Echinococcus granulosus* thioredoxin glutathione reductase; Grx, glutaredoxin; smTGR, *Schistosoma mansoni* thioredoxin glutathione reductase; TR, thioredoxin reductase. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars

the C-terminal redox center to approach the Grx active site. In both smTGR and egTGR, the Trp⁴⁰⁷ is missing, and Gln⁵⁰⁵ in egTGR and Lys⁵⁰⁶ in smTGR are the counterparts.

NADPH, FAD, and glutathione binding sites in egTGR

The crystal of free egTGR was obtained in the presence of 1 mM NADPH. However, NADPH (or NADP⁺) is not visible in the electron density. Nevertheless, the lining of the NADPH-binding pocket is the same as in smTGR, including several positively charged arginine residues [260, 317, 322, 413], implying that the NADPH-binding sites of both enzymes are highly comparable. The Cys residues that form part of the active site at the Grx (Cys³¹-Cys³⁴) and TR domains (Cys¹⁵⁶-Cys¹⁶¹) were found to be oxidized.

Each monomer has a cleft, which hosts the cofactor FAD. The FAD is bound in an extended conformation as in eukaryotic pyridine nucleotide thiol-disulfide oxidoreductases. As in unliganded smTGR, egTGR Tyr²⁹⁶ is positioned perpendicularly to the re-side of FAD and, in the van der Waals, contacts with isoalloxazine ring. Tyr²⁹⁶ was suggested to play a role to protect FAD from NADPH access (3). Residues from both subunits interact with FAD through hydrogen bonds, particularly at the interface, similar to smTGR and other TRs (4).

Different glutathione-binding sites, catalytic or regulatory, have been proposed based on different smTGR structures. A covalently bound GSH was observed between Leu³⁹⁷ and Thr⁴⁰⁴ on the two-turn helix of smTGR (3). In the egTGR structure, Leu³⁹⁷ and Thr⁴⁰⁴ also adopted a two-turn helix, but the Cys⁴⁰¹ is missing, suggesting that the glutathionylation of this residue in smTGR may be lineage specific or a

crystallization artifact. A solvent exposed positively charged region present in smTGR and GRs, but absent in TRs, has been proposed to participate in oxidized glutathione (GSSG) binding and reduction (4). The same positively charged region is observed in egTGR (residues Lys¹²⁶, Lys¹³⁰, and Arg⁴⁴⁹) (Supplementary Fig. S1; Supplementary Data are available online at www.liebertpub.com/ars). We have previously shown that TGR without a functional Grx domain is unable to reduce GSSG (10, 11). Additional experiments using a Grx-less TGR did not detect any residual GR activity even at 1 μ M of the Grx-less TGR (data not shown), suggesting that the role of this positively charged region would be to stabilize the reduced C-terminal redox active center as observed before (3).

The glutathione-binding site of the Grx domain resembles that of smTGR (3). When GSH is overlaid into the egTGR Grx pocket, the γ -glutamyl moiety of GSH interacts with Asp⁸⁴, Ser⁸⁵, and Gln⁸⁶, while Gln⁶⁰ and Lys²⁵ bind the glycine moiety of GSH. These residues are completely conserved among TGRs. A third Cys residue of the Grx domain, Cys⁸⁸, is only 5 Å away from Cys³¹ of Grx. When GSH is modeled in the pocket, the S γ of Cys⁸⁸ is only 3.3 Å away from the oxygen atom of the Glu residue of GSH. This suggests that Cys⁸⁸ may have a functional role previously suggested.

Au^I compounds are potent inhibitors of TGR

We tested Au^I-MPO and Au^ICl(PPh₃) for their capacity to inhibit TR and GR activities of wild-type TGR. Initial time-course experiments indicated that GR inhibition was fast (Supplementary Fig. S2A), while TR inhibition was

TABLE 1. DATA COLLECTION AND REFINEMENT STATISTICS

Data collection	Native	Gold complex
Space group	P4 ₃ 2 ₁ 2	P4 ₃ 2 ₁ 2
Cell dimensions		
<i>a</i> , <i>b</i> , <i>c</i> (Å)	108.7, 108.7, 257.1	109.3, 109.3, 258.7
α , β , γ (°)	90, 90, 90	90, 90, 90
Resolution (Å)	50–2.7 (2.8–2.7) ^a	49.6–2.88 (3.03–2.88)
<i>R</i> _{merge} ^b	9.8 (100)	6.2 (64.8)
<i>R</i> _{pim} ^c	4.4 (58.6)	6.2 (64.8)
<i>I</i> / σ	17.7 (1.0)	16.8 (2.4)
Completeness (%)	99.4 (97.9)	99.2 (99.5)
Redundancy	7.4 (5.5)	13.3 (13.9)
Refinement		
Resolution (Å)	47.8–2.7 (2.76–2.70)	49.6–2.88 (2.95–2.88)
No. of reflections	42,994 (2591)	36,052 (2572)
<i>R</i> _{working} / <i>R</i> _{free} ^d	20.0/26.0 (31.4/37.9)	18.8/24.0 (32.2/35.8)
R.m.s deviations		
Bond lengths (Å)	0.013	0.017
Bond angles (°)	1.6	1.5
Ramachandran plot		
Most favored region (%)	95.3	91.6
Generously allowed region (%)	3.8	6.8
Disallowed region (%)	1.0	1.6

^aAll data (outer shell).

^b $R_{\text{merge}} = \sum_{hkl} \sum_i |I_i(hkl) - \langle I(hkl) \rangle| / \sum_{hkl} \sum_i I_i(hkl)$.

^c R_{pim} (precision-indicating R_{merge}) = $\sum_{hkl} [(1/(N-1))^{1/2} \sum_i |I_i(hkl) - \langle I(hkl) \rangle|] / \sum_{hkl} \sum_i I_i(hkl)$.

^d $R_{\text{work}} = \sum_{hkl} |F_{\text{obs}}| - |F_{\text{calc}}| / \sum_{hkl} |F_{\text{obs}}|$. $R_{\text{free}} = \sum_{\text{Test}} |F_{\text{obs}}| - |F_{\text{calc}}| / \sum_{\text{Test}} |F_{\text{obs}}|$, where “Test” is a test set of about 5% of the total reflections randomly chosen and set aside.

FIG. 3. Inhibition of TGR activities by gold compounds. (A) TR inhibition. The remaining TR activity (DTNB assay) as a function of inhibitor concentration is plotted. 1.5 nM of active Sec-containing wild-type egTGR was used in all assays. Reaction mixes containing enzymes, inhibitors, and NADPH were preincubated for 3 min, and the assay was then initiated by the addition of DTNB. For each compound, independent curves were performed three times; representative curves are shown. (B) GR inhibition. The remaining GR activity (GR assay) as a function of inhibitor concentration is plotted. 1.5 nM of active Sec-containing wild-type egTGR was used in all assays. Reaction mixes containing enzymes, inhibitors, and NADPH were preincubated for 3 min, and the assay was then initiated by the addition of GSSG. A control with yeast GR (SIGMA G3664) at 10 nM was carried out in parallel. For each compound, independent curves were performed three times and representative curves are shown. egTGR preparation contained a [Sec-containing TGR (active)]/[truncated TGR (inactive)] ratio of 0.17. DTNB, 5,5'-dithiobis(2-dinitrobenzoic acid); GR, glutathione reductase; GSSG, oxidized glutathione.

time dependent (Supplementary Fig. S2B). However, short preincubation of the enzyme with NADPH and gold inhibitors previous to the addition of 5,5'-dithiobis(2-dinitrobenzoic acid) (DTNB) resulted in maximal TR inhibition (Supplementary Fig. S2C). These results would suggest that gold binds to a site close to DTNB-binding site, which is different to the GSSG-binding site.

Au^I-MPO inhibited TGR activities slightly better than auranofin, and approximately five times more efficiently than Au^ICl(PPh₃). Inhibition with Au^I-MPO was highly efficient: ~75–80% inhibition of TR and GR activities was achieved at

FIG. 4. TGR inhibition by Au^I-MPO is reverted by DTT but not by GSH. (A) Reaction mixes containing 1.5 nM of active wild-type egTGR, NADPH, and 20 nM inhibitor were preincubated for 3 min, and chromatographed by size exclusion (SEC) on a PD10 column. The TR activity (DTNB assay) was measured before and after SEC. Time courses for the treated enzyme before and after SEC are shown. The time course for the enzyme in the absence of the inhibitor is shown for comparison. (B) Mixes containing 2 nM active Sec-containing wild-type TGR, 100 μM NADPH, and 200 nM Au^I-MPO were preincubated for 3 min, treated with either 100 μM DTT or 100 μM GSH, desalted using a Zeba™ desalting spin column, and assayed for GR activity. A control without inhibitor was included. egTGR preparation contained a [Sec-containing TGR (active)]/[truncated TGR (inactive)] ratio of 0.17. DTT, dithiothreitol.

a 1:1 concentration ratio of Au^I-MPO:TGR (Fig. 3). However, total inhibition of the enzyme required a large excess of inhibitor (~50:1). The inhibition data did not fit a tight-binding inhibitor (Morrison equation) and suggest the existence of two gold-binding sites with different affinities.

To further characterize the inhibition, we preincubated NADPH, enzyme, and Au^I-MPO mixes at the Au^I-MPO:TGR concentration ratios of 10:1 in the enzyme reaction buffer, for 3 min, and then applied the mixes to a PD10 column to remove unbound inhibitor. Inhibition persisted after removal of the excess inhibitor (Fig. 4A). Furthermore, no activity was

FIG. 5. The gold-binding site in egTGR. The two subunits of egTGR are depicted in green and cyan, respectively. The gold ion is shown as orange sphere. Gold binding Cys⁵¹⁹-Cys⁵⁷³, N-terminal Cys¹⁶¹-Cys¹⁵⁶ active site, acid base catalyst dyad His⁵⁷⁰-Glu⁵⁷⁵, and FAD are shown as balls and sticks. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars

recovered even after allowing a 1-h equilibration of the TGR eluate (*i.e.*, allowing for inhibitor/enzyme desorption), indicating that Au^I-MPO:TGR binding is strong. We then examined whether inhibition was reversible by dithiothreitol (DTT) or GSH. The inhibited enzyme treated with DTT and then desalted was active. However, gold inhibition was not affected by GSH, the most abundant thiol *in vivo* (Fig. 4B).

Neither egTGR activities nor its inhibition by gold was affected by the [NADPH]/[NADP⁺] ratios from 10 to 0.1 (data not shown).

The structure of TGR in complex with Au^I-MPO

The crystal structure of egTGR in complex with Au^I-MPO was determined at 2.88 Å in the presence of 1 mM NADPH. No major conformational change occurred on Au^I-MPO binding. The complex structure revealed that only one Au^I was ligated to Cys⁵¹⁹ and Cys⁵⁷³ (Fig. 5). Similar to the Au^I-

smTGR complex, the electron density of scaffold of the gold compounds was not observed. Mutation of these Cys residues to Ser resulted in a 75–80% decrease of TR and GR activities (Table 2), mainly due to a decrease in k_{cat} . The activities of these mutants were inhibited by gold, indicative of an additional gold-binding site. In contrast to the Au^I-smTGR complex (5), the redox center Cys¹⁵⁶-Cys¹⁶¹ was not bound to gold, but forming a disulfide instead.

The Fo-Fc electron-density map suggested that the FAD isoalloxazine had an alternative bend butterfly conformation, implying that FAD was partially reduced (32). To explore how Au impacts on the reduction of FAD, spectrophotometric experiments were performed. The visible spectra (300–800 nm) were collected for TGR in the presence and absence of Au^I-MPO. No differences were found in the oxidized and reduced spectra at the Au^I-MPO:TGR molar ratios ranging from 0.2 to 2 (Supplementary Fig. S3). To gain further information regarding additional gold-binding sites, egTGR was treated with NADPH and Au^I-MPO (10:1 concentration ratio) and then analyzed by mass spectrometry (Supplementary Fig. S4). Gold was bound to Cys⁵¹⁹ and Cys⁵⁷³ and to a minority proportion of Cys³⁴⁷. The peptide containing the Cys¹⁵⁶-Cys¹⁶¹ was oxidized as in the crystal structure. The Sec-containing peptide was not detected either native or bound to gold.

Cys⁵¹⁹ and Cys⁵⁷³ could play an important role in the egTGR catalytic cycle. We modeled the C-terminal tail in the position where the GCUG redox center would be reduced. C-terminal tails in TGR structures are mostly disordered. In a reduced smTGR structure, the C-terminal tail was visible (PDB: 2X80C) (3), the GCUG tetrapeptide was stabilized by the long helix of residues 196–218, on the opposite side of the C-terminal active center in the interdomain space of TGR. Based on this smTGR structure, the flexible C-terminal tail starting with residue Ala⁵⁹⁰ can easily swing into the interdomain groove between the alpha chain Tyr⁵⁰³ to Leu⁵⁰⁷ and the loop of beta chain His⁵⁷⁰ to Thr⁵⁷⁶ without any clashes (Fig. 6A). The residues before Ala⁵⁹⁰ of the C-terminal loop can still interact with the guiding bar.

The GCUG tetrapeptide would insert into a “stabilizing cleft” mainly composed of main chain atoms of residues 505–507, Cys⁵⁷³, Pro⁵⁷¹, and His⁵⁷⁰ (Fig. 6). There are no large residues in the cleft causing any clashes and the docking surface would nicely complement the N-terminal active site. During the

TABLE 2. THE KINETIC PARAMETERS OF WILD-TYPE egTGR, AND CYS⁵¹⁹→SER, CYS⁵⁷⁴→SER egTGR MUTANTS REACTING WITH THE SUBSTRATES DTNB AND GSSG IN THE PRESENCE OF SATURATING NADPH

	DTNB k_{cat} (s^{-1})	DTNB K_M (μM)	DTNB k_{cat}/K_M ($s^{-1} \mu M^{-1}$)
Wild-type egTGR	55 ± 4	38 ± 3.6	1.45
Cys ⁵¹⁹ →Ser	12 ± 1.0	34 ± 2.6	0.35
Cys ⁵⁷⁴ →Ser	10.2 ± 0.9	33 ± 2.5	0.31
	GSSG k_{cat} (s^{-1})	GSSG K_M (μM)	GSSG k_{cat}/K_M ($s^{-1} \mu M^{-1}$)
Wild-type egTGR	28 ± 2	15 ± 1.6	1.87
Cys ⁵¹⁹ →Ser	5.6 ± 0.4	12 ± 1.2	0.47
Cys ⁵⁷⁴ →Ser	5.0 ± 0.5	13 ± 1.5	0.38

In the case of GSSG, the parameters were determined in the presence of GSH to avoid hysteresis. The selenium content of wild-type egTGR and Cys⁵¹⁹→Ser and Cys⁵⁷⁴→Ser mutants was 17%, 15%, and 15%, respectively.

DTNB, 5,5'-dithiobis(2-dinitrobenzoic acid); egTGR, *Echinococcus granulosus* thioredoxin glutathione reductase; GSSG, oxidized glutathione.

FIG. 6. Interaction between the egTGR C-terminal GCUG tetrapeptide and the N-terminal active site disulfide. The egTGR is shown in *ribbon* representation: cyan subunit (A); yellow subunit (B). (A) Superposition of the C-terminal active site tails of egTGR (*same* coloring) and smTGR monomers (*magenta*). Normally, the C-terminal active site is stabilized by the guiding bar and a B chain helix on the opposite side of the C-terminal active center. During the thiol exchange, the loop could swing into the stabilizing cleft and bring the Sec⁵⁹⁶ close to C-terminal active center. This C-terminal movement would be rather small and involve only residues from Ala⁵⁹⁰ to Gly⁵⁹⁷. The other part of C-terminal loop still interacts with the stabilizing bar. (B) During the reduction, the GCUG tetrapeptide inserts into the stabilizing cleft and is stabilized by interactions with Cys⁵⁷³ and other residues. This would position the Sec⁵⁹⁶ close to His⁵⁷⁰ at 3.5 Å, facilitating the thiol interchange. Our and previous data suggested that the reducing equivalents are transferred from reduced Cys¹⁵⁶ through His⁵⁷⁰-Glu⁵⁷⁵ dyad to the C-terminal Sec⁵⁹⁶. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars

dithiol–disulfide exchange between N-terminal and C-terminal redox centers, the C-terminal loop needs to be stabilized to perform the reaction. Our model shows that Cys⁵⁷³ can form a potential hydrogen bond or van der Waals contacts with the main chain oxygen of Cys⁵⁹⁵ (Fig. 6B). This is in agreement with the important role of Cys⁵⁷³ from our data. The impact of Cys⁵¹⁷ is secondary. Cys⁵¹⁷ could be important in stabilizing both Cys⁵⁷³ and residues of guiding bar loop by hydrogen bonds or van der Waals forces due to the close proximity.

The N-terminal active site disulfide is shield by Ser¹¹⁹, Ile¹⁶², Tyr²¹², Thr⁴⁴¹, and His⁵⁷⁰ (Supplementary Fig. S5). Due to the size and shape of the C-terminal Gly residue, the penultimate Sec⁵⁹⁶ could not directly access the N-terminal active center. The stabilizing cleft seems to be the only place that can accommodate the GCUG active center and position the Sec⁵⁹⁶ residue close to the N-terminal active center.

Our model would bring the GCUG tetrapeptide 3.5 Å to the His⁵⁷⁰, which is the acid-base catalyst for the reduction of the N-terminal active site disulfide. Sec⁵⁹⁶ is 7 Å away from Cys¹⁶¹, the interchanging thiol of the N-terminal active center. During the reaction, the reduced Cys¹⁶¹ thiolate could directly attack Sec⁵⁹⁶, which is situated in the stabilizing cleft, while the His⁵⁷⁰ rotates out of way. We have shown before that His residues often shuffle the positions during the dithiol–disulfide interchange in the catalytic cycles of ferredoxin–thioredoxin reductase (17).

Au^I-MPO kills *E. granulosus* larval worms *in vitro*

To test whether Au^I-MPO was active against parasites, we assessed the effect of Au^I-MPO against *E. granulosus* larval worms (protoscoleces) *in vitro*. As a control for *in vitro* studies, we used MPO, an organic parental compound of Au^I-MPO, which did not display any TGR inhibition. Incubation with 10 and 20 μM Au^I-MPO killed larval worms within 24 and 4 h, respectively, but protoscoleces survived at 2 and 5 μM concentrations of Au^I-MPO. Treatment for 24 h at 5 μM Au^I-MPO led to an ~60% decrease in TGR activities, with respect to MPO-treated protoscoleces. These results, summarized in Table 3, indicate a correspondence between TGR inhibition and *in vitro* killing of larval worms and that the inhibitor was able to cross the protoscolex tegument. Figure 7 shows the effect of Au^I-MPO on larval worms at 10 μM Au^I-MPO.

The disorganization of the parenchyma of the larval worms and the conspicuous loss of the crown of hooks observed on treatment were very similar to those observed with the Au^I compound auranofin (11). Interestingly, this phenotype is not observed with oxadiazole N-oxides, which also are TGR inhibitors that kill larval worms (40).

Since TGR inhibition leads to an imbalance of redox homeostasis in flatworm parasites (31, 33), we reasoned that treatment with sublethal concentrations of Au^I-MPO would lead to compensatory changes in gene expression and affect its proteome. However, this treatment did not revealed major changes (Supplementary Table S1), suggesting that a deeper coverage is required to detect significant proteome changes on gold treatment.

Discussion

Bacterial yeast and plant NADPH-dependent TRs consist of FAD-binding and NADPH-binding domains (18, 50). In these enzymes, the reducing equivalents are transferred from

active site disulfide–dithiol motif directly to thioredoxin. In mammals, TRs are selenoproteins, containing a flexible C-terminal tail to shuffle the electrons to their substrates. Additional complexity is observed in the parasite TGR, which is a fusion protein of TR and Grx enzymes. Its TR domain is similar to the whole mammalian TR. Recently, structural studies of smTGR further elucidated the catalytic cycle of TGRs (3–5). The structural comparison of egTGR and smTGR with human TRs suggests that they also share the same reaction mechanism. This has also been proposed in previous studies involving mouse TGR (43, 45).

The unique property of TGR, compared to TRs, is that this enzyme uses a flexible C-terminus to transport the electrons to Grx. egTGR structures clarify the GSH and thioredoxin binding sites. Thioredoxin is reduced by the C-terminal active site, and GSH can only be reduced by the Grx domain, which in turn is reduced by the C-terminal active site. Based on our previous and current findings, we suggest that the C-terminal tail, which is stabilized by a guiding bar (21), is more flexible than that of mammalian TR1. This flexibility may be required for direct reduction of Grx.

Thioredoxin and the Grx domain of TGR interact differently with the TR domain of TGR and would be docking on different TR sites (Fig. 2A). This domain arrangement would facilitate NADPH-independent electron transfer between the Grx domain and thioredoxin through the Sec-containing C-terminal tail, independent of NADPH as has been hypothesized by Angelucci *et al.* (3). To test this hypothesis, we examined whether GSH can reduce Trx *via* TGR using the insulin reduction assay. Insulin reduction was observed in reaction mixes with NADPH, TGR, and Trx, but not with GSH, TGR, and Trx (Supplementary Fig. S6), suggesting that the Sec-containing redox center and the Grx domain would not function equilibrating the two redox systems.

It is of interest whether TGR possesses additional glutathione-binding sites, glutathionylation sites, and Cys residues involved in catalysis or regulation of TGR activities. The Cys⁸⁸ residue of egTGR was found in close proximity to the Grx redox active site, and it was glutathionylated at high GSSG concentrations (11), suggesting a physiological role for this residue. In mouse TGR, a third Cys¹⁰⁵ residue in the Grx domain is also present (19). Superposition of the Grx domains of mouse and egTGRs showed that these two Cys residues are in the similar location. This implied that the Grx of mammalian TGRs may share the same mechanisms as egTGR. Although a Ser residue is present in smTGR at the homologous position, it is not unusual to observe differences in auxiliary resolving Cys positions among related thiol ox-

idoreductases. Similarly, Cys⁴⁰¹ residue was found to be glutathionylated in one of the smTGR structures (3), but this residue is absent in egTGR. It has also been proposed that a cluster of positively charged residues present in the TR domain of smTGR would be involved in an additional glutathione-binding site and responsible for the marginal GSSG reduction observed in the Sec → stop smTGR mutant (4). egTGR has this basic region, but experiments with the Grx-less TGR revealed that its TR module is devoid of GR activity. Common to all studied flatworm TGRs is the hysteretic behavior (*i.e.*, a lag time before the enzyme is fully active) at high GSSG concentrations (52). In egTGR, this phenomenon is mostly abolished by a Cys³⁴ → Ser mutation at the Grx redox active site, suggesting that this Cys residue could be reversibly glutathionylated (10). Collectively, these data suggest that flatworm TGRs are complex enzymes that share common features, but may also exhibit lineage-specific differences.

The crystal structure of smTGR in complex with auranofin showed three Au ions ligated to smTGR. One Au ion coordinated between the two N-terminal active site Cys residues on the si-face of FAD cofactor, a second one coordinated Cys⁵²⁰ and Cys⁵⁷⁴, and a third one in the putative NADPH binding pocket (5). It has also been observed that an Au⁺ ion ligated to the active site Cys residues next to FAD in trypanothione reductase and human GR (15, 48). The crystal structure gold-egTGR complex and mass spectrometry of gold-treated TGR revealed that these Cys were forming a disulfide bond. Unlike trypanothione reductase or GR, the active sites of TGR or TR1 are buried in the interface of the homodimers. Furthermore, the binding of Au requires the Cys residues to be reduced, and the reduced state of TGR is transient and short lived and would make TGR inhibition rather inefficient. The Au^I-MPO:egTGR crystal was obtained at equimolar concentrations, while the study that described the structure of smTGR with auranofin used an excess of auranofin, which might have led to nonspecific binding. A single Au bound to Cys⁵¹⁹ and Cys⁵⁷³ was identified in the gold-egTGR crystal. These Cys residues are conserved in the vast majority of TGRs and mammalian TR1 (Fig. 8). Furthermore, these residues are only 13 Å away from the TGR N-terminal active disulfide and 9 Å away from Glu⁵⁷⁵ of the catalytic dyad of TGR. Binding of Au may perturb the electron transfer pathway. Cys⁵¹⁹ and Cys⁵⁷³ are too distant to form a disulfide bond and have been found to be reduced in smTGR (51). This implies that the thiol form of Cys⁵¹⁹ and Cys⁵⁷³ may be an easy target for Au ligation, leading to egTGR inhibition. Cys → Ser mutations of these residues affected electron flow, decreasing TGR

TABLE 3. EFFECTS OF Au^I-MPO ON PROTOSCOLECES VIABILITY AND TR AND GR ACTIVITIES

	Viability at 4 h (%)	Viability at 24 h (%)	TR activity after 24 h ($\mu\text{M DTNB min}^{-1} \text{mg}^{-1}$)	GR activity after 24 h ($\mu\text{M GSSG min}^{-1} \text{mg}^{-1}$)
Control (10 μM MPO)	100	100	2.0	1.7
Au ^I -MPO 2 μM	100	100	1.6	1.5
Au ^I -MPO 5 μM	100	95	0.8	0.8
Au ^I -MPO 10 μM	70	5	Not assayed	Not assayed
Au ^I -MPO 20 μM	0	0	Not assayed	Not assayed

Viability was determined by exclusion of vital dye eosin.

GR, glutathione reductase; TR, thioredoxin reductase.

FIG. 7. Au^{I} -MPO effect on *E. granulosus* larval worms. Protoscoleces were incubated *in vitro* at 37°C, 5% CO_2 in DMEM with 10 μM Au^{I} -MPO, a TGR inhibitor, or its parental compound (10 μM MPO) in dimethylsulfoxide as a control. (A) Control protoscoleces after 24 h of culture. (B) Treated protoscoleces after 24 h of culture. The scale bar represents 100 μm . Treated protoscoleces showed disorganization of the parenchyma and the loss of the crown of hooks (highlighted by arrows). To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars

activities to ~20–25%, in agreement with a functional role for these residues. In smTGR, Cys → Ala mutants in these residues also affect DTNB and GSSG reduction although less markedly (28).

It has been shown that the C-terminal loop of hTR1 adopted different conformations to accommodate Trx interaction in different redox states. To bind Trx, the GCUG peptide should flip to the surface. In all these cases, the C-terminal active site is more than 20 Å away from the N-terminal active center. We know that the electrons are transferred from $\text{NADPH} \rightarrow \text{FAD} \rightarrow \text{N-terminal redox center} \rightarrow \text{C-terminal redox center}$. However, how the C-terminal active site is reduced by the N-terminal active center is still unknown. Our data suggest that the catalysis reaction involves Cys⁵⁷³ and Cys⁵¹⁹. The reduction of 75–80% activity of Ser mutants precluded their direct participation in the catalytic cycle. Rather, Cys⁵⁷³ and Cys⁵¹⁹ must play a crucial role in stabilizing the GCUG tet-

rapeptide to facilitate the reaction. Our modeling provides a plausible mechanism showing that Cys⁵⁷³ can play an important role in maintaining the position and conformation of the GCUG tetrapeptide in the “stabilizing cleft,” so that Sec⁵⁹⁶ can be in close proximity with Cys¹⁶¹ and His⁵⁷⁰, and directly perform the dithiol–disulfide exchange. This explains why Cys⁵⁷³ and Cys⁵¹⁹ are an ideal gold inhibitor binding site.

The inhibition of TGR by gold suggested the existence of more than one gold-binding site, with one of them of high sensitivity to gold. Mass spectrometry revealed that in addition to Cys⁵¹⁹ and Cys⁵⁷³, Cys³⁴⁷ was marginally bound to gold. This surface-exposed cysteine is distant from both active sites, so this gold binding may be nonspecific. Cys³⁴⁷ was not bound to gold in the structure and it is not conserved. We cannot rule out the possibility of gold binding to the Sec-containing carboxy-terminal peptide, since this peptide was not detected in the structure or by mass spectrometry.

FIG. 8. Sequence alignment of TRs and TGRs. Human and mouse TRXR3 correspond to TGRs (*i.e.*, contain an N-terminal Grx domain). The Cys⁵¹⁹, Cys⁵⁷⁴, and Sec⁵⁹⁷ residues are highlighted (numbering corresponding egTGR). The mitochondrial TR from *C. elegans* (TRXR2), *P. falciparum*, and insect TRs are Cys-containing enzymes. All TRs and TGRs, except *F. hepatica*, contain the Cys residue at the position homologous to Cys⁵¹⁹ in egTGR. All Sec-containing TRs and TGRs, except *C. elegans* cytosolic TR (TRXR1), contain a Cys residue at the position homologous to Cys⁵⁷³ in egTGR. CAEEL, *Caenorhabditis elegans*; DROME, *Drosophila melanogaster*; ECHGR, *Echinococcus granulosus*; FASHE, *Fasciola hepatica*; Grx, glutaredoxin; HYMMI, *Hymenolepis microstoma*; PLAF, *Plasmodium falciparum*; SCHMA, *Schistosoma mansoni*; TR, thioredoxin reductase.

Noteworthy, a 75–80% inhibition of the activity is achieved by a nearly 1:1 relationship of active enzyme to gold, implying that the selenium-less protein present in the wild-type egTGR is not binding gold. This would suggest that Sec is involved in the inhibition mechanism.

In this study, we used three different gold compounds, the reference gold compound auranofin, Au^I triphenylphosphine, and a novel Au^I-triphenylphosphine complexed with the bioactive coligand pyridine-2-thiol N-oxide (Au^I-MPO). This latter compound was found to be more potent than its parental form and slightly more potent than auranofin, indicating that the scaffold can be optimized for TGR inhibition. The capacity of Au^I-MPO and auranofin to kill *E. granulosus* protoscoleces *in vitro* was very similar (11). Au^I-MPO was previously noted for its potent antiproliferative effect against trypanosomatids (49). In that study it was shown that DNA was not the main target for the compound (as it is the case of cisplatin). Instead, NADH-fumarate reductase activity was inhibited by Au^I-MPO, probably due to the fact that pyridine-2-thiol N-oxide is an effective inhibitor of this enzyme, which is absent in flatworms. However, trypanothione reductase was not assayed in this study as a target for this compound. Treatment of protoscoleces with the Au^I-MPO sublethal dose led to a reduction in TGR activities after 24 h, similar to what was observed in *S. mansoni* (31). A recent work in *Taenia crassiceps* (a flatworm parasite of the same class as *E. granulosus*) showed that the addition of auranofin to the larval form of the parasite led to a concentration-dependent decrease of the internal glutathione concentration, formation of glutathione–protein complexes, and export of GSSG to the culture medium (33). Thus, most likely the primary target of gold compounds in flatworms is TGR, although we cannot rule out other targets. A previous report showed that Au^I-MPO has low toxicity to mammalian cells (49).

Gold compounds have been used for medical uses since ancient times, and in the last century they became important for rheumatoid arthritis treatment (30). This and other studies indicate that gold compounds can be repurposed for treating flatworm-caused diseases, and contribute to our understanding of their biochemical effects. Overall, our studies revealed insights into the structural basis of the TGR reaction mechanism and inhibition of this enzyme by selected gold compounds. Of particular relevance is the finding that the residues Cys⁵¹⁹ and Cys⁵⁷³ constitute a primary site for binding gold and their substitution affects TGR activities. This study also highlights common and specific features of TGRs that may be relevant when considering a rational drug design for flatworms, and that gold compounds are promising prodrugs that can be optimized through scaffold adjustments.

Materials and Methods

Cloning, expression, and purification of *E. granulosus* TGR

The constructs for expressing wild-type full-length *E. granulosus* TGR (egTGR), the Sec⁵⁹⁶→Cys egTGR mutant, the Grx-less TGR mutant, and egThioredoxin were previously generated. Cys⁵¹⁹→Ser and Cys⁵⁷³→Ser egTGR mutants were generated by site-directed mutagenesis using the overlap extension method. Recombinant proteins were

produced and purified as previously described (11), following protocols optimized for expression of Sec-containing TR (6). The selenium content of wild-type, Cys⁵¹⁹→Ser, Cys⁵⁷³→Ser, and Grx-less egTGRs was 17, 15, 15, and 18%, respectively, as determined by atomic absorption using a plasma emission spectrometer (Jarrell-Ash 965 ICP) in Chemical Analysis Laboratory, University of Georgia, and used to correct selenoprotein concentration. For crystallization, the Sec⁵⁹⁶→Cys mutant was used. Following Ni Sepharose Fast Flow column (GE Healthcare) purification, the eluted protein was concentrated to 2 ml and applied onto a Superdex G200 (GE Healthcare) column equilibrated with buffer consisting of 10 mM Tris-HCl pH 8.0, 100 mM NaCl, and egTGR was collected and concentrated to 1 mg ml^{−1} for crystallization.

Enzymatic assays

Reduction of DTNB with concomitant NADPH oxidation was determined by the increase in absorbance at 412 nm due to the formation of 5'-thionitrobenzoic acid (TNB) ($\epsilon = 13,600 \text{ M}^{-1} \text{ cm}^{-1}$) (7). The reaction mixtures contained 100 μM NADPH and 5 mM DTNB.

The GR activity was assayed as the NADPH-dependent reduction of GSSG, which is followed by the decrease in absorbance at 340 nm due to NADPH oxidation ($\epsilon = 6200 \text{ M}^{-1} \text{ cm}^{-1}$) (14). The reaction mixtures contained 100 μM NADPH and 1 mM GSSG and 1 mM GSH to avoid conditions of hysteresis (11, 39).

The insulin interchain disulfide reduction was performed as described in an article (24). Assay mixes contained 1.0 mg ml^{−1} insulin, 10 nM egTGR, 60 μM egThioredoxin (EUB56960.1), and 1 mM GSH or 200 μM NADPH in 100 mM potassium phosphate, pH 7.0. The reaction was followed for 1 h by the increase in absorbance at 620 nm due to the precipitation of free insulin β -chain.

Synthesis of gold compounds

Au^I-MPO was synthesized and characterized according to a previously reported procedure (49). Briefly, 0.2 mmol of each AuCl(PPh₃) and pyridine-2-thiol N-oxide sodium salt (NaMPO) were heated under reflux in 20 ml methanol for 6 h under N₂ using Schlenk techniques. The white grayish solid was filtered off the reaction medium and washed with water, ethanol, and ethyl ether. Elemental analyses, electrospray ionization (ESI)-MS, UV-vis, FTIR, ¹H and ³¹P NMR spectroscopic results agreed with those previously reported (49). Pyridine-2-thiol N-oxide sodium salt and Au^ICl(PPh₃) were commercially available.

Inhibition studies

Stock solutions of Au^ICl(PPh₃) and Au^I-MPO were prepared by dissolving the compounds in dimethylsulfoxide at 40°C to a stock solution of 1 mM concentration. The compounds were tested in the nM– μM concentration range. Except otherwise specified, wild-type egTGR was used at a 1.5 nM final concentration in all assays, and NADPH, enzyme, and inhibitor mixes were preincubated for 3 min and the reaction started by the addition of DTNB or GSSG for TR and GR assays, respectively, and followed for 5 min. All assays were performed in duplicate. In every case, a reference

progress curve without an enzyme was performed to control for reactions between substrates and inhibitors. The percentage of TR or GR inhibition was calculated as follows: % Inhibition = $100 - (v_i/v_o) \times 100$, where v_i and v_o correspond to the initial velocities of TNB formation or NADPH consumption ($\mu\text{M}/t$ (s) with and without inhibitor, respectively. To characterize the enzyme-inhibitor binding, NADPH-enzyme-inhibitor mixes were preincubated as described above and then the mixes were applied to a PD10™ column to remove the unbound inhibitor. The TR activity of the eluates was evaluated by adding NADPH and DTNB. Control experiments without either inhibitors or TGR were carried out as control. To test whether inhibition was revertable by low molecular weight thiols, experiments were carried out with mixes containing 2 nM wild-type egTGR, 100 μM NADPH and 200 nM Au^I-MPO were preincubated for 3 min, treated with either 100 μM DTT or 100 μM GSH, desalted using a Zeba™ desalting spin column and assayed for GR activity.

Visible spectra of TGR

The 300–800 nm visible spectra were obtained on a Varian Cary 50 Bio spectrophotometer for the oxidized and NADPH-reduced egTGR using 20 μM egTGR in the absence or presence of Au^I-MPO at the inhibitor:enzyme ratios from 0.2 to 2.

Crystallization, data collection, and processing

Screening for crystallization conditions took place at 16°C using commercial kits from Hampton Research (Crystal Screen, Crystal Screen 2, Index, PEG Rx1/Rx2, PEG/Ion and PEG/Ion 2). 0.1 μl 10 mg ml⁻¹ protein solution was mixed with an equal amount of reservoir solution and equilibrated against 80 μl reservoir solution. Clusters of needle-shaped crystals appeared after 7 days in Crystal Screen condition at 16°C. Fine-tuning the pH in the range 7.5–9.5 and the PEG 3350 concentration in the range 12–25% produced crystals that were suitable for diffraction. A suitable single crystal for data collection was finally obtained after 10 days in a condition consisting of 0.1 M lithium sulfate, 0.1 M Tris-HCl, pH 7.5, and 18% (w/v) polyethylene glycol 3350 at 16°C. The crystallization condition of the complex is same to the native, and Gold was added to the drop of well.

The harvested crystals were quickly frozen and mounted on the goniometer in a nitrogen stream at -173°C. Data were collected using the Pilatus detector at ID-24c of the Advanced Photon Source, Argonne National Laboratory, at a wavelength of 0.98. The data were indexed, integrated, and scaled with HKL-2000 (37). The data collection statistics is listed in Table 1.

Molecular continuum electrostatic surface potentials

Molecular continuum electrostatic surface potentials were calculated in PDB2PQR (20) and the Adaptive Poisson–Boltzmann Solver (8) with solvent accessible molecular potential isosurface visualization in Jmol (www.jmol.org) using a red-white-blue (negative to positive) gradient, followed by rendering with PovRay (Persistence of Vision Raytracer Pty. Ltd). Ligands and waters were removed for the calculations.

Modeling of TGR C-terminal loop

The position of TGR C-terminal tail was investigated and manually adjusted on computer modeling program, O (29). There were two criteria for positioning the C-terminal tail. First, the GCUG active center needs to be close to the N-terminal active disulfide without any clashes. Second, the C-terminal loop should have minimal movement from its position in the reduced smTGR (PDB: 2X8C), in which the Sec was 13 Å away from the N-terminal active-site disulfide. The position of GCUG active center was further refined using energy minimization using program CNS (12).

In vitro culture of larval worms

Eighty thousand protoscoleces from *E. granulosus sensu stricto* (G1 strain, assessed by COX1 amplification) (16), obtained from asceptical puncture of a single hydatid cyst from bovine lung, were washed several times with PBS and then incubated at 37°C, 5% CO₂, in DMEM supplemented with antibiotics and 20 mM HEPES, pH 7.3. For assessing the effect of the inhibitors on larval worm viability, 2000 protoscoleces were treated with 2, 5, 10, and 20 μM concentrations of Au^I-MPO. Protoscoleces were observed under the microscope every 4 h and endpoint viability assessed by exclusion of the vital dye eosin at 24 h (9). The infected bovine viscera were kindly provided by UREXPORT (material normally discarded under the control of the Government Secretary of Agriculture and Livestock).

Proteomic experiments

For proteomic studies, 10,000 protoscoleces were incubated as detailed above, with vehicle (dimethyl sulfoxide) or sublethal concentration of Au^I-MPO (2 and 5 μM) for 24 h. Aqueous soluble and insoluble protein extracts were prepared from cultured protoscoleces by resuspension of the lyophilized pellets into the RapiGest™ SF surfactant (Waters), 0.1% diluted in 50 mM ammonium bicarbonate, pH 8.0 (46). The proteins were subsequently reduced by adding DTT (final concentration: 5 mM) for 30 min at 60°C, and alkylated with iodoacetamide (final concentration: 15 mM) for 30 min in the dark at 20°C. The protein samples were then digested with trypsin (Trypsin Gold, Mass Spectrometry Grade; Promega) at the enzyme-to-protein ratio of 1:50 (w/w) overnight at 37°C. After digestion, the RapiGest SF surfactant was removed with trifluoroacetic acid, as previously described (46). The resulting peptides were desalted using an OA-SIS® HLB Cartridge column (Waters) and analyzed by liquid chromatography separation coupled with tandem mass spectrometry strategy (LC-MS/MS) (34). MS analyses were performed using an ESI quadrupole time-of-flight Ultima API mass spectrometer (Micromass) coupled to a capillary liquid chromatography system (CapLC; Waters). The peptides were separated in a NanoEase C18 (75 μm ID) capillary column by elution with a water/acetonitrile 0.1% formic acid gradient. Data were acquired in a data-dependent mode, and multiple charged peptide ions (+2, +3, and +4) were automatically mass selected and dissociated in MS/MS experiments. LC and ESI conditions included a 200 nL/min flow, a nanoflow capillary voltage of 3.5 kV, a block temperature of 100°C, and a cone voltage of 100 V. Protein identification based on peptide MS/MS data was performed using Mascot

software (Matrix Science) with the following parameters: a maximum of one missed cleavage, fixed carbamidomethyl alkylation of cysteines, variable oxidation of methionine, mass tolerance for the monoisotopic peptide window was set to ± 0.1 Da, and MS/MS tolerance window was set to ± 0.1 Da. All tandem mass spectra were searched against *E. granulosus* deduced proteome annotation available on www.genedb.org (47).

For mass spectrometry analysis of wild-type egTGR treated with Au^I-MPO, 2 nM Sec-containing egTGR was treated with 20 nM Au^I-MPO and 100 μ M NADPH for 1 min and desalted using a Zeba spin desalting column, 40K, 0.5 ml (Thermo). The sample was digested with trypsin and passed through a ZipTip® (Millipore) column and peptides analyzed on an LTQ VELOS nano-ESI (Thermo Scientific). The egTGR preparation contained a [Sec-containing TGR]/[truncated TGR] ratio of 0.17.

Structure coordinates have been deposited in the Protein Data Bank under accession codes: 5W1J for egTGR alone, and 5W1L for egTGR/gold complex.

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Author Disclosure Statement

No competing financial interests exist.

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Address correspondence to:

Prof. Gustavo Salinas
Worm Biology Laboratory
Institut Pasteur de Montevideo
Mataojo 2020
Montevideo 11400
Uruguay

E-mail: gsalin@fq.edu.uy

Dr. Shaodong Dai
Department of Biomedical Research
National Jewish Health
Denver, CO 80206

E-mail: dais@njhealth.org

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Abbreviations Used

DTNB = 5,5'-dithiobis(2-dinitrobenzoic acid)
 egTGR = *Echinococcus granulosus* thioredoxin
 glutathione reductase
 GR = glutathione reductase
 Grx = glutaredoxin
 GSH = glutathione
 RMSD = root mean square deviation
 Sec = selenocysteine
 smTGR = *Schistosoma mansoni* thioredoxin
 glutathione reductase
 TGR = thioredoxin glutathione reductase
 TNB = 5'-thionitrobenzoic acid
 TR = thioredoxin reductase