Mapping the Catalytic Cycle of *Schistosoma mansoni* Thioredoxin Glutathione Reductase by X-ray Crystallography

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Schistosomiasis is the second most widespread human parasitic disease. It is principally treated with one drug, praziquantel, that is administered to 100 million people each year; less sensitive strains of schistosomes are emerging. One of the most appealing drug targets against schistosomiasis is thioredoxin glutathione reductase (TGR). This natural chimeric enzyme is a peculiar fusion of a glutaredoxin domain with a thioredoxin selenocysteine (U)-containing reductase domain. Selenocysteine is located on a flexible C-terminal arm that is usually disordered in the available structures of the protein and is essential for the full catalytic activity of TGR. In this study, we dissect the catalytic cycle of *Schistosoma mansoni* TGR by structural and functional analysis of the U597C mutant. The crystallographic data presented herein include the following: the oxidized form (at 1.9 Å resolution); the NADPH- and GSH-bound forms (2.3 and 1.9 Å, respectively); and a different crystal form of the (partially) reduced enzyme (3.1 Å), showing the physiological dimer and the entire C terminus of one subunit. Whenever possible, we determined the rate constants for the interconversion between the different oxidation states of TGR by kinetic methods. By combining the crystallographic analysis with computer modeling, we were able to throw further light on the mechanism of action of *S. mansoni* TGR. In particular, we hereby propose the putative functionally relevant conformational change of the C terminus after the transfer of reducing equivalents from NADPH to the redox sites of the enzyme.

Schistosomes are human platyhelminth parasites causing Schistosomiasis, a severe disease still classified among the major causes of mortality in tropical and subtropical countries, affecting more than 200 million people (1). The only drug employed to fight the disease is praziquantel, whose efficacy is restricted to the adult stages of the parasite and whose mechanism of action is still incompletely clarified (2, 3). Because this drug is administered to 100 million people every year, some less sensitive strains have already been isolated, and given the massive drug administration, resistance might become a serious problem (3).

Because of this, the search for a new drug against Schistosomiasis is a necessity and a priority according to the World Health Organization (4). The main evidence suggesting that targeting the thiol redox pathway of the parasite may represent a profitable starting point for rational drug design is as follows: (i) schistosomes, living in the human bloodstream, are subjected not only to endogenous reactive oxygen species but are also exposed to radicals produced by the host immune response; and (ii) the thiol redox pathway employed by the worms for the enzymatic reduction of the reactive oxygen species is different from its human counterpart (5, 6).

In mammals, two proteins belonging to the pyridine nucleotide disulfide oxidoreductase family, thioredoxin reductase (TR) and glutathione reductase (GR), are the head antioxidant enzymes shuttling electrons through the thioredoxin (Trx) and the GSH systems, respectively (5). Trx peroxidases in addition to Trxs are the other components of the Trx system; the GSH system involves GSH peroxidase (Gpx) and glutaredoxin (Grx), an oxidoreductase acting on S-glutathionylated substrates (7, 8). TRs and GRs are homodimeric NADPH-dependent flavoproteins that are structurally highly similar. The two proteins share the main fold and the architecture of the binding sites for NADPH and FAD. However, TRs differ from GRs because of the presence of a selenocysteine residue (Sec, U) located in a GCUG tetrapeptide at the C terminus of the protein. This additional C-terminal extension is required for the full catalytic activity of the enzyme (9–11). Thioredoxin glutathione reductase (TGR) is another selenoprotein belonging to the above-mentioned family of oxidoreductases. Characterization of mouse TGR demonstrated that the enzyme can reduce components of both mammalian Trx and GSH systems. In mammals, TGR exerts a wide range of catalytic activities though its selective overexpression in testis after puberty suggests its involvement in very specific functions related with sperm maturation (12).

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3 The abbreviations used are: TR, thioredoxin reductase; TGR, thioredoxin glutathione reductase; SmTGRfl, *S. mansoni* TGR full length (U597C mutant); SmTrx, *S. mansoni* thioredoxin; GR, glutathione reductase; Grx, glutaredoxin; PDB, Protein Data Bank; Sec, selenocysteine.
A similar enzyme has been identified in *Schistosoma mansoni* (SmTGR) (6). The schistosomal antioxidant system lacks the typical mammalian TR and GR enzymes, which are both replaced by SmTGR; this peculiarity revealed that SmTGR may be a target of choice for the development of new antischistosomal drugs (5, 13). Moreover, TGR enzymes with similar roles have been isolated in other human parasites, such as *Taenia* (14) and *Echinococcus* (15). All these parasites can be killed, *in vivo* and *ex vivo*, by auranofin, a gold compound that specifically inhibits Sec- or Cys-dependent reductases (16).

Crystallographic studies on SmTGR have recently highlighted the structural basis for the multienzymatic function of this enzyme. SmTGR is a homodimeric flavoprotein, where each subunit results from the fusion of a typical TR domain with a glutaredoxin (Grx) domain, the latter being located at the C-terminal tail of each subunit, which is close to the FAD active site of the adjacent subunit, contains the Sec residue (17).

The catalytic mechanism of TGR has not been fully elucidated, although an hypothesis has been published for the mouse enzyme (18). Functional analysis performed on SmTGR and on different TGRs proved that the penultimate Sec is essential for the reduction of thioredoxin and the glutaredoxin domain (6, 12, 15). Reduction of glutathione under physiological conditions is probably dependent on the redox exchange with glutaredoxin and thus is also inhibited in Sec-lacking enzymes, even though we observed residual GR activity in a C-terminally truncated variant of SmTGR (17). This residual activity is only observed in the SmTGR variant that lacks the C-terminal arm and is not shared by the full-length U597C mutant (see supplemental material); thus, we believe that in the WT enzyme the GR activity is mostly accomplished by the Grx domain.

We present in this study a structural characterization of some key intermediates belonging to the catalytic cycle of SmTGR by means of x-ray crystallography and computer modeling, as described in Fig. 1. We provide evidence for the reactions in Fig. 1, showing from a structural point of view the steps that allow electron transfer from NADPH to *S. mansoni* thioredoxin (SmTrx) and to the Grx domain of SmTGR with the participation of the C-terminus of the enzyme.

The evidence presented below includes the structural and functional characterization of the full-length U597C mutant of SmTGR (SmTGRfl, see Fig. 1). The enzyme was crystallized in the presence of the following: (i) CuSO₄ to obtain a fully oxidized state (Structure 1 (1.9 Å)); (ii) NADPH (Structure 2 (2.3 Å)); (iii) reduced glutathione GSH (Structure 4 (1.9 Å)). Moreover, we obtained the three-dimensional structure of SmTGRfl, in a different crystal form (Structure 3 (3.1 Å)), which contains one dimer, and not one protomer, in the asymmetric unit. The crystallographic analysis combined with the results obtained by computer modeling (Model 1 and Model 2) and by rapid kinetics experiments allows us to postulate and discuss the possible N-terminus of the enzyme. The two domains work either coupled or independently to catalyze the reduction of a large number of oxidized substrates. The C-terminal tail of each subunit, which is close to the FAD active site of the adjacent subunit, contains the Sec residue (17).

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![Structure 2 / Structure 3](image)
functionally relevant movements of the C terminus during the redox cycle of the enzyme.

**EXPERIMENTAL PROCEDURES**

**Cloning, Expression, and Purification of the SmTGR Mutant**

The gene for full-length TGR, with the mutation U597C, was obtained starting from the gene of truncated SmTGR optimized for *Escherichia coli* expression (17), with appropriate DNA primers. The gene was cloned in a pGEX4T-1 commercial vector (GE Healthcare) and expressed in BL21(DE3) *E. coli* cells (Novagen). The best results, in terms of solubility and protein yield, were obtained by incubation of cells at 20 °C overnight with 20 μM FAD, without isopropyl-1-thio-β-D-galactopyranoside induction. Successful expression was confirmed by SDS-PAGE, highlighting a band at about 90 kDa. The protein was purified by standard affinity chromatography; the GST tag was cleaved by thrombin (Sigma) and removed according to the manufacturer's instructions. Protein purity was >98% as assessed by SDS-PAGE, and concentration was determined by the FAD absorption peak (ε<sub>max</sub> = 11.3 mm<sup>-1</sup> cm<sup>-1</sup>). SmTGR<sub>fl</sub> was dialyzed in 20 mM Tris-HCl, pH 7.4, 50 mM NaCl, 100 μM FAD (to saturate the enzyme), concentrated to 10 mg/ml using an Amicon ultrafiltration filter device (Millipore), and stored at −80 °C.

**Protein Crystallization**

Crystallography of Oxidized SmTGR<sub>fl</sub>—A crystal of the SmTGR<sub>fl</sub>, grown in 0.1 M Hepes, pH 7.4, 20% PEG 3350, 0.2 M KI, and 5 mM β-mercaptoethanol, was picked up and incubated in the same well solution with 2 μM CuSO<sub>4</sub> and without β-mercaptoethanol. After 48 h, the crystal was frozen under liquid nitrogen, after adding 30% PEG 200 as cryoprotectant.

Crystallography of SmTGR<sub>fl</sub>-NADPH Complex—120 μM of purified SmTGR<sub>fl</sub> in 20 mM Tris, pH 7.4, 100 mM NaCl was initially incubated for 24 h with 300 μM plumbagin (Sigma), 400 μM NADPH, in anaerobiosis condition. The mixture was then concentrated to 10 mg/ml before crystallization trials were set up. The protein solution was mixed with an equal amount of the reservoir solution containing 0.1 M Hepes, pH 7.0, 20% PEG 3350, 0.2 M KSCN, and 5 mM GSH. Crystals grew for 1 month in the standard sitting drop vapor diffusion method and were cryoprotected with the same reservoir solution adding 30% PEG 200.

Crystallography of the SmTGR<sub>fl</sub>-GSH Complex—Crystals were grown by sitting drop vapor diffusion. Crystals grew in conditions similar to those of oxidized SmTGR<sub>fl</sub> (see above). 5 mM GSH replaced β-mercaptoethanol. Crystals grew in 1 week and were cryoprotected with the same reservoir solution containing 30% PEG 200.

Crystallography of SmTGR<sub>fl</sub> with Reduced and Ordered C Terminus—Crystallization conditions were initially screened by the Phoenix nanoliter dispensing robot (ArtRobbins) and then manually refined. Crystallization was achieved at 293 K by the sitting drop vapor diffusion technique. 10 mg/ml protein samples were equilibrated in 1 mM NADPH in 20 mM Tris-HCl, 100 mM NaCl, pH 7.4. Drops of 2 μl were mixed with an equal amount of the reservoir solution containing 0.2 M magnesium formate, 20% PEG 6000, 0.1 M Hepes, pH 7.0–7.5, plus 5 mM β-mercaptoethanol. Crystals grew in 1–2 weeks and were cryoprotected with the same reservoir solution adding 30% PEG 200.

**Data Collection, Processing, and Refinement**

Oxidized Form, SmTGR<sub>fl</sub>-NADPH and SmTGR<sub>fl</sub>-GSH Complexes; Structures 1, 2, and 4—The best data sets for the oxidized and for the NADPH-bound form and for SmTGR(U597C) have been collected at ESRF (Berlin, Germany) at 100 K at 1.9 and 2.3 Å resolution, respectively; and for the GSH-bound form, the best data set was collected at ESRF at 1.9 Å resolution. Data analysis was performed with HKL2000 suite (19) indicating that all crystals belonged to the C2 space group, isomorphous to the SmTGR truncated form (17).

**SmTGR<sub>fl</sub> with Reduced and Ordered C Terminus; Structure 3—**Diffraction data were collected at 3.1 Å resolution at Bessy (Berlin, Germany) and analyzed using the HKL2000 suite (19). The three-dimensional structure was solved by molecular replacement (Phaser, 20) using as a search model one protomer of truncated SmTGR structure (PDB code 2v6o) (17). The structure was refined using REFMAC5 and fitted to generated electron density maps using Coot. Refinement was performed until a final R/R<sub>free</sub> of 22.0 and 29.3%, respectively, was reached. The final model showed one dimer in the asymmetric unit with a FAD molecule bound to each subunit. The quality of the model was assessed with PROCHECK (22). The statistics of crystallographic data collection and model refinement are shown in Table 1. The structure was deposited at RCSB protein data bank (PDB code 2X8C).

**Modeling of the C Terminus of SmTGR<sub>fl</sub> onto the Grx Domain; Model 1—**The C-terminal residues, Cys<sup>257</sup> and Gly<sup>598</sup>, visible in Structure 3, were superimposed to the Cys and Gly residues of the GSH bound to the Grx domain in the GSH-bound form of the same SmTGR (Structure 4). The model was obtained mainly by rotation of the C-terminal arm at the level of Lys<sup>598</sup> ψ angle. Small adjustments of the main chain were performed to obtain the best superposition of the last two residues of the protein with the analogue residues of the bound GSH, avoiding clashes between amino acid side chains. Idealization of the model (Refmac5, 20) was done at the end to ensure the absence of unfavorable contacts between atoms. The model, after optimization, was evaluated with PROCHECK (22).

**Modeling of SmTGR<sub>fl</sub>-SmTrx Complex; Model 2—**The structures of oxidized and reduced SmTrx (PDB codes 2XBI and 2XBQ)<sup>4</sup> and Structure 3 of SmTGR<sub>fl</sub> (PDB code 2X8C) were superimposed to the C-terminal arm of SmTGR<sub>fl</sub> using REFMAC5 (20). When present, NADPH and GSH were excluded from the first rounds of refinement and added only after convergence of R and R<sub>free</sub> factors. COOT (21) was used to build all the models of protein and ligands. The quality of the model was assessed with PROCHECK (22). The statistics of crystallographic data collection and model refinement are shown in Table 1. The structure was deposited at RCSB protein data bank (PDB code 2X8C).

<sup>4</sup> G. Boumis, F. Angelucci, A. Bellelli, M. Brunori, D. Dimastrogiovanni, and A. E. Miele, manuscript in preparation.
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TABLE 1
Crystallographic data collection and refinement statistics

| Structure | Structure 1 | Structure 2 | Structure 3 | Structure 4 |
|-----------|-------------|-------------|-------------|-------------|
| Data collection | C2 | C2 | P21,21,21 | C2 |
| Space group | 141.6, 102.8, 59.2 Å | 141.6, 102.6, 60.0 Å | 84.5, 87.4, 185.5 Å | 141.6, 102.9, 59.1 Å |
| Resolution | 40.0/1.9 Å | 40.0/2.3 Å | 30.0/3.1 Å | 40.0/1.9 Å |
| Rmerge | 0.07 (0.32) | 0.1 (0.4) | 0.1 (0.5) | 0.1 (0.4) |
| Completeness | 90.1% (92.6%) | 100% (99.9%) | 99.3% (99.2%) | 100% (99.7%) |
| Redundancy | 3.0 (2.8) | 8.1 (7.8) | 7.1 (7.0) | 5.7 (5.7) |
| Refinement | Overall | 18.6 Å² | 22.5 Å² | 43.5 Å² | 14.6 Å² |
| No. of reflections | 52,728 | 33,294 | 4936 | 100% (99.9%) |
| No. of atoms | 52,728 | 33,294 | 9174 | 9031 |
| Ligand/ion | 106 | 249 | 143 | 139 |
| Water | 255 | 185 | 0 | 365 |
| B-Wilson | 19.0 | 35.1 | 63.4 | 14.6 |
| Bond angles | 1.064° | 1.302° | 1.164° | 0.978° |
| Root mean square deviations | 0.008 Å | 0.011 Å | 0.009 Å | 0.007 Å |
| Bond lengths | 1.064 Å | 1.302 Å | 1.164 Å | 0.978 Å |

Model quality

Ramachandran plot
Most favored | 91.4% | 91.0% | 86.3% | 92.6% |
Additional allowed | 8.4% | 9.0% | 13.5% | 7.2% |
Generously allowed | 0.2% | 0% | 0.3% | 0.2% |

* Values in parentheses refer to the highest resolution shell.

Docked using a rigid body method with shape complementarity to model a plausible complex. The structures were used as input for the SUMMA server (23). The server calculated the best complementary surface and was instructed to freely perform energy minimization only on SmTGRfI terminal arm (residues 509–598) and on the active site stretch of SmTrx (residues 33–38). Idealization of the model was automatically performed at the end to ensure the absence of unfavorable contacts between atoms. An S–S bond was built between SmTrx Cys34 and SmTGRfI Cys596 with rotamers compatible with the reduced SmTrx protein.

Reductive Half-reaction, Stopped-flow Experiments—Stopped-flow experiments were carried out using an Applied Photophysics (Leathered, UK) in aerobic conditions at 25 °C. Time-dependent spectra were followed using the diode array detector, and single wavelength kinetic traces were recorded with a photomultiplier.

Oxidative Half-reaction, Spectrophotometric Experiments—SmTGRfI (3.0, 5, and 10 μM) were anaerobically reduced with 2 eq of NADPH to produce EH2 at 25 °C in 0.1 M NaCl, 0.1 M Tris, pH 7.4, 1 mM EDTA. The reduced enzyme samples were then mixed with 2 eq of SmTrx, and the reactions were followed until completion.

RESULTS

Structure 1, Oxidized SmTGRfI—The three-dimensional structure of SmTGRfI in the oxidized state was determined at 1.9 Å resolution. The statistics of the diffraction and refinement data are summarized in Table 1. The refined model consists of one protomer per asymmetric unit. The map gave the possibility to fit residues from 6 to 593. The overall architecture of Structure 1 is the same as that previously reported for the truncated form of the enzyme (PDB code 2v60 (17)). The catalytic sites, i.e. the Cys28–Cys31 couple of the Grx domain and the Cys154–Cys159 of the enzyme (PDB code 2v6o (17)). The catalytic sites, i.e. the Cys28–Cys31 couple of the Grx domain and the Cys154–Cys159 of the enzyme (PDB code 2v6o (17)).

Structure 2, the SmTGRfI-NADPH Complex—The structure of SmTGRfI in complex with NADPH and two molecules of GSH was determined at 2.3 Å resolution. The statistics of the diffraction and refinement data are summarized in Table 1. The refined model contains one protomer per asymmetric unit, one FAD, one NADPH, and two GSH molecules. The map gave the possibility to fit residues from 6 to 592. Overall, Structure 2 differs from Structure 1 mainly for slight side chain movements of some residues in the FAD and NADPH binding pockets. In particular, the two cysteine residues on the si-face of the isoalloxazine ring of FAD were found in both redox states due to a double conformation of Cys154 (each with 0.5 occupancy), although Cys159 maintains the same position. Thus, the sulfur-sulfur distance is 3.1 Å in the reduced state and 2.1 Å in the oxidized state. Moreover, Cys159 is at 3.2 Å from C4 and C10 of the isoalloxazine ring of FAD and might be implicated in a charge transfer complex, as already observed in human GR (PDB code 3djg) (10). The reduced Cys154 forms an H-bond with a water molecule, kept in place by His571, that may have a role in the stabiliza-
formed by one α-helix (Leu<sup>197</sup>–Thr<sup>204</sup>), a β-strand (Lys<sup>227</sup>–Leu<sup>230</sup>), and the nucleotide moiety of the FAD. The Cys of GSH is engaged in a disulfide bond with Cys<sup>402</sup> (2.1 Å). The rest of the molecule is stabilized by polar contacts with Lys<sup>227</sup>, Gly<sup>228</sup>, Arg<sup>229</sup>, and Leu<sup>230</sup>. Moreover the carboxylate group of the γ-glutaminyk end is at 2.9 Å from the FAD adenine amine group (Fig. 2, panel B).

The binding of one GSH molecule in the TR domain is associated with a clear change in the conformation of the region between residues 397 and 404, as compared with the unliganded structure (Fig. 2, panel B) (17). The presence of GSH forces the loop 397–404 to turn around two pivot residues (Leu<sup>397</sup> and Thr<sup>404</sup>) and to acquire a 2-turn α-helix structure. As a result, the hydrophobic side chains of Val<sup>400</sup> and Leu<sup>401</sup> are exposed to the solvent, and GSH takes the place previously occupied by the loop (Fig. 2, panel B). The presence of NADPH/NADP<sup>+</sup> in its binding site might be somehow correlated with the glutathionylation of Cys<sup>402</sup>, given the following: (i) attempts to co-crystallize the enzyme with NADPH in the absence of GSH were always unsuccessful; (ii) the change in conformation of the above mentioned loop is close to the gate of the NADPH-binding site (Fig. 2, panel B). At the moment, we are unable to propose a rationale to the concomitant presence of the two ligands, especially in view of the fact that Cys<sup>402</sup> is not a member of a redox Cys couple.

Finally, in this structure, the Cys<sup>28</sup>–Cys<sup>31</sup> couple on the Grx domain is reduced. This is probably due to the excess GSH present in the medium (5 mM).

Structure 3, SmTGRfl with Reduced and Ordered C Terminus—The structure of SmTGRfl in P<sub>2</sub>2<sub>1</sub>2<sub>1</sub> space group was solved at 3.1 Å resolution. The statistics of the diffraction and refinement data are summarized in Table 1. The main differences between this structure and the others reported so far (PDB codes 2v6o and 3h4k for Structures 1, 2, and 4) are due to the presence of the physiological dimer in the asymmetric unit and to the structuring of the reduced C-terminal arm, whose density is visible in one of the two subunits. The low resolution of Structure 3 does not permit an accurate estimate of the redox state of the two Cys couples at the FAD active site (Cys<sup>154</sup>–Cys<sup>159</sup>) and at the Grx redox site (Cys<sup>28</sup>–Cys<sup>31</sup>) because of the following. (i) The slight configurational change of Cys<sup>154</sup> with respect to Cys<sup>159</sup> upon reduction (~1.0 Å, see Structure 2) would be hard to detect in view of the fact that the electron pair is shared between the FAD and the Cys<sup>154</sup>–Cys<sup>159</sup> couple, leading to the latter being only partially reduced (as in Structure 2), consistent with the rapid kinetic experiments. (ii) Both the Grx domains are characterized by high mobility indicated by the high B factors (see below). Too weak electron density did not permit the assignment of the first five residues at the N terminus of both subunits, as well as of the last five residues at the C-terminal portion of subunit A. On the contrary, a stronger electron density allowed the building of the entire C-terminal segment of subunit B, implying a structural asymmetry in the organization of the two subunits. A second evidence of the same asymmetry comes from the different thermal factors of the two Grx domains (residues 7–104) as follows: the Grx portion of subunit A was rather less mobile (overall (B) = 57 Å<sup>2</sup>) than the Grx domain of subunit B (overall (B) = 88 Å<sup>2</sup>).
The terminal tetrapeptide GCCG, as it appears in the electron density map, is fully reduced, with the two cysteine residues (Cys$^{596}$–Cys$^{597}$) being in a trans position with respect to the main chain and pointing their sulfur atoms toward the solvent (Fig. 3, panel A). The positioning of the C terminus is permitted by a kink centered on Lys$^{586}$. This conformation is stabilized by several H-bonds and salt bridges (listed in Table 2), mostly with residues of the B subunit but also with one residue of the A subunit. Hence the C terminus of the B subunit lies in proximity of the FAD-binding site of the A subunit, at 13 Å from the Cys$^{154}$–Cys$^{159}$ redox couple. This distance could be easily bridged by small rearrangements of the polypeptide chain to be made compatible with the electron transfer (Fig. 3, panel A).

Structure 4, the SmTGRfl-GSH complex—in the absence of NADPH, only one molecule of GSH per protomer was found located at the redox site on the Grx domain, as already quoted for Structure 2. The three-dimensional structure of SmTGRfl in complex with GSH was determined at 1.9 Å resolution. A summary of the statistics of the diffraction and refinement is presented in Table 1. The refined model consists of residues from 6 to 593 of one protomer per asymmetric unit.

The Cys$^{154}$–Cys$^{159}$ couple is partially reduced as it occurs in Structure 2, probably due to the presence of GSH in the crystallization conditions. We assume that the reversible electron transfer between FADH$_2$ and Cys$^{154}$–Cys$^{159}$ occurs also in this structure, in the absence of NADP$^+$. The distance between the sulfur atoms of Cys$^{28}$ and Cys$^{31}$ is 3.2 Å, suggesting that these Cys are also reduced. Electron density of the GSH molecule was detected in the proximity of Cys$^{28}$ (Fig. 3, panel E). The average B factor of the GSH ligand is slightly higher (by ~5 Å$^2$) than that of surrounding residues. The GSH pocket is composed of three segments of the polypeptide...
chain, each capable of binding (noncovalently) one of the three residues of GSH. Amino acids from Asp34 to Glu86 contour the γ-glutamyl moiety, by clamping it with two H-bonds. The stretch 70–72 forms two H-bonds with GSH orienting the sulfur atom toward the redox active site of the Grx domain (3.4 Å distance from Cys78 sulfur). Finally, Gln60 and Lys25 bind the glycine moiety of the ligand (Fig. 3, panel D).

Model 1, C Terminus of SmTGRfl onto the Grx Domain—Using Lys386 as pivot residue and after superposition of Structures 3 and 4, we have modeled the possible movement of the C-terminal arm to internally shuttle electrons from FAD to the Grx domain. Without changing the conformation of the extended arm as refined in Structure 3, the last two residues of the C terminus Cys597–Gly598 were placed into the GSH-binding site of the Grx domain (Fig. 3, panels E and F). This was obtained by simple rotation on the ψ angle of Lys596 of subunit B, bringing the terminal arm onto the redox site of the Grx domain of subunit A (Fig. 3, panels E and F). The model clarifies the functional role of the TGR dimer. Indeed, the dimerization is made necessary by the fact that the Grx domain of each subunit is much closer to the C-terminal arm of the opposite subunit (~20 Å) than to the C-terminal arm of the same subunit (~65 Å; distances were measured between Cys28 of both subunits and Lys596 of subunit B as found in Structure 3).

Model 2, SmTGRfl–SmTrx Complex—Our interest was to find a docking surface for the external substrate SmTrx. Thus, we carried out a surface complementarity search between the two proteins, followed by automated cycles of restrained refinement to minimize the energy and regularize the geometry, avoiding possible clashes (see “Experimental Procedures”). SmTrx can be docked into SmTGRfl in a surface cleft at the bottom of the TR domain, opposite to the Grx domain (Fig. 3, panels B and C). This cleft, formed by residues belonging to the C terminus of subunit B and to the FAD-binding site of subunit A, has a complementary surface to the stretch from Trp31 to Lys38 of SmTrx, which contains the active site couple Cys34–Cys78. In particular, Trp35 of SmTrx fits into a hydrophobic pocket formed by residues of subunit A (Val155, Ile160, Leu164, and Leu208); Lys38 of SmTrx is engaged in a salt bridge with Asp137 of SmTGRfl subunit B; and Cys34 (the most external of the active site Cys couple) ends up at 2.0 Å distance from Cys596 of SmTGRfl subunit B (Fig. 3, panels B and C). The geometry of this mixed disulfide bond is superimposable to the mixed S–S bond found in the crystallographic complex of E. coli TR–Trx (PDB code 1F6M (27)), supporting the plausibility of this model. Another independent support to this model comes from the structures of Trx from other species, which show that the Trp adjacent to the catalytic site is engaged in hydrophobic contacts upon dimerization (28).

Functional Observations Relevant to the Structural Changes—The catalytic cycle of SmTGR is complex, and the exploration of the individual reaction steps is not an easy task, except for the bimolecular reduction and oxidation reactions by substrates. The former reaction was studied by time-resolved spectroscopy, by systematically varying the concentrations of both the enzyme and NADPH. The reaction time courses recorded in this type of experiment are biphasic and span from the spectrum of the oxidized FAD to that of a partially reduced form. Indeed, despite the large difference in the reduction potentials of FAD and NADPH+, we never succeeded in obtaining a complete reduction of the enzyme (Fig. 4, panel A). The time courses recorded at two significant wavelengths (460 and 560 nm) for five concentrations of NADPH and two concentrations of SmTGR were fitted to a two-step sequential mechanism using the integrated equation developed by Bateman (29). The wavelengths were selected as representative of the reduced FAD derivatives based on the better characterized homologues, GR and lipoamide dehydrogenase (30, 31). The two rate constants obtained were both second order, with values of 4 × 106 and 1 × 106 M⁻¹ s⁻¹ (Fig. 4, panel B). Absorbance changes at 680 nm, possibly indicative of the transient formation of a charge transfer complex between NADPH+ and FADH2 (24), were looked for but not found; the time course at this wavelength could be interpreted in terms of the same two kinetic processes described above, indicating that the charge transfer complex does not accumulate in SmTGRfl.

Our interpretation is the following. The first reaction step is the transfer of two electrons from NADPH to the primary redox center of the enzyme, constituted by FAD and the couple Cys154–Cys159, rate-limited by complex formation, and hence second order. Because the primary redox center of the enzyme has the ability to accept four electrons, rather than two, a charge transfer state is populated as the product, in which the electrons move back and forth between the FAD and the Cys couple, thus explaining the incomplete reduction of the former. This mixed valence state exchanges electrons with the C-terminal redox couple. The second reaction step again corresponds to the transfer of two electrons from a new molecule of NADPH to those enzyme molecules in which the primary redox center is fully oxidized because of the internal electron transfer, and it is thus second order. It entails little further reduction of the FAD, because it mainly occurs on those enzyme molecules in which the cofactor has been reoxidized by the C terminus. The observed spectroscopic changes (and the pertinent second-order rate constants) are thus assigned to the reaction steps leading from the oxidized enzyme (Structure 1) to the two-electron reduced intermediate (presumably in Structure 2), and from this to the four-electron reduced species (that may assume either Structure 2 or 3; see under “Discussion”). The time-resolved spectra and the overall behavior of the reaction are reminiscent of those recorded by Bauer et al. (32) under similar experimental conditions on TR from Drosophila melanogaster.

**Table 2**

| Polar contacts between the C-terminal arm and the TR domain of SmTGRfl, based on Structure 3 |
|---------------------------------------------------------------|
| C-terminal residue (subunit B) | Subunit A | Subunit B | Distance |
|--------------------------------|----------|----------|---------|
| V583(O)                        | H502(N)  |          | 3.0     |
| T584(OG)                       | S587(OG)|          | 2.9     |
| K585(N)                        | V500(O)  |          | 2.9     |
| K585(O)                        | G588(N)  |          | 2.8     |
| K585(NZ)                       | E327(OE1)|          | 2.8     |
| K585(NZ)                       | Q327(OE1)|          | 2.8     |
| P591(N)                        | N504(O)  |          | 3.3     |
| V593(N)                        |          |          |         |
| G598(O)                        | K124(NZ) |          | 2.4     |

Catalytic Cycle of *Schistosoma mansoni* TGR
Re-oxidation of SmTGR (reduced by stoichiometric amounts of NADPH) by the physiological substrate SmTrx can also be followed by absorbance spectroscopy (Fig. 4, panel C). The U597C mutant used in this study yielded time courses well described by a second-order rate constant of $0.8 \times 10^4 \text{M}^{-1} \text{s}^{-1}$ (Fig. 4, panel D). This value is approximately 2 orders of magnitude lower than that reported by Kuntz et al. (5) for the wild type enzyme, consistent with the important role played by Sec in the reduction of the physiological substrate. Both the oxidation and reduction half-cycles of SmTGR are rate-limited by second-order kinetic processes, and accumulation of a Michaelis type complex does not occur at the substrate concentrations used.

**DISCUSSION**

SmTGR is one of the most promising drug targets against Schistosomiasis (33, 34), the second most prevalent parasitic disease after malaria; thus the structural characterization of intermediates in the catalytic cycle is relevant. In this study, we solved several structures of this enzyme in complex with some of its substrates, and it would be highly desirable to assign these structures to the reaction intermediates. A partial description of the enzymatic cycle has been proposed for mouse TGR, mainly based on the better characterized cycle of TRs and on a three-dimensional model of mouse TGR (18, 35). Our structures fit the known or presumed catalytic intermediates of mouse TGR as follows; Structure 1 clearly corresponds to a unique species of SmTGR (the oxidized one). Structures 2 and 4 have been obtained in the presence of excess reductants, thus the species probably prevalent in the crystallization medium was the one called EH$_4$ in TRs, with reducing equivalents stored on the FAD/Cys$^{154}$–Cys$^{159}$ site and the C terminus (in SmTGR two more electrons can be stored on the Grx domain, but we shall maintain the usual nicknames for clarity). Structures 2 and 4 are essentially identical to each other except for the presence or the absence of NADP$^+$ and one GSH. Because there is no evidence that the overall structure of SmTGR is affected by the redox state of the disordered C-terminal residues, we believe that Structures 2 and 4 are also compatible with EH$_2$, the two-electron reduced intermediate bearing the reducing equivalents on the FAD/Cys$^{154}$–Cys$^{159}$ site. Structure 3 is peculiar having one ordered C terminus per dimer, which is clearly reduced; thus this structure is compatible with EH$_4$ and possibly also with the minor fraction of EH$_4$ in which the electrons have been internally transferred from the FAD/Cys$^{154}$–Cys$^{159}$ to the C terminus (see Fig. 1).

Before discussing these assignments in greater detail, we should justify our decision to use a variant of the enzyme in which the C-terminal Sec$^{597}$ has been replaced by cysteine (SmTGR$^{\text{fl}}$) by the following: (i) the inherent difficulty to obtain suitable amounts of the highly purified Sec-containing wild type enzyme (35–37); (ii) the structure of a Sec-containing form of rat TR, the only reported so far (36), shows the stereochemistry of the entire C-terminal arm to be very similar to that found by us (see below); and (iii) the functional characterization of SmTGR$^{\text{fl}}$, reported in the *supplemental material*, indicates that the enzyme has fully functional FAD and Grx redox sites and an active C-terminal arm, as demonstrated by the ability to
Electron Flow within the TR Domain, from NADPH to the C Terminus (Figs. 2 and 3)—The catalytic cycle starts with the transfer of two electrons from NADPH to fully oxidized SmTGR (Structure 1). The product of this reaction is usually called EH₂. Kinetic experiments demonstrate that the reduction of FAD follows a bimolecular process with a rate constant $k_1 = 4 \times 10^{4} \text{M}^{-1} \text{s}^{-1}$ (Fig. 4, panels A and B). The reduction, as judged by the absorption spectra, is not complete because of the reversible internal electron transfer to Cys¹⁵⁴–Cys¹⁵⁹. The fact that less than 50% of the FAD is reduced agrees very well with the partial reduction of the Cys¹⁵⁴–Cys¹⁵⁹ couple observed in Structures 2–4.

Structure 2 clearly shows that binding of the reducing substrate to the enzyme occurs as described in previous studies on flavoenzymes from other organisms, where the nicotinamide and the isoalloxazine ring are oriented in a stacked position to favor electron transfer (Fig. 2, panel A) (26, 38). The recognized role of Tyr¹⁹⁶ (39), acting as a “gate” for NADPH binding, is confirmed in our structure. In fact, the phenolic ring of Tyr¹⁹⁶, which points toward the re-face of the isoalloxazine ring of FAD in a T-shape configuration (see Structure 1 and also Ref. 17 for the oxidized truncated form), swings out parallel to the FAD ring in the “open” conformation, allowing occupancy of the pocket by NADPH (Fig. 2, panel A).

The structure of EH₂ is most likely similar or identical to our Structures 2 and 4 depending on whether the intermediate considered contains NADP⁺ or not. Reduction is associated with a shift of the Cys¹⁵⁴ side chain, which moves from a position compatible with the formation of a disulfide bridge with Cys¹⁵⁹ (2.0 Å in Structure 1) to a rotamer 3.1 Å distant from Cys¹⁵⁹, compatible with the reduced state of the redox couple. The latter rotamer of Cys¹⁵⁴ is also stabilized by a water molecule at 2.7 Å from the sulfur atom, in turn kept in place by His²⁷¹ from the related symmetric subunit (Fig. 2, panel A); this residue is known to be relevant to the enzymatic activity of homologue enzymes (24, 25).

Once the Cys¹⁵⁴–Cys¹⁵⁹ couple has been reduced, electrons may be picked up by the oxidized C-terminal arm. We do not know whether this occurs before, after, or independently of the binding of the second molecule of NADPH. The reduced state of the C terminus may adopt an ordered conformation, at least in one protomer of the dimer, as it occurs in Structure 3 and in a structure of the Sec-containing TR1 from rat in its reduced state (36) (Fig. 3, panel A).

Even though the exact sequence of events is unknown, EH₂ binds to a second molecule of NADPH and is converted to the four-electron reduced enzyme (EH₄) in a bimolecular process with the apparent second-order rate constant $k_2 = 1 \times 10^{4} \text{M}^{-1} \text{s}^{-1}$ (Fig. 4, panel B). EH₄, as populated at the end of the second reduction step, presents the reduced C terminus and may therefore adopt Structure 3. In agreement with Bauer et al. (32), Structure 3 is supposed to be ready to transfer reducing equivalents to its substrates, such as Trx and/or the Grx domain.

Electrons Exit from the TR Domain, from the C terminus to Either SmTrx or the Grx Domain (Fig. 3)—Based on Models 1 and 2 on Structures 3 and 4, we describe in this section the major structural features involved in electron transfer sustaining the TR and Grx activities of the enzyme (see Fig. 1).

The TR activity of TGR follows the mechanism proposed for the mammalian enzymes, a key role being played by the protein C-terminal arm, which is postulated to shuttle electrons from Cys¹⁵⁴–Cys¹⁵⁹ to the incoming Trx (Fig. 1) (9, 36). Looking at Structure 3, this arm is solvent-exposed and ~13 Å apart from the isoalloxazine ring, in a position suitable to supply reducing equivalents to SmTrx (Fig. 3, panel A). The C-terminal arm is probably quite mobile in solution, and one can assume that only a further slight movement is sufficient to shuttle electrons from Cys¹⁵⁴–Cys¹⁵⁹ to Trx.

Electrostatic interactions between the C-terminal arm of one subunit and a positive surface on the symmetric subunit (contributed by Lys¹²⁴, Lys¹₂₈, and Arg⁴⁵⁰, see Fig. 3, panel A) may suggest a mechanism whereby the motion of the former depends on its oxidation state. We presume that the C terminus is contacting the external surface of enzyme when reduced, although it slides back into the FAD-binding site upon oxidation (the net charge of the C terminus dropping from −2 to −1, Sec being completely ionized at physiological pH). Starting from the observation that the C-terminal is flexible, we propose a model (Model 2) for the complex between SmTGRfl and SmTrx (Fig. 3, panels B and C) whereby Trx is positioned in its putative binding site on SmTGR surface (as described previously by Cheng et al. (36) for human TR1). After energy minimization of the docked complex, Cys⁵⁹⁷ of SmTGRfl is 2.0 Å from Cys³⁴ of SmTrx, thereby suggesting the formation of a mixed disulfide bond. The second-order rate constant for electron transfer from SmTGR to SmTrx is 0.8 × 10⁸ M⁻¹ s⁻¹ (Fig. 4, panel D), compatible with the turnover of the U597C mutant but significantly lower than that estimated for the wild type enzyme (5).

The C-terminal arm of SmTGR is also involved in the reduction of the Grx domain and thus in the Grx activity of the enzyme as demonstrated by experiments on TGR from different organisms (6, 15, 35). In TGR, the Grx domain could be reduced either by GSH in vitro, as common to Grx proteins (8), or by the C terminus through the reducing equivalents supplied by FAD (see Fig. 1) (18). A computer model (35) predicted the Grx domain of TGR to be located in the same position as the Trx-binding site, which was not unreasonable because the Grx proteins belong to the Trx superfamily. However, our structural data strongly argue against this hypothesis. Until now, we crystallized three different variants of SmTGR in four different space groups (this paper and Refs. 13, 17). The data show different crystallographic contacts but always the same overall architecture, suggesting the structure in solution and in the crystal is the same. If the relative orientation of the Grx domain with respect to the TR domain is maintained, structural motions compatible with internal electron transfer are those bringing the mobile C terminus onto the Grx redox site. In fact,

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5 F. Angelucci, D. Dimastrogiovanni, G. Boumis, M. Brunori, A. E. Miele, F. Saccoccia, and A. Bellfelli, unpublished results.
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Structure 4 gives some hints on this movement. Here, GSH is bound to the Grx domain active site, with its −SH at 3.1 Å far from Cys28 of the protein (Fig. 3, panel D). We hypothesize that the C-terminal arm of SmTGR occupies the same site of GSH on the adjacent subunit (Fig. 3, panels E and F). To substantiate this hypothesis, we built a model (Model 1, Fig. 3, panels E and F) as follows: by rotating the C-terminal arm around the Cα–Cβ bond of Lys586 (see “Experimental Procedures”), the Cys597–Gly598 pair of subunit B superimposes to GSH bound to the Grx domain of subunit A (Structure 4).

Consistent with this hypothesis, some structural features can be underlined as follows: (i) from Lys586 onward, the C terminus (as seen in Structure 3) is unstructured and anchored by only a few contacts with the rest of the protein (see “Results” and Table 2); (ii) the last two residues of SmTGR, i.e. Sec-Gly, resemble the Cys-Gly moiety of GSH (except for the selenium domain in TGR enzymes could be vital for parasites that spend their life in the host organism, thus under constant oxidative attack. Under these conditions, sudden fluctuations of the GSH concentration may impair Grx activity and increase the percentage of protein glutathionylation, thus hindering some vital activities of the parasite (8, 42). The peculiar intramolecular electron pathway of TGR, from NADPH to the internal Grx domain through the C terminus, would ensure deglutathionylation of target proteins independently of GSH, hence helping the parasites to circumvent the host oxidative stress.

Assuming the above scenario to be reliable, it is natural to ask why the presence of TGR is associated with the absence of GR in parasitic flatworms? As SmTGR is not an efficient GR (5), one may wonder whether the schistosomes may derive part of their reduced GSH from the erythrocytes they ingest. It has been estimated that schistosome females consume about 330,000 erythrocytes per hour and males about 39,000 (43). Human erythrocytes contain about 3 mM reduced GSH, and thus a female schistosome could in principle obtain GSH from its diet at the remarkable rate of over 60 pmol/h (and should eliminate an equivalent amount of GSSG). It would be interesting to assess this hypothesis by direct measurement, as it may bear implications for therapy.

Concluding Remarks—The data reported in this study describe the complete catalytic cycle of SmTGR, a complex enzyme, based on structural data and modeling. We describe the possible structure of the main intermediate species and assign at least two kinetic constants to the pertinent reduction steps. A crucial question is how the relevant oxidizing substrate, either the Grx domain or the external Trx, is selected; a detailed functional study of wild type SmTGR is required to settle this issue. This study defines some cornerstones on the following points: (i) the external Trx and the Grx domain do not compete for the same binding site; (ii) electrons are selectively delivered to their acceptor by the C-terminal arm shuttling between either of two alternative positions; (iii) it is possible, at least in theory, that TGR may transfer electrons from GSH to Trx (or vice versa) via the Grx domain and the C-terminal arm, thus bypassing NADPH.

The structural and evolutionary complexity of SmTGR suggests that the function of the enzyme is peculiar and that linking the two principal thiol-reducing pathways may offer selective advantages to the schistosome. TGR has been proposed to have evolved from the gene of TR1 (18, 40). Thus, we look at SmTGR as a modified TR, and we observe that most of the biochemical weaponry necessary for the complex and distinctive functions of TGR is already present in TRs, e.g., in both types of reductase the function of the C-terminal arm is that of reaching the redox site of Trx, but only in TGR does this appendage acquire the function of reducing the internal Grx domain.

A recent paper (41) reports the presence of TGR only in parasitic flatworms but not in the free-living species of the same phylum where separated GR and TR exist. A possible explanation for this finding is that the internal reduction of the Grx domain in TGR enzymes could be vital for parasites that spend all their life in the host organism, thus under constant oxidative attack. Under these conditions, sudden fluctuations of the GSH concentration may impair Grx activity and increase the percentage of protein glutathionylation, thus hindering some vital activities of the parasite (8, 42). The peculiar intramolecular electron pathway of TGR, from NADPH to the internal Grx domain through the C terminus, would ensure deglutathionylation of target proteins independently of GSH, hence helping the parasites to circumvent the host oxidative stress.

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