Monothiol Glutaredoxin-1 Is an Essential Iron-Sulfur Protein in the Mitochondrion of African Trypanosomes

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African trypanosomes encode three monothiol glutaredoxins (1-C-Grx). 1-C-Grx1 occurs exclusively in the mitochondrion, and 1-C-Grx2 and -3 are predicted to be mitochondrial and cytosolic proteins, respectively. All three 1-C-Grx are expressed in both the mammalian bloodstream and the insect drion, and 1-C-Grx2 and -3 are predicted to be mitochondrial impaired cell growth. Treatment of wild-type bloodstream in vitro whereas 1-C-Grx2 and 1-C-Grx3 are monomeric proteins. 1-C-Grx1 exists as a noncovalent homodimer, 2S cluster that required GSH as an additional ligand. Both bloodstream and procyclic trypanosomes were refractory to point to an essential role of the mitochondrial 1-C-Grx1 in different subcellular compartments; for instance, yeast Grx3 and Grx4 are nuclear proteins, whereas Grx5 is mitochondrially localized (for a review see Ref. 2). The mitochondrial isoform of 1-C-Grx seems to be a key component of the evolutionarily conserved machinery that incorporates iron-sulfur clusters (ISC) into the apoproteins (3). For instance, yeast and zebrailfaki Grx5 are unable to maturate iron-sulfur proteins (4, 5). Consequently, the yeast mutant does not grow under respiratory conditions, accumulates intracellular iron, and is hypersensitive toward oxidative stress (1, 4). In vertebrates, Grx5 deficiency also impairs heme synthesis (5, 6). Escherichia coli has a single 1-C-Grx (Grx4) that is probably essential (7, 8). Different eukaryotic and prokaryotic 1-C-Grx can functionally substitute for Grx5 in yeast mitochondria (9). Very recently, it was shown that 1-C-Grx from evolutionarily distant organisms are able to bind iron-sulfur clusters (10, 11).

Trypanosomatids, to which belong the causative agents of tropical diseases such as African sleeping sickness (Trypanosoma brucei gambiense and Trypanosoma brucei rhodesiense), Nagana cattle disease (Trypanosoma congolense and Trypanosoma brucei brucei), Chagas disease (Trypanosoma cruzi), and the different forms of leishmaniasis encode three putative monothiol glutaredoxins. 1-C-Grx1 and 1-C-Grx2 are single domain proteins with a predicted mitochondrial presequence, whereas 1-C-Grx3 is a thioredoxin/1-C-Grx hybrid with no evident targeting signal. Recombinant T. b. brucei 1-C-Grx1 has been shown to be susceptible to specific thiolation, suggesting a possible reox redox regulation (12). Complementation studies in Grx5-deficient Saccharomyces cerevisiae targeting the T. brucei proteins into the mitochondria reveal that only 1-C-Grx1 modestly rescues the mutant phenotype (13). The striking lack of functional conservation in the trypanosomal proteins, in comparison with various prokaryotic and eukaryotic 1-C-Grx (5, 9, 14), suggests a significant divergence of the parasite proteins

Monothiol glutaredoxins (1-C-Grx)3 were first discovered in yeast (1), but the proteins occur in organism of all phyla. 1-C-Grx contain a single cysteine in their putative active site (CGFS motif) and are either single domain proteins or form part of multidomain arrangements such as tandem repeats (e.g. protein kinase C-interacting cousin of thioredoxin, the so-called PICOT protein) or fusions to thioredoxin-like domains (e.g. yeast Grx3 and Grx4 and Arabidopsis thaliana Grx3). The proteins occur in different subcellular compartments; for instance, yeast Grx3 and Grx4 are nuclear proteins, whereas Grx5 is mitochondrially localized (for a review see Ref. 2). The mitochondrial isoform of 1-C-Grx seems to be a key component of the evolutionarily conserved machinery that incorporates iron-sulfur clusters (ISC) into the apoproteins (3). For instance, yeast and zebrailfaki Grx5 are unable to maturate iron-sulfur proteins (4, 5). Consequently, the yeast mutant does not grow under respiratory conditions, accumulates intracellular iron, and is hypersensitive toward oxidative stress (1, 4). In vertebrates, Grx5 deficiency also impairs heme synthesis (5, 6). Escherichia coli has a single 1-C-Grx (Grx4) that is probably essential (7, 8). Different eukaryotic and prokaryotic 1-C-Grx can functionally substitute for Grx5 in yeast mitochondria (9). Very recently, it was shown that 1-C-Grx from evolutionarily distant organisms are able to bind iron-sulfur clusters (10, 11).

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1,4-dithiothreitol; ESI-MS, electrospray ionization mass spectrometry; ISC, iron-sulfur cluster; KO, knockout; MOPS, 4-morpholinopropane sulfonic acid; MTS, mitochondrial targeting sequence; PAC8, puromycin N-acetyltansferase resistance; PBS, phosphate-buffered saline; PC, procyclit; Tet, tetracycline; TR, trypanothione reductase; UTR, untranslated region.
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and raises the question of the function(s) of the proteins in trypanosomatids.

Here we report on the structural and cell biological characterization of the three 1-C-Grx in the different life and growth stages of African trypanosomes. We show that the concentration of these proteins is highest in parasites that are grown to the stationary/starvation phase. For the mitochondrial 1-C-Grx1, we provide strong evidence that the homodimeric protein can coordinate an iron-sulfur center using GSH as ligand, fulfilling an essential role in the iron and redox homeostasis of the parasite.

**EXPERIMENTAL PROCEDURES**

**Reagents**—All enzymes employed in the molecular biology experiments were purchased from MBI Fermentas. Kits for the purification of genomic DNA, gel extraction, and mini- and midi-preps were from Qiagen or Macherey-Nagel. Oligonucleotides were synthesized by MWG Biotech, Operon, or Meta-bion. *E. coli* strains DH5-α and Novabluve served as host cells in all genetic procedures. Glutathione disulfide, menadione, and 5,5′-dithiobis-(2-nitrobenzoic acid), used for thiol determinations (15), were purchased from Sigma. DNase, complete mini protease inhibitor mixture, and recombinant *Aspergillus niger* glucose oxidase were from Roche Applied Science and hydrogen peroxide and isopropyl-D-thiogalactopyranoside from Merck. Antibiotics were purchased from Sigma and Invitrogen. Polyclonal guinea pig antibodies against *T. brucei* His6-1-C-Grx1, untagged 1-C-Grx2, and 1-C-Grx3 were produced by Eurogentec. The antisera against *T. cruzi* trypanothione reductase (TR) and *T. brucei* 1-C-Grx1 were purified (13). Mouse monoclonal anti-c-Myc (clone 9E10) antibodies were purchased from Roche Applied Science. Anti-guinea pig and anti-rabbit IgG secondary antibodies conjugated with horseradish peroxidase were obtained from Santa Cruz. Alexa Fluor®488-labeled goat anti-guinea pig IgG and anti-mouse IgG were purchased from Molecular Probes™ (Invitrogen). His-tagged 1-C-Grx1 was prepared essentially as described (13). Before induction, the cultures were kept at 4 °C for 30 min, 200 μM isopropyl-β-D-thiogalactopyranoside was added and the cells were incubated at 4 °C overnight. The pH of all buffers was 7.0.

**Cloning, Expression, and Purification of His-tagged 1-C-Grx2 and 1-C-Grx3**—The coding regions of 1-c-grx2 and 1-c-grx3 were amplified from *T. brucei* (strain 427) (16) genomic DNA with the primers given in the supplemental table. The amplicon size of 1-c-grx2 was 27786 bp. 1-c-grx2 was amplified essentially as described (13). Before induction, the cultures were kept at 4 °C for 30 min, 200 μM isopropyl-β-D-thiogalactopyranoside was added and the cells were incubated at 4 °C overnight. The pH of all buffers was 7.0.

**Gel Chromatography and Mass Spectrometric Analysis of 1-C-Grx1 Treated with GSSG or Hydrogen Peroxide**—In a total volume of 5 ml of PBS buffer, pH 7.4, 50 μM tag-free 1-C-Grx1 (expressed from a modified pET9d vector (New England Biolabs) containing a tobacco etch virus (TEV) protease-cleavable thioredoxin-His6 fusion vector (kindly provided by Gunther Stier, EMBL, Heidelberg, Germany)) was incubated with 500 μM GSSG overnight at 4 °C and with 500 μM H2O2 for 2 h at room temperature, respectively. The latter reaction was stopped by adding 0.1 unit of bovine liver catalase (Fluka). The GSSG-treated sample was subjected to two PD-10 columns (GE Healthcare) to remove the low molecular mass components and stored at 4 °C for 3 days. Both 1-C-Grx1 samples contained 0.3 free thiol/protein subunit as shown by Ellman’s reaction (15). The protein solutions were concentrated in Amicon 10-kDa concentrators (Millipore) to 3.6 and 4.0 mg/ml for the
subsequent size exclusion chromatography. A Superose 12 HR 10/30 column (GE Healthcare) was pre-equilibrated in 100 mM NaCl, 50 mM NaH₂PO₄ pH 8.0. 200-µl samples of the oxidized 1-C-Grx1 species as well as of the untreated fully reduced protein, corresponding to about 0.8 mg of protein, were chromatographed at a flow rate of 0.2 ml/min at 8 °C. The low molecular weight gel filtration calibration kit (GE Healthcare) served for standardization. The elution fractions were analyzed by SDS-PAGE under nonreducing and reducing conditions. The peak fractions were subjected to ESI-MS on an API-QSTAR™ Pulsar instrument (Applied Biosystems) with a high pressure liquid chromatography system (Agilent) on-line-coupled to the ESI-QTOF (quadrupole time-of-flight) instrument. 50 µl of a 10 µM protein solution was incubated with 20 mM iodoacetamide and injected into the mass spectrometer essentially as described previously (12).

Reconstruction Assay and [Fe-S] Cluster Stability—In vitro reconstitution of 1-C-Grx1 was performed as described (17). 100–200 µM 1-C-Grx1 was incubated under an argon atmosphere at room temperature with 0.01 molar equivalents of E. coli cysteine desulfinase IscS, 2–5 equivalents of cysteine, 2–4 equivalents of Fe(NH₄)₂(SO₄)₂, 1 mM GSH, 5 mM DTT, and 10 µM pyridoxal phosphate in 50 mM sodium phosphate buffer, pH 8.0, containing 200 mM NaCl. After 2–3.5 h, the protein was separated from the reaction components on Superose 12 HR 10/30 column (GE Healthcare) pre-equilibrated in 100 mM sodium phosphate buffer, pH 8.0, containing 200 mM NaCl, and 2 mM GSH. The molecular mass of the complex was determined by a standard curve provided by the supplier’s manual and by standard proteins. Absorbance at 280 and 420 nm was recorded. UV-visible spectra and the kinetics of cluster disassembly, measuring the absorbance at 420 nm in the presence or absence of 1 mM GSH, as well as of 0, 0.01, 0.1, 0.36, and 1 mM H₂O₂, were recorded on a Shimadzu UV-2100 spectrophotometer. Kinetic measurements were performed in 50 mM sodium phosphate buffer, pH 8.0, containing 200 mM NaCl at 25 °C.

Determination of Iron and Acid-labile Sulfide—Protein bound iron was quantified as described by Fish (18), and acid-labile sulfide was quantified according to Broderick et al. (19). Three determinations were carried out on samples from five independent reconstitution assays.

DNA Constructs for the Overexpression or Deletion of 1-c-grx1 in T. brucei—The full-length 1-c-grx1 coding region was amplified from pMM351-grx1 (clone 5) (13) using forward Grx1 HindIII and reverse Grx1 BamHI as primers (supplemental table) and cloned into the pHD1700 vector (Dr. Christine Clayton, Heidelberg, Germany) to generate pHD1700-1-c-grx1-c-myC₂. T. brucei cells were transfected with the NotI-linearized plasmid (see below). For disruption of the 1-c-grx1 alleles, blasticidin S-deaminase (BLA) genes were amplified with primers containing the respective restriction sites (supplemental table) from plasmids pHD 1034 and pHD 886 (Dr. Christine Clayton, Heidelberg, Germany), respectively. The 1-c-grx1 5’-UTR, the PAC- or BLA-gene, and the 1-c-grx1 3’-UTR were cloned stepwise into the SacI/XbaI, BamHI/PstI, and HindIII/XhoI restriction sites, respectively, of pBluescript II SK(+) (+). The complete knock-out (KO) cassettes were excised with SacI/XhoI, purified from an agarose gel, and used to transfect T. brucei cells as described below. All PCRs and genetic engineering procedures were carried out using Pfu DNA polymerase and E. coli DH5-α or Novablue (Novagen) strains, respectively. The integrity of all constructs was verified by DNA sequencing (MWG Biotech).

Cell Lines, Culture Conditions, and Transfection—The T. brucei cell line 449 (referred to here as wild type, encoding one copy of the Tet repressor protein, Phleostrain) (20) and cell line 514–1313 (encoding the T7-RNA Pol and two copies of the Tet repressor protein, PhleoR and NeoR, respectively) (21) employed in this work derive from strain 427 (16). Bloodstream cells were grown in HMI-9 medium (22) at 37 °C in a humidified atmosphere with 5% CO₂ and procyclic T. brucei in MEM-Pro medium (Biochrom) containing 7.5 µg/ml hemin at 27 °C. Both culture media were supplemented with 10% (v/v) fetal calf serum (Biochrom, unless otherwise stated), 50 units/ml penicillin, and 50 µg/ml streptomycin. Depending on the resistance gene harbored by the trypanosomes, the medium of bloodstream and procyclic cells contained 0.2 and 0.5 µg/ml phleomycin, respectively, and/or 2.5 and 15 µg/ml G-418, respectively.

3 × 10⁷ Trypanosomes were transfected with 10–100 µg of the DNA constructs according to standard techniques (23, 24) in an ECM 630 electroporator (BTX). Single clones of bloodstream and procyclic cells were obtained in 24-wells culture plates by splitting the cultures immediately after transfection and by limiting dilution 24 h after transfection, respectively. Selection of stable transfectants was initiated about 16 h after electroporation by adding the respective antibiotics.

Generation and Growth Phenotype Analysis of 1-C-Grx1-overexpressing T. brucei Cells—T. brucei bloodstream (cell line 449) and procyclic (cell line 514-1313) were transfected with pHD1700-1-c-grx1-c-myc₂ and selected with 10 and 50 µg/ml hygromycin, respectively. Ectopic expression was induced with tetracycline (0.01, 0.1, 1, 10, 100, and 1000 ng/ml) and evaluated by Western blotting as described below. Bloodstream parasites were inoculated at 1 × 10⁵ cells/ml in 10 ml of fresh medium with (Tet-induced) or without (control) 1 µg/ml tetracycline. The cells were counted daily, and the cultures were diluted to the initial cell density with fresh medium with or without tetracycline. In the case of procyclic parasites, the starting cell density was 5 × 10⁵ cells/ml. The medium was not replaced during cultivation, and the cell density was assessed at different time points. Sustained expression of 1-C-Grx1-c-Myc₂ was achieved by adding 1 µg/ml tetracycline every 48 h.

Generation and Analysis of 1-c-grx1 Knock-out T. brucei Cell Lines—Bloodstream and procyclic T. brucei cells were transfected with the BLA-KO cassette and grown in the presence of
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5 and 10 μg/ml blasticidin, respectively. In procyclic cells, replacement of the second 1-c-grx1 allele was attempted in two ways. A loss of heterozygosity was induced by stepwise increasing the concentration of blasticidin (10, 70, 150 and 300 μg/ml). The cells were seeded at 5 × 10⁵ cells/ml, cultivated for 4 to 7 days in the presence of the selecting drug, and diluted back to the initial cell density before increasing the concentration of the antibiotic. Alternatively, 1 × 10⁶ cells/ml were grown for 1 week in the presence of 300 μg/ml blasticidin, the culture was diluted 1:2, and cultivation was continued. In the second approach, BLA-resistant cells were transfected with the PAC-KO cassette and grown in the presence of puromycin (2 μg/ml) and blasticidin (10 μg/ml).

To generate 1-c-grx1 conditional KOs of procyclic T. brucei, cell line 514-1313 Tbl1-c-grx1 T¹ HYG (clone B5) displaying a tightly regulated expression of the ectopic copy of 1-c-grx1 was first transfected with the BLA-KO cassette. Cell cloning in a 24-well microculture plate was performed as described above in the presence of 10 μg/ml blasticidin. Loss of the second allele was achieved by transfecting a BLA-resistant cell line with the PAC-KO cassette in the presence of 1 μg/ml tetracycline (replaced every 48–72 h), 10 μg/ml blasticidin, and 2 μg/ml puromycin. The successful replacement of the 1-c-grx1 gene was verified by PCR and Western blot analysis (described below). Genomic DNA was subjected to PCR using oligonucleotides complementary to the open reading frames upstream and downstream of the 1-c-grx1 gene in combination with 1-c-grx1 specific primers (see supplemental table). Genomic DNA from wild-type cells served as negative control. PCR products were analyzed on a 1% agarose gel. Procyclic cells with a single allele replaced and wild-type parasites (control) were grown continuously, and the cell density was determined daily. The growth of procyclic trypanosomes with a conditional-1-c-grx1-KO genotype was followed in medium with Tet-free cer- tified fetal calf serum (Cambrex). Steady expression of 1-C-Grx1-c-Myc2 expression, were harvested by centrifugation at 2000 × g for 10 min at 27 °C and incubated for 15 min at 27 and 37 °C in 10 ml of fresh medium containing 0.2 and 0.75 μM MitoTracker® Red CMXRos (Molecular Probes), respectively. Subsequently, the cells were washed with PBS, suspended in 10 ml of fresh medium, cultivated for 20 min, and again washed. After fixation in 1 ml PBS, 4% (w/v) paraformaldehyde for 18 min at room temperature, the cells were pelleted at 5000 rpm for 1 min and washed three times with 500 μl of PBS, resuspended in 800 μl of PBS, and allowed to settle on a 4-well culture slide (200 μl/well; BD Falcon™) by overnight incubation at 4 °C. The cells were permeabilized with 0.2% (v/v) Triton X-100 in PBS for 20 min, washed twice with PBS, and blocked for 20 min with 0.5% (w/v) gelatin in PBS. After washing with PBS, the slides were incubated for 1 h with purified guinea pig anti-1-C-Grx1 or mouse monoclonal anti-c-Myc (1:400 in PBS), washed again with PBS, and then incubated for 1 h with the secondary antibodies (Alexa Fluor® 488-labeled goat anti-guinea pig IgG and anti-mouse IgG, dilution 1:250). The parasite DNA was stained with 500 ng/ml (50 μl/well) 4′,6-diamidino-2-phenylindole dihydrochloride. The cells were examined under a Leica DMRXA fluorescence microscope at ×1000 magnification, and images were recorded with a digital charge-coupled device camera (Hamamatsu).

**Experiments Involving Iron Homeostasis and Oxidative Stress**—Constant expression of 1-C-Grx1-c-Myc2 in bloodstream T. brucei was achieved by adding 1 μg/ml tetracycline 24 h prior to and throughout the experiments. The initial cell density was adjusted to 1 × 10⁶ and 5 × 10⁵ cells/ml for the assays involving iron and oxidative challenges, respectively. In all experiments, untreated Tet-induced and noninduced cells served as controls. Upon addition of the different stressors, the parasites were incubated under optimal culture conditions, and viable cells were counted in a Neubauer chamber after 16–24 h.

**Data Analysis**—If not stated otherwise, the experimental data presented here refer to the analysis of at least two different clones in at least three independent experiments. A two-tailed Student’s t test was applied for statistical analysis.

**RESULTS**

**Subunit Composition of the T. brucei Monothiol Glutaredoxins**—The genes encoding T. brucei 1-C-Grx1, 1-C-Grx2, and 1-C-Grx3 were cloned and overexpressed in E. coli both as N-terminal His-tagged proteins and as tag-free proteins. Gel chromatography of the His-tagged proteins revealed 1-C-Grx1 dimers (13), but 1-C-Grx2 and 1-C-Grx3 occurred in mixtures of monomeric and oligomeric forms (not shown). To evaluate whether the oligomerization was an artifact caused by the N-terminal histidine stretch, the tag-free proteins were prepared and subjected to gel chromatography. In the case of 1-C-Grx1 the tag-free protein, containing two free thiol groups per monomer, also eluted with an apparent molecular mass of 31–33 kDa, corresponding to a dimer (theoretical mass of the subunit 16.1 kDa; Fig. 1). The noncovalent nature of the dimer was verified by SDS-PAGE under nonreducing conditions, which revealed only the monomer (Fig. 1C). Because 1-C-Grx1 is susceptible to specific thiolation of Cys²⁸⁴ and can form an intramolecular disulfide bridge when treated with GSSG (12), we considered whether the oligomeric state of the protein was dependent on its redox state. 1-C-Grx1 was treated with GSSG...
or H$_2$O$_2$ after which removal of the low molecular mass components resulted in samples with 0.3 free thiol group/monomer instead of the 2 free cysteines present in the untreated protein.

Treatment of the protein with either GSSG or H$_2$O$_2$ led to the appearance of two major peaks with apparent masses of about 32,000 and 48,000 or 44,000 Da as well as high molecular mass species (Fig. 1, D and E). SDS-PAGE under nonreducing conditions revealed that all high molecular mass fractions represent covalent polymers. In contrast, both the 32,000- and the 44/48,000-Da fractions contained nearly exclusively monomeric protein, independent of the presence or absence of DTT (not shown). To reveal the molecular nature of the protein species, the samples were subjected to ESI-MS analysis (Table 1). The fraction eluting from the gel chromatography column with a mass of 32,000 Da (Fig. 1D, elution fraction F3) showed for the GSSG-treated 1-C-Grx1 a main peak (16,362 Da) that corresponded to the reduced protein carboxamidomethylated at both cysteines. In the case of the H$_2$O$_2$-treated 1-C-Grx1, a second prominent peak at 16,337 Da corresponded to 1-C-Grx1 with two oxygen atoms and one carboxamidomethyl group bound. This species most probably represents 1-C-Grx1, which was oxidized to the sulfenic acid state at Cys$^{104}$ and alkylated at Cys$^{181}$ (12). The fractions of the GSSG- or H$_2$O$_2$-treated 1-C-Grx1 species that eluted with an apparent mass of 48,000 and 44,000 Da (Fig. 1D, elution fraction F1), respectively, contained exclusively or nearly exclusively the protein with intramolecular disulfide bridge (16,246 Da). A monomeric peak was never observed for the native protein; therefore, we can rule out the possibility that the 44–48-kDa peaks correspond to a trimeric species (see also next paragraph).

Instead, the formation of the intramolecular disulfide in 1-C-Grx1 likely induced a conformational change in the protein that altered its elution profile toward a species with apparent higher molecular masses.

Tag-free, reduced 1-C-Grx2 and 1-C-Grx3 eluted from the Superose 12 HR (Fig. 1A) as well as a Hi-load 26/60 Superdex 75 column (not shown) in a single peak with an apparent mass of 18–20 and 28–32 kDa, respectively (Fig. 1B). These masses are higher than the theoretical masses of 12.6 and 24.5 kDa for the subunits but are clearly below the mass for the respective dimers, which may indicate an overall non-globular structure. Such unusual behavior upon gel chromatography has been described for other glutaredoxins. A plant protein was reported to run with apparent masses of 34,000 and 17,000 Da for the dimer and monomer in comparison with the theoretical masses of 25,000 and 12,500 Da, respectively (26).

Identification of the [Fe-S] Cluster in 1-C-Grx1—His$_6$-1-Cys-Grx1 was purified as a slightly colored protein. In addition to the absorption maximum around 280 nm, the protein displayed peaks at 320 and 420 nm (Fig. 2A). These spectral properties resembled those described before for glutaredoxins coordinating [2Fe-2S] clusters (10, 11, 25, 26) and became more evident after in vitro reconstitution of the cluster (Fig. 2A). Assembly (not shown) and stabilization of the Fe-S cluster in 1-C-Grx1 proved to require GSH as cofactor (Fig. 2B). Hydrogen peroxide triggered the disassembly of the cluster from 1-C-Grx1 in a concentration-dependent manner (Fig. 2C). By colorimetric methods we determined that 0.78 ± 0.29 mol of iron and 1.15 ± 0.22 mol of acid-labile sulfides were bound per mol of monomeric protein, which is consistent with the presence of one [2Fe-2S] center/protein dimer. The molecular mass of the com-
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EIS-MS analysis of 1-C-Grx1 species after treatment with GSSG or H$_2$O$_2$

1-C-Grx1 was incubated with 500 nM GSSG or H$_2$O$_2$, subjected to gel chromatography (see Fig. 1D), and analyzed by ESI-MS as described under “Experimental Procedures.” Prior to ESI-MS analysis, elution fractions F1 (12.5–13 ml), F2 (13–13.5 ml), and F3 (13.5–14 ml) were treated with iodoacetamide. Carboxamidomethylation (CM), or glutathionylation (GS), or over-oxidation to a sulfonic acid (2 oxygen molecules, (2O) of free thiols (SH) caused an increase of the protein mass by 57, 305, and 32 Da, respectively. S-S, intramolecular disulfide. The main masses observed in each fraction and the respective modifications are given in bold letters.

| Treatment | Mass (Da) | Cys modifications |
|-----------|-----------|-------------------|
| None      | 16,247    | 2X SH (untreated control) |
| H$_2$O$_2$ |           |                   |
| F3        | 16,361    | 2X CM             |
|           | 16,363    |                   |

**Table 1**

**Gene Deletion of 1-c-grx1 in Procyclic T. brucei**—We next attempted to replace the 1-c-grx1 allele by a classical double-KO approach (Fig. 4A). In the case of bloodstream T. brucei, we have not been able thus far to isolate any viable cell line lacking a 1-c-grx1 allele. With procyclic trypanosomes, 1-c-grx1 single knock-out clones were obtained. In most of these clones, replacement of one allele with the blasticidin resistance gene correlated with a significantly lower level of 1-C-Grx1 (Fig. 4B). This depletion of 1-C-Grx1 had no effect on the growth rate (not shown). To delete the second allele, two strategies were followed. In the first approach, the blasticidin concentration was increased in steps up to 300 µg/ml (30-fold higher than the concentration commonly used) to induce a loss of heterozygosity. This resulted in resistant cell lines all of which had retained a copy of 1-c-grx1 and expressed the protein at levels of about 50% when compared with wild-type cells (not shown). If the blasticidin concentration was increased directly from 5 to 300 µg/ml, no viable clones were obtained. The respective experiment was carried out in parallel with single-KO cells for the glyoxalase II gene, which encodes an enzyme that is not essential for procyclic cells. In this case, cells with a double-KO genotype were obtained. Because in both approaches the genetic background of the strain and the maximum concentration of the selection marker were identical, it is likely that the stepwise addition allowed genomic rearrangements to retain a copy of 1-c-grx1 and overcome drug toxicity by gene duplica-
tion or increased transcription of the resistance gene. This was not the case when a high concentration of the selecting agent was added immediately. The second approach was based on conventional gene replacement by another resistance marker. Substitution of the second allele with a puromycin cassette resulted in procyclic cells that were resistant to both blasticidin and puromycin (BLAR and PACR) but still harbored a copy of the \(1\)-\(c\)-grx1 gene (Fig. 4C) and expressed the protein at levels similar to single-KO or wild-type cells (Fig. 4D). The refractoriness of \(T.\ brucei\) to \(1\)-\(c\)-grx1 gene silencing or deletion is a strong indication that the protein is indispensable for the parasite.

**Conditional Knock-out of \(1\)-\(c\)-grx1 in Procyclic \(T.\ brucei\)**—Finally, we tried to generate conditional KO cell lines containing a tetracycline-inducible copy of \(1\)-\(c\)-grx1-c-myc2. To assure a tightly regulated expression of the ectopic gene, bloodstream and procyclic parasites harboring two copies of the Tet repressor protein (cell line 514-1313) were chosen as host cells. The cells were transfected with the \(1\)-\(c\)-grx1-c-myc2 construct (Fig. 5A), which integrated in the untranscribed spacer of the ribosomal RNA locus. Western blot analyses of protein extracts from transformed parasites grown in the absence of tetracycline displayed a faint band with the expected molecular mass of 1-C-Grx1-c-Myc2 indicating a minor leakiness of the expression system. After 24 h of growth in the presence of tetracycline, the cells showed a 5–10-fold higher concentration of 1-C-Grx1-c-Myc2 when compared with the authentic protein (Fig. 5B). The correct subcellular localization of 1-C-Grx1-c-Myc2 was verified by immunofluorescence microscopy. Procyclic and bloodstream \(T.\ brucei\) overexpressing 1-C-Grx1-c-Myc2 revealed an identical staining pattern for anti-
c-Myc and the mitochondrial marker (Fig. 5C). The signal obtained with the anti-1-C-Grx1 serum also overlapped with that of the mitochondrion. In addition, a weak and diffuse nuclear staining was observed. Because this was not the case with the anti-c-Myc monoclonal antibody, it was most likely due to a cross-reaction with other proteins (e.g. see in Fig. 5B, the unspecific band detected in cell extracts). Taken together, the bloodstream and procyclic cell lines showed a Tet-responsive expression and proper compartmentalization of the protein expressed from the ectopic gene copy. For bloodstream trypanosomes, deletion of a single 1-c-grx1 allele was again not successful, not even when 1 μg/ml tetracycline was added freshly to the medium every 48 h to guarantee a steady level of recombinant 1-C-Grx1-c-Myc2. In this respect, we cannot rule out that insufficient and/or unstable PAC and BLA cannot rule out that insufficient and/or unstable PAC and BLA
(1-c-grx1-c-myc2Δ1-c-grx1::BLA/1-c-grx1::PAC) were obtained starting from a single-KO cell line resistant to blasticidin. The cells were transfected with the 1-c-grx1-myc2 construct and then in the presence of tetracycline with the PAC cassette and finally were cultivated in the presence of tetracycline, blasticidin, and puromycin (Fig. 6A). In these conditional KO clones, depletion of 1-C-Grx1-c-Myc2 was subsequently achieved by growing the cells in a medium supplemented with Tet-free fetal calf serum. Because of the phase-dependent expression of 1-C-Grx1, the growth behavior and the degree of 1-C-Grx1-c-Myc2 depletion were evaluated in logarithmically growing parasites (Fig. 6, B and C) as well as in cells harvested in the stationary phase (Fig. 6, D and E). The growth behavior of cells depleted of 1-C-Grx1-c-Myc2 and of the corresponding overexpressing controls was identical (Fig. 6B), even if the concentration of 1-C-Grx1-c-Myc2 was only about one-fifth of the authentic 1-C-Grx1 in log-phase wild-type parasites (Fig. 6C, clone cKO3 and wt). Also upon long-term cultivation, the growth behavior of the conditional KO cell lines was independent of tetracycline (Fig. 6D). Under this experimental setting, 1-C-Grx1-c-Myc2 could be lowered to 29 ± 7% (n = 5) of the level of the authentic protein in wild-type cells (Fig. 6E), which corresponds to a minimum concentration of 1-C-Grx1-c-Myc2 in the mitochondrion of cKO procyclic cells of about 3 μM. 5–7 Days after tetracycline withdrawal, 1-C-Grx1-c-Myc2 expression started to recover (Fig. 6E). Moreover, in the absence of tetracycline, all conditional KO clones displayed the bands below the c-Myc-tagged protein are probably due to cross-reactions of this batch of antiserum because they were also detected in extracts of wild-type cells (see Fig. 5B). The black and gray arrows mark the position of wild-type and 1-C-Grx1-c-Myc2, respectively.

FIGURE 6. Analysis of a conditional double knock-out of 1-c-grx1 in procyclic T. brucei. A, DNA from wild-type parasites (wt) and the conditional KO cell lines cKO-1 and cKO-3 was subjected to PCR with different primer combinations (see Fig. 4, A and C, and the supplemental table). In the clones, PCR fragments of 1150 and 1213 bp (primer pairs U/r and f/D, respectively) were not obtained, proving that the 1-c-grx1 alleles had been successfully replaced. PCR with the primer pair f/r amplified a fragment of 540 bp of the 1-c-grx1 gene, which in the conditional KO cell lines corresponds to the ectopically integrated 1-c-grx1-c-myc2. M, molecular size standards (GeneRuler™ 1-kb DNA ladder, MBI Fermentas). B, the conditional KO clones that had been continuously grown in the presence of tetracycline were washed three times with tetracycline-free medium and seeded at a cell density of 5 × 10^6 cells/ml in fresh medium containing 1 μg/ml tetracycline (cKO-1, --; cKO-3, --) or without tetracycline (cKO-1, --; cKO-3, --). Every 48 h, cell viability was assessed by light microscopy, samples were withdrawn for Western blot analysis, and the cultures were diluted to 5 × 10^5 cells/ml with fresh medium. C, Western blot analysis of cKO1 and cKO3 grown in the absence (Tet –) or presence (Tet +) of tetracycline. TR served as loading control. wt and re represent extracts from wild-type parasites and 5 ng of recombinant His6-1-C-Grx1, respectively. D, long term cultivation of conditional KO cell lines (n = 5) in the presence (––) or absence (––) of 1 μg/ml tetracycline. Every 5 days, the cells were counted, samples were withdrawn for Western blot analysis, and the cultures were diluted to the initial cell density of 5 × 10^5 cells/ml. E, the levels of 1-C-Grx1-c-Myc2 and 1-C-Grx3 were followed by Western blot analysis with anti 1-C-Grx1 and 1-C-Grx3 antibodies in the clones cKO-17 and cKO-20 grown in the absence of tetracycline. Extracts from cells harvested at day 0 show the level of 1-C-Grx1-c-Myc2 prior to withdrawal of Tet. The numbers below the blots give the amount of 1-C-Grx1-c-Myc2 and 1-C-Grx3, respectively, relative to the level in wild-type trypanosomes normalized against the TR signal. The bands below the c-Myc-tagged protein are probably due to cross-reactions of this batch of antiserum because they were also detected in extracts of wild-type cells (see Fig. 5B). The black and gray arrows mark the position of wild-type and 1-C-Grx1-c-Myc2, respectively.
1-C-Grx1-c-Myc2 levels that were higher than those in non-induced wild-type cell lines harboring a Tet-inducible 1-c-grx1-c-myc2 copy (compare for instance Fig. 6E, day 10, with Fig. 5B, PC 1A4 Tet −, α-Grx1). Probably the transcriptional control of the ectopic gene by the Tet repressor got lost at least partially. This is a common phenomenon reported for essential genes controlled with this system (24, 30–33). To evaluate whether the intracellular concentration of 1-C-Grx1 correlates with that of the other monothiol glutaredoxins in T. brucei, extracts from the 1-C-Grx1-c-Myc2-depleted conditional KO cell lines were subjected to Western blot analyses with anti-1-C-Grx2 and anti-1-C-Grx3 sera. Under these conditions, expression of 1-C-Grx2 (not shown) resembled that in wild-type cells (Fig. 3B), whereas the 1-C-Grx3 level was slightly increased (190 ± 48%, n = 4) when the content of 1-C-Grx1-c-Myc2 was lowest (Fig. 6E). The
failure to obtain parasites devoid of 1-C-Grx1 strongly suggests that despite their homology and probably mitochondrial co-localization, 1-C-Grx2 cannot functionally replace 1-C-Grx1. The slight up-regulation of 1-C-Grx3 in cells with lowered 1-C-Grx1 content may be an indirect effect, or it may reflect a cross-talk between the cytosolic and mitochondrial monothiol glutaredoxins. Taken together, 1-C-Grx1 probably plays an essential and specific function in the mitochondrion of \textit{T. brucei}.

\textbf{Involvement of 1-C-Grx1 in the Iron and Redox Metabolism of \textit{T. brucei}—} The role of 1-C-Grx1 in the iron and redox homeostasis of the mammalian infective form was studied in wild-type and 1-C-Grx1 overexpressing parasites. As shown in Fig. 5, 1-C-Grx1-c-Myc2 can be expressed at a 5–10-fold concentration of that of the authentic protein and is targeted to the mitochondrion. This overexpression did not affect the growth (Fig. 7A) or morphology (not shown) of the parasites when cultivated under optimal conditions. Parasites containing the 1-c-grx1-c-myc2 gene were subsequently grown in the absence and presence of tetracycline and treated with Fe^{3+}, the iron chelator defereroxamine (Dfx), shown to deplete cellular iron (34), hydroxyl peroxide, and the redox cycler menadione (2-methyl-1,4-naphthoquinone) (35), respectively. The addition of 100 \(\mu\)M Fe^{3+} did not significantly affect the growth of the parasites independent of the 1-C-Grx1 level. In contrast, trypanosomes overexpressing 1-C-Grx1 displayed significant growth retardation in the presence of 100 \(\mu\)M Dfx (Fig. 7, B and C). The cells also showed enhanced sensitivity toward hydrogen peroxide, either given as a bolus or generated continuously by glucose oxidase. In procyclic cells overexpressing 1-C-Grx1-c-Myc2 a comparable sensitivity toward hydrogen peroxide was observed (not shown). Although wild-type and 1-C-Grx1-overexpressing parasites were more sensitive toward treatment with 10 \(\mu\)M menadione compared with 100 \(\mu\)M hydrogen peroxide (not shown), overexpression of 1-C-Grx1 did not enhance the sensitivity of the parasites against the quinone, in contrast to the effect observed for hydrogen peroxide (Fig. 7B). This suggests that menadione has a different mode of action to exert its cytotoxic effect caused by oxidative stress and/or iron depletion in bloodstream \textit{T. brucei} (Fig. 7, B and C).

In addition, we studied the impact of the different stressors on the 1-C-Grx1 levels in wild-type \textit{T. brucei} (Fig. 7D). Exposure of bloodstream cells to 100 \(\mu\)M hydrogen peroxide resulted in a 2-fold up-regulation of 1-C-Grx1. In contrast, in cells treated with 100 \(\mu\)M Dfx, the 1-C-Grx1 content was lowered by 50\%, whereas exogenous iron did not affect the level of the protein. This again suggests a physiological role of the mitochondrial 1-C-Grx1 in the iron and redox metabolism of \textit{African} trypanosomes.

\textbf{DISCUSSION} African trypanosomes possess three distinct 1-C-Grx. Whereas the parasite 1-C-Grx2 and 1-C-Grx3 like homologues from yeast, plasmodia, and bacteria are monomeric proteins (1, 8, 37), \textit{T. brucei} 1-C-Grx1 forms noncovalent homodimers. As shown recently (12), treatment of the protein with GSSG leads to the specific thiolation of Cys^{188}, which triggers the formation of an intramolecular disulfide bridge with Cys^{104} (12). In a 1-Cys-d-peroxiredoxin, glutathionylation of the catalytic cysteine induces a switch from a noncovalent dimer to the monomeric form (38). As shown here, exposure of 1-C-Grx1 to GSSG or \(\text{H}_2\text{O}_2\) did not induce dissociation of the noncovalent dimer. Besides nonspecific covalent polymers, the protein appeared in the combined effect of Dfx and hydrogen peroxide (Fig. 7C). Independent of the overexpression of 1-C-Grx1-c-Myc2, a combination of oxidative stress (100 \(\mu\)M \(\text{H}_2\text{O}_2\) or 1 milliunit/ml glucose oxidase) and iron depletion (25 or 100 \(\mu\)M Dfx) had a more deleterious effect on parasite viability and proliferation than the respective single treatment (Fig. 7C). For instance, a highly cytotoxic effect was observed when the parasites were incubated for 18 h in the presence of 100 \(\mu\)M Dfx and either 1 milliunit/ml glucose oxidase or 100 \(\mu\)M \(\text{H}_2\text{O}_2\). Parasites subjected to oxidative stress in the presence of lower concentrations of the iron chelator (25 or 5 \(\mu\)M Dfx) were generally arrested. However, 1-C-Grx1-overexpressing cells treated with 100 \(\mu\)M \(\text{H}_2\text{O}_2\) and 25 \(\mu\)M Dfx or with 100 \(\mu\)M Dfx and 1 milliunit/ml glucose oxidase showed a significantly stronger growth impairment than the noninduced cultures. Taking together, high levels of 1-C-Grx1-c-Myc2 appear to exacerbate the cytostatic or cytotoxic effect caused by oxidative stress and/or iron depletion in bloodstream \textit{T. brucei} (Fig. 7, B and C).

FIGURE 7. Phenotypical analysis of 1-C-Grx1-overexpressing and wild-type bloodstream \textit{T. brucei}. A growth curve of two cell lines containing an inducible copy of 1-C-Grx1-c-Myc2 in the presence (---) or absence (---) of 1 \(\mu\)g/ml tetracycline. Wild-type parasites (cell line 449) grown in the presence (---) and absence (---) of tetracycline served as control. The number of viable cells was determined every 24 h. \(8\%, \) sensitivity of trypanosomes that overexpress 1-C-Grx1-c-Myc2 toward different stresses. Overexpression of 1-C-Grx1-c-Myc2 was induced by adding tetracycline (1 \(\mu\)g/ml) 24 h prior to and during the course of the experiment. The initial cell density was 5 \(\times\) 10^7 and 1 \(\times\) 10^7 cells/ml in the case of iron and oxidative challenges, respectively. 100 \(\mu\)M Dfx, 100 \(\mu\)M iron (Fe^{3+}), as well as \(\text{H}_2\text{O}_2\), glucose oxidase, and menadione at the depicted concentrations were added to the cultures. Tet-induced and noninduced cells without treatment showed identical growth (none) and are set to 100\%. Cell growth and viability were evaluated at 16–24 h after stress induction. The relative cell density (%) refers to the growth of the Tet-induced cells to the respective noninduced cells. The values are the mean of three experiments that differed by less than 5\%. \(C\) combined effect of Dfx and hydrogen peroxide on bloodstream \textit{T. brucei} overexpressing 1-C-Grx1-c-Myc2. 1 \(\mu\)g/ml tetracycline was added 24 h prior to and during the course of the experiment. Noninduced parasites were included as control. The initial cell density was 5 \(\times\) 10^6 cells/ml and is depicted with a gray bar (Co, control) and a dashed line. The white and black bars give the mean cell densities of Tet-induced and noninduced parasites, respectively, after 18 h growth in the presence of 0, 5, 25, and 100 \(\mu\)M defereroxamine, 100 \(\mu\)M \(\text{H}_2\text{O}_2\), and 1 milliunit/ml glucose oxidase. The data represent the mean of three independent experiments. Asterisks denote significant differences (\(n = 6, \rho < 0.1, \) two-tailed t test). D, one representative experiment of three showing the expression of 1-C-Grx1 in wild-type bloodstream \textit{T. brucei} subjected to different stress conditions. Parasites in the late log phase of growth (\(C\)) were harvested and diluted in fresh medium to a density of 5 \(\times\) 10^7 and 1 \(\times\) 10^7 cells/ml for the experiments involving iron and oxidative challenges, respectively. 100 \(\mu\)M \(\text{H}_2\text{O}_2\), 2 milliunits/ml glucose oxidase (GOD), 100 \(\mu\)M FeCl_3 (Fe^{3+}), and 100 \(\mu\)M Dfx, respectively, were added to the cultures. Nontreated cultures served as control (\(C\)) for oxidative stress treatment and \(C\) for experiments involving iron homeostasis. After 20 h, viable cells were counted, and the extract of 4 \(\times\) 10^5 cells was subjected to Western blot analyses with 1-C-Grx1 and TR antibodies. The amount of 1-C-Grx1 in cells exposed to different stresses was estimated by densitometric analysis and is expressed relative to that in the corresponding untreated control. The 1-C-Grx1 content in the control cultures \(C\) and \(C\) is also compared with that in the inocula (\(C\)).
Leishmania Interestingly, the orthologous proteins from molecular disulfide between Cys\(^{181}\) and Cys\(^{104}\) triggered by exposure to oxidants is a reversible state that could protect 1-C-Grx1 (especially Cys\(^{104}\)) against irreversible over-oxidation. Interestingly, the orthologous proteins from Leishmania major, Leishmania infantum, T. cruzi, and T. congolense contain only a single cysteine that corresponds Cys\(^{104}\) in \(T. brucei\) 1-C-Grx1. Because an intramolecular disulfide cannot be formed, it remains to be investigated whether and/or how these proteins undergo a redox regulation.

Very recently, two new 1-C-Grx from yeast (named Grx6 and Grx7) were shown to dimerize noncovalently in a GSH- and GSSG-independent manner (11). The proteins appear to represent a novel subgroup of the 1-C-Grx family. In contrast to most 1-C-Grx, they share several structural and catalytic (e.g. transhydrogenase activity) features with dithiol glutaredoxins (11). Yeast Grx6 and Grx7 are much more distantly related to the \(T. brucei\) 1-C-Grx1 than are yeast Grx3–5, which indicates that the oligomeric structure of monothiol glutaredoxins is unpredictable.

Several dithiol (17, 26, 39) and, very recently, monothiol (10, 11) Grx proteins have been reported to complex a [2Fe-2S] cluster employing GSH as thiol cofactor. As shown here for the first time for a trypanosomatid protein, 1-C-Grx1 can coordinate one [2Fe-2S] center/protein dimer using GSH as the non-protein ligand. For the glutaredoxins studied thus far, ISC assembly to the apoprotein leads to protein oligomerization, \textit{i.e.} conversion of a monomer or dimer into the corresponding dimer and tetramer, respectively (10, 11, 25, 26). Interestingly, 1-C-Grx1 appears to be the first example of a glutaredoxin-type protein in which the incorporation of the [2Fe-2S] cluster into the homodimeric apoprotein does not cause a change in the oligomeric structure. Our data did not allow us to decide whether binding of the cluster is accompanied by local or large conformational changes in the dimer. Picciocchi \textit{et al.} (10) suggested that the ability to coordinate an ISC is an evolutionarily conserved feature of monomeric 1-C-Grx presenting a conserved CGFS motif. However, \(T. brucei\) 1-C-Grx1 and yeast Grx6 disprove this generalization. The proteins have a CAYS and CSYS active site motif, respectively, and exist as dimers in the apo-state. Instead, the structural motif common to all 1-C-Grx capable of binding an ISC seems to be the absence of a proline residue at the active site, which, in the case of dithiol Grx, was shown to be a key factor for cluster incorporation (17, 26). In line with this statement, \(S. cerevisiae\) Grx7 with its CPYS motif does not coordinate an ISC (11).

Both the mitochondrial 1-C-Grx1 and the 1-C-Grx3 are abundant proteins in both life stages of \(T. brucei\). In contrast, 1-C-Grx2, the second putative mitochondrial protein, was detectable only in stationary phase trypanosomes. The levels of all three monothiol glutaredoxins were highest in parasites harbored in the stationary and starvation growth phases. This expression pattern is unusual considering the down-regulation of RNA and protein during stationary phase reported for several housekeeping enzymes in different trypanosomatids (40–42) and as observed here for TR. Because in kinetoplastids gene expression is almost exclusively controlled at the post-transcriptional level (43), the high protein concentration in stationary parasites may be due to an increased half-life or translation of the corresponding transcript and/or protein. A comparable up-regulation in the stationary phase was reported for Grx4, the single monothiol glutaredoxin of \(E. coli\) (8), whereas the transcript levels of \(S. cerevisiae\) and \(S. pombe\) achieved a maximum in the exponential growth phase and a minimum in stationary phase cells (1, 44, 45). Taking into account that 1-C-Grx1 can complex an ISC and its biological function is linked to the iron metabolism of trypanosomes, the strong enrichment in nondividing stationary parasites suggests that the protein may suit for the rapid \textit{de novo} synthesis of iron-containing proteins when the cells resume growth.

Despite a variety of strategies, it was not possible to silence the expression of the authentic 1-C-Grx1 in bloodstream cells, and in procyclic \(T. brucei\), the maximum achievable depletion was by 70%. In procyclic parasites, the chromosomal alleles could be replaced but only if an ectopic copy of 1-c-grx1 was expressed. Down-regulation of 1-C-Grx1-c-Myc\(_2\) expression in these cells was not accompanied by an up-regulation of 1-C-Grx2, which also has an N-terminal sequence that is recognized by the mitochondrial import machinery of yeast (13). This clearly demonstrates that the two 1-C-Grx proteins lack functional redundancy and points to an indispensable function of 1-C-Grx1 in the organelle. 1-C-Grx from different phyla have been shown to efficiently substitute for the mitochondrial Grx5 in yeast and to participate in ISC biogenesis (5, 9, 14, 46). Interestingly, \(T. brucei\) 1-C-Grx1, but not 1-C-Grx2, partially rescued the phenotype of grx5-deficient yeast cells when targeted to the mitochondria (13), suggesting that the parasite protein plays a similar role in ISC biogenesis. In \(T. brucei\), two components of the ISC biosynthetic pathway have been characterized thus far. Both the cysteine desulfurase (TbiscS52) and a scaffold protein (TbiscU) are essential for procyclic cells (47), which adds value to the relevance of this metabolism for African trypanosomes.

Overexpression of an ectopic copy of 1-c-grx1 resulting in a 5–10-fold concentration compared with the authentic protein, did not affect the proliferation rate and morphology of bloodstream cells when grown under optimal conditions. In contrast, a 1.5-fold overexpression of the mitochondrial Grx5 in \(S. pombe\) impaired the growth of the fission yeast (44), although the underlying mechanism was not investigated. Interestingly, overexpression of 1-C-Grx1 in \(T. brucei\) resulted in an increased sensitivity of the parasite toward the iron chelator Dfx. The unphysiologically high concentration of 1-C-Grx1 may augment the depletion of the “free” iron pool due to an increased synthesis of ISC proteins and/or to iron sequestration in the form of the [Fe-S]-1-C-Grx1 complex. The 2-fold down-regulation of 1-C-Grx1 in wild-type bloodstream parasites upon treatment with Dfx could thus be a compensatory mechanism to balance the cellular iron levels.
Overexpression of 1-C-Grx1 also sensitized trypanosomes against H$_2$O$_2$-mediated oxidative stress. One mechanism as to how hydrogen peroxide damages cells is via oxidation of ISCs with release of iron and initiation of Fenton chemistry (48, 49).

As shown *in vitro*, formation of the [Fe-S]-1-C-Grx1 complex requires the reduced protein, and the complex is rapidly destroyed by exposure to H$_2$O$_2$ (estimated half-life, ~40 min for 100 μM H$_2$O$_2$). In addition, treatment of apo-1-C-Grx1 with H$_2$O$_2$ or GSSG leads to formation of an intramolecular disulfide probably accompanied by strong conformational changes. Under conditions of oxidative stress, both reactions may occur *in vivo*, which would shift the equilibrium of holo-/apo-1-C-Grx1 to an inactive oxidized form of the protein with the concomitant impairment of ISCs biosynthesis. In the case of cells overexpressing 1-C-Grx1, this condition may be worsened due to heavy metals and reactive oxygen species down-regulated in accordance with a probable shift to inactive oxidized apoprotein.

Strikingly, exposure of wild-type trypanosomes to hydrogen peroxide induced a 2-fold increase of the 1-C-Grx1 levels. This can be interpreted as a physiological response to hydrogen peroxide challenge.

In conclusion, the three monothiol glutaredoxins occur in parasites overexpressing 1-C-Grx1, this phenotype was augmented in accordance with a probable shift to inactive oxidized apoprotein.

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