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Cloning, Expression, and Characterization of a Dithiol Glutaredoxin from *Trypanosoma cruzi*

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Abstract

Glutaredoxins play an important role in cellular functionality. A putative dithiol glutaredoxin is encoded in the genome of *Trypanosoma cruzi*. We cloned the gene and obtained the recombinant protein, which behaved as a typical thioltransferase. Activity was variable and dependent on the nature of reducer or oxidant agent used, or both. Epimastigote extracts exhibited similar activity, suggesting the occurrence of the protein in the parasite. Results support a redox scenario in *T. cruzi*, with glutaredoxin being involved mainly in reduction of glutathione disulfide as well as in deglutathionylation of target proteins. *Antioxid. Redox Signal.* 12, 787–792.

Introduction

Trypanosoma cruzi is the causative agent of Chagas disease. As usual for aerobic organisms, the parasite is exposed to several reactive oxygen species (ROS): superoxide anions, hydrogen peroxide, and myeloperoxidase-derived products. These chemical species are generated during the host-defense reaction and also as by-products of the aerobic metabolism. The ability of *T. cruzi* to cope with such oxidative conditions appears oddly weak. Although trypanosomatids possess an iron-containing superoxide dismutase to scavenge phagocyte-derived superoxide anions (17), they lack catalase, and their glutathione peroxidase-like system exhibits low efficiency (10, 26). Whereas catalase and selenocysteine-containing glutathione peroxidases are the major hydroperoxide-metabolizing enzymes in host organisms (3, 21), in members of the family Trypanosomatidae, the peroxide metabolism involves mainly a glutathionyl derivative of spermidine, trypanothione [N^1,N^8-bis (glutathionyl)-spermidine, $T(SH)_2$] (7, 14). A system linking three distinctive oxidoreductases is able to catalyze the $T(SH)_2$-dependent hydroperoxide removal. These enzymes are trypanothione reductase (TR) (7, 14), a thioredoxin-related protein called tryparedoxin, tryparedoxin peroxidase (TXNPx), and peroxiredoxin-type proteins (or glutathione peroxidase type) (10, 28). Additionally, a classic thioredoxin was described and characterized in *T. cruzi* (24), but neither glutathione reductase nor thioredoxin reductase is found in these microorganisms (11, 23).

Glutaredoxins (Grxs) are ubiquitous oxidoreductases belonging to the thioredoxin family of proteins (18). They are classified as monothiolic or dithiolic Grxs after the respective occurrence of the conserved motifs CGFS or CXYC into the redox active site. Dithiolic Grxs reduce glutathionylated compounds or intramolecular disulfide bonds by a monothiol or a dithiol mechanism (6). Among the many functions proposed for dithiolic Grxs, they were first identified as electron donors of ribonucleotide reductase in the generation of deoxyribonucleotides (8, 18). They also were characterized as substituting thioredoxin as reducer of phosphoadenylylsulfate reductase in bacteria, plants, and yeasts (18). Furthermore, Grxs can function as general glutathione-dependent protein disulfide oxidoreductases (6), catalyzing the reduction of glutathionylated compounds (5), as well as glutathionylation and deglutathionylation of specific proteins (4). The present work reports the molecular cloning, expression, and purification of a dithiolic Grx from *T. cruzi* and functional studies supporting the protein as a key component of the redox metabolic scenario in the parasite. This is the first time that this kind of Grx has been characterized in trypanosomatids.

Molecular Cloning and Heterologous Expression of *T. cruzi* Dithiol Grx

In the *T. cruzi* CL Brener database (http://tcruzidb.org), we identified two putative *grx* genes (Tc00.1047053506475.116 and Tc00.1047053511431.40) having 97.8% identity between them. Both genes code for an identical protein at the level of an amino acid sequence; which shows 22 to 30% identity with other reported Grxs. Sequence alignment detailed in Fig. 1

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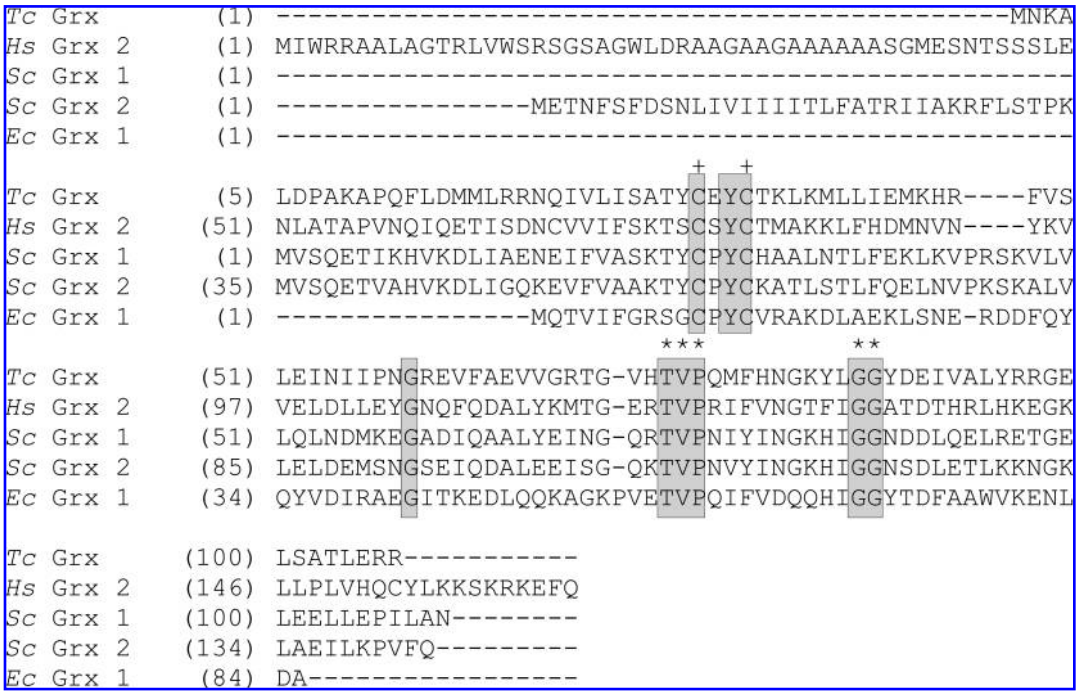

FIG. 1. Alignment of amino acid sequences of dithiol glutaredoxins. The displayed sequences are *T. cruzi* Grx (*Tc* Grx, XP_817200.1), *E. coli* Grx 1 (*Ec* Grx 1, YP_001729828.1), *Saccharomyces cerevisiae* Grx 1(*Sc* Grx 1, NP_09895.1), *S. cerevisiae* Grx 2 from (*Sc* Grx 2, NP_10801.1), and human Grx 2 (*Hs* Grx 2, NP_932066.1). Residues that are identical are presented in black background. $^{+}$Cysteines (C) in the active site, and *residues involved in glutathione-binding site.

shows that the protein coded in *T. cruzi* contains structures typically found in dithiol Grxs, as the active-site CXYC motif (18), and domains TVP and GG, reported to be involved in glutathione binding (20).

Based on one of the reported nucleotide sequences for *Grx* (Tc00.1047053506475.116), we designed specific oligonucleotides (see Materials and Methods) for amplifying the gene with polymerase chain reaction (PCR) from genomic DNA of *T. cruzi* CL Brener. The amplified product was cloned and sequenced to confirm its identity. The gene is predicted to encode a protein of 108 amino acids, with a molecular mass of 12.44 kDa and a calculated isoelectric point of 9.26. The gene was cloned into pRSET-A and expressed in *Escherichia coli* BL21(DE3) cells to produce a recombinant protein fused to a six-histidine tag at the N-terminus. The protein was purified onto a Co^{2+} affinity resin column, concentrated and analyzed with sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS-PAGE). The recombinant *Tc*Grx exhibited a molecular mass of ~15 kDa (Fig. 2), a value in agreement with that expected for the expressed fused protein. The amino acids sequence of the pure recombinant enzyme, determined with MALDI-TOF-MS, completely matched the sequence deduced from the gene, confirming the identity of *Tc*Grx.

Functional Properties of *Tc*Grx

HEDS reduction is the enzymatic assay most commonly used for characterizing Grxs (18). In this assay, HEDS and GSH spontaneously react to form a mixed disulfide, which is a suitable substrate for reduced Grx. Recombinant *Tc*Grx was able to deglutathionylate the mixed disulfide, after being reduced by either GSH, $T(SH)_2$, or GspSH, with similar activity in all cases (Table 1). As also detailed in Table 1, the couple $T(SH)_2$/*Tc*Grx exhibited significantly higher activity as a reducer of GSSG compared with the previous assay. GspSH also was effective as an electron donor for *Tc*Grx-directed GSSG reduction; the reductive activity assayed with this compound

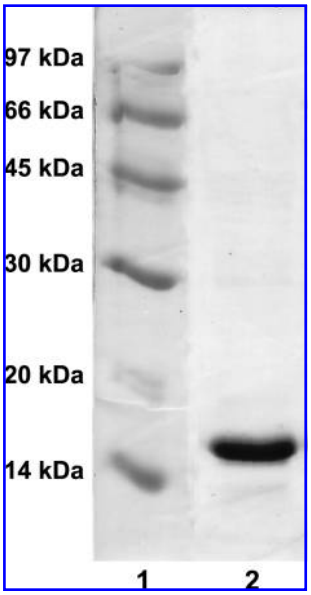

FIG. 2. Expression and purification of *Tc*Grx. The electrophoretic analysis was performed in a 15% (wt/vol) SDS-PAGE. Lane 1, molecular mass markers (GE-Health Care); lane 2, purified and concentrated *Tc*Grx (8 μg).

Table 1. Comparison of Specific Enzyme Activities of *Tc*Grx in Systems Involving Different Reducer and Oxidant Agents

Oxidant	*Reducer*	*Activity* ($U \cdot mg^{-1}$)
HEDS (1 m*M*)	GSH (0.1 m*M*)	0.036
	$T(SH)_2$ (0.1 m*M*)	0.033
	GspSH (0.2 m*M*)	0.067
GSSG (1 m*M*)	$T(SH)_2$ (0.1 m*M*)	9.13
	GspSH (0.2 m*M*)	0.67

One unit (U) of activity is defined as the amount of enzyme that catalyzes the oxidation of 1 μmol NADPH per minute.

was 28-fold lower compared with the use of $T(SH)_2$ (Table 1). The evolution of NADPH consumption in the couple system for GSSG reduction by *Tc*Grx was dependent on the concentration of the latter. As illustrated in Fig. 3, increasing concentration of *Tc*Grx in the reaction mixture augmented the rate of NADPH oxidization. Furthermore, the system behaved with specificity to reduce GSSG, as no activity was detected in the assay to reduce insulin or redox proteins, such as GPxI and TXNPx from *T. cruzi* (data not shown). The latter agrees with previous work (18), indicating that not all dithiol Grxs are able to catalyze the reduction of intramolecular disulfide bonds by a dithiol mechanism. No activity was observed when we used dihydrolipoamide as the electron donor, suggesting that the system is quite specific for reducers derived from glutathione, between others found in the parasite.

Further to explore the occurrence and functionality of Grx in *T. cruzi*, we sought to determine the HEDS-reduction assay by using a soluble extract of epimastigote cells. The Grx-related thioltransferase activity thus measured was of 9 mU/mg or 2.7 mU per 10^8 cells, which is in the range of specific activities found in crude extracts from other sources (22). The activity determined in epimastigotes could be significantly higher for an *in vivo* functionality of Grx in mediating reduction of GSSG, if the data shown in Table 1 are considered.

S-glutathionylation of proteins has been reported as a regulatory mechanism operating in prokaryotes and eukaryotes, the functionality of which underlies the reversibility of the reaction (4, 27, 29). We explored whether the deglutathionylating properties of *Tc*Grx could be extended to modified enzymes. We found that *Triticum aestivun* glyceraldehyde-3-P dehydrogenase (*Ta*GapC) was sensitive to oxidation by GSSG, with loss of the enzyme activity. Interestingly, modified *Ta*GapC recovered its activity after deglutathionylation mediated by *Tc*Grx (Fig. 4). The latter support a role of *Tc*Grx in a mechanism of regulation of enzymes by reversible glutathionylation. It would be of interest to explore further the identification of specific targets for regulation in trypanosomatids.

Concluding Remarks and Future Directions

We were able to produce a highly pure recombinant dithiol Grx from *T. cruzi*, which exhibited thioltransferase activity. A similar activity could be detected by using *T. cruzi* crude extracts, thus revealing the *in vivo* occurrence of the protein. The recombinant dithiol *Tc*Grx did not use TXNPx or GPxI from *T. cruzi* to reduce *t*-bOOH, suggesting that the protein would not be directly involved in hydroperoxide detoxification. A similar result was recently reported by Filser *et al.* (8) for a monothiol glutaredoxin from *T. brucei*. Nevertheless, *Tc*Grx was able to mediate GSSG reduction mainly by using GspSH or $T(SH)_2$ as reducers, which envisages a functional role of the protein in the maintenance of GSH in its reduced state. The activity with $T(SH)_2$ was significantly higher than that with the former reducer. However, the physiological relevance of the activity with GspSH should not be thrust aside, because it has been demonstrated that levels of accumulation of the redox intermediate are of importance in different organisms (13, 15). The functionality of a system using $T(SH)_2$ or GspSH as electron donors for *Tc*Grx

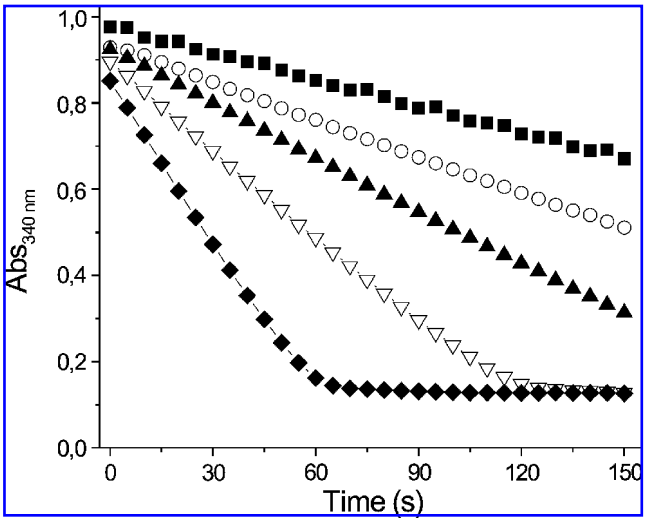

FIG. 3. Reduction of oxidized glutathione catalyzed by *Tc*Grx. Curves of NADPH oxidation in the enzymatic system containing 100 μM $T(SH)_2$, 1 mM GSSG and different concentrations of *Tc*Grx: (■) 0 μM; (○) 0.156 μM; (▲) 0.312 μM; (▽) 0.625 μM; (◆) 1.25 μM. The assay was performed in buffer 100 mM Tris-HCl pH7.5, 2 mM EDTA, 300 μM NADPH and 1μM TR, in a final volume of 50 μL.

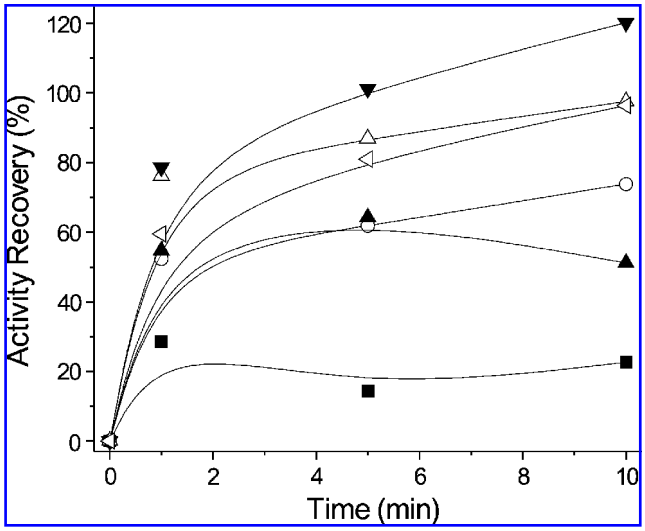

FIG. 4. Recovery of GapC activity catalyzed by *Tc*Grx. Evolution of percentage of activity recovery for pre-oxidized GapC incubated with: (■) 0 mM GSH, 0 μM *Tc*Grx; (○) 10 mM GSH, 0 μM *Tc*Grx; (▲) 10 mM GSH, 0.62 μM *Tc*Grx; (△) 10 mM GSH, 1.25 μM *Tc*Grx; (▼) 10 mM GSH, 2.5 μM *Tc*Grx; (▷) 10 mM GSH, 5 μM *Tc*Grx.

to reduce GSSG seems clearly relevant in trypanosomatids, which lack glutathione reductase (11, 23).

Another relevant role for *Tc*Grx is based on its capacity in reducing glutathionylated substrates, mainly proteins. Thus, *Tc*Grx seems to be involved in a mechanism able to regulate enzymes by glutathionylation/deglutathionylation. Such a mechanism could play a critical role in the parasite, not only in redox regulation of enzymes but in repairing oxidative damage. Identification of enzymes that are targets for this regulation remains as an issue of interest for future research. As a whole, results suggest that the redox metabolic scenario in *T. cruzi* should be revisited, considering Grx as a key functional protein in the parasite.

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Author Disclosure Statement

No competing financial interests exist.

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Abbreviations Used

GpxI = glutathione peroxidase I
Grx = glutaredoxin
GSH = reduced glutathione
GspSH = reduced glutathionylspermidine
GSSG = oxidized glutathione
HEDS = hydroxyethyl disulfide
*Ta*GapC = *Triticum aestivum* glyceraldehyde 3-P dehydrogenase
t-bOOH = tert-butyl hydroperoxide
TR = trypanothione reductase
$T(SH)_2$ = trypanothione
TXNPx = tryparedoxin peroxidase

(Appendix follows →)

Appendix: Materials and Methods

1. Cloning and heterologous expression of grx

The putative gene encoding *grx* was amplified from *T. cruzi* (CL Brener) genomic DNA with PCR, by using the oligonucleotides primers GGATCCATGAACAAAGCTTTAGATCC and GAATTCTCACCGCCTCTCCAA, which contain restriction sites *Bam*HI and *Eco*RI (underlined). The oligonucleotide primer pair was designed accordingly to a highly conserved sequence region of the putative *grx* genes obtained from the *T. cruzi* database (http://tcruzidb.org). PCR was performed under the following conditions: 95°C for 5 min; 30 cycles of 95°C for 1 min, 65°C for 1 min, and 72°C for 1 min; then 72°C for 10 min.

The amplified *grx* gene was cloned into pGEM-T Easy vector (Promega, Buenos Aires, Argentina), being *E. coli* Top 10 F_1 cells (Invitrogen, Buenos Aires, Argentina) transformed with the plasmid pGEM-T Easy/*Tcgrx*. The identity of the *grx* gene was confirmed by sequencing. DNA preparation of the recombinant plasmid pGEM-T Easy/*Tcgrx* was digested with *Bam*HI and *Eco*RI, to release and subclone the gene into the pRSET-A vector (Invitrogen).

Escherichia coli (BL21) DE3 cells transformed with pRSET-A/*Tcgrx* were grown in 500 ml of Terrific Broth medium containing 100 μg/ml ampicillin, at 37°C and 200 rpm in an orbital shaker up to $DO_{600\,nm}$ 0.8–1. The expression was induced with IPTG (1 m*M* final concentration), followed by incubation at 26°C and 180 rpm for 16 h. After harvesting by centrifugation, the cell pellet was resuspended in 20 m*M* Tris-HCl pH 8.0, 300 m*M* NaCl (binding buffer), and disrupted by sonication. Cell debris was removed by centrifugation, and the soluble fraction was loaded onto IDA-Co^{2+} column pre-equilibrated with binding buffer. The column was washed with binding buffer containing 30 m*M* imidazole, and His_6-*Tc*Grx was eluted by increasing imidazole in the buffer to 100 m*M*. The fractions containing *Tc*Grx were pooled, concentrated, and desalted by ultrafiltration.

2. Protein methods

Purity of the recombinant protein was analyzed with SDS-PAGE (16) by using the Bio-Rad minigel apparatus and Coomassie Brilliant Blue to stain protein bands. Protein contents were determined by the method of Bradford (2), with BSA as standard.

3. MALDI-TOF

Amino acids sequence of the purified *Tc*Grx was analyzed with MALDI-TOF mass spectrometry in the Unit of Analytical Biochemistry and Proteomics at the Pasteur Institute (Montevideo, Uruguay). In brief, the samples were faded, washed, and resuspended in 30 m*M* NH_4HCO_3, pH 8.0; and incubated with trypsin (sequencing grade; Promega). The peptides were extracted with 60% (vol/vol) acetonitrile, 0.1% (wt/vol) trifluoroacetic acid. Then the samples were concentrated with speed-vac and finally desalted with a reverse-phase column. The elution was directly loaded on the equipment plate with a matrix solution (α-cyano-4-hydroxycinnamic acid in 60% (vol/vol) acetonitrile and 0.2% (wt/vol) trifluoroacetic acid (1). Peptide profiles were analyzed with the Mascot server (www.matrixscience.com).

4. Enzymatic assays

Thioltransferase activity was measured spectrophotometrically by monitoring the consumption of NADPH at 340 nm and 30°C, with a Multiskan Ascent one-channel vertical light-path filter photometer (Thermo Electron Co., Buenos Aires, Argentina). The standard reaction medium (50 μl final volume) contained 100 m*M* Tris-HCl pH 7.5, 2 m*M* EDTA, and 300 μM NADPH, and the different coupled reactions were analyzed after the following specific additions. HEDS reduction assay (8, 19) used 1 m*M* GSH or 100 μM $T(SH)_2$ or 200 μM GspSH as reducing agents and different concentrations of *Tc*Grx (0–5 μM). Similar conditions were used to measure activity in soluble extracts of *T. cruzi* epimastigote cells. For the latter measurement, the purified recombinant *Tc*Grx was replaced in the medium with an extract obtained after resuspending the pellet from 3×10^9 epimastigote cells in 500 μl of 100 m*M* Tris-HCl pH 7.5, 2 m*M* EDTA, followed by disruption by sonication and centrifugation. The reduction of GSSG with $T(SH)_2$ and GspSH as electron donors was performed according to Lüdeman *et al.* (19), by using 100 μM TS_2 or 200 μM GspSH, 1 μM *Tc*TR, 1 m*M* GSSG, and different concentrations of *Tc*Grx (0–1.25 μM).

The activity of *Tc*Grx mediating transference of reducing equivalents between *Tc*TXNPx and *Tc*GPxI to reduce *t*-bOOH was followed as described previously by Flohé *et al.* (9), by using 100 μM TS_2, 1 μM *Tc*TR, 5 μM *Tc*TXNPx or 5 μM *Tc*GPxI, 70 μM *t*-bOOH, and different concentrations of *Tc*Grx up to 5 μM.

Reduction of the two interchain disulfide bonds of insulin catalyzed by recombinant *Tc*Grx was analyzed in an assay adapted from that previously described by Holgrem (12), by using 100 μM TS_2, 1 μM *Tc*TR, 100 μM bovine insulin, and different concentrations of *Tc*Grx up to 5 μM.

The ability of *Tc*Grx to deglutathionylate proteins was assayed by using *Ta*GapC as a target. The plant enzyme was inactivated by oxidative treatment with 10 m*M* GSSG in standard reaction medium. Oxidized *Ta*GapC was then incubated under the same conditions with 10 m*M* GSH and variable concentrations of *Tc*Grx. At different times, aliquots were withdrawn and assayed for GapC activity, according to protocols previously described (25).

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