The Monothiol Single-Domain Glutaredoxin Is Conserved in the Highly Reduced Mitochondria of *Giardia intestinalis*\(^\dagger\)

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*Giardia intestinalis* is a parasitic protozoan that was considered amitochondriate until recently (7). Although typical ATP-producing mitochondria are not present, related organelles, named mitosomes, were discovered in this organism (29). Mitosomes and mitochondria have a number of similarities; most notably, both are surrounded by a double membrane (29), they have a common mode of protein import and maturation (6, 24), and both harbor key components of the FeS cluster assembly machinery (29). These similarities indicate that mitosomes are highly reduced forms of mitochondria (7, 30), even though alternative evolutionary scenarios are still being discussed (17).

FeS clusters are cofactors of a number of FeS proteins involved mainly in electron transport, energetic metabolism, synthetic pathways, and biological sensing (12). Most importantly, the FeS protein Rli1 is indispensable for FeS cluster biogenesis. Consequently, the biogenesis of FeS clusters is an essential process for all cells from bacteria to human cells (15). In most nonplant eukaryotes, the crucial part of this biosynthetic pathway occurs in the mitochondrion or mitochondrial-related organelles (14, 24, 28). Studies of *Saccharomyces cerevisiae* mitochondria showed that the FeS cluster assembly is centered on IscU, a metallochaperone that serves as a scaffold for a new FeS cluster. The cysteine desulfurase IscS (in *S. cerevisiae* named Nis1) forms a heterodimer with Isd11. This heterodimer catalyzes the mobilization of sulfur for the FeS cluster. The delivery of iron is most likely regulated by frataxin (1). Reducing equivalents, which are required during FeS cluster biogenesis, are provided by a short electron transport chain, including the mitochondrial [2Fe2S] ferredoxin and ferredoxin:NADH reductase. Finally, a transient FeS cluster is transferred from IscU (IscU1/2) to apoproteins by the action of the Hsp70 (Ssq1) and Hsp40 (Juc1) chaperones and the proteins IscA (IscA1/2) and Iba57 (15). This last step also requires a monothiol class glutaredoxin (Grx5) with a characteristic CGFS active site motif (20). This class of glutaredoxins catalyzes the reduction of disulfide bonds in proteins converting glutathione (GSH) to GSH disulfide. It has been recently demonstrated that dimeric monothiol glutaredoxins can coordinate a [2Fe2S] cluster via the cysteine residue of the active site of each monomer and the cysteines of two GSH molecules (20). Although the exact role of Grx5 remains to be elucidated, it was hypothesized that, in the final step of iron sulfur cluster biogenesis, the FeS cluster that is transiently formed on an IscU scaffold protein is transferred to a Grx5 dimer in a GSH-dependent manner and is subsequently passed on to the target apoproteins (20).

The ability of *Giardia* mitosomes to assemble FeS clusters on apoferrredoxin has been demonstrated (29), indicating that all necessary components of the FeS cluster assembly machinery are present in these organelles. However, only three components known from *S. cerevisiae* mitochondria have been shown to be localized to mitosomes so far (IscS, IscU, and

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The proteomic approach. The mitosomal localization of Gigrx was confirmed by expression of the tagged protein in *Giardia* cells. The protein contains an unusually long N-terminal extension, which is involved in targeting the protein to the organelle, while the C-terminal part contains conserved residues required for the coordination of an FeS cluster. Phylogenetic analysis showed the close relationship between Gigrx and its mitochondrial homologues, which is consistent with the proposed mitochondrial origin of the iron-sulfur cluster assembly machinery in *G. intestinalis*.

MATERIALS AND METHODS

Reagents. Acetonitrile, α-cyano-4-hydroxycinnamic acid, diithiothreitol (DTT), Na-tosyl-l-lysine chloromethyl ketone, leupeptin, polyvinylidene difluoride membranes, protein A-Sepharose, reduced and oxidized GSH, sucrose, Tris, trifluoroacetic acid, trypsin, CaCl2, KCl, and NH4HCO3 were purchased from Sigma-Aldrich.

Cell cultivation. The *G. intestinalis* strain WB (American Type Culture Collection [ATCC 30957]) was grown in TYI-S-33 medium supplemented with 10% heat-inactivated bovine serum (PAA Laboratories, GmbH, Austria), 0.1% bovine bile, and antibiotics (13).

Preparation of mitosome-rich fractions. *Giardia* trophozoites were harvested, washed twice in ST buffer (250 mM sucrose, 0.5 mM KCl, 10 mM Tris [pH 7.2]), and suspended in ST buffer with 50 μg/ml Na-tosyl-l-lysine chloromethyl ketone and 10 μg/ml of leupeptin. The cells were disrupted by sonication and centrifuged at 680 × g for 10 min and at 2,760 × g for 20 min to remove unbroken cells, nuclei, and cytoskeletal residues. The supernatant was centrifuged at 50,000 × g for 30 min. The high-speed supernatant was used for preparation of the cytosolic fraction by centrifugation at 250,000 × g for 30 min. The high-speed pellet was resuspended in 0.5 ml of ST buffer and layered on a discontinuous sucrose gradient consisting of 1 ml each of 25%, 30%, 35%, 40%, 45%, 50%, 55%, and 60% sucrose in 25 mM Tris-HCl, pH 7.2. The gradient was centrifuged for 22 h in a Beckman SW40 rotor at 120,000 × g and 4°C. Fractions (0.5 ml each) were collected and analyzed by immunoblotting using polyclonal rabbit anti-GiiseU antibody (29). The concentrations of sucrose in the collected fractions were determined by refractometry.

2DE PAGE. The mitosome-rich fraction was washed with ST buffer, solubilized in 7 M urea, 2 M thiourea, 4% CHAPS [3-(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate), 0.5% Bio-Lyte ampholyte (Bio-Rad), 100 mM DTT, bromophenol blue, and 40 mM Tris, pH 8.8, and subjected to isoelectric focusing on 17-cm, pH 3 to 10 and pH 4 to 7 immobilized pH gradient strips (Bio-Rad). After the focusing, the gel strips were equilibrated twice for 15 min each with 6 M urea, 20% glycerol, 50 mM Tris (pH 8.2), 2% sodium dodecyl sulfate (SDS), 65 mM DTT, and bromophenol blue. The second dimension was run on 13.5% SDS-polyacrylamide gel electrophoresis (PAGE) gels, and the separated proteins were stained with Coomassie brilliant blue G250. Gel images were analyzed using PDQuest two-dimensional (2D) analysis software (Bio-Rad).

Trypsin digestion and mass spectrometry. Protein spots were manually excised from the 2D gels, washed twice with 25 mM NH4HCO3, dehydrated in acetonitrile, dried, and subjected to in-gel digestion by rehydration with 10 μg/ml trypsin, 25 mM NH4HCO3, and 5 mM CaCl2. Digestion was performed at 37°C overnight. Tryptic peptides were extracted by subsequent washing with 50% acetonitrile-0.1% trifluoroacetic acid and 100% acetonitrile-0.1% trifluoroacetic acid. The washes were pooled, desalted with a C18 ZipTip (Millipore), and analyzed in a Voyager-DE PRO matrix-assisted laser desorption ionization–time of flight mass spectrometer (Applied Biosystems). A saturated solution of α-cyano-4-hydroxycinnamic acid in 50% acetonitrile and 0.1% trifluoroacetic acid was used as the matrix. The spectra were analyzed using an in-house Mascot search engine (Matrix Science, London, United Kingdom) with a local implementation of GiardiaDB v1.1 proteins and translated coding sequences.

Sequence analyses. Putative cleavage sites for mitochondrial processing peptides were predicted with PsortII (http://psort.im.s.u-tokyo.ac.jp/) and MitoProt II (http://iah2.helmholtz-muenchen.de/iah/mitoprot.html). The Gigrx sequence was aligned to the glutaredoxin sequences of *S. cerevisiae* (NCBI accession no. Q02784), Caulobacter sp. strain K31 (NCBI accession no. YP_001685901), and *Synechocystis* sp. strain PCC 6803 (NCBI accession no. NP_440398) using ClustalW (28) and manually edited with BioEdit (10). For phylogenetic reconstruction, glutaredoxin-related sequences were identified by BLAST searches (http://blast.ncbi.nlm.nih.gov/Blast.cgi), aligned using ClustalX, and manually edited with BioEdit (10). The Bayesian method was used for detailed analysis of glutaredoxin clades. Bayesian searches of tree space were performed with MrBayes (23) using the JTT amino acid substitution matrix with one invariant I rate category and four variable Γ rate categories. Four Monte Carlo Markov chains, each with 100,000 generations, were performed, with trees sampled every 100 generations. For compilation of Bayesian consensus topologies, a “burn-in” of 600 trees was used.

Selectivte translation of *G. intestinalis* and immunofluorescence analysis. A gene coding for the complete *G. intestinalis* glutaredoxin (gigrx2, 202), GiardiaDB identification number GL5803_2013; http://www.giardia-db.org/hgiardi.db/) and its truncated form coding for amino acids 65 to 202 (gigrx2, 202), numbers according to Fig. 3) were amplified by PCR using the forward primers GigrxF1 (5'-CATGATATTAGACCAAAATACGGG-3') and GigrxF2 (5'-CATATCATGCGGATGTTCTCCTG-3') and reverse primer GigrxR (5'-CATTGGTACCGGCTGATACATCACCACACCTT-3'). cDNA was used as template, which was prepared as previously described (6). PCR products were inserted into the plasmid pONDRA-HA (6). Cells were transfected and selected as described in reference 25. *G. intestinalis* cells expressing recombinant proteins fused with a hemagglutinin (HA) tag at the C terminus were fixed and stained for immunofluorescence microscopy with mouse monoclonal anti-HA antibody (6). In double-labeling experiments, GiiseU was detected as a mitosomal marker protein using rabbit polyclonal anti-GiiseU antibody (29). Alexa Fluor-488 (green)-donkey anti-mouse antibody and Alexa Fluor-594 (red)-donkey anti- rabbit antibody (Invitrogen) were used as secondary antibodies (6). The slides were examined with an Olympus IX81 microscope equipped with an MT20 illumination system. The images were processed using ImageJ 1.41e software (NIH).

Protein expression and purification, gigrx1–202 and gigrx65–202 were inserted into the pET42b and pQE30 (Qiagen) vectors, respectively, for expression of the proteins with a C-terminal (pET42) or N-terminal (pQE) hexahistidine tag in *Escherichia coli*. The expression and purification of Gigrx1–202 were performed under denaturing conditions using Ni-nitriotriacetic acid affinity chromatography according to the manufacturer’s protocol (Qiagen, GmbH, Hilden, Germany). The expression of Gigrx65–202 was induced by 0.5 mM isopropyl-β-D-thiogalactopyranoside, and the bacteria were grown at 20°C overnight using Luria-Bertani medium supplemented with 0.5 mM ferric ammonium citrate and GSH. Gigrx65–202 was purified by Ni-nitriotriacetic acid affinity chromatography under native conditions (Qiagen, GmbH, Hilden, Germany). The active mitosomal processing peptide (GPP) was prepared and cleavage of Gigrx1–202 was tested as described in reference 24.

Immunoblot analysis. Samples of cell fractions were separated using 13% SDS-PAGE, and, after transfer to a nitrocellulose membrane, giardial glutaredoxin was detected using polyclonal rat antibody, which was raised against recombinant Gigrx99–202 by Moravian Biotechnology Ltd. (Czech Republic). The expression and processing of HA-tagged Gigrx in *Giardia* were followed using mouse monoclonal anti-HA antibody (Exbio Prague a.s., Czech Republic).

Immunoprecipitation and N-terminal amino acid sequencing. C-terminally HA-tagged GiiseU was immunoprecipitated from the mitosome-rich fraction of *G. intestinalis* expressing Gigrx1–202 using mouse monoclonal anti-HA antibody and protein A-Sepharose as described in reference 19. Immunoprecipitated GiiseU was separated by SDS-PAGE (13% gel), transferred onto a polyvinylidene fluoride membrane, and subjected to N-terminal protein sequencing by Edman degradation (Institute of Organic Chemistry and Biochemistry, Academy of Sciences of the Czech Republic).

Analytical methods. UV-visual spectra of freshly purified Gigrx65–202 were monitored at room temperature between 260 and 700 nm using a Shimadzu UV-1601 spectrophotometer.

RESULTS

Isolation of the mitosome-rich fraction and identification of glutaredoxin by mass spectrometry. An enrichment of *Giardia intestinalis* mitosomes in subcellular fractions was achieved by centrifugation of the high-speed sediment of sonicated cells in a discontinuous sucrose gradient (25% to 60% [vol/vol] sucrose) (Fig. 1). Experiments using Percoll or metrizamide gra-

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Patients did not lead to mitosome separation in our experiments (data not shown). Seventeen fractions were collected, and the separation of mitosomes was followed by immunoblot analyses of each fraction using IscU as a mitosomal marker protein (Fig. 1). Fractions 5 and 6 (44 to 49% sucrose) with the highest signal for IscU were pooled and further used for 2D gel separations (Fig. 2). Twenty-five of the most abundant spots were selected for mass spectrometry, among which four known mitosomal proteins (GiiscS, GiiscU, Hsp70, and Cpn60) were identified (Fig. 2; see Table S1 in the supplemental material for the complete list of identified proteins). Tryptic peptide sequences obtained from two protein spots on 2D gels corresponded to a glutaredoxin-related protein (Gigrx) annotated at GiardiaDB (www.giardiadb.org/giardiadb; identification no. GL50803_2013). The first protein displayed an approximate molecular size of 14.6 kDa, with pI 6, and the second protein was 14.9 kDa, with pI 5.3. All tryptic fragments derived from both proteins were localized to the C-terminal part of Gigrx, whose predicted molecular size was 22.1 kDa and pI value was 7.7 (Fig. 3). The first peptide determined by mass spectrometry started at Thr 81.

**Gigrx contains an N-terminal extension which is involved in targeting the protein to mitosomes.** A comparison of the predicted amino acid sequences of Gigrx with its mitochondrial and bacterial orthologs revealed an N-terminal extension of about 98 amino acids preceding the conserved Grx domains (Fig. 3). Such an extension may serve as the targeting sequence, which is required for the translocation of a protein to mitosomes and which is cleaved off by a mitosomal processing peptidase (24). However, all known mitosomal targeting sequences are rather short (24). Indeed, two programs designed for the prediction of mitochondrial N-terminal sequences, PsortII and MitoProt II, predicted the first 19 to 23 amino acids of the extension as putative mitochondrial targeting sequences and suggested either SRG/LI (PsortII) or TRY/AS (MitoProt II) motifs as putative cleavage sites for the mitochondrial processing peptidase. However, the calculated size of Gigrx, processed according to predicted cleavage sizes, was about 20 kDa, which did not correspond to the form of Gigrx observed on 2D gels. Thus, we raised a rat anti-Gigrx polyclonal antibody to verify which form(s) of Gigrx is present in mitosomes. Immunoblot analysis confirmed that Gigrx is present as a 14.5-kDa protein. Neither the protein corresponding to the molecular sizes of the predicted processed forms (about 20 kDa) nor the full-length preprotein (22 kDa) was observed (Fig. 4).

We next prepared a construct for the expression of full-length Gigrx (Gigrx1–202) with a C-terminal HA tag in *Giardia*. In transformed cells overexpressing Gigrx1–202 we observed two forms of Gigrx corresponding to the preprotein (upper panel of Fig. 4), with one protein migrating as a 14-kDa protein and the other as a 15-kDa protein. However, the second protein was not further analyzed.

![Fig. 1](https://example.com/fig1.png) **FIG. 1.** (A) Purification of the mitosome-rich fraction of *Giardia*. Separation of a high-speed pellet of sonicated cells on a discontinuous sucrose gradient (25 to 60%) is shown in the inset. Numbers correspond to collected fractions. The curve corresponds to protein amounts in the fractions. (B) Western blot analysis of collected fractions using rabbit polyclonal anti-GiiscU antibody.

![Fig. 2](https://example.com/fig2.png) **FIG. 2.** 2D electrophoresis of the mitosome-rich fraction. A representative pH 3 to 10 gradient 2D gel is shown, with four mitosomal marker proteins (HSP70, CPN60, IscS, and IscU) and two spots of identified glutaredoxins.
band) and mature Gigrx (lower band) (Fig. 5). To identify the preprotein cleavage site, mature tagged Gigrx was immuno-precipitated from the mitosomal cellular fraction. The N-terminal sequencing of the mature protein by Edman degradation revealed the cleavage site to be between glycine 77 and leucine 78 (SRG/LE motif) (Fig. 3).

To test this protein cleavage experimentally, E. coli-produced Gigrx1–202, which was fused with a hexahistidine tag at the C terminus, was incubated with recombinant S. cerevisiae mitochondrial and giardial mitosomal processing peptidases (24). However, no cleavage of the Gigrx preprotein was observed at either of the in silico-predicted sites or the cleavage site determined for the immunoprecipitated mature protein (see Fig. S1 in the supplemental material).

Transcription of the gene coding for Gigrx, including the N-terminal extension, was confirmed by means of reverse tran...
bacteria were grown overnight at 20°C in Luria-Bertani media.

The isolated Gigrx was brown, as noticed previously (20).

Asp 86 of Thr 168, and Asp 183, corresponding to Lys 23, Thr 71, and leucine 78, was which experimentally determined for the mature Gigrx. Thus, we investigated whether the complete N-terminal extension is required for targeting this protein or whether the shorter Gigrx version can mediate this function.

Two constructs for the expression of Gigrx1–202 and Gigrx65–202 were prepared, and their cellular localizations were compared by immunofluorescence microscopy (Fig. 5). Gigrx1–202 clearly colocalized with the mitosomal marker protein GiiscU. The label corresponded with organelles organized into rodlike structures between two nuclei and with separate tiny organelles scattered mainly within the posterior part of the body, which is a typical distribution of Giardia mitosomes (27). In contrast, no mitosomal localization of Gigrx65–202 was observed. These findings were corroborated by immunoblot analysis of cellular fractions. While Gigrx1–202 in the form of the preprotein as well as the mature protein was associated with the mitosome-rich fraction, Gigrx65–202 was exclusively detected in the cytosol (Fig. 5).

Taken together, these results showed that the Gigrx preprotein contains a long N-terminal targeting presequence of 77 amino acids, which is required for protein translocation into the organelle and which is absent in the mature Gigrx.

**Domain structure and phylogeny of G. intestinalis glutaredoxin.** Sequence analysis of the C-terminal part of Gigrx (100 to 202 amino acids) revealed the presence of a single glutaredoxin domain with a monothiol consensus CGFS motif at the active site (Fig. 3). This structure is typical for mitochondrial glutaredoxins such as Grx5 in *S. cerevisiae* and those found in alphaproteobacteria, which exhibited a high sequence identity to Gigrx (52.4% and 44.2% for *Caulobacter* sp. strain K31 and *S. cerevisiae* Grx5, respectively). The affinity of Gigrx to mitochondrial orthologs is apparent from the phylogenetic analysis based on the Bayesian method, in which Grx5-type glutaredoxins formed a distinct mitochondrial cluster (Fig. 6). Two other major clusters of glutaredoxins, which are present in various cell compartments of eukaryotes, were also resolved: dithiol glutaredoxins, which typically possess a CPYC consensus motif, and multidomain monothiol glutaredoxins, which contain an N-terminal thioredoxinlike domain and multiple C-terminal monothiol Grx domains (2, 16). This analysis suggests a mitochondrial origin of Gigrx, which likely shares ancestry with other mitosomal proteins involved in iron-sulfur cluster biosynthesis in *G. intestinalis* (26).

**Homodimeric Gigrx coordinates the FeS cluster.** The predicted Gigrx sequence contains all the residues crucial for the binding of GSH and coordination of the FeS cluster: Lys 120, Thr 168, and Asp 183, corresponding to Lys 23, Thr 71, and Asp 86 of *Synechocystis* monothiol glutaredoxin SyGrx3p (20). Therefore, we tested the possible formation of an FeS cluster on Gigrx. The C-terminal part of Gigrx with a His tag at the N terminus was expressed in *E. coli* and affinity purified to homogeneity. The isolated Gigrx was brown, as noticed previously for other FeS cluster-binding glutaredoxins (20). Optimal recovery of the colored protein was obtained when induced bacteria were grown overnight at 20°C in Luria-Bertani medium supplemented with ferric ammonium citrate and GSH. The UV-visible absorption spectra of recombinant Gigrx displayed a prominent peak at 410 nm and shoulders at 320, 510, and 590 nm (Fig. 7). This spectral profile corresponded to [2Fe2S] glutaredoxins of *Synechocystis* and other organisms (20). However, the absorption decreased in time, indicating instability of the FeS cluster. The cluster stability increased with addition of GSH and DTT, while GSH disulfide had little effect. The addition of EDTA led to the loss of the absorbance at 320 to 590 nm.

When freshly isolated Gigrx was analyzed by gel filtration under native conditions, two protein subpopulations were isolated, with molecular masses of about 17.8 and 37.6 kDa. The absorbance at 410 nm was associated only with the 37.6-kDa protein. SDS-PAGE analysis of both denatured subpopulations showed a single protein with the lower molecular mass (Fig. 8). These results indicate that the FeS cluster is coordinated by a homodimeric Gigrx structure.

**DISCUSSION**

Proteomic analysis of the mitosome-enriched cellular fraction together with localization studies by immunofluorescence microscopy of *Giardia intestinalis* revealed the presence of a CGFS-type glutaredoxin (Gigrx) in the mitosomes of this parasitic protist. Eukaryotes contain a series of glutaredoxins across various cellular compartments, where they carry out site-specific redox reactions. According to the consensus motif found in the active site of the glutaredoxin domain and the type of reaction they mediate, the proteins are classified into dithiol and monothiol groups. Dithiols use two cysteyl groups with the consensus sequence CPYC in the reduction of protein disulfides, while monothiols (CGFS) rely purely on the N-terminal cysteinyl residue of the motif. Whereas the former exist as single-domain proteins, the latter can also build up multidomain proteins with up to three monothiol glutaredoxin domains and a thioredoxin domain. For example, in *Arabidopsis thaliana* approximately 50 genes encode glutaredoxinlike proteins with specific functions in various cellular compartments. In contrast, Gigrx is the only glutaredoxinlike protein which is present in the genome of *Giardia intestinalis*. This glutaredoxin type (also named Grx5) is specifically present in mitochondria. Several lines of evidence suggested that Grx5 is involved in the formation of the FeS clusters required for the maturation of FeS proteins. The genomic knockout of Grx5 in *S. cerevisiae* led to the impaired function of enzymes containing an FeS cluster in their catalytic sites (22). Another phenotype of Grx5-deficient mitochondria was an increase in iron load on the FeS scaffold protein IscU (Isu1 in *S. cerevisiae*), suggesting that Grx5 is required for inserting the prebuilt FeS cluster into the target apoproteins (18). More recently, it was suggested that CGFS-type Grxs are an alternative scaffold to accommodate transient [2Fe2S] clusters in chloroplasts (3). Although its precise biochemical role is not clear, it seems that Grx5 is involved in the later steps of FeS cluster biogenesis. The presence of Grx5 in the highly reduced proteome of the *G. intestinalis* mitosome underlines its importance for FeS cluster assembly, the only known function of these organelles. Unlike mitochondria, mitosomes have no known FeS proteins except...
for those which are members of the FeS cluster assembly machinery (IscU, IscA, and [2Fe2S] ferredoxin). Thus, Gigrx is likely required either for the functioning of the FeS cluster assembly machinery itself or for the synthesis of FeS clusters or compounds, which can then be exported from the organelle to the cytosol for the maturation of extramitochondrial FeS proteins (14).

Our experiments showed that the Gigrx homodimer coordinates FeS clusters with spectral properties corresponding to those of [2Fe2S] clusters as in other monothiol CGFS-type glutaredoxins (20). The cysteine of the CGFS motif has been shown to be essential for the assembly of an FeS cluster and also for dimerization of the protein (20). Mutation of this cysteine in SyGrx3p of *Synechocystis* led to the formation of a monomeric colorless protein (20). The [2Fe2S] cluster bridging two Grx monomers is anchored by four cysteinyl residues. Two cysteinyl residues are provided by the cysteines of the CGFS motif of each monomer, while the free thiol groups of GSH donate the other two cysteine residues. GSH is also required for the ligation of FeS clusters in other FeS cluster binding glutaredoxins, such as the CGYC-type dithiol Grx-C1 in *Populus tremula* (8). Interestingly, an earlier study reported the absence of GSH in *G. intestinalis* (4). Although high-pressure liquid chromatography analysis of the whole-cell lysate led to the proposal that cysteine is the major low-molecular-weight thiol in this organism, our search in the *Giardia* genome revealed the presence of genes coding for two key enzymes required for GSH synthesis, glutamate-cysteine ligase (GiardiaDB identification no. GL50803_16001) and GSH synthase (GiardiaDB identification no. GL50803_15429). Thus, GSH is likely present in *Giardia*, probably at low concentrations which were not detected in the previous study (4).
Cell localization studies showed that the translocation of Gigrx required an N-terminal extension of 77 amino acids, which is not present in the mature protein immunoprecipitated from *G. intestinalis* mitosomes. The presence of the transcript coding for the complete preprotein including the N-terminal extension was verified by reverse transcription-PCR. No other forms of Gigrx were observed in these mitosomes. The extension is unusually long in comparison to known mitosomal targeting presequences, which usually comprise 10 to 18 amino acid residues (24). The composition of the Gigrx extension is also unique. While multiple positively charged residues are found within the extension of Gigrx, only a single such residue is found in close proximity to the cleavage site in other mitosomal proteins. This feature is related to the unique structure of the mitosomal processing peptidase in *Giardia*, which has evolved toward processing a limited set of substrates (24). Neither mitosomal nor mitochondrial processing peptidases were able to cleave the Gigrx preprotein in vitro. As a result, the mechanism by which Gigrx is processed upon its translocation to the organelles remains a puzzle.

Recent phylogenetic analysis (16) as well as this report revealed that monothiol CGFS-type Grxs are highly conserved across eukaryotic lineages, being present in organelles of endosymbiotic origin. Interestingly, except for those in *G. intestinalis*, genes coding for CGFS-type Grxs were not found in mitosome-harboring organisms, including *Entamoeba* and *Cryptosporidium* species, and no gene coding for this Grx type was identified in the genome of hydrogenosome-possessing *Trichomonas vaginalis*. A similar glutaredoxin with a CGFT motif was found in the genome of the microsporidium *Giardia*. The presence of an FeS cluster were associated only with the higher-molecular-mass protein (1), while no absorption within this range was obtained with the lower-molecular-mass protein (2). A similar glutaredoxin with a CGFT motif was found within the extension of Gigrx, only a single such residue is found in close proximity to the cleavage site in other mitosomal proteins. This feature is related to the unique structure of the mitosomal processing peptidase in *Giardia*, which has evolved toward processing a limited set of substrates (24). Neither mitosomal nor mitochondrial processing peptidases were able to cleave the Gigrx preprotein in vitro. As a result, the mechanism by which Gigrx is processed upon its translocation to the organelles remains a puzzle.

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Fig. 7. UV-visible spectrum of recombinant Gigrx indicate presence of FeS cluster. N-terminal truncated Gigrx was produced in E. coli and purified to homogeneity. An absorption peak at 410 nm and shoulders at 320, 510, and 590 nm (arrows) were observed, which are characteristic for a [4Fe4S] cluster coordinated by CGFS-type monothiol glutaredoxins. GSSG, GSH disulfide.
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