Supplementary Data

Supplementary Materials and Methods

Reagents

Molecular biology reagents were purchased from Fermentas (Thermo-Fisher Scientific) and Invitrogen. Dithiotreithol (DTT), glutathione (GSH), glutathionylspermidine (Gsp), $FeCl_3$, $Fe(NH_4)_2(SO_4)_2$, β-mercaptoethanol, 5,5′-dithio-bis-2-nitrobenzoic acid (DTNB), 4,4′-dithiodipyridine (DTDPy), ethylenediaminetetraacetic acid (EDTA), pyridoxal-5′-phosphate (PLP), isopropyl-β-D-thiogalactopyranoside (IPTG), phenylmethylsulfonyl fluoride (PMSF), *N*-α-tosyl-L-lysine chloromethyl ketone (TLCK), and most chemicals (analytical grade or higher) were obtained from Sigma-Aldrich or AppliChem. Antibiotics were purchased from Invitrogen and Sigma-Aldrich. Oligonucleotides (Supplementary Table S2) were synthesized by MWG Biotech or IDT. The kits for DNA purification were from Macherey-Nagel, Promega, Sigma-Aldrich, or VivoScience (Uruguay). All protein purification resins and columns were from General Electric Healthcare Life-Sciences (GE). The basic HMI-9 medium and additives were purchased from Sigma-Aldrich, and fetal calf serum was from PAA.

Plasmids

The coding region of the mature (residues Q42-L184; *Tb*1-C-Grx1 wild-type [WT]) and truncated (residues M77-L184; *Tb*1-C-Grx1Δ76) forms of *Trypanosoma brucei brucei* 1-C-Grx1 was amplified by polymerase chain reaction (PCR) from pQE30/*Tb*1-C-Grx1 (8) with oligonucleotides A/B and C (Supplementary Table S2) that introduced a 5′-*Nco*I and a 3′-*Kpn*I restriction site, respectively. The amplicons were inserted into the pET-trx1b (kindly provided by Günther Stier; EMBL), yielding the *Escherichia coli* expression plasmids pET-trx1b/*Tb*1-C-Grx1WT and pET-trx1b/*Tb*1-C-Grx1Δ76. Single-cysteine mutants C104S (primer D) and C181S (primer E) were generated with the pQE30/*Tb*1-C-Grx1 vector (8) as a template and the QuikChange® Site-Directed Mutagenesis Kit (Stratagene) and/or by PCR. This commercial kit in combination with primers F and G (Supplementary Table S2) and pHD1700-1-C-Grx1-cMyc$_2$ (3) as a template were used to generate the C104S mutant in the trypanosomal vector. The pET-trx1b was used for the production of tag-free proteins. Here, the gene of interest is cloned downstream of a His-tagged *E. coli* thioredoxin sequence and a 3C-type tobacco etch virus (TEV) protease recognition sequence. Expression from the pQE30 plasmids resulted in N-terminally His_6-tagged proteins. Cloning of dithiol *Tb*2-C-Grx1 was previously reported (2). *E. coli* DH5-α served as host cells. The correctness of the plasmids was confirmed by DNA sequencing (Molecular Biology Unit, Institut Pasteur Montevideo).

Expression and purification of recombinant proteins

Tag-free *Tb*1-C-Grx1 and *Tb*2-C-Grx1. *E. coli* BL21 (DE3) cells were transformed with pET-trx1b encoding the mature (WT) or truncated (Δ76) forms of *Tb*1-C-Grx1. Transformed cells were grown at 37°C and 220 rpm in a 2YT medium. At an OD_{600} ~1.0, the cells were transferred to 4°C, and after 30 min, the expression was induced with 200 μM IPTG and the culture incubated for 16 h at 20°C and 200 rpm. Cells were harvested by centrifugation (4000 *g*, 10 min, 4°C), and resuspended in 50 m*M* sodium phosphate, pH 7.8, 300 m*M* NaCl (buffer A) plus 1 m*M* PMSF, 40 μg/ml TLCK, 150 n*M* pepstatin, 4 n*M* cystatin, 0.1 mg/ml aprotinin, and 1 mg/ml of lysozyme (lysis buffer). After disruption by sonication, the lysate was incubated with DNase (Invitrogen) and 10 m*M* $MgCl_2$ for 1 h at 4°C and then centrifuged at 20,000 *g* for 1 h. The supernatant was cleared with 0.45 μm filters (Millipore) and loaded onto a HisTrap column (GE) equilibrated with buffer A. The column was washed with 20 m*M* imidazol in buffer A, and the proteins were eluted with 500 m*M* imidazol in buffer A. The fusion protein was dialyzed overnight (4°C) against buffer A in the presence of DTT 5 m*M* and His-tagged 3C-type TEV at a 1:35–70 (mg/ml) protease: protein ratio (see below). This procedure allows the simultaneous cleavage of the fusion protein into His-tagged EcTrx and nontagged *Tb*1-C-Grx1, while imidazol is diluted >2000-fold. The digest was then applied onto a second HisTrap, and the flow through was collected, concentrated *via* ultrafiltration (5-kDa filter cut-off), and run on a HiLoad 26/60 Superdex 75 prep-grade column (GE) equilibrated with 100 m*M* sodium phosphate buffer, pH 7.4, and 150 m*M* NaCl (buffer B). Fractions containing the protein of interest (as assessed with molecular-weight standards) were collected, concentrated to 1-2 mg/ml, and stored in aliquots at 4°C or at −20°C after addition of 10% glycerol. The mature tag-free 1-C-Grx1 (starting at Gln42) and its Δ76 mutant (starting at Met77) contain an N-terminal GAMG and GA vector-derived stretch. *Tb*2-C-Grx1 was expressed and purified according to Ceylan *et al.* (2).

His-tagged WT, C104S, and C181S *Tb*1-C-Grx1. The procedure was essentially as reported in Filser *et al.* (8), with minor modifications. Briefly, *E. coli* Novablue or BL21 (DE3) cells were transformed with pQE30/*Tb*1-C-Grx1. Growth conditions, induction, and purification by Ni^{2+}-affinity chromatography were performed as described for the tag-free proteins. The apoprotein was prepared by treatment with 5 m*M* DTT and 2 m*M* EDTA for 30 min at room temperature (RT) and buffer exchange (buffer B) on a HiTrap-desalting column. Whenever needed, further purification was done by preparative size-exclusion chromatography (SEC) on a HiLoad 26/60 Superdex 75 prep-grade column (GE), as described above. The proteins produced from this plasmid possess a 12-residue-long N-terminal extension ($MRGSH_6SG$) preceding Gln42.

Preparation of *Tb*1-C-Grx1Δ76 for nuclear magnetic resonance analysis. To prepare ^{15}N and $^{15}N/^{13}C$ uniformly labeled protein, *E. coli* BL21 (DE3) cells transformed with pET-trx1b/*Tb*1-C-Grx1Δ76 were grown in an M9 minimal medium containing 4 g/l [^{13}C]glucose and/or 1 g/l $^{15}NH_4Cl$ as the only carbon and nitrogen source, respectively. The labeled protein was expressed and purified as described above, except that the final SEC step was omitted. The buffer was exchanged to 50 m*M* sodium phosphate, pH 7.0, 150 m*M* NaCl, and 10 m*M* DTT in H_2O/D_2O (90:10% v/v), using a HiTrap desalting column. Finally, *Tb*1-C-Grx1Δ76 was concentrated up to 1 m*M* by ultrafiltration.

3C-type TEV protease. The protein is expressed from a modified pTH24 vector developed by van den Berg *et al.* (18) in *E. coli* Rosseta (DE3) pLysS cells. Transformed cells were grown in the 2YT medium supplemented with ampicillin (100 μg/ml) and chloramphenicol (30 μg/ml) at 37°C and 220 rpm. At an OD_{600} of $\sim$0.6, the expression was induced with 1 m*M* IPTG, and the culture was incubated for 16 h at 20°C and 230 rpm. The cells were harvested and lysed as described above. After sonication, 1% (v/v) Triton X-100 was added, and the cell debris removed by centrifugation at 20,000 *g* (60 min at 4°C). The lysate was cleared by filtration (0.45 μm filter) and loaded onto a HisTrap column equilibrated with buffer A. The protein was purified as described previously. The eluted protein was treated with 2 m*M* EDTA, 5 m*M* DTT, and the buffer exchanged with a HiTrap column equilibrated in 25 m*M* sodium phosphate, pH 7.0, 200 m*M* NaCl, 2 m*M* DTT, 2 m*M* EDTA, and 10% glycerol. Aliquots of 1 mg TEV protease were stored at −80°C.

E. coli cysteine desulfurase. *E. coli* BL21 (DE3) cells were transformed with a pET28a(+) plasmid encoding *E. coli* cysteine desulfurase (*Ec*IscS; 1). Transformed bacteria were grown on a Terrific Broth medium with 50 μg/ml kanamycin at 37°C and 220 rpm. At an OD_{600} of $\sim$0.8, the medium was supplemented with 10 μ*M* PLP, and the expression was induced overnight with 150 μ*M* IPTG at 15°C and 180 rpm. The protein was purified as essentially described above. After elution from the Ni^{2+}-affinity column, the protein was incubated with 100 μ*M* PLP for 1 h and then dialyzed against buffer A overnight at 4°C (dilution ratio > 2000). The dialyzed protein was concentrated; 10% (v/v) glycerol was added; and argon-flushed aliquots were stored at −80°C.

Analytical techniques

All purified proteins were controlled by absorption and fluorescence spectroscopy, denaturing gel electrophoresis (sodium dodecyl sulfate–polyacrylamide gel electrophoresis), analytical SEC, and mass spectrometry. The protein concentration was determined as described in the main text. Thiols were quantified with DTNB (ε_{412nm} = 14,150 M^{-1} cm^{-1}) (15) or DTDPy (ε_{324nm} = 21,400 M^{-1} cm^{-1}) (6). Far UV–visible circular-dichroism (CD) spectra were collected with 10–100 μ*M* reduced protein in 20 m*M* sodium phosphate, pH 7.0, in 0.1-cm path-length cuvettes (Hellma) in the absence of DTT, while near-UV CD spectra were acquired in buffer B with 1.0-cm cuvettes (Hellma). The scan speed was set at 20–50 nm/min and bandwidth at 0.2 nm, and three to five scans were averaged for each experiment. Buffer scans under identical conditions were subtracted from the spectra. The oligomeric state of apo- and holoproteins was analyzed by gel chromatography on an analytical Superdex 75 10/300 GL column (GE) equilibrated in buffer B at 0.5 ml/min and RT, coupled to an ÄKTA–FPLC system (GE) with online UV–visible detection. Columns were calibrated under identical run conditions with SEC molecular-weight standards (75–6.5 kDa; GE kit). In addition, the hydrodynamic radius was determined by dynamic light scattering (Malvern Nano S) in buffer B at 25°C with protein solutions ranging 0.1–10 mg/ml. The molecular mass of the native proteins was determined in an MDS SCIEX 4800 MALDI TOF/TOF instrument (Applied Biosystems) using sinapinic acid as the matrix and cytochrome c as an external standard.

Nuclear magnetic resonance spectroscopy

Experimental and calculation details are presented in the main text.

Isolation, reconstitution, and characterization of holoproteins

Holoproteins were purified from recombinant *E. coli* cells expressing WT or Cys-mutants of His-tagged *Tb*1-C-Grx1 as described above, except for three modifications: (i) 100 μ*M* $FeCl_3$ was added to the culture medium before IPTG induction; (ii) all buffers used during purification were extensively degassed and bubbled with argon; and (iii) the protein eluted from the Ni^{2+}-affinity chromatography was stored under an argon atmosphere and protected from light. The fresh eluate was loaded onto an analytical SEC column equilibrated with degassed buffer A, coupled to an ÄKTA-FPLC system. As a control, an identical fraction of the fresh eluate was treated with 5 m*M* EDTA for 30 min at RT and chromatographed under the same conditions. ISC was reconstituted onto apo-*Tb*1-C-Grx1 *in vitro* with the tag-free and His-tagged protein as reported previously (1, 3) and described in the main text.

Supplementary References

1. Berndt C, Hudemann C, Hanschmann E-M, Axelsson R, Holmgren A, and Lillig CH. How does iron-sulfur cluster coordination regulate the activity of human glutaredoxin 2?. *Antioxid Redox Signal* 9: 151–157, 2007.
2. Ceylan S, Seidel V, Ziebart N, Berndt C, Dirdjaja N, and Krauth-Siegel RL. The dithiol glutaredoxins of african trypanosomes have distinct roles and are closely linked to the unique trypanothione metabolism. *J Biol Chem* 285: 35224–35237, 2010.
3. Comini MA, Rettig J, Dirdjaja N, Hanschmann E-M, Berndt C, and Krauth-Siegel RL. Monothiol glutaredoxin-1 is an essential iron-sulfur protein in the mitochondrion of African trypanosomes. *J Biol Chem* 283: 27785–27798, 2008.
4. Delaglio F, Grzesiek S, Vuister GW, Zhu G, Pfeifer J, and Bax A. NMRPipe: a multidimensional spectral processing system based on UNIX pipes. *J Biomol NMR* 6: 277–293, 1995.
5. Dolinsky TJ, Czodrowski P, Li H, Nielsen JE, Jensen JH, Klebe G, and Baker NA. PDB2PQR: expanding and upgrading automated preparation of biomolecular structures for molecular simulations. *Nucleic Acids Res* 35: W522–W525, 2007.
6. Egwim IO and Gruber HJ. Spectrophotometric measurement of mercaptans with 4,4′-dithiodipyridine. *Anal Biochem* 288: 188–194, 2001.
7. Farrow NA, Muhandiram R, Singer AU, Pascal SM, Kay CM, Gish G, Shoelson SE, Pawson T, Forman-Kay JD, and Kay LE. Backbone dynamics of a free and phosphopeptide-complexed Src homology 2 domain studied by 15N NMR relaxation. *Biochemistry* 33: 5984–6003, 1994.
8. Filser M, Comini MA, Molina-Navarro MM, Dirdjaja N, Herrero E, and Krauth-Siegel RL. Cloning, functional analysis, and mitochondrial localization of *Trypanosoma brucei* monothiol glutaredoxin-1. *Biol Chem* 389: 21–32, 2008.
9. Kay LE, Xu GY, Singer AU, Muhandiram DR, and Formankay JD. A gradient-enhanced HCCH-TOCSY experiment for recording side-chain 1H and 13C correlations in H2O samples of proteins. *J Magn Reson Ser B* 101: 333–337, 1993.

10. Keller RLJ (ed.). *The Computer Aided Resonance Assignment Tutorial*. Editorial Goldau, Switzerland: Cantina Verlag, 2004.
11. Lescop E, Schanda P, and Brutscher B. A set of BEST triple-resonance experiments for time-optimized protein resonance assignment. *J Magn Reson* 187: 163–169, 2007.
12. Löhr F, Hänsel R, Rogov VV, and Dötsch V. Improved pulse sequences for sequence specific assignment of aromatic proton resonances in proteins. *J Biomol NMR* 37: 205–224, 2007.
13. Marion D, Kay LE, Sparks SW, Torchia DA, and Bax A. 3-Dimensional heteronuclear NMR of N-15-labeled proteins. *J Am Chem Soc* 111: 1515–1517, 1989.
14. Pervushin K, Riek R, Wider G, and Wüthrich K. Attenuated T2 relaxation by mutual cancellation of dipole-dipole coupling and chemical shift anisotropy indicates an avenue to NMR structures of very large biological macromolecules in solution. *Proc Natl Acad Sci U S A* 94: 12366–12371, 1997.
15. Riener CK, Kada G, and Gruber HJ. Quick measurement of protein sulfhydryls with Ellman's reagent and with 4,4'-dithiodipyridine. *Anal Bioanal Chem* 373: 266–276, 2002.
16. Sattler M, Schleucher J, and Griesinger C. Heteronuclear multidimensional NMR experiments for the structure determination of proteins in solution employing pulsed field gradients. *Prog Nucl Magn Reson Spectrosc* 34: 93–158, 1999.
17. Takeda M, Hallenga K, Shigezane M, Waelchli M, Löhr F, Markley JL, and Kainosho M. Construction and performance of an NMR tube with a sample cavity formed within magnetic susceptibility-matched glass. *J Magn Reson* 209: 167–173, 2011.
18. van den Berg S, Löfdahl PA, Härd T, and Berglund H. Improved solubility of TEV protease by directed evolution. *J Biotechnol* 121: 291–298, 2006.