Glutathionylation of Trypanosomal Thiol Redox Proteins

Johannes Melchers¹, Natalie Dirdjaja‡, Thomas Ruppert§, and R. Luise Krauth-Siegel†

From the Biochemie-Zentrum and the Zentrum für Molekularbiologie, Universität Heidelberg, 69120 Heidelberg, Germany

Trypanosomatids, the causative agents of several tropical diseases, lack glutathione reductase and thioredoxin reductase but have a trypanothione reductase instead. The main low molecular weight thiols are trypanothione (N²,N⁶-bis(glutathionyl) spermidine) and glutathionyl-spermidine, but the parasites also contain free glutathione. To elucidate whether trypanosomes employ S-thiolation for regulatory or protection purposes, six recombinant parasite thiol redox proteins were studied by ESI-MS and MALDI-TOF-MS for their ability to

versatile overoxidation of cystine residues and the formation of one GSH from one GSSG molecule without NADPH consumption. Accumulation of glutathionylated proteins under oxidative stress conditions has been reported for different cell types (1–3). In addition, glutathionylation of abundant proteins under basal conditions may serve as a glutathione store (4). The specific mechanisms causing formation of protein-SSG intermediates are largely unknown. In contrast, it is well established that glutaredoxins (thiol transferases) are specific and efficient catalysts of protein-SSG deglutathionylation (3, 5–7).

Trypanosomes and Leishmania are the causative agents of severe tropical diseases such as African sleeping sickness (Trypanosoma brucei gambiense and Trypanosoma brucei rhodesiense), Chagas disease (Trypanosoma cruzi), and the different forms of leishmaniasis (Leishmania donovani, Leishmania major, Leishmania mexicana). All of these parasites have in common that the ubiquitous glutathione reductase is replaced by a trypanothione reductase. The flavoenzyme maintains trypanothione (N²,N⁶-bis(glutathionyl)spermidine) and glutathionylspermidine (Gsp)² in the reduced state (8, 9). The parasite-specific trypanothione is synthesized from glutathione and spermidine in two consecutive steps. In the first reaction, Gsp is formed, which reacts with a second glutathione molecule to trypanothione (10, 11). The cellular concentration of Gsp in T. brucei is about 50 μM (12). In the insect parasite Crithidia fasciculata, the Gsp concentration is higher and can rise up to 2.5 mM in the stationary phase. In addition, the ratio between trypanothione and Gsp strongly depends on the growth stage of the parasites (13). Gsp, but not trypanothione, is also formed in Escherichia coli when the bacteria enter the stationary phase (14). Although trypanothione forms the basis of the parasite thiol metabolism, trypanosomatids contain also significant levels of free glutathione. In logarithmic and nearly stationary culture forms of bloodstream T. brucei, the concentration of free glutathione is 1.2 mM and 170 μM, respectively (15), which is even higher than the respective trypanothione concentrations (340 and 100 μM). In T. brucei isolated from infected mice, as well as in cultured procyclic parasites, the GSH concentration is 200–300 μM (12). Promastigote L. donovani contain up to 1.8 mM GSH (16). Thus, the question arises whether the parasites employ glutathione only for the

---

¹This work was supported by the Deutsche Forschungsgemeinschaft (Sonderforschungsbereich 544 “Control of Tropical Infectious Diseases,” Project B3). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. 1–9.

¹ The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AJ619696.

§ To whom correspondence should be addressed: Biochemie-Zentrum der Universität Heidelberg, Im Neuenheimer Feld 504, 69120 Heidelberg, Germany. Tel.: 49-6221-544187; Fax: 49-6221-545586; E-mail: luise.krauth-siegel@bzh.uni-heidelberg.de.

2 The abbreviations used are: Gsp, glutathionylspermidine; Grx, glutaredoxin; Gip, glutaredoxin-like protein; Grx1, mono-Cys-glutaredoxin 1; ESI-MS, electrospray ionization mass spectrometry; MALDI-TOF-MS, matrix-assisted laser desorption ionization/time-of-flight mass spectrometry; m/z, mass to charge ratio; Trx, thioredoxin; TS₂, trypanothione disulfide; PxlII, trypanoxin peroxidase III (glutathione peroxidase-type enzyme); DTNB, 5,5′-dithiobis-(2-nitrobenzoic acid); DTT, dithiothreitol; MES, 4-morpholineethanesulfonic acid; QTOF, quadrupole time-of-flight.
synthesis of glutathionylspermidine conjugates or also for the glutathionylation of thioredoxin proteins.

*T. brucei* mono-Cys-glutaredoxin 1 is a glutaredoxin-like protein with a CAYS motif replacing the CPYC active site of classical glutaredoxins. The biological function of the protein is not yet known. Related proteins from other organisms form mixed disulfides with glutathione (7, 17, 18), which may indicate a function in redox signaling. Yeast cells that are deficient in mono-Cys-glutaredoxin 5 (Grx5) are more sensitive toward oxidative stress and the protein has been shown to be involved in the biosynthesis of iron sulfur clusters (19). *T. brucei* peroxidase III is a cysteine homologue of the classical selenocysteine-containing glutathione peroxidases (20). The enzyme catalyzes the trypanothione/tryparedoxin-dependent reduction of a variety of hydroperoxides and is essential for both the mamalian and the insect stages of *T. brucei* (21, 22). *T. brucei* thioredoxin catalyzes reactions typical for thioredoxins such as the reduction of ribonucleotide reductase and insulin disulfide (23). The parasite thioredoxin is probably kept reduced by the spontaneous reaction with trypanothione, because the completely sequenced genomes of *T. brucei* as well as *T. cruzi* and *L. major* do not reveal any gene for a thioredoxin reductase (24). Tryparedoxins are small parasite-specific dithiol proteins with a CPC active site motif. They belong to the super family of proteins with a CXXC motif, which comprises thioredoxins and glutaredoxins (25). *T. brucei* tryparedoxin transfers electrons from trypanothione onto different peroxidases (see above), as well as ribonucleotide reductase (26), and is involved in the parasite replication of mitochondrial (kinetoplastid) DNA (27, 28). *T. brucei* glyoxalase II catalyzes the hydrolysis of lactoyltrypanothione, which is formed from methylglyoxal and trypanothione (29). Trypanothione reductase, the key enzyme of the trypanothione metabolism, is essential for the parasites (30). It is responsible for maintaining a reducing intracellular milieu by catalyzing the NADPH-dependent reduction of trypanothione disulfide. Here we report on the thiolation of specific cysteine residues as revealed by ESI- and MALDI-TOF-MS analyses of the parasite proteins after treatment with GSSG or Gsp disulfide.

**EXPERIMENTAL PROCEDURES**

**Materials**—Recombinant *T. brucei* thioredoxin (Trx) with N-terminal His6 tag was prepared as described (31). Recombinant glutathione peroxidase-type *T. brucei* peroxidase III (PxIII) with N-terminal His6 tag (20), *T. brucei* glyoxalase II with N-terminal His6 tag (29), *T. brucei* tryparedoxin (32), and *T. cruzi* trypanothione reductase (33) were provided by Dr. Tanja Schlecker, Dr. Thorsten Irsch, and Edith Ro¨ckel. Samples of human and *Drosophila melanogaster* thioredoxin reductases were kind gifts of Drs. R. Heiner Schirmer and Stephan Gromer. Trypsin, chymotrypsin, GluC, and LysC (sequencing grade) as well as GSSG were purchased from Roche Applied Science. Gsp disulfide and trypanothione disulfide (TS2) were obtained from Bachem. α-Cyano-4-hydroxycinnamic acid, iodoacetic acid, trifluoroacetic acid, and DTNB were from Sigma. Insulin–zinc complex from bovine pancreas was purchased from Serva. A stock solution of 172 μM was freshly prepared in 100 mM potas-

**Glutathionylation of Trypanosomal Proteins**

*T. brucei* mono-Cys-glutaredoxin 1 is a glutaredoxin-like protein with a CAYS motif replacing the CPYC active site of classical glutaredoxins. The biological function of the protein is not yet known. Related proteins from other organisms form mixed disulfides with glutathione (7, 17, 18), which may indicate a function in redox signaling. Yeast cells that are deficient in mono-Cys-glutaredoxin 5 (Grx5) are more sensitive toward oxidative stress and the protein has been shown to be involved in the biosynthesis of iron sulfur clusters (19). *T. brucei* peroxidase III is a cysteine homologue of the classical selenocysteine-containing glutathione peroxidases (20). The enzyme catalyzes the trypanothione/tryparedoxin-dependent reduction of a variety of hydroperoxides and is essential for both the mamalian and the insect stages of *T. brucei* (21, 22). *T. brucei* thioredoxin catalyzes reactions typical for thioredoxins such as the reduction of ribonucleotide reductase and insulin disulfide (23). The parasite thioredoxin is probably kept reduced by the spontaneous reaction with trypanothione, because the completely sequenced genomes of *T. brucei* as well as *T. cruzi* and *L. major* do not reveal any gene for a thioredoxin reductase (24). Tryparedoxins are small parasite-specific dithiol proteins with a CPC active site motif. They belong to the super family of proteins with a CXXC motif, which comprises thioredoxins and glutaredoxins (25). *T. brucei* tryparedoxin transfers electrons from trypanothione onto different peroxidases (see above), as well as ribonucleotide reductase (26), and is involved in the parasite replication of mitochondrial (kinetoplastid) DNA (27, 28). *T. brucei* glyoxalase II catalyzes the hydrolysis of lactoyltrypanothione, which is formed from methylglyoxal and trypanothione (29). Trypanothione reductase, the key enzyme of the trypanothione metabolism, is essential for the parasites (30). It is responsible for maintaining a reducing intracellular milieu by catalyzing the NADPH-dependent reduction of trypanothione disulfide. Here we report on the thiolation of specific cysteine residues as revealed by ESI- and MALDI-TOF-MS analyses of the parasite proteins after treatment with GSSG or Gsp disulfide.

**EXPERIMENTAL PROCEDURES**

**Materials**—Recombinant *T. brucei* thioredoxin (Trx) with N-terminal His6 tag was prepared as described (31). Recombinant glutathione peroxidase-type *T. brucei* peroxidase III (PxIII) with N-terminal His6 tag (20), *T. brucei* glyoxalase II with N-terminal His6 tag (29), *T. brucei* tryparedoxin (32), and *T. cruzi* trypanothione reductase (33) were provided by Dr. Tanja Schlecker, Dr. Thorsten Irsch, and Edith Röckel. Samples of human and *Drosophila melanogaster* thioredoxin reductases were kind gifts of Drs. R. Heiner Schirmer and Stephan Gromer. Trypsin, chymotrypsin, GluC, and LysC (sequencing grade) as well as GSSG were purchased from Roche Applied Science. Gsp disulfide and trypanothione disulfide (TS2) were obtained from Bachem. α-Cyano-4-hydroxycinnamic acid, iodoacetic acid, trifluoroacetic acid, and DTNB were from Sigma. Insulin–zinc complex from bovine pancreas was purchased from Serva. A stock solution of 172 μM was freshly prepared in 100 mM potas-

**Glutathionylation of Trypanosomal Proteins**

*T. brucei* mono-Cys-glutaredoxin 1 is a glutaredoxin-like protein with a CAYS motif replacing the CPYC active site of classical glutaredoxins. The biological function of the protein is not yet known. Related proteins from other organisms form mixed disulfides with glutathione (7, 17, 18), which may indicate a function in redox signaling. Yeast cells that are deficient in mono-Cys-glutaredoxin 5 (Grx5) are more sensitive toward oxidative stress and the protein has been shown to be involved in the biosynthesis of iron sulfur clusters (19). *T. brucei* peroxidase III is a cysteine homologue of the classical selenocysteine-containing glutathione peroxidases (20). The enzyme catalyzes the trypanothione/tryparedoxin-dependent reduction of a variety of hydroperoxides and is essential for both the mamalian and the insect stages of *T. brucei* (21, 22). *T. brucei* thioredoxin catalyzes reactions typical for thioredoxins such as the reduction of ribonucleotide reductase and insulin disulfide (23). The parasite thioredoxin is probably kept reduced by the spontaneous reaction with trypanothione, because the completely sequenced genomes of *T. brucei* as well as *T. cruzi* and *L. major* do not reveal any gene for a thioredoxin reductase (24). Tryparedoxins are small parasite-specific dithiol proteins with a CPC active site motif. They belong to the super family of proteins with a CXXC motif, which comprises thioredoxins and glutaredoxins (25). *T. brucei* tryparedoxin transfers electrons from trypanothione onto different peroxidases (see above), as well as ribonucleotide reductase (26), and is involved in the parasite replication of mitochondrial (kinetoplastid) DNA (27, 28). *T. brucei* glyoxalase II catalyzes the hydrolysis of lactoyltrypanothione, which is formed from methylglyoxal and trypanothione (29). Trypanothione reductase, the key enzyme of the trypanothione metabolism, is essential for the parasites (30). It is responsible for maintaining a reducing intracellular milieu by catalyzing the NADPH-dependent reduction of trypanothione disulfide. Here we report on the thiolation of specific cysteine residues as revealed by ESI- and MALDI-TOF-MS analyses of the parasite proteins after treatment with GSSG or Gsp disulfide.

**EXPERIMENTAL PROCEDURES**

**Materials**—Recombinant *T. brucei* thioredoxin (Trx) with N-terminal His6 tag was prepared as described (31). Recombinant glutathione peroxidase-type *T. brucei* peroxidase III (PxIII) with N-terminal His6 tag (20), *T. brucei* glyoxalase II with N-terminal His6 tag (29), *T. brucei* tryparedoxin (32), and *T. cruzi* trypanothione reductase (33) were provided by Dr. Tanja Schlecker, Dr. Thorsten Irsch, and Edith Röckel. Samples of human and *Drosophila melanogaster* thioredoxin reductases were kind gifts of Drs. R. Heiner Schirmer and Stephan Gromer. Trypsin, chymotrypsin, GluC, and LysC (sequencing grade) as well as GSSG were purchased from Roche Applied Science. Gsp disulfide and trypanothione disulfide (TS2) were obtained from Bachem. α-Cyano-4-hydroxycinnamic acid, iodoacetic acid, trifluoroacetic acid, and DTNB were from Sigma. Insulin–zinc complex from bovine pancreas was purchased from Serva. A stock solution of 172 μM was freshly prepared in 100 mM potas-
Glutathionylation of Trypanosomal Proteins

with different concentrations of low molecular weight disulfides as described above. Iodoacetamide was added to a final concentration of 9 mM to alkylate residual free cysteine residues. The modified and unmodified proteins were digested with trypsin (T. brucei Grx1, T. brucei glyoxalase II, T. brucei tryparedoxin, T. cruzi trypanothione reductase), GluC (T. brucei Grx1, T. brucei PxIII, T. brucei glyoxalase II), chymotrypsin (T. brucei Trx), and LysC (T. brucei Trx) at a protease:protein ratio of 1/10 (w/w). The reaction was allowed to proceed for 2 h at 37 °C, and the resulting peptides were analyzed by MALDI-TOF-MS as described in the next section.

Mass Spectrometric Analysis—The intact masses of modified and unmodified proteins were determined by ESI-MS on an API-QSTAR™ Pulsar instrument (Applied Biosystems) with a high pressure liquid chromatography system (HPLC; Agilent) on-line-coupled to the ESI-QTOF instrument. 100 μl of a 1 to 10 μM protein solution diluted with water was loaded onto a 50 Poros R1 trapping column. After 1.5 min of washing (0.1% trifluoroacetic acid, 0.4 ml/min), the protein was eluted into the Poros R1 trapping column. After 1.5 min of washing (0.1% trifluoroacetic acid, 0.4 ml/min essentially as described in Ref. 36. The QTOF instrument was calibrated with apomyoglobin (Sigma). The mass accuracy was better than 100 ppm. For relative quantification of thiolation, mixtures of known composition were evaluated, changing the amount of thiolated protein against the unmodified protein. The modified and unmodified proteins were digested with trypsin (T. brucei Grx1, T. brucei glyoxalase II), chymotrypsin (T. brucei Trx), and LysC (T. brucei Trx) at a protease:protein ratio of 1/10 (w/w). The reaction was allowed to proceed for 2 h at 37 °C, and the resulting peptides were analyzed by MALDI-TOF-MS as described above.

pH Dependence of the Reaction of T. brucei Mono-Cys-Glutaredoxin 1 with GSSG or Gsp Disulfide—Reduced Grx1 was prepared as described above. The protein was eluted from the PD10 column in 50 mM Tris/HCl, pH 9.5. The 50 μM Grx1 solution was titrated with 50 mM MES to pH values between 9 and 5 (at lower pH values the protein precipitated). 0.5 mM GSSG or Gsp disulfide was added, and the reaction mixture was incubated at room temperature for 4 h. The reaction was stopped by freezing the sample, and the intact mass of the protein was determined by ESI-MS. The degree of thiolation was estimated by comparing the peak intensities of modified and unmodified protein species.

Formation of an Intramolecular Disulfide between Cys104 and Cys181 in Grx1—50 μM Reduced Grx1 was treated with 2 mM GSSG, Gsp disulfide, TS2, or hydroxyethyl disulfide at room temperature for 24 h in 50 mM ammonium bicarbonate/HCl, pH 7.5. Excess low molecular weight reagents were removed by size exclusion chromatography on a PD10 column. In addition, reduced Grx1 was incubated with 100 or 500 μM hydrogen peroxide at room temperature for 20 min, and the reaction was stopped by the addition of 50 units of bovine catalase. The thiol content of the protein samples was determined with DTNB as described above. Remaining free thiols were alkylated by adding 15 mM iodoacetamide, and the intact mass of the protein was determined by ESI-MS. The incorporation of carboxamidomethyl groups showed cysteine residues that are not involved in an intramolecular or a mixed disulfide. To further analyze formation of an intramolecular disulfide, the tryptic digests of the treated Grx1 samples were subjected to MALDI-TOF-MS and examined for a fragment containing the linked Cys104 and Cys181 peptides. The identity of the disulfide-forming fragment was confirmed by MALDI-TOF-MS peptide sequencing and by the fact that the respective peak disappeared after the addition of 15 mM DTT.

Assaying Potential Catalytic Activities of T. brucei Grx1—To analyze whether Grx1 can catalyze the glutathionylation of other proteins, 50 μM reduced Grx1 in 50 mM ammonium bicarbonate/HCl, pH 7.5, was incubated with an equimolar concentration of reduced peroxidase III or thioredoxin and 0.5 mM GSSG. After 2, 5, 10, 20, 30 min, and 4 h, aliquots were subjected to ESI-MS. To measure a possible deglutathionylation activity, T. brucei thioredoxin was glutathionylated as described above, and excess GSSG was removed on a PD10 column. 10 μM glutathionylated thioredoxin in 50 mM ammonium bicarbonate/HCl, pH 7.5 and 8.0, was treated with 10 μM reduced Grx1. The ability of Grx1 to reduce protein disulfides was studied by incubating 50 μM reduced Grx1 with an equal concentration of oxidized tryparedoxin in 50 mM ammonium bicarbonate/HCl, pH 7.5, followed by ESI-MS.

Deglutathionylation and Reduction of Grx1—To elucidate whether other thiol proteins catalyze the deglutathionylation and/or reduction of the intramolecular disulfide bridge of Grx1,
Glutathionylation of Trypanosomal Proteins

MARCH 23, 2007 • VOLUME 282 • NUMBER 12
JOURNAL OF BIOLOGICAL CHEMISTRY 8681

50 μM reduced Grx1 in 50 mM ammonium bicarbonate/HCl, pH 7.5, was treated with 2 mM GSSG, which led to a mixture of glutathionylated protein and the protein with an intramolecular disulfide. 50 μM reduced T. brucei tryparedoxin or 5 μM D. melanogaster thioredoxin reductase and 200 μM NADPH in 50 mM ammonium bicarbonate/HCl, pH 7.5, were added. After 10 min, 15 mM iodoacetamide was added, and the samples were subjected to ESI-MS. To reveal whether GSH and trypanothione are able to reduce the intramolecular or the mixed disulfide of the protein, Grx1 treated with GSSG or Gsp disulfide was incubated with either 3 mM GSH or 0.8 mM TS2. 2 μM T. cruzi trypanothione reductase, and 4 mM NADPH in 50 mM ammonium bicarbonate/HCl, pH 7.5, and processed as described above.

Circular Dichroism Spectroscopy—50 μM Reduced Grx1 was incubated with 2 mM GSSG for 24 h at room temperature and desalted on a PD10 column. CD spectra of reduced (14 μM) and oxidized (10 μM) Grx1 were recorded at 20 °C from 195 to 300 nm on a JASCO J-810 spectropolarimeter in 5 mM potassium phosphate, 100 mM KCl, pH 7.5, with a 1-mm path length cuvette. Data were collected every nanometer with an averaging time of 2 s and a bandwidth of 1.5 nm averaging over four repeated scans. Thermal denaturations of reduced (112 μM) and oxidized (20 μM) Grx1 were followed at 222 nm by measuring the change in ellipticity at increasing temperatures from 20 to 95 °C at a speed of 0.5 °C/min. Data were recorded for each degree with an 8-s averaging time and a 1.5-nm bandwidth.

Reduction of Insulin Disulfide by Glutathionylated T. brucei Thioredoxin—T. brucei Trx was reduced as described above and incubated with 5 mM GSSG for 72 h at 4 °C. Excess glutathione was removed by centrifugation through a 10-kDa cutoff Amicon filter. Reduction of insulin disulfide was measured essentially as described by Casagrande et al. (37). In a total volume of 800 μl of 100 mM potassium phosphate, pH 7.0, 2 mM EDTA, the assays contained 160 μM insulin, 200 μM NADPH, and 1 or 2 μM of glutathionylated and untreated Trx, respectively. The reactions were started by adding 353 nm human thioredoxin reductase, and the absorption decrease was followed at 340 nm.

RESULTS

Intact Mass Determination of Thiolated T. brucei Mono-Cys-Grx1—Recombinant Grx1 was treated with GSSG and subjected to ESI-MS as described under “Experimental Procedures.” A, untreated protein. 17,330.1 Da corresponds to the theoretical mass of the His-tagged protein (17,330.9 Da for the reduced and 17,328.9 Da for the protein with an intramolecular disulfide bond). The observed masses were 17,328.6 ± 0.5 and 17,330.7 ± 0.3 Da, respectively (mean ± S.D. of six intact mass determinations). B–E, Grx1 incubated with 0.25 mM (B), 0.5 mM (C), 1 mM (D), or 5 mM (E) GSSG. The peak at 17,636 Da (17,636:0 ± 0.3 Da; mean ± S.D. of six intact mass determinations) shows a mass shift of 306 Da with respect to the free protein, which corresponds to the binding of one molecule of GSH. Treatment with millimolar concentrations of GSSG (D and E) leads to binding of a second glutathione molecule (17,941 Da). The small peaks at slightly higher masses that accompany the main peaks are probably oxidation products of a glutathionylated protein and the protein with an intramolecular disulfide bond. The observed masses were 17,328.6 ± 0.5 and 17,330.7 ± 0.3 Da, respectively (mean ± S.D. of six intact mass determinations). F, reduced Grx1 treated with 0.5 mM TS2. No mixed disulfide is formed. G, reduced protein after incubation with 0.5 mM Gsp disulfide. The small peak at 17,763.3 Da corresponds exactly to one Gsp molecule bound to Grx1 (17,330 + 433 Da). The most prominent new peak, at 17,795.5 Da, has a 32-Da higher mass, which is probably caused by the additional incorporation of two oxygen atoms. The peak at 18,196 Da represents Grx1 modified by two Gsp molecules. The ESI-MS spectrum of the reduced unmodified protein shows 15 charge states from +11 to +25 with a maximum at +21. Interestingly, in the samples treated with the low molecular weight disulfides, the charge distribution for the unmodified protein showed a maximum of +15. This indicates that formation of the intramolecular disulfide bridge lowers the number of charge states. The mono- and bis-glutathionylated protein species occur in 13 charge states, from +13 to +25. Grx1 modified by one or two Gsp (G) molecules yielded 12 and 8 charge states from +14 to +25 and from +18 to +25; the maximum of all modified species of Grx1 was at +21. The reduced number of charge states observed for the protein with two bound Gsp molecules is only due to the low amount of this species in the mixture. When Grx1 was treated with 20 mM Gsp disulfide, which resulted in the protein with two bound Gsp molecules becoming the main product, the same charge states as in the case of Grx1 modified by a single Gsp molecule were observed.

Peak intensities can be used for the relative quantification of protein species if ion suppression is excluded. To elucidate whether the glutathionylation of Grx1 suppresses the ionization/detection of one protein species by the other in the mix-
Glutathionylation of Trypanosomal Proteins

ture, Grx1 was modified at both cysteine residues by treating the protein with 20 mM GSSG for 48 h at 4 °C. Different ratios of unmodified and bis-glutathionylated Grx1 were mixed and subjected to ESI-MS. In the first series of experiments, the unmodified protein was kept constant, and bis-glutathionylated Grx1 was varied. The percentage of bis-glutathionylated Grx1 detected in the spectrum was depicted against the theoretical percentage of this species in the mixture (see supplemental Fig. 1A). In the second series, bis-glutathionylated Grx1 was kept constant, and the free protein was varied (see supplemental Fig. 1B). The slopes of the lines obtained were 0.96 and 1.02, respectively, which shows that glutathionylation of both cysteine residues in Grx1 does not significantly affect the ionization efficiency. Because Grx1 with two bound glutathione molecules is formed only at millimolar concentrations of GSSG, even under extreme oxidative stress conditions (with a cellular GSH/GSSG ratio of 2 (38)), glutathionylation should be specific for one cysteine residue. Treatment of reduced Grx1 with 0.5 mM TS2 did not result in any protein modification (Fig. 1). Glutathionylation of Trypanosomal Proteins

The ESI-MS spectrum of Grx1 treated with 0.5 mM Gsp disulfide is shown in Fig. 1G. The peak at 17,763.3 Da corresponds to the protein with one bound Gsp molecule (433 Da). The most prominent peak with a mass of 17,795.5 Da is consistent with the incorporation of one Gsp molecule plus an additional mass of 32 Da. Another minor peak at 18,196 Da corresponds to the protein with two bound Gsp molecules. As described above for the quantification of the glutathionylated protein, Grx1 was thiolated by two Gsp molecules, and different mixtures of free and modified protein were analyzed by ESI-MS. The slopes of the calibration lines were 0.74 and 1.25 when modified and free Grx1, respectively, were varied (see supplemental Fig. 1, C and D). This indicates that binding of two Gsp molecules to Grx1 lowers the ionization efficiency by about 25% and that the protein species with a mass of 17,795.5 Da is indeed the most prominent one in the reaction mixture (Fig. 1G). The intact mass peaks of all untreated and thiolated forms of Grx1 are accompanied by minor peaks with higher masses that probably reflect oxidation products (Fig. 1, A–G). Interestingly, only in the case of Grx1 with one bound Gsp, the 32-Da higher mass (17,795.5 Da peak in Fig. 1G) is the most prominent peak. Several lines of evidence support the interpretation that this species represents the protein with Gsp bound at Cys181 and Cys104 being oxidized to a sulfonic acid. 1) The reactivity of both cysteine residues was lost when Cys181 was modified by Gsp. Treatment of the protein sample with iodoacetamide caused a peak shift of 57 Da for the mono-modified protein species but not in the case of mono-modified Grx1 with the additional 32-Da mass. 2) The peak at 18,196.4 Da matches the theoretical mass of Grx1 modified by two Gsp molecules, and a peak with an additional 32 Da was not observed for this species. 3) A protein species that lacks Gsp but carries the additional 32 Da did also not occur (see Table 4). The latter two species could occur if methionine residues were oxidized. Taken together, these data strongly suggest that the binding of one Gsp molecule to Grx1 leads to the oxidation of the second cysteine to a sulfonic acid. Treatment of Grx1 with 0.5 mM hydroxyethyl disulfide also generated monothiolated protein (not shown). Incubation of reduced Grx1 with 2 mM Gsp disulfide at room temperature for 24 h led mainly to the thiolation of both cysteines, which indicates that high concentrations of disulfide and long incubation times support this unspecific reaction (see Table 4).

pH Dependence of the Thiolation of T. brucei Mono-Cys-Glutaredoxin 1—Grx1 was allowed to react with GSSG and Gsp disulfide at pH values between 5.0 and 9.0. As described in the previous section and shown in supplemental Fig. 1, the binding of two glutathione or Gsp molecules affects the ionization and detection of Grx1 by less than 5% and by about 25%, respectively. This allowed us to estimate the relative amounts of modified and unmodified protein species from the ESI-MS spectra (Fig. 2). A minor thiolation of both cysteines was observed at pH 5.0, which probably reflects reaction of the unfolded protein, because recombinant Grx1 starts to denature at pH values ≤5.0. At pH 9.0, where cysteine residues should be present in the reactive thiolate state, almost exclusively mono-thiolated protein was obtained. In the case of Gsp disulfide, modification of the protein increased hyperbolically from pH 5.0 to 9.0. In comparison, incorporation of a glutathione moiety showed a maximum around pH 5.5 to 6.0, which then slightly decreased and remained constant up to pH 9.0. This was not due to the redox potential of the reagents, because glutathione and trypanothione have practically identical values (8). Probably the pK values of the leaving thiol groups play a major role. The pK of GSH is 8.2, and that of trypanothione is 7.4 because the secondary amine in the spermidine bridge favors deprotonation of the thiol groups (39). The pK of Gsp is not known. Because the spermidine moiety in Gsp has two positive charges, its pK value may be even lower than that of trypanothione. Gsp is probably a better leaving group than glutathione (38), which would favor its reaction with the protein kinetically. The higher degree of modification by Gsp may also be caused by favorable electrostatic interactions. Deprotonation of protein residues at
high pH values may still allow an interaction with Gsp but weaken an interaction with the overall negatively charged glutathione. The fraction of protein with two bound glutathione or Gsp molecules was low at pH 5.0 and negligible at pH 9.0 (Fig. 2). Therefore, over the whole pH range studied, thiolation of Grx1 occurs at a single site.

Mono-Cys-Glutaredoxin 1 Is Thiolated at Cys181—As outlined in the previous section, incubation of Grx1 with 0.25 or 0.5 mM GSSG leads to the modification of the protein by one GSH molecule (Fig. 1, B and C). T. brucei Grx1 possesses two cysteinyl residues at positions 104 and 181. To evaluate whether the Cys-containing proteolytic peptides are in principle detectable in modified form, the protein was treated with iodoacetamide, GSSG, or Gsp disulfide under conditions in which both cysteine residues were modified. The full-range spectra of the GluC-derived peptides of fully alkylated (see supplemental Fig. 3) and fully glutathionylated Grx1 (Figs. 3 and 4) as well as the protein with two bound Gsp molecules (Fig. 4A) revealed that the peaks of the modified Cys104-containing peptides have much higher intensities than the Cys181-containing peptides. The peak intensity of the 90–115 peptide with bound Gsp ($m/z$ 3358.2) is more than an order of magnitude higher than that of the 167–184 peptide with bound Gsp ($m/z$ 2481.6) in the same GluC digest (Fig. 4A). The same is true for the 90–115 ($m/z$ 3231.1) and 167–184 peptides ($m/z$ 2354.3) with bound glutathione (Fig. 4B). To disclose whether inefficient cleavage of Grx1 by GluC is responsible for the low peak intensities of the Cys181-containing peptides, the completely modified Grx1 species were also digested with trypsin. Again, the peaks for the glutathionylated or alkylated Cys104-containing peptides had much higher intensities than the glutathionylated Cys181-containing peptides (not shown).

To elucidate which of the two cysteines is glutathionylated in the mono-modified protein, Grx1 was treated with 0.5 and 1 mM GSSG, the remaining free thiols were alkylated by iodoacetamide, and the protein was digested with trypsin or GluC (The sequences of the Cys-containing peptides are depicted in Table 1). Treatment of Grx1 with 0.5 mM GSSG caused glutathionylation of Cys181. The ions at 2049 and 2354 $m/z$ correspond to the free and glutathionylated 167–184 peptide (Fig. 5B and Table 2). These ions did not appear in the GluC digest of the protein that was treated only with iodoacetamide (Fig. 5A). Under these conditions, a glutathionylated Cys104-containing peptide was not observed (Fig. 6B; for the full-range spectrum.

FIGURE 3. Full-range MALDI-TOF-MS spectrum of the GluC peptides of Grx1 glutathionylated at both cysteine residues. The reduced protein was incubated with 20 mM GSSG and digested with GluC without prior alkylation of the remaining free thiol groups as described under “Experimental Procedures.” Intact mass determination by ESI-MS confirmed that the protein was completely modified by two glutathione molecules. The intensities of the peaks at 3231.1 and 4921.3 $m/z$, which represent the glutathionylated Cys181-containing 167–184 and 101–141 peptides, are much higher than those of the glutathionylated Cys104-containing 90–115 and 101–141 peptides, respectively. The relative peak intensities of the glutathionylated forms are higher than that of the free peptide, whereas the intensity of the peak for the glutathionylated Cys104-containing peptide is much lower than that of the free peptide.
Glutathionylation of Trypanosomal Proteins

see supplemental Fig. 4). Upon treatment of the protein with 1 mM GSSG before alkylation, both glutathionylated and alkylated Cys-containing peptides were observed in the GluC as well as in the trypic digests (Fig. 6C, for the full-range spectrum, see supplemental Fig. 5 and Table 3). The intensities of the peaks in the MALDI-TOF-MS spectra do not reflect the ratio of the peptide species in the digest. This is evident from the analysis of a 1:1 mixture of fully carboxamidomethylated and fully glutathionylated GluC peptides. In the case of Cys104, the peak intensities for both peptides (90–115 and 101–141) were much higher than those for the peak intensities of both modified peptides with bound glutathione were very low in comparison with the free peptide (not shown). This finding again underlines the specific glutathionylation of Cys181.

When Grx1 was digested with trypsin, cleavage at Lys177 was not complete, and two Cys181-containing peptides were obtained (Table 3). The unmodified singly charged 178–182 and 176–182 peptide ions occur at 549 and 792 m/z. The smaller ion was shown by MALDI-TOF-MS in an experiment with decreased ion suppression. With the general settings of the mass spectrometer, the peptide appeared only in glutathionylated form (854 m/z). Reaction of Grx1 with 0.5 or 1 mM GSSG yielded for the two trypic Cys181-containing peptides new peaks at 854 and 1097 m/z that are consistent with glutathionylation of the smaller (549 + 305 m/z) and the larger (792 + 305 m/z) fragment. Again, the peptide mixture of Grx1 treated with 1 mM but not with 0.5 mM GSSG showed glutathionylation of Cys104 in addition to Cys181. Also, when the protein treated with 0.5 mM GSSG was digested with trypsin without prior alkylation of the remaining free thiol groups, Cys104 became modified because of the reaction of the free peptide in solution. The addition of DTT to Grx1 that had been treated with 0.5 mM GSSG and iodoacetamide before digestion abolished the glutathione-containing peaks for the Cys181-containing peptide in accordance with GSSG forming a mixed disulfide with the protein (Table 3).

T. brucei Mono-Cys-Grx1 was treated with 0.5 mM Gsp disulfide, remaining free thios were alkylated with iodoacetamide, and the protein was cleaved by trypsin or GluC. At pH 7.5, the MALDI-TOF-MS anal-

TABLE 1
Possible Cys-containing peptides of *T. brucei* mono-Cys-glutaredoxin 1

Grx1 was cleaved with trypsin or GluC as described under "Experimental Procedures." In the case of trypsin, all Cys-containing peptides with one misсleavage site were selected. Because in the GluC digestion both detected Cys104 peptides contained two misсleavage sites, all peptides with two misсleavage sites were considered. The cysteine residues are given in bold letters.

| Position | Cys  | Tryptic peptides | Detected |
|----------|------|-----------------|----------|
| 178–182  | 181  | GITCR           | +        |
| 176–182  | 181  | DKGITCR         | +        |
| 97–108   | 104  | GLPEAPMCAYSK    | +        |
| 97–109   | 104  | GLPEAPMCYKR     | +        |
| 88–108   | 104  | SEDVTFIKGLPEAPMCAYSK | + |

| GluC peptides |
|---------------|
| 101–115       | 104  | APACAYSKMDVLE  | −        |
| 167–184       | 181  | SQDLKMLKRDGITYCRDL | + |
| 101–120       | 104  | APACAYSKMDVLEAGL | −        |
| 90–115        | 104  | DTVTFIKGLPEAPMCAYSKMDVLE | − |
| 90–120        | 104  | DTVTFIKGLPEAPMCAYSKMDVLEAGL | − |
| 154–184       | 181  | PFGGLDXTIVEMLESGDLKMLRDGITYCRDL | − |
| 101–138       | 104  | APACAYSKMDVLEAGLYTSFSDVLAHPVRSYKVE | + |
| 101–141       | 104  | APACAYSKMDVLEAGLYTSFSDVLAHPVRSYKVEV | + |
| 73–115        | 104  | DEIMVKKKIDDTIKSEDVTFIKGLPEAPMCAYSKMIDVLE | − |

* C terminus of the protein.
The peaks at 2040 and 2110 m/z represent other peptides of the protein. The origin of the ion at 2132 m/z is not known. A, Grx1 was alkylated with iodoacetamide and digested by GluC. The ion at 2106 m/z corresponds to that of the carboxamidomethylated Cys181-containing 167–184 peptide. B, after treatment of reduced Grx1 with 0.5 mM GSSG, two new peaks appear. The peak at 2049 m/z represents the free Cys181-containing peptide. The peak at 2354 m/z corresponds to the glutathionylated peptide with a shift of 248 Da when compared with the carboxamidomethylated peptide. C, treatment of reduced Grx1 with 0.5 mM Gsp disulfide yields a peptide at 2481 m/z in accordance with the binding of Gsp to Cys181.

The predicted and detected ions of Cys-containing GluC peptides of Grx1 are listed in Table 2. The mass-to-charge ratios of the ions were determined using MALDI-TOF-MS. The ions with mass-to-charge ratios higher than 4000 Da were not detectable. Instead, the ions occurred in alkylated form (Fig. 7; for the full-range spectra of the GluC and tryptic digests, see supplemental Figs. 7 and 8). Taken together, the intact mass determinations as well as the analysis of the tryptic and GluC peptides of thiolated Grx1 revealed that GSSG and Gsp disulfide preferably modify Cys181 in the C-terminal part of the protein.

As shown in Fig. 4A, treatment of Grx1 with 20 mM Gsp disulfide causes thiolation of both cysteine residues. Interestingly, the two Cys104-containing GluC peptides 90–115 and 101–141 not only occurred with bound Gsp (3358 and 5049 m/z) but also as ions of the respective glutathionylated peptides (3231 and 4921 m/z). To a minor degree this also occurred in the case of Gsp bound to Cys181 (2482 and 2354 m/z). An impurity of the commercial Gsp disulfide by GSSG can be ruled out. The ESI-MS spectrum of free Gsp disulfide did not reveal any peak for GSSG (not shown).

In addition, treatment of T. brucei thioredoxin with 20 mM Gsp disulfide yielded exclusively the protein with bound Gsp (see supplemental Fig. 2). These data indicate that Gsp when bound to Cys181 of Grx1 can undergo a slow hydrolysis of the amide bond connecting glutathione and spermidine, which results in the glutathionylated form of the peptide. The deconvoluted ESI-MS spectrum of Grx1 treated with 20 mM Gsp disulfide confirms this observation. In addition to the expected mass of 18,195 Da for the protein with two bound Gsp molecules, the spectrum showed a peak at 18,068 Da that reflects the protein with one Gsp and one glutathione molecule bound. Long range storage of the protein sample leads to the slow hydrolysis of the spermidine also from Gsp bound to Cys181 (see supplemental Fig. 9).

**Cys104 and Cys181 of Grx1 Can Form an Intramolecular Disulfide Bridge**—The ESI-MS spectrum of a Grx1 sample stored for 2 weeks at 4°C showed a mass of 17,330 Da (Fig. 1A, Table 4). This species mainly represents the reduced protein, because addition of iodoacetamide caused a mass shift of 114 Da in accordance with the alkylation of two cysteine residues (Table 4). When 50 μM Grx1 was incubated with 2 mM GSSG for 24 h, desalted, and then alkylated, the ESI-MS spectrum revealed three masses. The lowest one (17,328 Da) represents the protein with an intramolecular disulfide bridge (Table 4). This oxidized protein form can easily be distinguished from the reduced protein because, as described above, the mass of the reduced but not the oxidized form would have been shifted to 17,444 Da by the alkylation of the two cysteine residues. The third peak (17,693 Da) represents monogluthationylated and

---

**Table 2**

| GluC peptide | Cys residue | Modification | Observed m/z | Calculated m/z |
|--------------|-------------|--------------|--------------|---------------|
| 167–184      | 181         | None         | 2049.2       | 2049.1        |
| 167–184      | 181         | CM           | 2106.2       | 2106.1        |
| 167–184      | 181         | GS–          | 2354.3       | 2354.4        |
| 167–184      | 181         | Gsp–         | 2481.5       | 2481.7        |
| 90–115       | 104         | None         | 2925.5       | 2925.5        |
| 90–115       | 104         | CM           | 2982.5       | 2982.5        |
| 90–115       | 104         | GS–          | 3231.1       | 3230.8        |
| 90–115       | 104         | Gsp–         | 3338.2       | 3338.1        |
| 101–141      | 104         | None         | 4616.2       | 4616.3        |
| 101–141      | 104         | CM           | 4673.0       | 4673.3        |
| 101–141      | 104         | GS–          | 4920.9       | 4921.6        |
| 101–141      | 104         | Gsp–         | 5048.9       | 5048.9        |

*Monoisotopic mass-to-charge ratios of the singly charged peptide ions are given for peptides up to 4000 m/z. Because peptides with mass-to-charge ratios higher than 4000 do not show single isotope peaks, the average m/z is given.*
Glutathionylation of Trypanosomal Proteins

monocarboxamidomethylated Grx1. Incubation of Grx1 with 2 mM Gsp disulfide (Table 4), trypanothione disulfide or hydroxyethyl disulfide for 24 h (not shown) also caused formation of the intramolecular disulfide. ESI-MS analysis as well as thiol determinations with DTNB indicated that the intramolecular TS₂ is less efficient in inducing formation of the protein disulfide bond than the intermolecular glutathione, Gsp, and hydroxyethyl disulfides. Vice versa this shows that Grx1 is able to reduce these low molecular weight disulfides.

As described above, recombinant Grx1 does not contain a significant amount of protein with intramolecular disulfide bridge; only long range storage generates some intramolecular disulfide together with unspecific protein dimers and polymers. The presence of a mixed disulfide at Cys¹⁸¹ obviously triggers formation of the intramolecular disulfide. Treatment of Grx1 with 100 μM hydrogen peroxide for 20 min at room temperature only slightly affected the thiol content of the protein. Incubation with 500 μM H₂O₂ lowered the free thiol groups to about 0.6 per protein molecule (data not shown). ESI-MS analysis showed that treatment with hydrogen peroxide does not lead to the incorporation of oxygen into Grx1, but it induces also formation of the intramolecular disulfide. Incubation of the GSSG-treated Grx1 with DTT regenerated the fully reduced protein.

To further analyze the formation of the intramolecular disulfide, the GSSG-treated and subsequently alkylated Grx1 sample was cleaved by trypsin, and the peptides were analyzed by MALDI-TOF-MS. Two ions (1813 and 2056 m/z, data not shown) were obtained that correspond to the Cys¹⁸¹-containing 178–182 and 176–182 peptides linked to the Cys¹⁰⁴-containing 97–108 peptide by a disulfide bridge. Both peaks disappeared upon addition of DTT. The identity of the peptides forming the disulfide bond was confirmed by sequencing the larger fragment by MALDI-TOF-TOF-MS. Cys¹⁰⁴-Cys¹⁰⁴- or Cys¹⁸¹-Cys¹⁸¹-containing peptides were not observed, which corroborates the specific formation of the intramolecular disulfide bridge. Reaction of Grx1 with 0.5 mM Gsp disulfide also generated the protein with intramolecular disulfide bridge (17,328 Da), but the most prominent peak was the protein with one bound Gsp and an additional mass of 32 Da (Fig. 1G, Table 4). As outlined above, this species most probably represents Grx thiolated at Cys¹⁸¹ carrying a sulfenic acid residue at position 104.

### Table 3

MALDI-TOF-MS analysis of the tryptic Cys¹⁸¹- and Cys¹⁰⁴-containing peptides of Grx1 treated with GSSG

Grx1 was glutathionylated, alkylated by iodoacetamide (IAM), and digested with trypsin as described under “Experimental Procedures.” Binding of a carboxamidomethyl group (CM) and glutathione (GS−) leads to a mass increase of 57 and 305 Da, respectively.

| Treatment | Tryptic peptide | m/z a | Rel. int. b | Cys¹⁸¹ Tryptic peptide | m/z a | Rel. int. b | Cys¹⁰⁴ |
|-----------|----------------|------|------------|------------------------|------|------------|--------|
| 9 mM IAM  | 176–182        | 849.5| 1.0        | CM                     | 97–108| 1323.7     | 1.0    |
| 0.5 mM GSSG, 9 mM IAM | 176–182        | 792.5| 0.30       | None                   | 97–108| 1266.6     | 0.48   |
| 176–182  | 849.5          | 0.34 |            | CM                     | 97–108| 1323.7     | 0.52   |
| 176–182  | 1097.6         | 0.36 |            | GS−                    | 97–108| 1571.7     | 0.75   |
| 0.5 mM GSSG | 176–182        | 792.5| 0.36       | None                   | 97–108| 1266.6     | 0.25   |
| 176–182  | 854.4          | 0.64 |            | GS−                    | 97–108| 1571.7     | 0.75   |
| 178–182  | 1097.5         | 0.64 |            | GS−                    | 97–108| 1571.7     | 0.75   |
| 1 mM GSSG, 9 mM IAM | 176–182        | 792.5| 0.25       | None                   | 97–108| 1266.7     | 0.37   |
| 176–182  | 849.5          | 0.33 |            | CM                     | 97–108| 1323.7     | 0.30   |
| 178–182  | 854.4          | 0.42 |            | GS−                    | 97–108| 1571.8     | 0.33   |
| 176–182  | 1097.6         | 0.42 |            | GS−                    | 97–108| 1571.8     | 0.33   |
| 0.5 mM GSSG, 9 mM IAM, 15 mM DTT | 176–182        | 792.4| 0.39       | None                   | 97–108| 1266.7     | 0.54   |
| 176–182  | 849.5          | 0.61 |            | CM                     | 97–108| 1323.7     | 0.46   |

a Monoisotopic mass-to-charge ratio of the singly charged peptide ions.
b The relative intensity (Rel. int.) of a peptide form was calculated from the peak areas of all species of this peptide given by the SNAP algorithm of the program Flex Analysis from Bruker. As outlined in the text, the values do not necessarily reflect the real content of this species in the mixture.
c The free singly charged peptide ion (792 m/z) is occasionally generated by breakage of the disulfide bond in the laser beam (see also Fig. 5, B and C).
d Because of incomplete cleavage at Lys¹⁷⁷, two Cys¹⁸¹-containing peptides were obtained. The smaller peptide (178–182) appears at 549.5 and 606.3 m/z in the free and carboxamidomethylated form, respectively. Because these ions were detected only in a separate experiment with decreased ion suppression, relative quantification of the peptide was not possible.
Circular Dichroism Spectroscopy—The circular dichroism spectra of reduced and GSSG treated Grx1 are identical (Fig. 8A). Formation of the intramolecular disulfide or glutathionylation does not significantly change the secondary structure of the protein. The pronounced minimum at 207 nm indicates relatively high loop content. Unfolding of Grx1 was studied by thermal denaturation. The melting point of the reduced protein was 59 °C, whereas the oxidized protein did not show an inflection point up to 95 °C (Fig. 8B). The shift by more than 25 °C to higher temperatures indicates that the oxidized protein has a more stable conformation than the reduced protein.

Reduction of the Intramolecular Disulfide and Deglutathionylation of T. brucei Mono-Cys-Grx1 by Different Thiol Systems—50 μM Grx1 was treated with 2 mM GSSG, which, after removal of excess GSSG on a PD10 column, results in a mixture of protein with intramolecular disulfide bridge and the protein with one and (to a minor degree) two bound glutathione molecules. This sample was incubated at pH 7.5 with an equimolar concentration of reduced T. brucei tryparedoxin for 20 min at room temperature followed by alkylation with iodoacetamide. The intact mass determination by ESI-MS revealed a single protein species with two bound carboxamidomethyl groups in accordance with Grx1 being completely reduced and deglutathionylated (Table 5). The trypanothione/trypanothione reductase system also reduced both the intramolecular disulfide and the protein disulfide with glutathione (Table 5) or Gsp (not shown). In contrast, D. melanogaster thioredoxin reductase catalyzed the reduction of the intramolecular disulfide but did not deglutathionylate the protein. Trypanothione reductase and NADPH alone had no effect. GSH reduced the protein disulfide, and the small amount of mixed disulfide between Cys104 and glutathione or Gsp but did not cleave the mixed disulfides at Cys181 (Table 5). Interestingly, treatment of the protein with bound Gsp with GSH caused a thiol/disulfide exchange at Cys181 resulting in the glutathionylated protein with an identical degree of modification (not shown).

### Table 4

| Treatment                 | Mass [Da] | Cys modifications |
|---------------------------|-----------|-------------------|
| None (control)            | 17,330    | None (2 × SH)     |
| 15 mM IAM                 | 17,444    | 2 × CM            |
| 2 mM GSSG, 15 mM IAM      | 17,328    | S-S               |
|                           | 17,444    | 2 × CM            |
|                           | 17,693    | 1 × Gs−, 1 × CM  |
| 2 mM Gsp disulfide, 15 mM IAM | 17,328   | S-S               |
|                           | 17,763    | 1 × Gsp−          |
|                           | 17,795    | 1 × Gsp−, 2 O    |
|                           | 18,195    | 1 × Gsp−, 1 × CM |

Circular Dichroism Spectroscopy—The circular dichroism spectra of reduced and GSSG treated Grx1 are identical (Fig. 8A). Formation of the intramolecular disulfide or glutathionylation does not significantly change the secondary structure of the protein. The pronounced minimum at 207 nm indicates relatively high loop content. Unfolding of Grx1 was studied by thermal denaturation. The melting point of the reduced protein was 59 °C, whereas the oxidized protein did not show an inflection point up to 95 °C (Fig. 8B). The shift by more than 25 °C to higher temperatures indicates that the oxidized protein has a more stable conformation than the reduced protein.
Glutathionylation of Trypanosomal Proteins

**TABLE 5**
Reduction and/or deglutathionylation of GSSG-treated Grx1 by different thiol systems

| Reducing system | Mass [Da] | Cys modifications |
|-----------------|----------|------------------|
| None            | 17,328   | S-S              |
| 50 μM reduced trypanothione | 17,444   | 2× CM            |
| 4 mM NADPH, 0.8 mM TS2, 2 μM trypanothione reductase | 17,444   | 2× CM            |
| 3 mM GSH        | 17,444   | 2× CM            |

**T. brucei Mono-Cys-Glutaredoxin 1 Lacks (De)Glutathionylation Activity**—As described under "Experimental Procedures," 50 μM reduced thioredoxin or peroxidase III was incubated for different times with 0.5 mM GSSG in the presence and absence of 50 μM reduced Grx1. The degree of glutathionylation of the proteins was the same with and without Grx1 (not shown). Thus, Grx1 became glutathionylated but did not catalyze the glutathionylation of these proteins. Glutathionylated thioredoxin (see below) was then treated with an equimolar concentration of reduced Grx1 and subjected to ESI-MS. No deglutathionylation activity of Grx1 was observed. ESI-MS analysis of reduced Grx1 incubated with oxidized trypanothione revealed that Grx1 is also unable to reduce the intramolecular disulfide of trypanothione. In addition, no intermolecular disulfide between Grx1 and trypanothione was observed.

**T. brucei Peroxidase III Is Thiolated at Cys97 or Cys95**—Peroxidase III possesses three cysteine residues at positions 47, 76, and 95 (20). To identify the site of thiolation, the reduced protein was treated with GSSG or Gsp disulfide, the remaining free cysteine residues were alkylated, and the protein was digested by GluC. The resulting peptides were analyzed by MALDI-TOF-MS. Table 7 gives the sequences of the possible Cys-containing GluC-peptides. Treatment of the reduced peroxidase with GSSG caused a mean increase of 30 Da for both the Cys47- and the Cys95-containing peptides (Table 8). In the folded protein, Cys76 is not modified by glutathione or Gsp. Glutathionylation of Cys76 was observed only when the protein sample containing excess GSSG was digested without prior alkylation of the remaining free thiols. Under these conditions, the free Cys76 peptide reacted with GSSG, which proves that the modified peptide is detectable if it is formed. The intact mass determination of the GSSG-treated peroxidase revealed the binding of a single glutathione molecule (Table 6). Thus, glutathione binds either to Cys47 or to Cys95 but not to both cysteine residues in the same protein molecule. To elucidate whether there is a preference for one of the two cysteines, the incubation time was decreased from 4 to 1 h. Again, both residues became modified, indicating that the two cysteines have similar reactivity toward GSSG. Reaction of the reduced PxIII with Gsp disulfide resulted in the selective modification of Cys95 (Table 8).

**TABLE 6**
ESI-MS analysis of T. brucei peroxidase III treated with different disulfides

| Treatment          | Mass [Da] | Cys modifications |
|--------------------|-----------|------------------|
| None (control)     | 18,967    | None (1× S-S, 1× SH) |
| GSSG               | 18,967    | None (1× S-S, 1× SH) |
| Gsp disulfide      | 19,272    | 2× SH, 1× S-S  |
| TS2                | 19,399    | 2× SH, 1× Gsp– |
| 3 mM GSH           | 18,966    | None (1× S-S, 1× SH) |

**TABLE 7**
Possible Cys-containing GluC peptides of peroxidase III

| Position | GluC peptides | Detected |
|----------|---------------|----------|
| 93–103   | FVCTKFKAE     | –        |
| 90–103   | IKFVCTKFKAE   | –        |
| 93–113   | FVCTKFKAEFPINAKINNGE | + |
| 56–89    | TATTLNYKQSGFTLAFPNQFGQQPNEE | +|
| 20–55    | VLDADIKPVNLQHHRSPILLYWASKCCKTVKQY | +|
| 56–92    | TATTLNYKQSGFTLAFPNQFGQQPNEEIR | +|

**T. brucei Peroxidase III Is Thiolated at Cys97 or Cys95**—Peroxidase III possesses three cysteinyl residues, namely the redox active Cys31–Cys34 couple and Cys68 in the C-terminal moiety of the protein (23, 31). ESI-MS analysis of the reduced protein treated with 0.5 mM GSSG or Gsp disulfide revealed monothiolated protein species (Fig. 9). To elucidate whether the peak intensities of modified and unmodified thioredoxin are comparable and thus reflect the relative amount of the respective species in...
Glutathionylation of Trypanosomal Proteins

**TABLE 8**
Peptide analysis of thiolated peroxidase III

50 μM *T. brucei* peroxidase III was treated with 0.5 mM GSSG or Gsp disulfide or was carboxamidomethylated and digested with GluC as described under "Experimental Procedures." The Cys-containing peptides were analyzed by MALDI-TOF-MS. Binding of a carboxamidomethyl group (CM), glutathione (GS—), and glutathionylspermidine (Gsp—) increases the mass of the peptide by 57, 305, and 433 Da, respectively. IAM, iodoacetamide.

| Cysteine | Treatment | GluC peptide | Rel. int. | m/z | Cys modification |
|---------|-----------|--------------|-----------|-----|-----------------|
| Cys<sup>47</sup> | None | 20–55 | 1.0 | 3978.8 | None |
| Cys<sup>47</sup> | GSSG, IAM | 20–55 | 0.57 | 3978.9 | None |
| Cys<sup>47</sup> | IAM | 20–55 | 0.24 | 4035.9 | CM |
| Cys<sup>47</sup> | IAM | 2107 and 2234 | 0.19 | 4284.1 | GS– |
| Cys<sup>95</sup> | Gsp disulfide, IAM | 20–55 | 1.0 | 4035.9 | CM |
| Cys<sup>95</sup> | GSSG, IAM | 56–89 and 56–92 | 1.0 and 1.0 | 3769.4 and 4139.9 | None |
| Cys<sup>95</sup> | GSSG, IAM | 56–89 and 56–92 | 0.07 and 0.1 | 3769.4 and 4139.9 | None |
| Cys<sup>95</sup> | GSSG, IAM | 56–89 and 56–92 | 1.0 and 0.9 | 4076.6 and 4447.2 | GS– |
| Cys<sup>95</sup> | GSSG, IAM | 56–89 and 56–92 | 1.0 and 1.0 | 3826.7 and 4196.9 | CM |
| Cys<sup>95</sup> | GSSG, IAM | 93–113 | 0.38 | 2386.3 | None |
| Cys<sup>95</sup> | GSSG, IAM | 93–113 | 0.30 | 2443.4 | CM |
| Cys<sup>95</sup> | GSSG, IAM | 93–113 | 0.32 | 2691.5 | GS– |
| Cys<sup>95</sup> | GSSG, IAM | 93–113 | 0.30 | 2386.5 | None |
| Cys<sup>95</sup> | GSSG, IAM | 93–113 | 0.33 | 2443.6 | CM |
| Cys<sup>95</sup> | GSSG, IAM | 93–113 | 0.29 | 2819.8 | Gsp– |

<sup>a</sup> The relative intensity (Rel. int.) of a peptide form was calculated from the peak areas of all species of this peptide given by the SNAP algorithm of the program Flex Analysis from Bruker. As outlined in the text, the values do not necessarily reflect the real content of this species in the mixture.

<sup>b</sup> Mass-to-charge ratios of the monoisotopic singly charged peptide ions are given except for the ion at 4447.2 m/z, which is the average mass-to-charge ratio.

<sup>c</sup> Because of incomplete cleavage at Glu<sup>89</sup>, two Cys<sup>76</sup>-containing peptides were obtained.

TABLE 10
Glutathionylation of Tryparedoxin—Reduced *T. brucei* thioredoxin was incubated with 5 mM GSSG for 72 h, which resulted in the complete glutathionylation of Cys<sup>68</sup> as revealed by MALDI-TOF-MS analysis of the modified protein. Reduction of insulin disulfide was followed in an assay system containing NADPH, human thioredoxin reductase, and either glutathionylated or unmodified *T. brucei* thioredoxin. Both protein species showed the same activity, which means that glutathionylation of Cys<sup>68</sup> does not affect the ability of thioredoxin to reduce protein disulfides. MALDI-TOF-MS analysis of the modified protein confirmed that thioredoxin reductase and NADPH do not reduce the mixed disulfide with glutathione.

**T. brucei** Tryparedoxin Does Not Form Mixed Disulfides with Glutathione—*T. brucei* tryparedoxin (25, 32) possesses only two cysteinyl residues that form the redox active dithiol/disulfide couple (Cys<sup>41</sup> and Cys<sup>44</sup>). ESI-MS analysis of reduced tryparedoxin treated with GSSG showed a single peak with a mass (15,757.8 ± 0.4 Da, mean ± S.D. of six intact mass determinations) that was 2 Da lower than the theoretical mass of the reduced protein (15,759.8 Da, not shown). This was a first indication that, as observed for thioredoxin (see above), GSSG causes the active site cysteine residues to form an intramolecular disulfide bond. Disulfide bond formation was subsequently confirmed by peptide analysis with MALDI-TOF-MS (not shown). Reduced untreated and GSSG-treated tryparedoxin were alkylated by iodoacetamide and digested with trypsin. The MALDI-TOF-MS analysis of the GSSG-treated sample revealed an ion at 1775 m/z that represents the 31–45 peptide (TVFLYFSASWCPPCR) in oxidized form. In the reduced protein (but not in the GSSG-treated protein) the active site cys-
Glutathionylation of Trypanosomal Proteins

TABLE 10
MALDI-TOF-MS analysis of the thiolated Cys-glutathione disulfide
50 μM T. brucei thioredoxin was treated with 0.5 mM GSSG or Gsp disulfide; the remaining free thiols were alkylated by iodoacetamide (IAM), and the protein was digested with LysC as described under “Experimental Procedures.” The mass shifts of 57, 305, and 433 Da with respect to the unmodified peptide correspond to the incorporation of one carbamidomethyl (CM), glutathione (GS–), and glutathionylserendipity (Gsp) moiety, respectively.

| Treatment | m/z* | Rel. int. | Cysteine modification |
|-----------|------|----------|-----------------------|
| IAM       | 1802.2 | 0.04 | None |
| GSSG, IAM | 1859.2 | 0.96 | CM |
| Gsp disulfide, IAM | 1802.1 | 0.04 | None |
| 1859.1 | 0.90 | CM |
| 2107.0 | 0.06a | GS– |
| 1801.9 | 0.12 | None |
| 1859.0 | 0.71 | CM |
| 2234.1 | 0.16a | Gsp– |

* Monoisotopic mass-to-charge ratio of the singly charged peptide ion.

Glutathionylation of T. brucei Glyoxalase II—T. brucei Glyoxalase II contains six cysteinyl residues (29). 50 μM reduced protein was treated with 0.5 mM GSSG at room temperature for 24 h and then subjected to ESI-MS. Even after this extended incubation time, glutathionylated protein species were not observed. The spectrum showed a peak with a mass of 34,083.6 Da, which corresponds to the calculated mass of glyoxalase II with all cysteines in disulfide form. The most prominent peak at 34,098.9 ± 0.3 Da (mean ± S.D.) had a 16-Da higher mass, which may have been caused by oxidation of a methionine residue. Exposure of glyoxalase II to 5 mM GSSG for 24 h resulted in an additional peak (34,710 Da) with a 611-Da higher mass, which is consistent with the incorporation of two glutathione moieties. The reactivity of two of the six cysteinyl residues of glyoxalase II toward glutathione must be similar because no mono-modified protein species was observed. Because glyoxalase II reacted with GSSG only under drastic conditions, glutathionylation of the protein under cellular conditions is unlikely and therefore was not further analyzed.

T. cruzi Trypanothione Reductase Does Not Form Mixed Disulfides with Glutathione—Trypanothione reductase has a total of seven cysteinyl residues (33). ESI-MS analysis of the GSSG (0.5 and 5 mM)–treated reduced enzyme did not show any peak compatible with the binding of glutathione. MALDI-TOF-MS analysis of the tryptic peptides revealed that GSSG induced formation of the intramolecular disulfide between the redox active Cys52 and Cys57 couple (not shown).

DISCUSSION

Glutathionylation of proteins is a protection mechanism against oxidative damage as well as a regulation mechanism of
enzyme activities (1–4). Here we show that T. brucei mono-Cys-glutaredoxin 1, the glutathione peroxidase-type tryparedoxidin peroxidase 13, and thioredoxin can be glutathionylated and S-glutathionylspermidinylated. In the case of T. brucei glyoxalase II, glutathionylation occurs only at unphysiologically high GSSG concentrations. T. brucei tryparedoxin and T. cruzi trypanothione reductase do not show any binding of glutathione. As expected, trypanothione disulfide does not form a stable mixed disulfide with any of the proteins.

Treatment of T. brucei mono-Cys-glutaredoxin 1 with 0.25 or 0.5 mM GSSG resulted in the specific attachment of one glutathione molecule at Cys181. At higher GSSG concentrations, the reaction became less specific, and both cysteine residues were modified. 0.5 mM Gsp disulfide also reacted preferentially with the putative active site cysteine corresponding to Cys43 of other organisms, which are reported to be the C-terminal part of Grx1 contrasted with data from mono-Cys-glutaredoxins investigated so far do not catalyze the glutathione-dependent reduction of hydroxethyl disulfide. Thus, a glutathione mixed disulfide at the active site cysteine probably does not occur as a catalytic intermediate. In T. brucei Grx1, Cys181 is sensitive to thiolation, and a mixed disulfide at this residue triggers formation of an intramolecular disulfide bridge. One may speculate that the glutathione bound to Cys181 interacts with the conserved residues, which could facilitate Cys181 to approach Cys117 and/or provide a putative docking site for the second glutathione molecule that attacks the mixed disulfide during catalysis. Because these studies used mutants in which all but the first active site cysteine had been replaced, a possible glutathione-mediated oxidation of another cysteine residue would not have been detected. Most of the residues supposed to be involved in the binding of glutathione to E. coli Grx3 or human Grx1 are conserved in the parasite protein (Fig. 10); but, as outlined above, neither glutathione nor Gsp binds to the active site cysteine. In contrast to the classical 2-Cys-glutaredoxins, all mono-Cys-glutaredoxins investigated so far do not catalyze the glutathione-dependent reduction of hydroxethyl disulfide.

Incubation of the reduced Plasmodium falciparum glutaredoxin-like protein 2 (Glp2) with 2 mM GSSG leads to the binding of one glutathione molecule (18), but which of the two cysteiny1 residues of the protein is modified has not been determined. When reduced Glp2 was treated with 2 mM GSH under aerobic conditions for 24 h, a mixture of mono- and bis-glutathionylated protein was obtained, which indicates that both cysteine residues are accessible. In P. falciparum Glp2 the second cysteine (Cys216) is the fourth residue from the C terminus as is the case for Cys117 in T. brucei Grx1 (Fig. 10). Formation of an intramolecular disulfide has not been investigated in P. falciparum Glp2, and from model studies it has been suggested that this formation would be unlikely (18).
Glutathionylation of Trypanosomal Proteins

protein is stored under aerobic conditions but is induced by thiolation of Cys\textsuperscript{181}. The reaction is probably not a simple oxidation event, because incubation with 100 μM hydrogen peroxide only partially generates the protein disulfide. Even after treatment with 500 μM hydrogen peroxide, the reaction is still not complete. Incubation of \textit{S. cerevisiae} Grx5 (7) and \textit{E. coli} Grx4 (17) with GSSG also leads to an intramolecular disulfide between residues that in the reduced proteins may be far apart from each other (40). Formation of an intramolecular disulfide could be part of the catalytic mechanism of these thiol redox proteins, although the second cysteine does not necessarily occupy a conserved position. The probable mechanism for disulfide bond formation is that binding of glutathione or Gsp to Cys\textsuperscript{181} induces a conformational change of Grx1. This brings the mixed disulfide in close proximity to the side chain of Cys\textsuperscript{104}, which attacks Cys\textsuperscript{181} eliminating glutathione under formation of the intramolecular disulfide. Interestingly, binding of Gsp (and to a lesser extent also of glutathione) to Cys\textsuperscript{181} also promotes oxidation of Cys\textsuperscript{104} to a sulfinate. Thiolation of Cys\textsuperscript{181} appears to lower the redox potential of the active site Cys\textsuperscript{104} increasing its reducing capacity. Taken together, the results show that the binding of glutathione or Gsp activates Grx1. Also, for yeast Grx5 it was reported that binding of one glutathione molecule may increase the reactivity of the second cysteine of the protein. In this case, however, it was suggested that glutathione is first bound to the conserved active site Cys\textsuperscript{60}, which increases the reactivity of Cys\textsuperscript{117} and leads to the transfer of glutathione onto Cys\textsuperscript{117}. This mixed disulfide then acts as an intermediate upon formation of the intramolecular disulfide (7). Another enzyme that has been reported to be activated by specific glutathionylation is the HIV-1 protease (43).

Both the intramolecular disulfide and the glutathione-mixed disulfide at Cys\textsuperscript{181} of \textit{T. brucei} Grx1 are reduced by \textit{T. brucei} trypararedoxin. Also high concentrations of trypanothione reduce the GSSG-treated protein species completely. The parasite trypararedoxins belong to the large family of small dithiol proteins represented by thioredoxins and glutaredoxins (25). Classical glutaredoxins are known for their ability to catalyze the deglutathionylation of proteins (1, 5, 44). For instance, \textit{E. coli} glutaredoxin 1 deglutathionylates Grx4 but does not reduce the intramolecular disulfide of the protein (17). In contrast, \textit{E. coli} thioredoxin reductase specifically cleaves the intramolecular disulfide of Grx4. Other glutathione-mixed disulfides are reduced by thioredoxin reductase; for example, the mitochondrial human Grx2 is deglutathionylated by human thioredoxin reductase (45). Our \textit{in vitro} studies show that \textit{D. melanogaster} thioredoxin reductase reduces the protein disulfide in \textit{T. brucei} Grx1 but not the mixed disulfide with glutathione. However, the reaction should not play a physiological role, because all known trypanosomatids lack a thioredoxin reductase gene (26). In conclusion, the trypanothione/tryparedoxin system of the parasites, which is the donor of reducing equivalents for the parasite synthesis of DNA precursors (26) and the detoxification of hydroperoxides by different peroxidases (20, 22, 46, 47), also keeps the mono-Cys-glutaredoxin 1 in the reduced state.

Reduction of the intramolecular disulfide bond of \textit{T. brucei} Grx1 by GSH is slightly more efficient than in the case of \textit{S. cerevisiae} Grx5. Treatment of 50 μM oxidized Grx1 with 3 mM GSH for 15 min led to the complete reduction of the intramolecular disulfide, whereas under comparable conditions only 60% of \textit{S. cerevisiae} Grx5 is reduced (7). Incubation of the mono-Cys-glutaredoxin-like protein Glp1 from \textit{P. falciparum} with 1 mM GSH did not result in GSSG formation. Rahlfs \textit{et al.} (48) suggested that the protein is not reduced by GSH but did not rule out the possibility that the recombinant protein is obtained with its single cysteine residue in the thiol state. Later it was shown that only 10% of freshly prepared recombinant Glp1 forms a dimer (18). Extended treatment of Grx1 with GSSG or Gsp disulfide leads also to some thiolation of Cys\textsuperscript{104}. Interestingly, GSH only reduces the mixed disulfides at Cys\textsuperscript{104}. At Cys\textsuperscript{181}, the degree of glutathionylation remains constant upon treatment with GSH, and Gsp attached to Cys\textsuperscript{181} is replaced by glutathione. Free glutathione appears to attack the sulfur atom of Cys\textsuperscript{181} in the mixed disulfide leading to an exchange but not to a loss of the modification.

\textit{T. brucei} Grx1 does not catalyze the deglutathionylation of \textit{T. brucei} thioredoxin. A corresponding observation has been made for \textit{E. coli} Grx4 that lacks deglutathionylation activity toward glutathionylated bovine serum albumin (17). Incubation of reduced yeast Grx5 with glutathionylated carbonic anhydrase results in reduced carbonic anhydrase and Grx5 with an intramolecular disulfide bridge (7). Because equimolar concentrations of the proteins were applied, this does not reflect a catalytic activity, but it shows that the redox potential of Grx5 is low enough to act as an electron donor in redox reactions with oxidized proteins (7). The inability of these mono-Cys-glutaredoxins to catalyze the deglutathionylation of proteins is not simply because they lack the second active site cysteine. Mutant human Grx1 and -2, which maintain only the first cysteine of the CXXC motif, exert wild-type catalytic efficiency upon protein deglutathionylation (41, 45).

Treatment of reduced \textit{T. brucei} glutathione peroxidase-type PxIII with 0.5 mM GSSG led to the thiolation of either Cys\textsuperscript{47} or Cys\textsuperscript{95}. The simultaneous modification of both cysteines was observed only at millimolar GSSG concentrations. It remains to be elucidated whether different stimuli can lead to the distinct modification of one of the two cysteine residues as has been shown for the HIV-1 protease (43). Functionally, PxIII is a trypanothione/tryparedoxin-dependent peroxidase, as are the 2-Cys-peroxiredoxins of the parasites (46, 47, 49). Human peroxiredoxins 1 and 2 are glutathionylated under cellular stress conditions (4, 50). Recombinant cytosolic \textit{T. brucei} peroxiredoxin can also be glutathionylated (data not shown), which shows that both trypanosomal peroxidases are susceptible to thiolation. The catalytic mechanism of PxIII involves the formation of an intramolecular disulfide bond between Cys\textsuperscript{47} and Cys\textsuperscript{95}. Based on the crystal structures of classical glutathione peroxidases (51), this would imply a large rearrangement of the structure.

---

4. T. Schlecker, M. A. Comini, J. Melchers, J. Ruppert, and R. L. Krauth-Siegel, submitted for publication.
5. C. Johansson, K. L. Kavanagh, A. Rojkova, O. Gileadi, F. Von Delft, C. Arrowsmith, J. Weigelt, M. Sundström, A. Edwards, and U. Oppermann (Structural Genomics Consortium) (2006) Protein Data Bank’s Structure Explorer (PDB code 2G53).
protein upon oxidation. Alternatively, the two cysteines are also in relative proximity in the reduced enzyme, and gluta-
thionylation of one residue interferes sterically with gluta-thionylation of the second one. In contrast to glutathione, Gsp reacts exclusively with Cys$^{39}$. The larger Gsp molecule or the charge differences may prevent binding to Cys$^{37}$. The peak intensity of the Gsp-modified protein was much higher than that of the glutathionylated protein. This could be because of the different thiol pK values and/or the specific interactions between the spermidine moiety of Gsp and the protein, as outlined above. Cys$^{76}$, the third cysteine of PxIII, did not bind glutathione or Gsp under all conditions tested.

In T. brucei thioredoxin, besides the redox active di-thiole/sulfide, position 68 is occupied by a cysteine residue. In the presence of 0.25 or 0.5 mM GSSG about 40 and 60%, respectively, of the total protein occurs in glutathionylated form. In contrast to Grx1 and PxIII, which reacted more readily with Gsp disulfide, T. brucei thioredoxin showed a slight preference for glutathione binding. Cys$^{68}$ is embedded between Arg$^{67}$ and Lys$^{69}$. The positive charges may favor the interaction with the negatively charged glutathione and cause an electrostatic repul-
sion of the positively charged Gsp. Such charge interactions have also been discussed in the case of human thioredoxin. Mammalian thioredoxins possess three additional conserved cysteine residues (Cys$^{61}$, Cys$^{68}$, and Cys$^{72}$ in the human protein). Treatment of human thioredoxin with 5 mM GSSG leads to glutathionylation of Cys$^{72}$ but not of Cys$^{68}$ (37), which cor-
responds to the residue thiolated in T. brucei thioredoxin. In the human protein, Cys$^{68}$ is flanked by two glutamate residues. In contrast, a lysine residue precedes Cys$^{72}$, which may trig-
ger the adduct formation by interacting electrostatically with the $\gamma$-glutamyl moiety of glutathione. Cys$^{72}$ is also involved in the formation of covalent dimers (52), and glu-
thationylation of the residue prevents dimerization of human thioredoxin (37). Prolonged storage of T. brucei thioredoxin generates covalent dimers (31). Because Cys$^{68}$ is the only additional cysteine in the protein it may be involved in dimer formation (53). Treatment of reduced T. brucei thiore-
doxin with GSSG does not cause dimer formation but glutata-
thionylation of Cys$^{68}$. The chloroplast thioredoxin f has been shown sensitive to glutathionylation. Modification of Cys$^{60}$ (which is neither conserved in the trypanosomal nor the mam-
malian proteins) results in an impaired activation of target enzymes (54). Thus, nonconserved cysteine residues in the C-terminal moiety of thioredoxins are susceptible to glutathio-
ylation affecting the protein functions. Glutathionylation of Cys$^{68}$ of T. brucei thioredoxin did not impair its ability to reduce insulin disulfide. In contrast, glutathionylation of Cys$^{72}$ lowered the ability of human thioredoxin to catalyze the reduc-
tion of insulin disulfide to 66% (37). Although the physiological function of Cys$^{68}$ in the T. brucei thioredoxin is not known, glutathionylation of the residue can prevent dimerization of the protein and/or the irreversible overoxidation of this residue.

To reveal whether glutathionylation of an individual cysteine residue is mainly determined by the primary or tertiary structure of the protein, Grx1 and PxIII were treated with GSSG and digested without prior blocking of the remaining free thiols. This resulted in the unspecific glutathionylation of all cysteine residues, which shows that the three-dimensional structure of the protein is much more decisive for the selective glutathiony-
lation than the physico-chemical properties of neighboring res-
ides. As expected and shown previously for human thiore-
doxin (37), the redox active cysteines (Cys$^{31}$ and Cys$^{34}$ in T. brucei thioredoxin) do not form a stable mixed disulfide when the reduced protein is treated with GSSG, but they are oxidized to the intramolecular disulfide. The same is true for the active site cysteines in T. brucei tryparedoxin and T. cruzi trypanothione reductase. In the crystal structure of trypano-
thione reductase (Protein Data Bank accession number 1NDA), none of the five cysteine residues (in addition to the active site dithiol/disulfide couple) is exposed to the solvent. This agrees with the observed failure of trypanothione reductase to be glu-
thionylated and renders regulation of the enzyme by thiola-
tion very unlikely.

As shown here, the small thiol proteins mono-Cys-glutare-
doxin 1 and thioredoxin, as well as the glutathione peroxidase-
type tryparedoxin peroxidase III of T. brucei, are accessible to specific and reversible glutathionylation. In addition, Gsp can form mixed disulfides with these proteins. Under cellular con-
ditions, reaction of protein cysteiny1 residues with the disulfide form of glutathione and/or Gsp is one mechanism; another one is the reaction of protein sulfenic acids with GSH. All in vitro studies on the glutathionylation of proteins employed reduced proteins and GSSG. In this work we used 250 or 500 $\mu$M GSSG. Such concentrations can occur in the cell under oxidative stress conditions and definitely reflect the physiological conditions better than the 2 or 5 mM GSSG (17, 18, 37, 54) or even 40 mM GSSG (43) used in related in vitro studies. Future studies will focus on the physiological conditions that lead to thiolation of the proteins in living parasites.

REFERENCES

1. Shelton, M. D., Chock, B. P., and Mieyal, J. J. (2005) Antioxid. Redox Signal. 7, 348–366
2. Chai, Y. C., Hendrich, S., and Thomas, J. A. (1994) Arch. Biochem. Biophys. 310, 264–272
3. Klett, P., and Lamas, S. (2000) Eur. J. Biochem. 267, 4928–4944
4. Fratelli, M., Demol, H., Puype, M., Casagrande, S., Eberini, I., Salmona, M., Bonetto, V., Mengozzi, M., Duffieux, F., Miclet, E., Bachi, A., Vandekerckhove, J., Gianazza, E., and Ghezzi, P. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 3505–3510
5. Gravina, S. A., and Mieyal, J. I. (1993) Biochemistry 32, 3368–3376
6. Shenton, D., Perrone, G., Quinn, K. A., Dawes, I. W., and Grant, C. M. (2002) J. Biol. Chem. 277, 16853–16859
7. Tamarit, J., Belli, G., Cabisco, E., Herrero, E., and Ros, J. (2003) J. Biol. Chem. 278, 25745–25751
8. Fairlamb, A. H., and Cerami, A. (1992) Annu. Rev. Microbiol. 46, 695–729
9. Krauth-Siegel, R. L., Bauer, H., and Schirmer, R. H. (2005) Angew. Chem. Int. Ed. Engl. 44, 690–715
10. Oza, S. L., Aryanayagam, M. R., Atcheson, N., and Fairlamb, A. H. (2003) Mol. Biochem. Parasitol. 131, 25–33
11. Comini, M., Menge, U., Wissing, J., and Flohe, L. (2005) Antioxid. Redox Signal. 7, 348–366
12. Ariyanayagam, M. R., and Fairlamb, A. H. (2001) Mol. Biochem. Parasitol. 115, 189–198
13. Shim, H., and Fairlamb, A. H. (1988) J. Gen. Microbiol. 134, 807–817
14. Tabor, H., and Tabor, C. W. (1975) J. Biol. Chem. 250, 2648–2654
15. Shahi, S. K., Krauth-Siegel, R. L., and Clayton, C. E. (2002) Mol. Microbiol. 43, 1129–1138
16. Wyllie, S., Cunningham, M. L., and Fairlamb, A. H. (2004) J. Biol. Chem. 279, 13150–13156
