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Stress-Induced Protein *S*-Glutathionylation and *S*-Trypanothionylation in African Trypanosomes—A Quantitative Redox Proteome and Thiol Analysis

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Abstract

Aims: Trypanosomatids have a unique trypanothione-based thiol redox metabolism. The parasite-specific dithiol is synthesized from glutathione and spermidine, with glutathionylspermidine as intermediate catalyzed by trypanothione synthetase. In this study, we address the oxidative stress response of African trypanosomes with special focus on putative protein *S*-thiolation.

Results: Challenging bloodstream *Trypanosoma brucei* with diamide, H_2O_2 or hypochlorite results in distinct levels of reversible overall protein *S*-thiolation. Quantitative proteome analyses reveal 84 proteins oxidized in diamide-stressed parasites. Fourteen of them, including several essential thiol redox proteins and chaperones, are also enriched when glutathione/glutaredoxin serves as a reducing system indicating *S*-thiolation. In parasites exposed to H_2O_2, other sets of proteins are modified. Only three proteins are *S*-thiolated under all stress conditions studied in accordance with a highly specific response. H_2O_2 causes primarily the formation of free disulfides. In contrast, in diamide-treated cells, glutathione, glutathionylspermidine, and trypanothione are almost completely protein bound. Remarkably, the total level of trypanothione is decreased, whereas those of glutathione and glutathionylspermidine are increased, indicating partial hydrolysis of protein-bound trypanothione. Depletion of trypanothione synthetase exclusively induces protein *S*-glutathionylation. Total mass analyses of a recombinant peroxidase treated with $T(SH)_2$ and either diamide or hydrogen peroxide verify protein *S*-trypanothionylation as stable modification.

Innovation: Our data reveal for the first time that trypanosomes employ protein *S*-thiolation when exposed to exogenous and endogenous oxidative stresses and trypanothione, despite its dithiol character, forms protein-mixed disulfides.

Conclusion: The stress-specific responses shown here emphasize protein *S*-trypanothionylation and *S*-glutathionylation as reversible protection mechanism in these parasites. *Antioxid. Redox Signal.* 00, 000–000.

Keywords: trypanothione, protein *S*-glutathionylation, oxidative stress, proteome, *Trypanosoma*

Introduction

PROTEIN *S*-GLUTATHIONYLATION describes the transient formation of a mixed disulfide between glutathione (GSH) and a cysteine residue. Because of its reversibility, this post-translational modification serves as biological redox switch changing the structure and/or function of the target and to protect proteins from irreversible overoxidation [for reviews, see Refs. (1, 14)]. Multiple mechanisms are proposed to lead to protein *S*-glutathionylation, including

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Innovation

Trypanothione, formed by the trypanothione synthetase-catalyzed linkage of two glutathione molecules with spermidine, is the central thiol in trypanosomatids. This study shows for the first time that oxidatively challenged African trypanosomes undergo protein *S*-thiolation. Remarkably, not only glutathione and glutathionylspermidine but the dithiol trypanothione forms protein-mixed disulfides as well, both *in vitro* and in the intact parasite. The finding that proteins of the heat shock response, motility, and calcium metabolism undergo reversible *S*-thiolation should stimulate investigations on putative redox control mechanisms within these essential parasite pathways.

activation of a protein thiol to a sulfenic acid, sulfenyl amide, or *S*-nitrosyl intermediate, which then reacts with GSH (1, 14). Although distinct glutaredoxins (Grxs) have been shown to promote the modification, protein *S*-glutathionylation is primarily a spontaneous process. A main function of Grxs is clearly to facilitate the reverse reaction, namely the removal of GSH from the mixed disulfide (1).

GSH is by far the most widely distributed low-molecular-weight thiol. Nonetheless, some organisms contain alternative thiols such as mycothiol in *Actinomycetes* and bacillithiol in *Firmicutes*, which also undergo reversible *S*-thiolation of proteins (69). Trypanosomatids, the causative agents of African sleeping sickness (*Trypanosoma brucei gambiense* and *T. b. rhodesiense*), Nagana cattle disease (*T. congolense* and *T. b. brucei*), American Chagas disease (*T. cruzi*), and the different forms of leishmaniasis (*Leishmania* spp.), lack the GSH/glutathione reductase (GR) and thioredoxin/thioredoxin reductase couples, which in most organisms maintain intracellular thiol redox homeostasis. These systems are replaced by trypanothione [N^1,N^8-bis(glutathionyl)spermidine, $T(SH)_2$] and trypanothione reductase [for reviews, see Refs. (17, 33, 34, 41)].

$T(SH)_2$ is synthesized from GSH and spermidine with glutathionylspermidine (Gsp) as intermediate. The dithiol apparently occurs exclusively in trypanosomatid organisms, whereas Gsp is also formed in bacteria such as *Escherichia coli* (66). In all pathogenic trypanosomatids studied so far, a single enzyme catalyzes the two consecutive steps. This trypanothione synthetase (TryS) has, in addition to its biosynthetic activity, amidase activity and thus can catalyze the hydrolysis of Gsp and $T(SH)_2$ (Fig. 1) (38, 51, 75).

$T(SH)_2$ is a direct reducing agent for various low-molecular-weight metabolites and proteins whereby many of the reactions are accelerated by tryparedoxin (Tpx), a distant relative of thioredoxin-type oxidoreductases (12). In the cytosol, the $T(SH)_2$/Tpx couple is the electron donor for ribonucleotide reductase, methionine sulfoxide reductase, as well as for detoxification of hydroperoxides catalyzed by 2-Cys-peroxiredoxins (Prx) and nonselenium glutathione peroxidase-type (Px) enzymes [for reviews, see Refs. (7, 33, 34, 41)].

Trypanosomatids also contain free GSH, which is regenerated from glutathione disulfide (GSSG) by thiol/disulfide exchange with $T(SH)_2$. *In vitro*, several *T. brucei* redox proteins are susceptible to protein *S*-glutathionylation and/or Gsp-*S*-thiolation (8, 43, 47). The latter modification has been reported for *E. coli* redox regulation as well (9). Treatment with trypanothione disulfide (TS_2) did not result in any protein *S*-trypanothionylation and suggested that trypanothione may not form stable protein-mixed disulfides (41, 43). Under these conditions, however, protein *S*-trypanothionylation could only take place by thiol/disulfide exchange.

Several proteomic strategies have been developed to identify proteins that are reversibly oxidized under stress

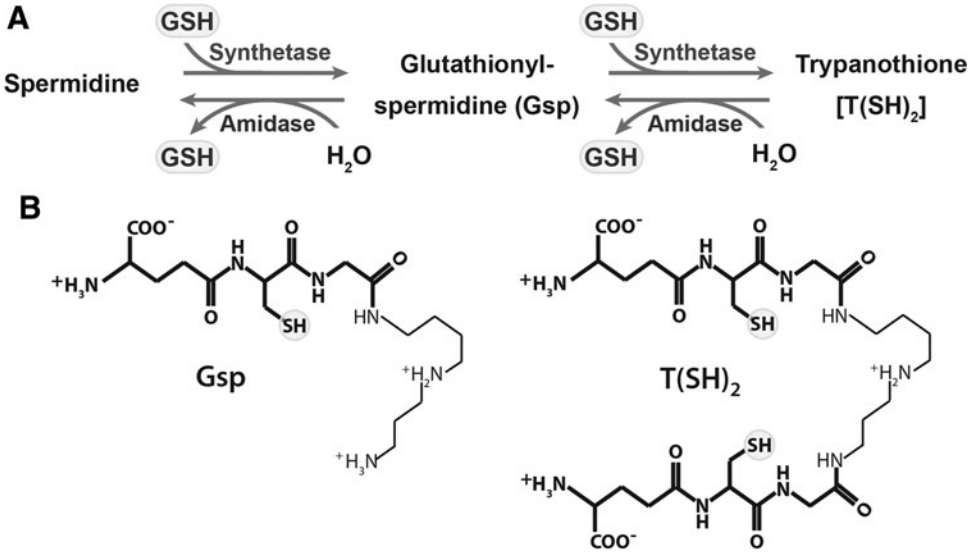

FIG. 1. Ligase and amidase activities of trypanothione synthetase and structures of parasite-specific thiols. (A) In trypanosomatids, glutathione (GSH) is linked to the polyamine spermidine to form glutathionylspermidine (Gsp), which then reacts with a second GSH molecule to form trypanothione [$T(SH)_2$, bis(glutathionylspermidine)]. Both steps are catalyzed by trypanothione synthetase. The bifunctional enzyme has a C-terminal synthetase and an N-terminal amidase domain and thus can hydrolyze the spermidine conjugates as well. **(B)** Structural formula of Gsp and $T(SH)_2$. The GSH moieties are depicted in *bold*. The sulfhydryl groups are highlighted by *circles*.

conditions (13, 35, 37, 42). *In vivo* studies targeting stress-induced protein *S*-glutathionylation are based on ^{35}S-labeling of the cellular GSH (19, 45) or biotinylated glutathione ethyl ester (65). To identify proteins modified by GSH even under basal conditions, Lind *et al.* (39) use the GSH/Grx couple to reduce protein-GSH-mixed disulfides. A similar approach identified *S*-glutathionylated proteins in *Plasmodium falciparum* (31). More recently, quantitative methods such as stable isotope labeling by amino acids in cell culture have been developed to compare protein abundance in two cell populations (48). The isotope-coded affinity-tagged thiol-trapping technique specifically allows identification and quantification of changes in the overall oxidized proteome (37). To quantitatively compare *S*-glutathionylated proteins in several samples, Su *et al.* combined the GSH/Grx reduction system with isobaric tagging (iTRAQ) (64). In *L. donovani*, iTRAQ labeling was applied to follow overall proteomic changes induced by oxidative and nitrosative stresses (59).

Bloodstream (BS) *T. brucei* multiply as free-living organisms in the blood and other body fluids of their mammalian host. In this study, we present the oxidized and *S*-thiolated proteomes of parasites upon exposure to different oxidative stresses. Depletion of TryS exemplifies that endogenous stresses also induce the post-translational modification. We show that not only GSH and Gsp but also the dithiol $T(SH)_2$ forms protein-mixed disulfides both in the parasite and *in vitro*.

Results

Distinct oxidative stresses induce protein S*-thiolation in BS* T. brucei

To get a first insight if trypanosomes undergo protein *S*-thiolation at all, BS *T. brucei* were treated with oxidative stressors and total protein *S*-thiolation was measured after labeling the released thiols with 2,3-naphthalene dicarboxaldehyde (NDA) (44).

First, the specificity of the reagent for different parasite thiols was evaluated (Fig. 2A). Free cysteine did not form a fluorophore, in accordance with published data (50). The fluorescence of GSH and γ-Glu-Cys derivatives increased proportionally up to an assay concentration of 100 μ*M* (not shown) and even 10 n*M* thiol was detectable. The cellular concentration of γ-Glu-Cys is below 10 μ*M* (Markus Wirtz, Heidelberg, personal communication) and also Gsp is only about 10 μ*M* (Fig. 8) and displayed very low fluorescence. Although $T(SH)_2$ yielded comparably low fluorescence, it is the predominant thiol of these parasites (33). Hence, the NDA assay provided a convenient measurement of total protein *S*-thiolation, reflecting protein-mixed disulfides formed with GSH and/or $T(SH)_2$.

Cells were lysed by 10% trichloroacetic acid (TCA) to quench any thiol redox reaction (25) and the pellet was washed twice with TCA before the proteins were redissolved in buffer, treated with tris(2-carboxyethyl)phosphine (TCEP), and the released thiols reacted with NDA. In unstressed parasites, marginal protein *S*-thiolation was observed. Five-minute

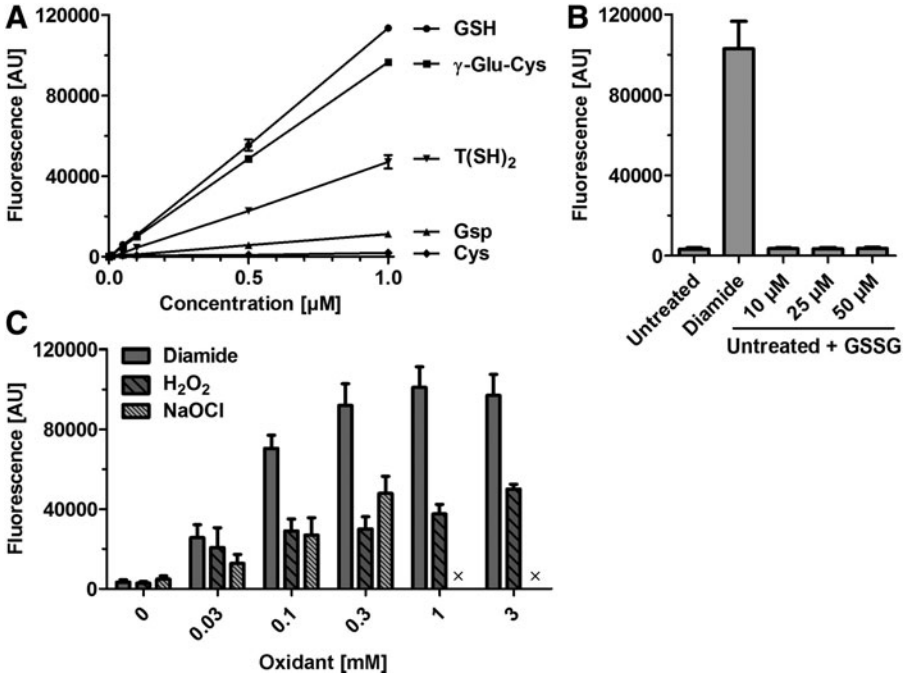

FIG. 2. Establishment of the NDA-based protein *S*-thiolation assay. (A) GSH, γ-Glu-Cys, $T(SH)_2$, Gsp, and Cys were derivatized with NDA and fluorescence was measured. **(B)** BS *Trypanosoma brucei* were lysed by 10% TCA in the absence and presence of 10, 25, and 50 μ*M* GSSG (corresponding to a theoretical cellular GSH concentration of 3, 9, and 17 m*M*, respectively). After centrifugation, the pellets were washed twice with 10% TCA, dissolved, and treated with TCEP. Proteins were precipitated and the supernatants subjected to the NDA assay. All samples revealed the same basal fluorescence demonstrating the quantitative removal of GSSG. For a positive control, cells were treated for 5 min at RT with 3 m*M* diamide. **(C)** BS *T. brucei* were exposed for 5 min at RT in PBS to different concentrations of diamide, hydrogen peroxide, or hypochlorite. Protein-bound thiols were measured in the NDA assay. In the presence of ≥1 m*M* hypochlorite, the parasites lysed and protein *S*-thiolation could not be measured (x). All values are the mean ± SD of at least three independent measurements. AU, arbitrary units. BS, bloodstream; NDA, 2,3-naphthalene dicarboxaldehyde; SD, standard deviation; TCA, trichloroacetic acid.

exposure of BS *T. brucei* to 3 m*M* diamide induced strong protein *S*-thiolation (Fig. 2B). To prove the removal of small disulfides from the TCA pellet, different concentrations of GSSG were added to the lysate of unstressed parasites before the samples were processed as described above. Irrespective of this treatment, the same basal fluorescence was measured, demonstrating the quantitative removal of free GSSG. For further controls, see Supplementary Figure S1 (Supplementary Data are available online at www.liebertpub.com/ars). Precipitating the proteins with acetone (44) or washing the pellet with acetone turned out to be unsuitable (not shown). Due to its insolubility in organic solvents, GSSG remained in the pellet and became, together with the protein-bound thiols, reduced and labeled by NDA.

BS *T. brucei* were challenged for 5 min with different oxidative agents (Fig. 2C). Diamide strongly induced the formation of protein-mixed disulfides. H_2O_2 caused concentration-dependent, but weaker, protein *S*-thiolation. Toward hypochlorite, the parasites displayed a narrow window of tolerance. Protein *S*-thiolation increased between 0.03 and 0.3 m*M* NaOCl, higher concentrations caused immediate lysis. A more pronounced cytotoxicity of hypochlorite compared with H_2O_2 has been described for other cells as well (63). Hypochlorite reacts with thiols very rapidly, causing disulfide formation or irreversible overoxidation (26). Taken together, exogenous oxidative agents cause protein *S*-thiolation in *T. brucei* with the degree of modification strongly depending on the individual stress.

Exposure of BS T. brucei *to diamide or H_2O_2 leads to time-dependent reversible protein* S*-thiolation*

Cells were incubated for different times with 3 m*M* diamide or 1 m*M* H_2O_2. The parasites displayed normal morphology over the whole observation period except for cells treated for 95 min with H_2O_2, which started to lyse (Fig. 3A, left). For parasites kept in phosphate-buffered saline (PBS), slight fluorescence that increased with time was observed, suggesting that starvation induces the formation of protein-mixed disulfides (Fig. 3A, right). Diamide caused intense protein *S*-thiolation already after 5 min. With H_2O_2, generation of protein-mixed disulfides was slower and less pronounced. Only after 95 min, *S*-thiolation reached similar values.

To address reversibility of the modification, cells were stressed and retransferred to standard culture conditions. After different times, the remaining protein-bound thiols were determined. Parasites that had been treated for 5 min with 3 m*M* diamide or 1 m*M* H_2O_2 retained ≥80% of cell density of the control (Fig. 3B, left), and within 5 min, protein *S*-thiolation was largely reversed (Fig. 3B, right). The modification was no longer reversible when the cells had been exposed for 45 min to 1 m*M* H_2O_2. The parasites lysed as soon as they were retransferred into the medium. In summary, short-term treatment of the parasites with diamide or H_2O_2 results in fully reversible protein *S*-thiolation.

Glutaredoxin 1 catalyzes protein dethiolation

T. brucei possesses two dithiol Grxs. Both proteins catalyze the deglutathionylation of model substrates, with cytosolic Grx1 displaying higher catalytic efficiency compared with mitochondrial Grx2 (8). In addition, Grx1 accounts for about 50% of the total capacity of BS *T. brucei* to reduce GSH-mixed disulfides (47). The absence of Grx1 or Grx2 does not affect the proliferation of BS *T. brucei* under culture conditions (47). Protein *S*-thiolation induced by 5-min treatment with 3 m*M* diamide or 1 m*M* H_2O_2 was virtually

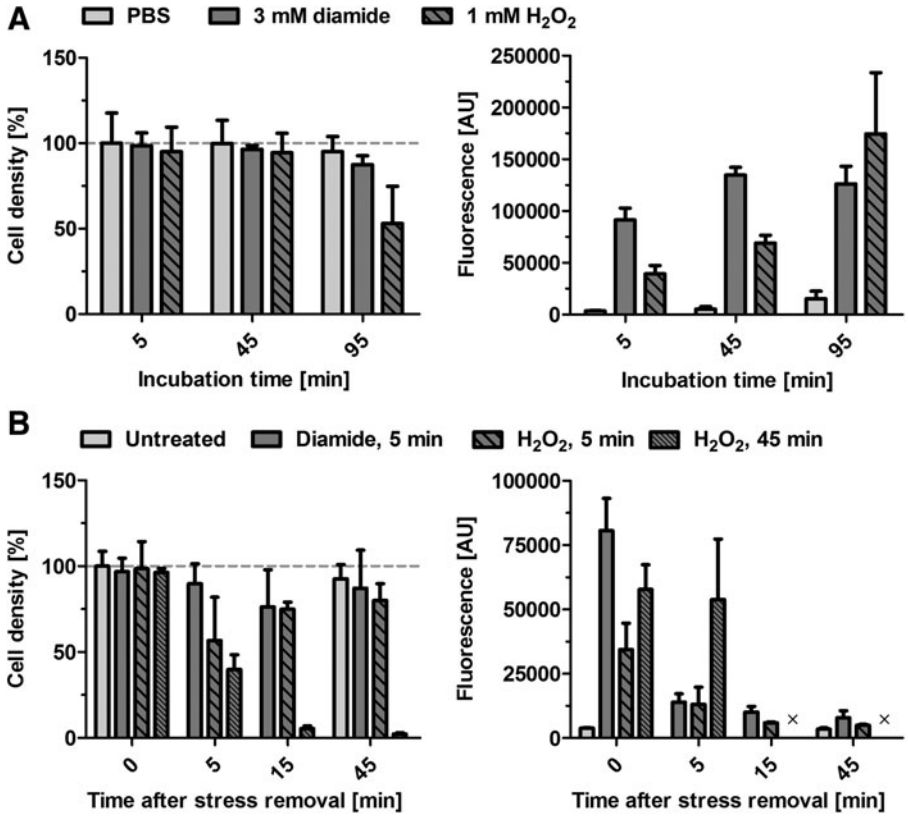

FIG. 3. Time-dependent reversible protein *S*-thiolation in oxidatively stressed cells. (A) BS *T. brucei* were treated at RT with 3 m*M* diamide or 1 m*M* hydrogen peroxide. After different times, the cells were counted and immediately lysed by TCA. The cell density of parasites kept in PBS was set to 100% (*left*) and formation of protein-mixed disulfides was measured in the NDA assay (*right*). **(B)** The parasites were stressed as described in **A**, and retransferred into 37 °C prewarmed medium. After different times, the cells were counted (*left*) and the remaining protein-bound thiols determined (*right*). Parasites treated with H_2O_2 for 45 min lysed when they were set back into medium and protein *S*-thiolation could not be measured (x). The values represent the mean of at least three independent measurements ± SD. PBS, phosphate-buffered saline.

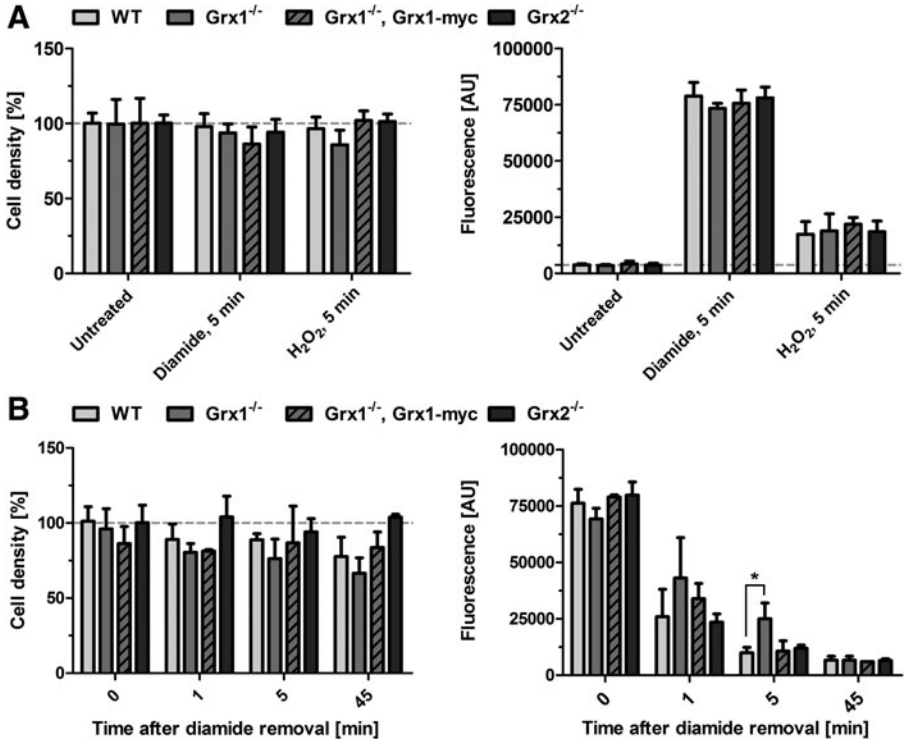

FIG. 4. BS *T. brucei* lacking cytosolic glutaredoxin (Grx)1, but not mitochondrial Grx2, show delayed protein dethiolation. (A) WT, Grx1$^{-/-}$, and Grx2$^{-/-}$ cells, as well as Grx1$^{-/-}$ mutants expressing an ectopic copy of myc-tagged Grx1, were treated for 5 min at RT with 3 m*M* diamide or 1 m*M* H_2O_2. **(B)** Parasites challenged with diamide were washed, counted, transferred into medium prewarmed to 37°C, and harvested after different times. The cell density of untreated parasites kept in PBS was set to 100% (*left*). Protein-bound thiols were quantified in the NDA assay (*right*). The values are the mean ± SD of at least three independent measurements. Cells lacking Grx1 displayed a slower dethiolation rate compared with the WT control (*p-value of 0.013). WT, wild-type.

identical in wild-type (WT) and Grx-deficient cells, indicating that neither Grx1 nor Grx2 plays a role in protein *S*-thiolation (Fig. 4A). Yet, reversion of the diamide-induced modification was slowed down in Grx1$^{-/-}$ cells compared with all other cell lines (Fig. 4B). Since after 5 min dethiolation was almost complete in WT parasites, we included a 1-min time point. Despite some variance, these data supported the slightly retarded dethiolation rate in Grx1$^{-/-}$ cells. Expression of an ectopic copy of Grx1 in the Grx1$^{-/-}$ mutant abolished the effect. The absence of Grx2 had no effect. Rapid reversion of protein *S*-thiolation after stress removal—even in the absence of Grx1—may be due to reduction of the mixed disulfides by $T(SH)_2$ (for details, see the Discussion section).

Overall oxidized thiol proteome of diamide-stressed BS T. brucei

Previous redox proteome analyses in trypanosomatids aimed at the interactome of the cytosolic Tpx I (55) and endosomal membrane-localized Tpx II (4) of *T. cruzi* under normal culture conditions or investigated changes in protein expression levels in *L. donovani* cultured in the presence of menadione (59).

In this study, we report on the overall oxidized and *S*-thiolated proteomes (next section) of BS *T. brucei* exposed to short-term oxidative stresses. To enrich proteins that were reversibly oxidized, we adapted a thiol-trapping method described for yeast cells (35) and quantified the proteins by mass spectrometry after stable isotope labeling (6), as summarized in Figure 5. Diamide-stressed and untreated cells were lysed by TCA precipitation. After blocking free thiols with *N*-ethylmaleimide (NEM) (21, 35, 37), reversibly oxidized cysteine residues (intra- or intermolecular protein disulfides and mixed disulfides with low-molecular-weight thiols, as well as sulfenic acid residues) were reduced by dithiothreitol (DTT) and the newly generated free thiols reacted with *N*-[6-(biotinamido)hexyl]-3′-(2′-pyridyldithio)-propionamide (HPDP-biotin). The biotinylated proteins were purified on avidin resin. The proteins were eluted by DTT, subjected to SDS-PAGE, in-gel alkylated by IAM, and digested with trypsin.

For relative quantification, peptides from stressed and control cells were labeled with normal (light) and deuterated (heavy) formaldehyde, mixed, and subjected to quantitative mass spectrometry (6). For each peptide pair, the ratio was calculated from the summed signal intensities of the isotopic pattern. The protein ratio ± stress was calculated as the median of all ratios of unique peptides. A protein was considered as stress dependently enriched if the light/heavy ratio was ≥1.5 in at least two of the three biological replicates.

In total, 84 proteins were reproducibly found oxidized in the diamide-stressed parasites and assigned to different functional classes (Supplementary Table S1). The largest group represented hypothetical proteins. This is not surprising since more than 60% of all *T. brucei* genes are annotated

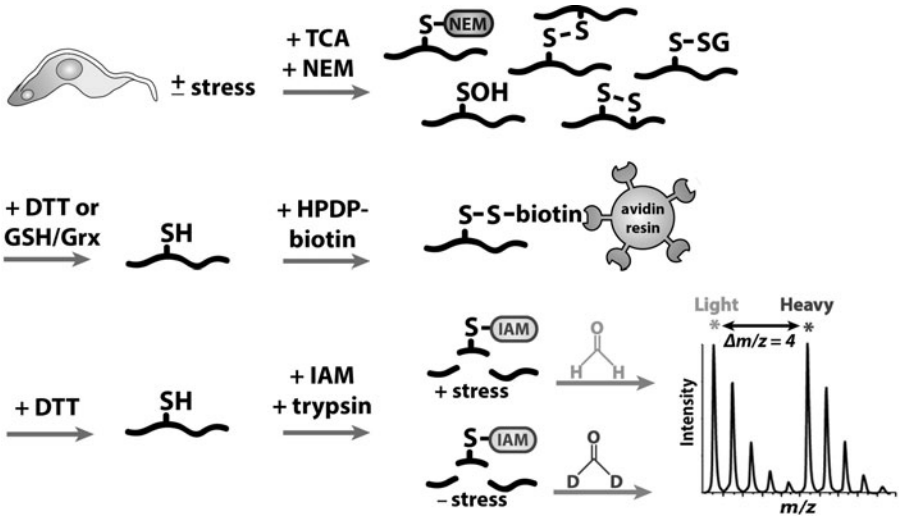

FIG. 5. Schematic overview of procedures to identify overall reversibly oxidized and *S*-thiolated proteins. BS *T. brucei* were incubated in PBS in the absence or presence of oxidative stressors and lysed by TCA precipitation. Free thiols were blocked with NEM. Reversibly oxidized protein thiols were reduced by either DTT (overall oxidized proteome) or GSH/Grx (*S*-thiolated proteome) and newly generated free cysteines reacted with HPDP-biotin. The labeled proteins were enriched by affinity chromatography on avidin-coated beads, eluted by DTT, and applied to SDS-PAGE. After in-gel alkylation with IAM and trypsin digestion, the peptides from stressed and control cells were dimethyl labeled using formaldehyde (light) and deuterated formaldehyde (heavy), respectively. The samples were mixed and subjected to ESI-MS analysis (for details, see the Materials and Methods section). NEM, *N*-ethylmaleimide.

to encode hypothetical proteins, which are conserved within trypanosomatids, but apparently do not have any counterpart outside the order of *Kinetoplastida* (62). Notably, our procedure did not enrich any cysteine-free protein, and for 56% of the proteins, at least one peptide with IAM-modified cysteine residue was identified. The oxidized proteome comprised proteins from the cytosol, ER, mitochondrion, glycosomes, nucleus, and lysosome. Both types of tryparedoxin peroxidases and Tpx, their physiological reductant, were identified, underlining the sensitivity of these proteins to reversible oxidation. Three heat shock protein (HSP) 70-type proteins were enriched. In response to oxidative stresses, HSP70 proteins form intermolecular disulfides with a variety of proteins (13). In addition, HSP40 proteins occur in all cellular compartments where they stimulate the ATPase activity of local HSP70s. In the oxidized proteome, we found only one of the 65 putative *T. brucei* HSP40s (18). It remains to be studied if its single, not conserved cysteine is involved in redox reactions, or if the protein was just copurified with the respective HSP70. Three protein disulfide isomerases (PDIs) were identified. In the functionally characterized PDI 2 (28), all four cysteines forming the two CXXC motifs were found to be IAM modified, consistent with their reversible oxidation in living cells.

The mammalian BS form of African trypanosomes relies exclusively on glycolysis for energy supply and the first seven enzymes of the pathway are compartmentalized in glycosomes (46). Four of the glycosomal and two cytosolic glycolytic enzymes were found in the oxidized proteome. Another enzyme of the energy metabolism that was enriched was arginine kinase, which catalyzes the reversible formation of phosphoarginine, a mobile energy carrier in invertebrates. *T. brucei* possesses three isoenzymes occurring in the flagellum, glycosomes, and cytosol (71). Three peptides covering C161, C222, and the active site C293 were found to be IAM labeled. The corresponding latter two cysteines form an intramolecular disulfide in *Limulus* arginine kinase (72). Overexpression of the enzymes confers *T. brucei* with increased resistance toward H_2O_2 in accordance with a role in the oxidative stress response (71).

The cytosolic adenosine kinase catalyzes the formation of AMP in the parasite adenosine salvage pathway (70). In one peptide, two cysteines, conserved in various species, were found to be alkylated by IAM, indicating that they may undergo disulfide bond formations. Adenosine kinase from *L. donovani* is reversibly oxidized upon diamide treatment with probable formation of an intramolecular disulfide (5). The identification of several paraflagellar rod proteins and alpha- and beta-tubulin, as well as microtubule-associated proteins, suggests that the highly dynamic meshwork may be redox regulated as well.

S-*Glutathionylated/thiolated proteome of oxidatively stressed parasites*

BS *T. brucei* were challenged with diamide or H_2O_2 and lysed by TCA precipitation. After blocking free thiols with NEM, the proteins were treated with NADPH/GR/GSH/Grx (57) (Fig. 5). Grx reacts with a protein-GSH-mixed disulfide, yielding the reduced protein. The mixed disulfide between Grx and GSH formed in this reaction is attacked by free GSH (re)generating the dithiol form of Grx and GSSG, which is finally reduced by GR (23). The dethiolated proteins were enriched and subjected to electrospray ionization mass spectrometry (ESI-MS) as described for the overall oxidized proteome. A protein was considered enriched if the light/heavy ratio was ≥1.5 in at least four of seven (diamide-treated cells) and three of five (H_2O_2 treatment) independent biological replicates.

From cells exposed for 5 min to 3 m*M* diamide, 15 proteins were enriched compared with the untreated control (Fig. 6 and

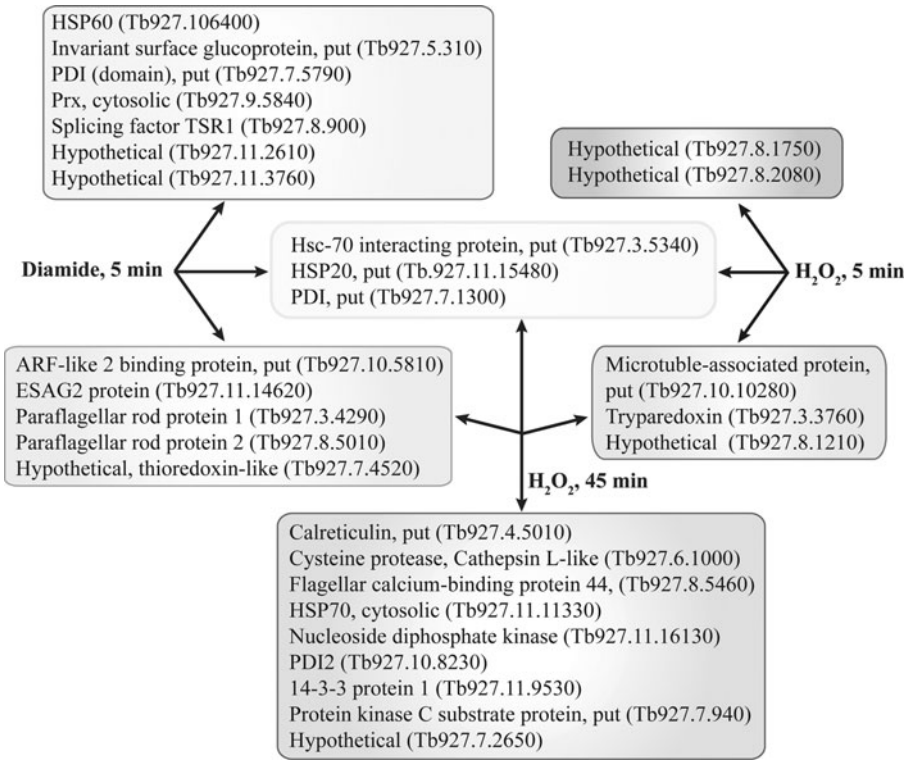

FIG. 6. Protein *S*-thiolation shows a stress-specific response pattern. Parasites were treated with 3 m*M* diamide for 5 min or with 1 m*M* H_2O_2 for 5 and 45 min. *S*-thiolated proteins were reduced by the GSH/Grx system, enriched by biotin trapping, and analyzed by quantitative ESI-MS as schematically summarized in Figure 5. Proteins specifically and commonly enriched under distinct stress conditions in comparison with untreated parasites are *boxed*. ESAG, expression site-associated gene; HSP, heat shock protein; PDI, protein disulfide isomerase; Prx, peroxiredoxin; put, putative.

Supplementary Table S2A). With one exception, all of them were identified also in the overall oxidized proteome (Supplementary Table S1). From parasites that were exposed for 5 min to 1 m*M* H_2O_2, eight proteins were enriched (Supplementary Table S2B), which reflected comparably lower overall protein *S*-thiolation (Fig. 3). H_2O_2 stress for 45 min revealed 20 putatively *S*-thiolated proteins (Supplementary Table S2C). Two hypothetical proteins were obtained in the 5-min, but not 45-min, H_2O_2 sample, suggesting an irreversible oxidation upon extended H_2O_2 exposure. Five proteins of the overall oxidized proteome of diamide-treated cells were enriched in the 45-min H_2O_2 sample, but only in some of the diamide analyses. Probably, these proteins are prone to *S*-thiolation, either to a minor extent or the mixed disulfide is a transient state that precedes protein disulfide formation. Notably, the GSH/Grx reducing system did not enrich any of the glycolytic enzymes found in the overall oxidized proteome. In other cells, these abundant enzymes were found to undergo *S*-glutathionylation upon oxidative stress (19, 61).

The majority of proteins were specifically *S*-thiolated under either diamide or H_2O_2 stress (Fig. 6). From H_2O_2-stressed cells as well as few of the diamide samples, Tpx was enriched. Tpx possesses only two cysteine residues forming the CPPC active site, which may suggest that the intramolecular disulfide formed in the stressed cells was reduced by the GSH/Grx couple. It is, however, also possible that Tpx becomes transiently *S*-thiolated. PDIs, which also have CXXC motifs, are accessible to *S*-glutathionylation. A functional implication could be lowering of the activity for the period that the cysteine residue is *S*-thiolated (67). The fact that only three proteins were enriched under all stress conditions (Fig. 6) emphasizes that protein *S*-thiolation in parasites strongly depends on the distinct oxidative stress applied.

In diamide-stressed parasites, the cytosolic Prx forms covalent dimers, whereas H_2O_2 causes the formation of overoxidized monomers

The cytosolic Prx was found enriched in the overall oxidized and *S*-thiolated proteomes of diamide-stressed, but not H_2O_2-treated, parasites (Fig. 6). To get an insight in the underlying mechanism, BS *T. brucei* were exposed to both oxidants and total lysates subjected to Western blot analysis using antibodies against the native or overoxidized Prx. Under nonreducing conditions, the Prx antibodies visualized in untreated parasites a single, monomeric protein band, in accordance with typical 2-Cys Prxs forming noncovalent homodimers. Exposing cells to 3 m*M* diamide caused within 1 min a shift to covalent homodimers (Fig. 7). The double band observed probably represents species with one or two intersubunit disulfide bridges plus putatively *S*-thiolated forms as shown *in vitro* for human Prx (52).

H_2O_2 induced covalent dimer formation if the cells were treated for 1 min with 0.1 m*M* oxidant. Remarkably, the upper dimer band was stronger, whereas in the diamide sample, the lower band was predominant, indicating that the oxidants led to distinct dimeric species. Longer incubation times or a higher concentration of H_2O_2 yielded exclusively a monomer band, which strongly reacted with the Prx-SO_3 antibody, indicating irreversible oxidation. Prx overoxidation was neither found in the control nor diamide-stressed cells. H_2O_2 oxidizes mammalian 2-Cys Prx to a covalent dimer, which progressively becomes overoxidized and then generates the overoxidized monomer (53). In our analysis, an overoxidized dimeric form was not detected probably because the stress applied resulted in fast sulfinic/sulfonic acid formation of the peroxidatic cysteine in both subunits. These results supported the proteome data (Supplementary Table S2), which revealed *S*-thiolated Prx only in diamide-stressed cells.

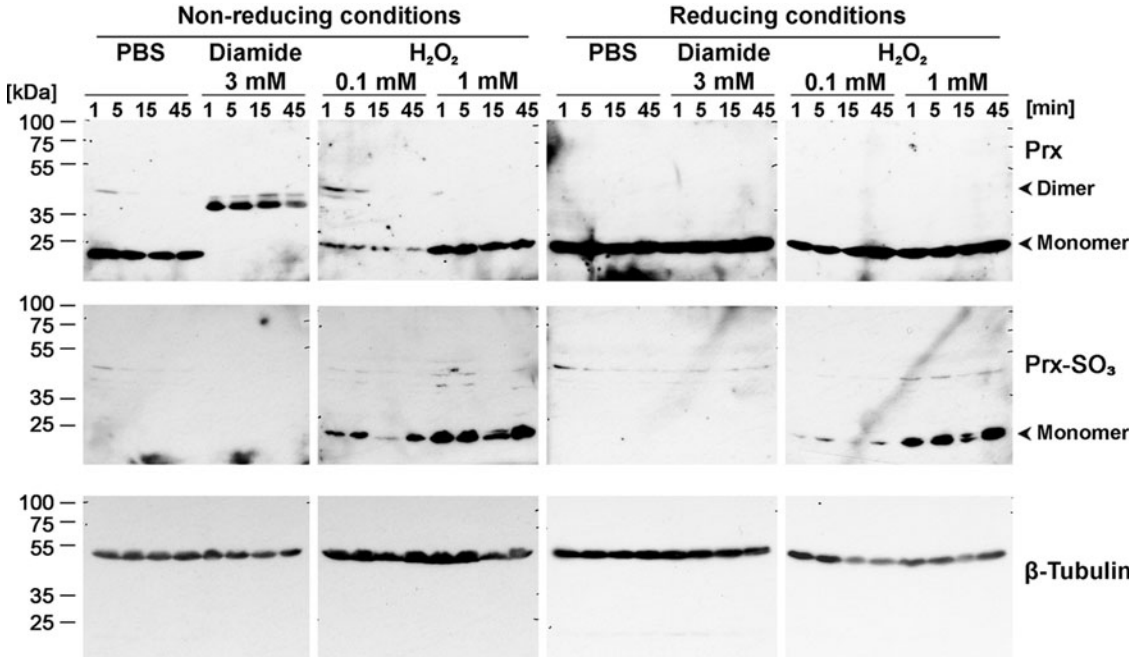

FIG. 7. Treatment of parasites with H_2O_2—but not diamide—results in overoxidation of the cytosolic Prx. Parasites were incubated at RT in PBS in the presence/absence of diamide or hydrogen peroxide. After different times, total cell lysates were subjected to SDS-PAGE under nonreducing and reducing conditions, followed by Western blot analysis with antibodies against *T. brucei* Prx, Prx-SO_3, and β-tubulin. For details, see the Materials and Methods section.

Upon diamide stress, virtually all low-molecular-weight thiols are protein-bound and trypanothione is partially hydrolyzed

To assess the fate of cellular GSH, Gsp, and $T(SH)_2$ under different stress conditions, the thiols were derivatized with monobromobimane (mBBr) and quantified by high performance liquid chromatography (HPLC) analysis. In untreated parasites, all three thiol species were almost exclusively present in free and reduced form. With about 350 μM, $T(SH)_2$ was the predominant cellular thiol. In addition, the parasites contained 10 μM Gsp and about 100 μM GSH, of which $\sim$5 μM was protein-bound (Fig. 8A). These data indicated low basal *S*-glutathionylation in the parasites, in accordance with the results of the NDA assay (Fig. 2B). Exposure for 5 min to 3 mM diamide caused the complete loss of free GSH, Gsp, and $T(SH)_2$. All three thiols became virtually completely protein-bound (Fig. 8B). In comparison, 5-min treatment with 1 mM H_2O_2 only slightly enhanced the level of protein-bound thiols (Fig. 8C). After 45 min of exposure to H_2O_2, protein *S*-thiolation increased (Fig. 8D), but was still lower than in diamide-stressed cells.

To verify the quantitative removal of free disulfides before the protein-bound thiols were released, unstressed cells and cells exposed for 5 min to 3 mM diamide were lysed by TCA precipitation and split into two parts. One part was kept untreated. The other one was mixed with 50 μM GSSG, 50 μM Gsp_2, and 100 μM TS2, corresponding to a theoretical cellular concentration of about 1.7 mM for each thiol. Subsequently, the pellets were washed with TCA, the proteins redissolved, treated with TCEP, again precipitated, and the freed thiols were mBBr labeled and analyzed by HPLC. In both cases, the two samples displayed an identical elution profile (Supplementary Fig. S1) demonstrating the complete removal of disulfides. Thus, the thiols measured in the stressed cells originated from protein-mixed disulfides.

Interestingly, in diamide-treated cells, the concentration of $T(SH)_2$ dropped to 220 μM, whereas that of GSH and Gsp concomitantly increased to 170 and 90 μM, respectively (compare Fig. 8A, B). Since under these conditions the parasite thiols almost exclusively formed protein-mixed disulfides, we suggest that an amide bond in protein-bound trypanothione is hydrolyzed, a reaction that may at least partially be due to the amidase activity of TryS (51, 75).

In vitro *trypanothiolation of* T. brucei *Px III*

Prereduced recombinant Px III was either kept untreated or incubated with $T(SH)_2$/diamide and $T(SH)_2/H_2O_2$, respectively, as described previously for *in vitro S*-glutathionylation (8), and subjected to intact mass analyses as outlined in the Materials and Methods section. Untreated Px III revealed the theoretical mass of the fully reduced protein (Fig. 9A). In the $T(SH)_2$/diamide-treated sample, the main peaks matched those for the protein with an intramolecular disulfide bridge and bound diamide. Several peaks corresponded to Px III forming mixed disulfides with one or two $T(SH)_2$ molecules (Fig. 9B).

In the $T(SH)_2/H_2O_2$-treated protein, the most prominent peaks corresponded to Px III with two additional oxygen atoms without and with bound $T(SH)_2$ (Fig. 9C). A covalent dimer observed in the stressed protein samples displayed *in vitro S*-trypanothionylation as well. For a detailed description of all protein species, see the legend of Figure 9 and Supplementary Table S3. These data revealed stable *in vitro S*-trypanothionylation of a recombinant protein upon diamide or H_2O_2 treatment.

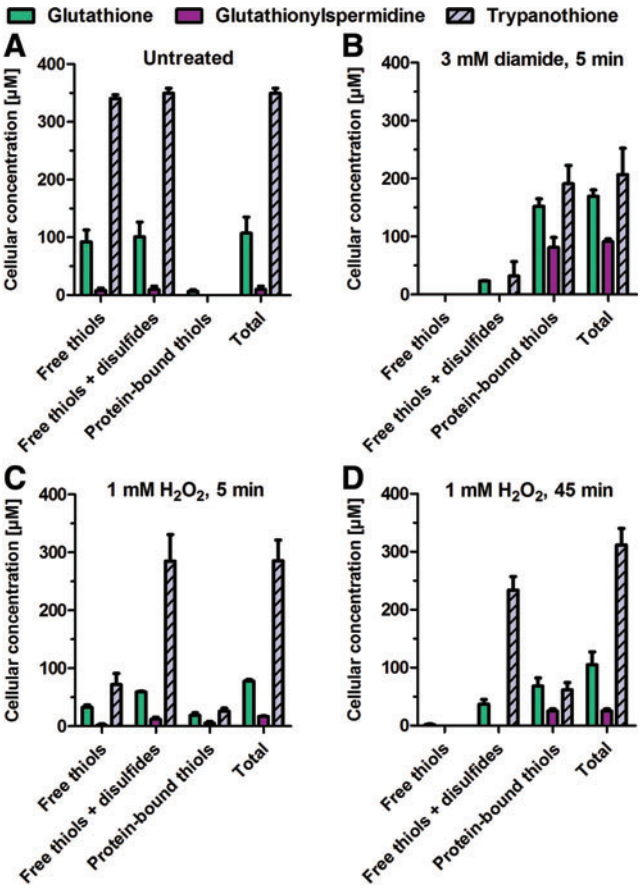

FIG. 8. In diamide-treated cells, the low-molecular-weight thiols become protein bound, whereas H_2O_2 stress results mainly in the formation of free disulfides. Parasites either **(A)** untreated or exposed to **(B)** 3 m*M* diamide for 5 min, **(C)** 1 m*M* H_2O_2 for 5 min, or **(D)** 45 min were counted and immediately lysed by TCA precipitation. Half of the supernatant was directly reacted with mBBr to label the free thiols. The other half was treated with TCEP, followed by mBBr derivatization, yielding the sum of free thiols and disulfides. To quantify protein-bound thiols, the TCA pellet was dissolved in buffer containing TCEP and the released thiols were labeled with mBBr. The thiol derivatives were separated and quantified by HPLC analysis. The values are the mean ± SD of three independent analyses. Total, sum of free thiols, disulfides, and protein-bound thiols. mBBr, monobromobimane. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars

Depletion of TryS induces protein S*-glutathionylation and GSH biosynthesis*

We generated a cell line that allowed the inducible RNA interference toward TryS (TryS RNAi). Forty-eight hours after induction, the parasites showed impaired growth compared with WT cells and the noninduced control (Fig. 10A). After 72 h, proliferation was arrested in accordance with published work (11). Western blot analysis revealed the downregulation of TryS already 24 h post induction. After 48 h, TryS was no longer detectable (Fig. 10B). Total *S*-thiolation was followed by the NDA assay (Fig. 10C), which in this case, represented specifically protein *S*-glutathionylation (Fig. 10E). Depletion of TryS proved to be an endogenous situation to which the parasite responded with protein *S*-glutathionylation (Fig. 10C).

TryS RNAi cells were induced for 24 h and then treated for 5 min with 1 m*M* diamide. This did not affect cell viability (not shown), but resulted in strong *S*-thiolation (Fig. 10D). The induced cells, however, displayed less total *S*-thiolation compared with the control. This is most likely due to the fact that $T(SH)_2$, which in stressed WT parasites forms protein-mixed disulfides (Fig. 8B), was strongly depleted. The rate of protein deglutathionylation was not affected (Fig. 10D). Apparently, the remaining free $T(SH)_2$ was still sufficient to cleave the GSH-protein-mixed disulfides.

As published previously (11, 75), TryS depletion resulted in a decline of Gsp and $T(SH)_2$ and strong increase in GSH (Fig. 10E). Within 24 h, the $T(SH)_2$ level dropped to 70 μM, and after 48 h, Gsp and $T(SH)_2$ were no longer detectable. The decrease of Gsp and $T(SH)_2$ was the result of their impaired biosynthesis. Degradation of the conjugates, as observed in diamide-stressed WT cells, can be ruled out. In the absence of TryS, both synthetase and amidase activities are missing. Obviously, the parasites try to compensate for the lack of $T(SH)_2$ by increasing the biosynthesis of GSH. The GSH/GSSG ratio did not change, but GSH became increasingly protein bound (Fig. 10E).

Discussion

In this work, we studied the oxidative stress response in African trypanosomes. Eighty-four proteins became reversibly oxidized upon challenging the parasites with diamide. Since the main limitation of global proteomics is sensitivity, we chose a procedure in which the oxidized proteins were enriched and then identified and quantified by their tryptic peptides. In other approaches, the proteins are first digested and only the oxidized cysteine peptides are enriched and analyzed by mass spectrometry (37, 42). This strategy reduces the complexity of the samples and directly provides oxidized cysteine residues. On the other hand, a cysteine peptide may be too small, too large, or present in several modifications, which would clearly lower the probability of its detection. This could at least partially explain why in oxidatively stressed *E. coli* or fission yeast only 23 or 27 proteins were found oxidized (37, 42).

The fact that the complete peroxidase system was enriched in the oxidized proteome of *T. brucei* corresponds to the situation in other organisms. In *Schizosaccharomyces pombe*, exposure even to mild H_2O_2 stress results in oxidation of two Prxs as well as glutathione peroxidase and thioredoxin (21). In HOCl-treated *E. coli*, thiol peroxidases are among the oxidized proteins (37), and also in stressed mammalian cells, Prxs are reversibly oxidized (13). *Kinetoplastida* possess an unprecedented number of HSPs (18). This suggests a key role of these proteins in adaptation to strong temperature shifts as well as the oxidative stress the parasites encounter in the mammalian host or the insect vector. Indeed, eight HSP-type proteins were found to be enriched in the oxidized proteome. Other prominent candidates were enzymes of the energy metabolism as well as proteins that play crucial roles in morphology and motility of parasites. The majority of proteins in the overall oxidized proteome are probably rather abundant. Nevertheless, other housekeeping cysteine-rich proteins such TryS, trypanothione reductase, and aldolase were not enriched, indicating some specificity.

Formation of protein-GSH-mixed disulfides is the most frequent post-translational modification of cysteine residues (1, 14). For *P. falciparum* (31) and *Chlamydomonas*

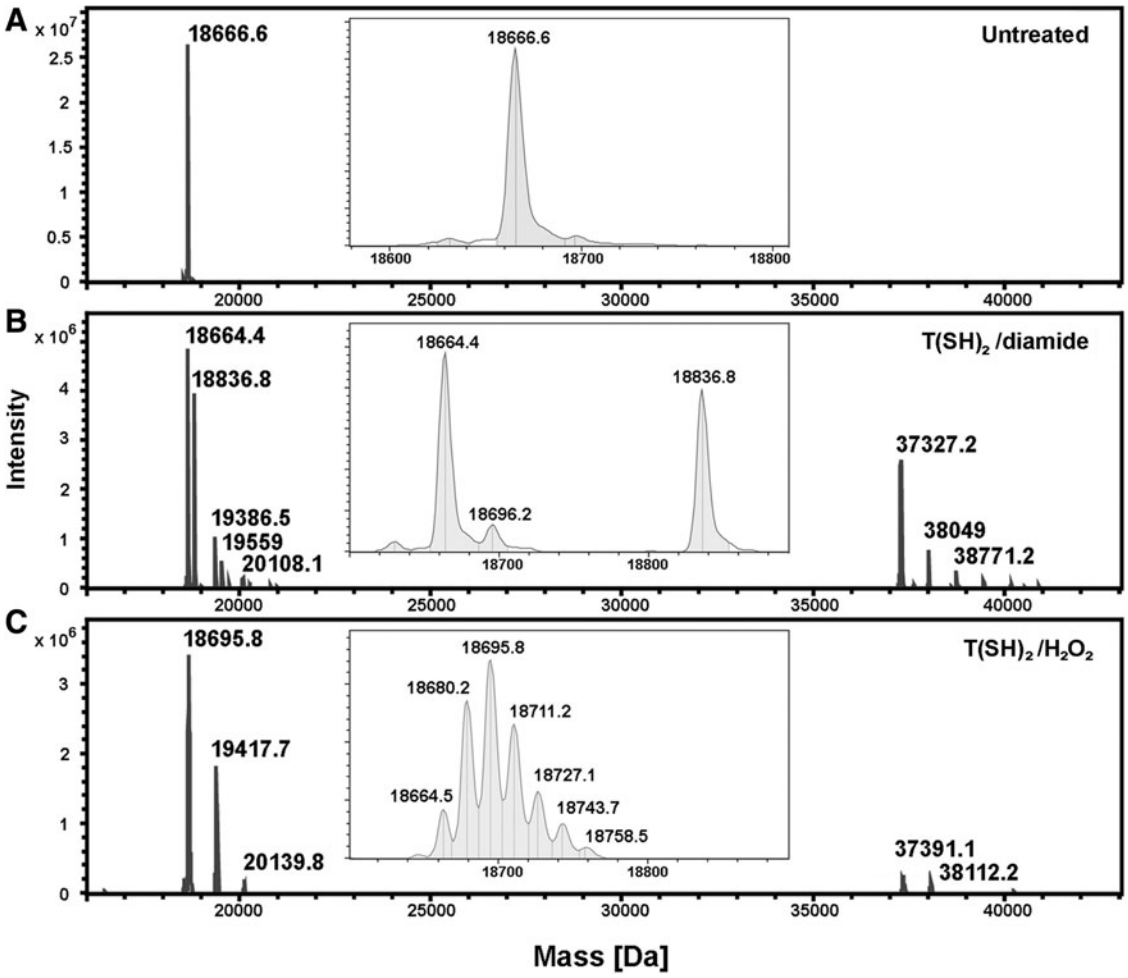

FIG. 9. Deconvoluted ESI-MS spectra of *in vitro S*-trypanothionylated *T. brucei* Px III. Recombinant Px III was prereduced with DTT and then treated with 5 m*M* $T(SH)_2$ and 8 m*M* of either diamide or H_2O_2 and subjected to total mass analysis. Covalent binding of oxygen, diamide, and trypanothione should result in a mass increase of 16, 172.2, and 719.8 Da, respectively. **(A)** The mass of 18666.6 Da observed for the untreated control corresponded to the theoretical mass (18667.4 Da) of Px III with all three cysteine residues in the thiol state. **(B)** Treatment of the protein with $T(SH)_2$/diamide yielded peaks at 18664.4 Da and 18836.8 Da, reflecting Px III with an intramolecular disulfide (theoretical mass 18665.4 Da) plus bound diamide (18837.6 Da), respectively. The peaks at 19386.5, 19559, and 20108.1 Da are in agreement with theoretical masses of Px III species with bound $T(SH)_2$ (19387.2 Da), $T(SH)_2$ plus diamide (19559.4 Da), or two $T(SH)_2$ molecules (20107 Da). **(C)** In the $T(SH)_2/H_2O_2$-treated sample, the main peaks displayed masses of 18695.8 and 19417.7 Da corresponding to the protein oxidized by two oxygen atoms plus either an intramolecular disulfide (theoretical mass 18697.4 Da) or a mixed disulfide with $T(SH)_2$ (19419.2 Da). The peak at 20139.8 Da corresponds to the respective protein species with two bound $T(SH)_2$ molecules (20139 Da). Covalent dimers of Px III formed in **(B)** and **(C)** were also partially *S*-trypanothionylated. The inserts provide an enlargement of the area around the most prominent peak. In the diamide-treated sample, a minor peak for the protein oxidized by two oxygen atoms occurs. In the case of the H_2O_2-treated sample, the mass of the untreated protein is detectable, but multiple species each differing by additional 16 Da are more prominent, indicating unspecific oxidation.

reinhardtii (78), 493 and 225 proteins, respectively, have been reported to be susceptible to *S*-glutathionylation. In *T. brucei*, we found 8–20 proteins putatively *S*-thiolated under distinct stress conditions. This was not due to a limited starting material. Using five times more cells than in the first approaches improved the peptide coverage of the individual protein, but did not increase the number of proteins identified. A main difference is that in our quantitative approach, proteins were identified exclusively if they displayed stress-induced oxidation. In mammalian cells, also only about 20 proteins have been found to be *S*-thiolated under diamide stress (19, 39).

BS *T. brucei* underwent protein *S*-thiolation upon diamide as well as short- and long-term H_2O_2 stress. Only three proteins, a putative HSP20, HSC70-interacting protein, and a PDI, were enriched under all three conditions. Other HSP-type and PDI proteins were found to be *S*-thiolated only upon a specific stress. In human PDI, one cysteine of the CXXC motifs can be *S*-glutathionylated, which impairs its isomerase activity and triggers the unfolded protein response (67). In breast cancer cells, PDI is a molecular chaperone of the estrogen receptor-alpha, protecting from oxidation, misfolding, and degradation. *S*-glutathionylation prevents the interaction and initiates cell death (76).

The cytosolic Prx, HSP60, and splicing factor TSR1 were found *S*-thiolated in diamide-stressed, but not H_2O_2-stressed, parasites. In T cells, corresponding proteins are also

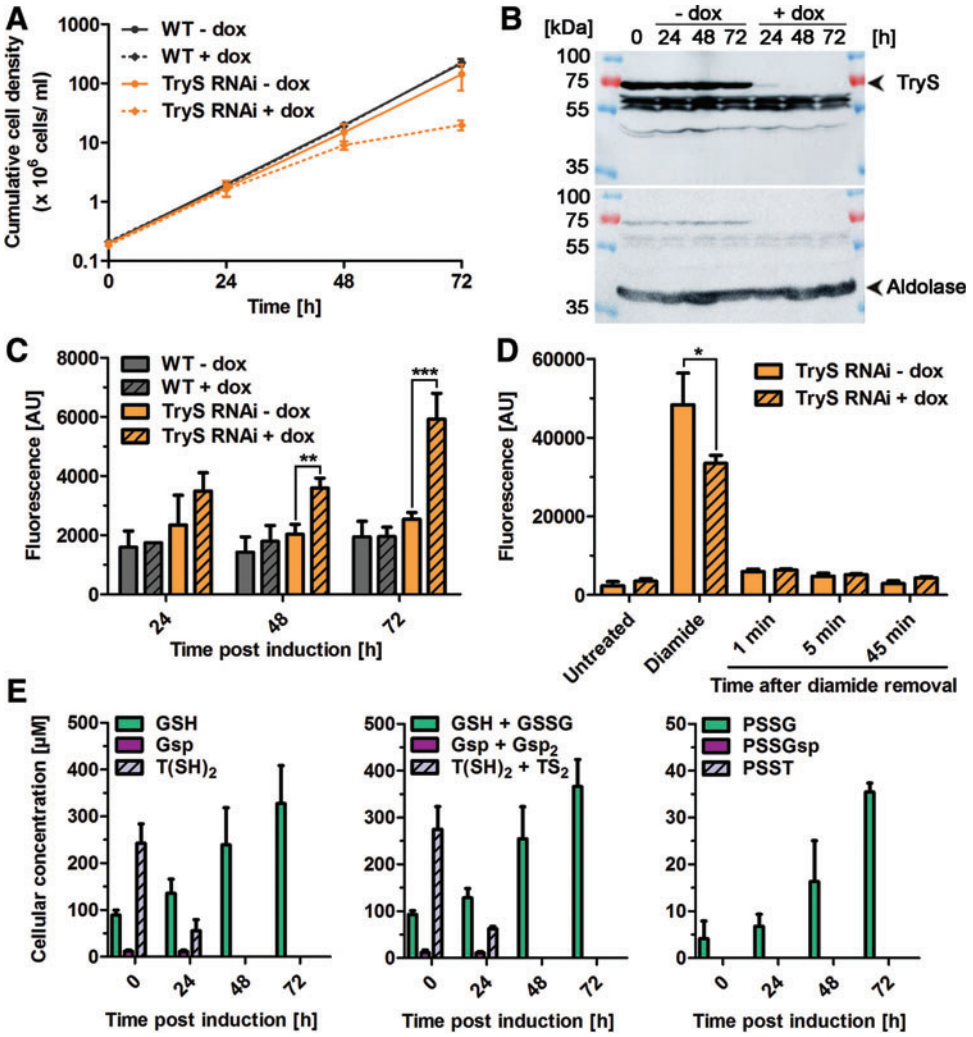

FIG. 10. Depletion of TryS causes protein *S*-glutathionylation, attenuates total *S*-thiolation upon diamide stress, and triggers the *de novo* synthesis of GSH. TryS RNAi cells as well as nontransfected 2T1 WT cells were grown in the absence or presence of doxycycline (dox). **(A)** Every 24 h, the cells were counted and the culture diluted to the initial density of 2×10^5 cells/ml. **(B)** Total lysates of TryS RNAi cells were subjected to Western blot analysis with antibodies against TryS as well as aldolase as loading control. The bands between 60 and 70 kDa are proteins that cross-reacted with the TryS antiserum. **(C)** Protein *S*-thiolation was measured in the NDA assay. **(D)** TryS RNAi cells cultured for 24 h ± dox were treated for 5 min at RT with 1 m*M* diamide in PBS, retransferred into medium, and incubated at 37°C. After different times, the remaining protein-bound thiols were measured in the NDA assay. **(E)** TryS RNAi cells +dox were lysed by TCA precipitation. Free thiols (GSH, Gsp, $T(SH)_2$), free thiols plus disulfides (GSSG, Gsp_2, TS_2), and protein-bound thiols (PSSG, PSSGsp, PSST) were quantified by HPLC analyses of mBBr-derivatized samples as outlined in the legend of Figure 8. All values are the mean ± SD of three independent experiments. **p*-value of 0.007; ***p*-value of 0.003; ****p*-value of 0.001. TryS, trypanothione synthetase. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars

S-thiolated only upon diamide treatment (19). Peskin *et al.* demonstrate *S*-glutathionylation of Prx2 when erythrocytes from Grx1 knockout mice are exposed to H_2O_2 (54). The H_2O_2 conditions applied in our analyses resulted in rapid irreversible oxidation of cytosolic Prx. Since overoxidation inactivates the enzyme (56), this may be one reason why the parasites were much more sensitive toward H_2O_2 than to diamide.

One of the most strongly enriched proteins from diamide-stressed cells was the serine–arginine-rich splicing factor TSR1. The protein plays an essential role in regulating splicing and mRNA stability in the parasite (24). At least two of the three cysteine residues of *T. brucei* TSR1 are conserved in orthologous proteins from other *Kinetoplastida*. It may be interesting to evaluate if, in addition to its extensive regulation by phosphorylation, TSR1 is amenable to redox regulation.

Several of the proteins found to be *S*-thiolated exclusively after 45 min of H_2O_2 treatment are involved in calcium signaling and/or cell death pathways. The question arises whether these modifications could contribute to rapid lysis observed when the parasites were put back to culture conditions. The ER-resident calreticulin is accessible to *S*-glutathionylation in mammalian cells (77). Another example is nucleoside diphosphate kinase (29). The *T. brucei* protein contains a single cysteine that directly precedes the catalytic histidine and is conserved in the trypanosomatid and yeast orthologs. These multifunctional enzymes are involved in

proliferation, differentiation, and cell death pathways. In human cancer cell lines, a nucleoside diphosphate kinase becomes *S*-glutathionylated upon H_2O_2 treatment, which impairs enzymatic activity (36).

The GSH/Grx couple is widely used in analyses aiming at the identification of *S*-glutathionylated proteins (31, 39, 57, 64). This is based on the fact that spontaneous dethiolation by GSH is very slow compared with the Grx-catalyzed reaction (39). Still, a distinct protein disulfide may react with GSH to form a mixed disulfide, which then acts as substrate for Grx. Indeed, the covalent homodimer of mammalian Prx2 has been shown to react with GSH to form GSH-mixed disulfides (54). Since the Grx-catalyzed reaction follows a monothiol mechanism, even a mutant lacking the second active site Cys, used in several studies (31, 39, 64), would not prevent this reaction. The C14S mutant of *E. coli* Grx1 can catalyze the reduction of thioredoxin disulfides by GSH (16). Thus, a protein disulfide that is generated under stress conditions and readily undergoes a thiol/disulfide exchange with GSH might be enriched in addition to proteins that were *S*-thiolated in the intact cell. Clearly, this should be a minor reaction since the overall oxidized proteome of diamide-treated parasites yielded almost six times more proteins than the respective GSH/Grx-based approach.

For only few *T. brucei* proteins, the cellular concentration has been determined experimentally. Therefore, we adapted proteome (68) and ribosome profiling (30) data to get an estimate. Most of the proteins found susceptible to *S*-thiolation appear to be relatively prominent ones. In comparison, HSP20, TSR1, and Tb927.11.3760 may be low abundance proteins. This is also the case for the two proteins found to be *S*-thiolated exclusively in parasites exposed for 5 min to 1 m*M* H_2O_2, of which one (Tb927.8.1750) has recently been annotated as putative cytokine-induced antiapoptosis inhibitor 1/FeS biosynthesis. Future work should reveal if these proteins could act as trypanosomal redox switches.

BS *T. brucei* contained about 350 μM $T(SH)_2$, 100 μM GSH, and 10 μM Gsp, in accordance with published data (33), which were present in reduced unbound form. Upon challenging the parasites with diamide, all three thiols were almost entirely protein-bound. When mammalian cells are exposed to diamide, *S*-glutathionylated proteins are also formed, but GSSG is the main product (25). Diamide causes the formation of GSSG, protein disulfides, and *S*-glutathionylated proteins (32). Protein thiols usually react at a lower rate compared with GSH. In trypanosomes, however, protein thiols may readily compete because the concentration of total free thiols is much lower than that of protein thiols. Protein *S*-thiolation was induced also by H_2O_2 and hypochlorite, which represent more physiological oxidants of metabolic relevance and/or as part of the host defense mechanisms. The finding that upon H_2O_2 treatment, the cellular thiols were mainly oxidized to free disulfides indicates that also in trypanosomes protein *S*-thiolation does not occur *via* thiol/disulfide exchange reactions.

Reaction of several parasite redox proteins, including Px III, with GSSG or Gsp_2 resulted in the formation of mixed disulfides, whereas TS_2 did not cause any modification (43). In this study, we show that treatment of recombinant Px III with $T(SH)_2$ in the presence of diamide or H_2O_2 results in *S*-trypanothionylation. That this modification was not observed in the previous work is probably due to the fact that the proteins were treated with TS_2 (43). Under these conditions, a mixed disulfide could form only by thiol/disulfide exchange. As reported by others (1, 14) and supported by our data, this is not a prominent mechanism for protein *S*-thiolation and, clearly, even less likely in the case of the bulky cyclic TS_2. Treatment of parasites with diamide or H_2O_2 results in activated protein thiols, which then can react with thiols to form the mixed disulfide (1, 14, 32). The—at first glance unexpected—stability of protein-$T(SH)_2$-mixed disulfides can be explained by steric constrains exerted by the protein, which prevent an attack by the remaining free thiol of bound $T(SH)_2$ and thus allow stable *S*-trypanothionylation.

In diamide-treated parasites, we observed a decrease of total trypanothione concomitantly with an increase in GSH and Gsp. Since free thiols were undetectable, we propose that protein-bound trypanothione became hydrolyzed. One candidate protein that could catalyze the reaction is TryS. Although the amidase activity is very low and dispensable for cell viability (75), *T. brucei* that express an amidase-dead-mutant display decreased *in vitro* growth and *in vivo* virulence. Wyllie *et al.* discuss that the low amidase activity of recombinant TryS could be readily explained if free $T(SH)_2$ and Gsp were not the natural substrates and suggest a physiological role by hydrolyzing trypanothione conjugates.

Hydrolysis of protein-bound trypanothione would generate GSH and Gsp, which—under stress conditions—could immediately react with other protein thiols and thus allow more proteins to be protected by *S*-thiolation. This mechanism might have physiological relevance since trypanosomes have a significantly lower total cellular thiol concentration compared with other eukaryotes containing millimolar GSH. In H_2O_2-treated cells, trypanothione was not degraded, most likely because the main product was TS_2. Based on the structure of *L. major* TryS (20), it is very unlikely that TS_2 is hydrolyzed by the enzyme. In addition, in the related *E. coli* Gsp synthetase/amidase, the catalytic Cys in the amidase domain is oxidized by H_2O_2 to sulfenic acid, which affects the amidase activity of the enzyme (9).

Grx1 accelerated protein dethiolation, but in cells lacking Grx1, protein-bound thiols were also rapidly removed. This suggests an alternative mechanism involving most likely $T(SH)_2$. A putative scenario is that after stress removal, protein-GSH-mixed disulfides react with $T(SH)_2$, yielding the reduced protein and a GSH-$T(SH)_2$-mixed disulfide, which is cleaved by attack of the remaining free thiol of $T(SH)_2$ either spontaneously or Tpx-mediated. Tpx does not catalyze GSH-dependent deglutathionylation reactions (8, 40). However, since its discovery, the parasite oxidoreductase is known to strongly accelerate the reduction of GSSG by $T(SH)_2$ (3, 22), a reaction that should comprise a glutathione–trypanothione-mixed disulfide intermediate as well. Thus, it is tempting to speculate that Tpx supports protein dethiolation by catalyzing the cleavage of protein or free trypanothione-containing mixed disulfides.

TryS RNAi induction for 24 h had no effect on reversibility of diamide-induced protein *S*-thiolation. Although the cellular $T(SH)_2$ level had already significantly dropped, the $T(SH)_2$/TR/NADPH system was probably still sufficient to reduce the protein-mixed disulfides. Indeed, the viability of TryS-depleted BS *T. brucei* with very low remaining $T(SH)_2$ is only affected if the parasites are exposed to strong H_2O_2 fluxes (11). TryS depletion increased the cellular GSH to

300–400 μM. In the absence of $T(SH)_2$, the parasites cannot regenerate GSH from GSSG, and GSH biosynthesis is presumably the only mechanism to produce reducing equivalents. Interestingly, in TryS-depleted cells, the level of GSSG did not increase, but GSH became increasingly protein-bound. Other eukaryotes actively export GSSG to maintain their reducing cytosolic milieu, a mechanism not reported for trypanosomes. Protein *S*-glutathionylation observed in TryS-depleted cells may thus contribute to maintain the cellular GSH/GSSG ratio.

In this study, we showed that African trypanosomes employ protein *S*-thiolation in response to exogenous and endogenous oxidative stresses. Future work will focus on identification of distinct protein residues that undergo *S*-thiolation and, especially, *S*-trypanothiolation. The aim is to get a deeper insight in both the mechanism and physiological role of this novel post-translational modification in trypanosomes.

Materials and Methods

Materials

HPDP-biotin (21341) and TCEP solution (77720) were purchased from Thermo Scientific. Soft release avidin resin (V2012) and trypsin (V5111) were obtained from Promega. NDA (70215) was ordered from Sigma-Aldrich and mBBr (596105) from Merck Millipore. GSSG was obtained from Serva and NADPH from Roth. All other reagents and the solvents were purchased from Sigma-Aldrich. $T(SH)_2$ and Gsp were enzymatically prepared and the disulfide form was generated by reaction with a stoichiometric concentration of H_2O_2 (10, 38). Recombinant *T. brucei* Px III was prepared as described (15). Human GR was a kind gift from Dr. Heiner Schirmer, Heidelberg University. Human Grx1 was purchased from Biomol.

Polyclonal rabbit antibodies against *T. brucei* Prx (15) and TryS (11) were generated previously. Rabbit antibodies against *T. brucei* aldolase were kindly provided by Dr. Christine Clayton, Heidelberg University, and the monoclonal mouse anti-β-tubulin antibodies were from Dr. Keith Gull, University of Oxford. Rabbit anti-Prx-SO_3 antibodies were purchased from Abcam. HRP-conjugated goat antibodies against rabbit and mouse IgGs were from Santa Cruz Biotechnology. The Super Signal West Pico Kit was from Thermo Scientific.

T. brucei *cell lines and cultivation*

If not stated otherwise, BS *T. b. brucei* 449 cells (Lister strain 427) were used. Grx1 and Grx2 knockout cell lines as well as $Grx1^{-/-}$ cells harboring an ectopic copy of *grx1-myc2* were generated previously (47). BS 2T1 cells (Lister 427, MITat1.2, clone 221a) allowing tetracycline-induced RNAi against TryS were generated. Specific RNAi targets against TryS and primers were selected using the RNAit software (http://trypanofan.path.cam.ac.uk/software/RNAit.html). A 458-bp fragment (corresponding to coding nucleotides 902–1359) from the *T. brucei* TryS gene (Tb927.2.4370) was amplified by PCR using gene-specific primers (forward: AGAAGAGTATTGGCCTCGCA; reverse: ACATCCACG TCTTCCACACA) and ligated into the $pRPa^{ISL}$ vector (2). The AscI-linearized construct $pRPa^{ISL}$-TryS (10 μg) was introduced into *T. brucei* BS 2T1 cells by nucleofection using program Z-001 and Basic Parasite Nucleofector solution (Lonza Cologne GmbH, Cologne, Germany) following the manufacturer's instructions. About 6 h after nucleofection, the appropriate drug selection was applied (2.5 μg/ml hygromycin) and cells were distributed into 24-well plates and stable lines isolated. TryS depletion was induced in selected clones by supplementing the growth medium with 1 μg/ml doxycycline. The parasites were cultivated in HMI-9 medium at 37°C as described previously (27). For all experiments, parasites were harvested at a density of $0.8–1.2 \times 10^6$ cells/ml and washed twice with ice-cold PBS.

Quantification of total cellular protein-bound thiols using NDA assay

To establish the procedure, 20 μl of GSH, γ-Glu-Cys, Gsp, $T(SH)_2$, or cysteine (0.1–10 μM in 280 mM Tris-HCl, pH 8.0 containing 3.5 mM TCEP, and 60 mM sulfosalicylic acid) was mixed with 180 μl NDA derivatization solution (freshly prepared from 1 ml of 10 mM NDA in dimethyl sulfoxide (DMSO), 7 ml of 50 mM Tris, and 1 ml of 0.5 M NaOH) and incubated for 30 min at room temperature (RT) in the dark (44). Fluorescence was measured in 96-well plates in a Victor X4 plate reader (Perkin Elmer) with excitation at 485 nm and emission at 535 nm. Per sample, 1×10^8 BS *T. brucei* were harvested and exposed to various stresses in PBS at 23°C. After centrifugation, cells were resuspended in 900 μl PBS, an aliquot was removed for cell counting, and the remaining parasites were immediately lysed by 10% ice-cold TCA for 30 min on ice. The pellet was washed twice with ice-cold 10% TCA in PBS. The proteins were dissolved in 56 μl of 500 mM Tris-HCl, pH 8.0, and kept for about 5 min in an ultrasonic bath to improve solubility. Insoluble debris was removed by centrifugation. The supernatant was mixed with 14 μl 25 mM TCEP and incubated for 30 min at RT. Proteins were precipitated by adding 30 μl 200 mM sulfosalicylic acid. After 30 min on ice and centrifugation, the supernatant was collected, and 20 μl-aliquots were subjected to the NDA assay. To follow the reversibility of protein *S*-thiolation, the stressed cells were washed with PBS and transferred into 37°C prewarmed HMI-9 medium, incubated for different times, and processed as described above.

Enrichment of reversibly oxidized proteins for proteome analysis

About 1×10^9 BS *T. brucei* were transferred into 1.8 ml PBS, split in two parts, and incubated for 5 min at 23°C with 3 mM diamide (+stress) or in PBS only (–stress). After centrifugation, the cells were resuspended in 900 μl PBS, an aliquot was removed for counting, and 100 μl ice-cold TCA was added. After 30 min on ice, the pellet was washed three times with ice-cold acetone, resuspended in 900 μl 50 mM Tris-HCl, 1 mM EDTA, pH 7.5, containing 20 mM NEM, and incubated for 30 min at RT. Insoluble aggregates were removed by centrifugation. Excess NEM was removed by TCA precipitation and acetone washing. The protein pellet was dissolved in 300 μl 50 mM Tris-HCl, 1 mM EDTA, pH 7.5, containing 20 mM DTT, and incubated for 45 min at 30°C. Proteins were precipitated by TCA, washed with acetone, dissolved in 180 μl 50 mM Tris-HCl, 1 mM EDTA, pH 7.5, containing 0.4 mM HPDP-biotin, and incubated for 45 min at 30°C in the dark. Unbound HPDP-biotin was removed by

TCA precipitation and acetone washing. The proteins were dissolved in 150 μl binding buffer (25 m*M* Tris-HCl, 5 m*M* EDTA, 50 m*M* NaCl, pH 8.8) and added to 20 μl avidin resin, previously washed five times with 200 μl binding buffer. After tumbling overnight at 4°C, the resin was washed once with 200 μl binding buffer, twice with 1 *M* NaCl in 25 m*M* Tris-HCl, pH 8.8, three times with 25 m*M* Tris-HCl, pH 8.8, and once with 7 m*M* Tris. Proteins were eluted in two steps each with 20 μl of 20 m*M* DTT in 7 m*M* Tris (35).

Enrichment of oxidized protein thiols susceptible to reduction by the GSH/Grx system

About 1×10^9 BS *T. brucei* were exposed at 23°C to three different stress conditions: 3 m*M* diamide for 5 min and 1 m*M* H_2O_2 for 5 and 45 min. After lysis by 10% TCA, the samples were treated as described in the previous section, except that DTT was replaced by GSH/Grx as reducing agent. The protein pellet was dissolved in 300 μl 50 m*M* Tris-HCl, 1 m*M* EDTA, pH 7.5, containing 1 m*M* GSH, 1 m*M* NADPH, 4 U/ml human GR, and 2.3 μ*M* human Grx1 and incubated for 15 min at 37°C (57).

Protein digestion, differential labeling, and mass spectrometric analysis

Proteins were separated by SDS-PAGE on a 10% gel. Electrophoresis was stopped when the proteins had entered 1–2 cm of the running gel. After Coomassie blue staining, each lane was cut into three pieces. Proteins were in-gel reduced, alkylated with IAM, and digested by trypsin essentially as described by Winkler *et al.* (73). The eluted peptides from stress-treated parasites were labeled with formaldehyde (light label) and control sample with deuterated formaldehyde (heavy label) (6). Both samples were mixed in a 1:1 ratio and peptides analyzed using an Orbitrap XL mass spectrometer (Thermo) coupled to an UltiMate 3000 RSLCnano System (Thermo). After trapping, samples were loaded on a C18 analytical column (nanoAcquity BEH130 C18, 1.7 μm × 250 mm, Waters) with 1% acetonitrile, 0.1% formic acid, and 5% DMSO in water and eluted with 90% acetonitrile, 0.1% formic acid, and 5% DMSO in water. The mass spectrometer was operated in data-dependent mode with the top 13 most intense ions selected for fragmentation by collision-induced dissociation. Fragment spectra were acquired with the ion trap detector.

The MS/MS spectra were matched to theoretical spectra obtained from the FASTA database for *T. brucei* (number of database entries: 9826) using the Mascot software, Version 2.4. Settings were adjusted to trypsin-digested peptides having up to one miss cleavage site. Derivatization of cysteines with IAM and NEM was specified as fixed and variable modifications, respectively. Deamidation of asparagine and glutamine, as well as oxidation of methionine residues, was specified as variable modifications. Mass tolerance for peptide molecular weight was set to 20 ppm and the MS/MS tolerance for peptide fragments to 0.5 Da. Protein hits were considered as identified if the Mascot score exceeded the significance level ($p > 0.01$). Peptide ratios were calculated from the summed isotopic signal intensities of CH_2O- and CD_2O-labeled peptide pairs (6) using the Proteome Discoverer 1.4 (Thermo Scientific). The protein ratio was calculated as the median of ratios of all unique peptides, which fulfilled the quality criteria implemented in the Proteome Discoverer.

Western blot analyses

To study the redox state of cytosolic Prx, BS *T. brucei* were exposed in PBS at 23°C to 3 m*M* diamide and 0.1 or 1 m*M* H_2O_2. After centrifugation, the cells were resuspended in PBS containing 20 m*M* NEM. SDS sample buffer without (nonreducing) or with (reducing) 2-mercaptoethanol was added and samples were boiled for 5 min. Total lysates from 10^7 cells were loaded per lane onto a 10% SDS gel. After electrophoresis, proteins were transferred onto a PVDF membrane, and probed with antibodies against Prx-SO_3 (recognizing the conserved active site peptide of Prxs with a sulfinic or sulfonic acid at the peroxidatic Cys (74); 1:1600), followed by HRP-conjugated goat antibodies against rabbit IgGs (1:10,000). After stripping, the membrane was incubated with antibodies against Prx (1:3000) and HRP-conjugated goat anti-rabbit antibodies. The blot was washed and developed with β-tubulin antibodies (1:2000), followed by HRP-conjugated goat antibodies against mouse IgGs (1:10,000). TryS expression in noninduced and induced 2T1 TryS RNAi cells was detected by Western blot analysis of total cell lysates as described above. The membrane was developed with antibodies against *T. brucei* TryS (1:1000) and HRP-conjugated goat anti-rabbit antibodies. After washing, the blot was treated with antibodies against aldolase (1:40,000) and HRP-conjugated goat anti-rabbit antibodies.

HPLC analysis of mBBr-labeled cellular thiols

About 1×10^8 BS T. *brucei* were exposed to various stress conditions in PBS at 23°C, washed with 1 ml PBS, and 5 μl of the suspension was removed for counting. After centrifugation, cells were resuspended in 90 μl PBS, 10 μl ice-cold TCA was added, and the suspension was incubated for 30 min on ice and centrifuged. The supernatant was kept on ice and the pellet was re-extracted twice with 50 μl 10% TCA in PBS. The supernatants were pooled, washed five times with ice-cold diethylether, and split into two parts. One sample was mixed with 300 μl 150 m*M* Tris-HCl, pH 8.3, and 100 μl 10 m*M* mBBr solution (freshly prepared from a 200 m*M* mBBr stock in acetonitrile). The other sample was treated with 4 μl 25 m*M* TCEP for 30 min at RT, followed by mBBr derivatization.

The pellet fraction was redissolved in 56 μl 500 m*M* Tris-HCl, pH 8.0, and treated with 14 μl TCEP. After addition of 30 μl 200 m*M* sulfosalicylic acid, the sample was incubated for 30 min on ice and centrifuged. Eighty microliters of supernatant was mixed with 130 μl 233 m*M* Tris-HCl, pH 8.3, and 50 μl 10 m*M* mBBr. After 15 min of incubation at RT in the dark, the reaction was stopped by adding 10 μl 5 *M* methanesulfonic acid. Excess mBBr was removed by dichloromethane extraction and the samples were subjected to HPLC analysis and fluorescence detection as described previously (60). Standards of GSH, Gsp, and $T(SH)_2$ were treated in parallel exactly like the samples. The amount of thiol was quantified by comparing integrated peak areas of the sample and standards. The cellular concentration was calculated by normalizing to the cell number and a cell volume of 58 fl (49).

In vitro *trypanothionylation*

Recombinant *T. brucei* Px III was treated with 20 m*M* DTT. An aliquot was kept on ice. The protein was washed

with ice-cold PBS on a 10 kDa cutoff 0.5 ml Amicon filter device. One hundred micromolar prereduced protein was treated for 2 h at RT with 5 m*M* $T(SH)_2$ and 8 m*M* of either diamide or H_2O_2. The reaction was stopped by adding 1% formic acid. The intact masses of (un)treated proteins were determined by ESI-MS on a QTof mass spectrometer (MAXIS, Fa Bruker) after desalting by HPLC (Agilent) essentially as described (58). The protein samples were diluted to 1 μM in 0.1% trifluoroacetic acid and 200 μl loaded onto a reversed-phase trapping column (0.8×2 mm, Poros R1; Applied Biosystems). After washing for 3 min with 0.3% formic acid at a flux of 300 μl/min and for 8 min with 4% isopropanol, 1% acetonitrile, and 0.3% formic acid at a flux of 40 μl/in, the proteins were eluted with 40% isopropanol, 5% acetonitrile 0.3% formic acid, followed by washing with 80% isopropanol, 10% acetonitrile, and 0.3% formic acid, at a flux of 40 μl/min. Protein mass spectra were deconvoluted using the MaxEnt algorithm implemented in the Compass DataAnalysis 4.2 software (Bruker).

Statistical data analyses

If not stated otherwise, statistical analyses were performed with *Prism* (GraphPad) and *Excel* (Microsoft) software. *p*-Values were determined by unpaired one-tailed Student's *t*-test.

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Author Disclosure Statement

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Abbreviations Used

BS = bloodstream
GR = glutathione reductase
Grx = glutaredoxin
GSH = glutathione
Gsp = glutathionylspermidine
HSP = heat shock protein
MS = mass spectrometry
NDA = 2,3-naphthalene dicarboxaldehyde
NEM = *N*-ethylmaleimide
PBS = phosphate-buffered saline
PDI = protein disulfide isomerase
Prx = peroxiredoxin
TCA = trichloroacetic acid
TCEP = tris(2-carboxyethyl)phosphine
TryS = trypanothione synthetase
WT = wild-type