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ORIGINAL RESEARCH COMMUNICATION

Protein *S*-Glutathionylation in Malaria Parasites

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Abstract

Aims: Protein *S*-glutathionylation is a widely distributed post-translational modification of thiol groups with glutathione that can function as a redox-sensitive switch to mediate redox regulation and signal transduction. The malaria parasite *Plasmodium falciparum* is exposed to intense oxidative stress and possesses the enzymatic system required to regulate protein *S*-glutathionylation, but despite its potential importance, protein *S*-glutathionylation has not yet been studied in malaria parasites. In this work we applied a method based on enzymatic deglutathionylation, affinity purification of biotin-maleimide-tagged proteins, and proteomic analyses to characterize the *Plasmodium* glutathionylome. ***Results:*** We identified 493 targets of protein *S*-glutathionylation in *Plasmodium*. Functional profiles revealed that the targets are components of central metabolic pathways, such as nitrogen compound metabolism and protein metabolism. Fifteen identified proteins with important functions in metabolic pathways (thioredoxin reductase, thioredoxin, thioredoxin peroxidase 1, glutathione reductase, glutathione *S*-transferase, plasmoredoxin, mitochondrial dihydrolipoamide dehydrogenase, glutamate dehydrogenase 1, glyoxalase I and II, ornithine δ-aminotransferase, lactate dehydrogenase, glyceraldehyde 3-phosphate dehydrogenase [GAPDH], pyruvate kinase [PK], and phosphoglycerate mutase) were further analyzed to study their ability to form mixed disulfides with glutathione. We demonstrate that *P. falciparum* GAPDH, PK, and ornithine δ-aminotransferase are reversibly inhibited by *S*-glutathionylation. Further, we provide evidence that not only *P. falciparum* glutaredoxin 1, but also thioredoxin 1 and plasmoredoxin are able to efficiently catalyze protein deglutathionylation. ***Innovation:*** We used an affinity-purification based proteomic approach to characterize the *Plasmodium* glutathionylome. ***Conclusion:*** Our results indicate a wide regulative use of *S*-glutathionylation in the malaria parasite and contribute to our understanding of redox-regulatory processes in this pathogen. *Antioxid. Redox Signal.* 15, 2855–2865.

Introduction

Redox-signaling pathways, protein structures, and enzymatic reactions are often controlled by thiol groups of cysteine residues acting as redox-sensitive switches both in proteins and in low-molecular-weight thiols such as the tripeptide glutathione. Glutathione (1–10 mM) (34) exists in a reduced (GSH) and an oxidized form (GSSG), with most of the glutathione being in the reduced form in unstressed cells. Glutathione is critical for maintaining a balanced intracellular redox state and for regulating oxidative signaling pathways (17). Protein *S*-glutathionylation represents the most abundant form of oxidative thiol modifications: The cysteine-sulfhydryl moiety of GSH forms a reversible mixed disulfide with a cysteine-sulfhydryl moiety of a protein (PSH), resulting in an *S*-glutathionylated protein (mixed protein glutathione disulfide, PSSG). Protein *S*-glutathionylation occurs during oxidative stress, but also under physiological conditions, and fulfills various important functions in redox regulation. Protein *S*-glutathionylation can initiate functional changes of enzymes, and regulates signaling transducers (11, 14). Numerous proteins such as carbonic anhydrase III, α-ketoglutarate dehydrogenase, as well as heat shock protein 70, c-Jun, and NF-κB have been reported to be reversibly activated or inhibited by *S*-glutathionylation of functionally or structurally critical cysteine residues [reviewed in (16)]. Further, during increased oxidative stress *S*-glutathionylation can protect thiol groups from overoxidation (49), and simultaneously functions as a storage form of glutathione inside the cell.

Besides thiol disulfide exchange between GSSG and a protein, protein *S*-glutathionylation can occur in the presence of GSH and oxidants. In this case, protein thiols are oxidized to sulfenic acids, which can react with GSH (1, 49). In addition, *S*-glutathionylation can be mediated by more reactive oxidized

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Innovation

The rapidly growing and multiplying malaria parasite *Plasmodium falciparum* needs to adapt efficiently to various hostile environments in human and mosquito. Therefore, antioxidant defense and redox regulatory processes play a central role in this pathogen. Here we provide the first systematic study on cellular targets of protein *S*-glutathionylation in *P. falciparum*. Using multidimensional protein identification technology, we identified 321 proteins with a predicted function and 172 hypothetical proteins as targets of *S*-glutathionylation. Further, we studied the *S*-glutathionylation of selected proteins systematically by protein immunoblotting and functional analyses. We also provide evidence that not only *Plasmodium* glutaredoxin1 (Grx1), but also thioredoxin 1 (Trx1) as well as plasmoredoxin (Plrx) are able to efficiently catalyze protein deglutathionylation. Protein *S*-glutathionylation can be considered as redox regulation, if several aspects are met: (i) efficient reversibility, (ii) specificity toward certain cysteine residues, (iii) must occur in response to a physiological stimulus in intact cells, and (iv) protein activity as well as the respective cell function should be changed by *S*-glutathionylation (16, 43). Most of these criteria have been demonstrated for the examined proteins, including *P. falciparum* (Pf) GAPDH and PfOAT. However, the role of *S*-glutathionylation in response to different physiological and pathophysiological stimuli, like oxidative and pharmacological stress, the differential susceptibility of protein sulfhydryls to *S*-glutathionylation, as well as the mechanisms leading to protein (de)glutathionylation in *P. falciparum in vivo* remain to be elucidated in detail.

forms of glutathione, for example, *S*-nitrosoglutathione, or can be catalyzed enzymatically by glutaredoxin *via* a monothiol-mechanism (33), as well as by glutathione *S*-transferase (47). However, the predominant mechanism *in vivo* remains unclear. The reverse reaction, called deglutathionylation, can be efficiently catalyzed by glutaredoxins and thioredoxins *via* a dithiol exchange mechanism (4, 20, 23, 24).

Malaria caused by *P. falciparum* is one of the deadliest diseases worldwide and affects nearly 250 million people annually, most of them being children in the world's most disadvantaged countries. During its developmental stages *Plasmodium* shows only minor transcriptional changes in response to external stimuli, suggesting that its proteins are mainly regulated by versatile post-transcriptional and post-translational modifications (21). *Plasmodium* possesses a functional glutathione system, including glutathione reductase (GR) (18), glutathione, a 2-Cys glutaredoxin (Grx1) (40), and a glutathione *S*-transferase (GST) (27). Thus, the parasite is enzymatically equipped to use protein *S*-glutathionylation as a redox-regulatory mechanism. However, despite its potential importance, protein *S*-glutathionylation has not yet been studied in *Plasmodium*.

Several recent articles describe the identification of *S*-glutathionylated proteins based on the enzymatic reduction of *S*-glutathionylated proteins by glutaredoxin, tagging with biotin-maleimide, affinity purification, and identification by proteomic analysis (2, 25, 32, 41). By using this specific and highly sensitive approach, we identified 321 proteins with a predicted function and 172 hypothetical proteins of the *P. falciparum* glutathionylome. Further, we studied the *S*-glutathionylation of selected proteins systematically by protein immunoblotting and functional analyses.

Results

Identification of S*-glutathionylated proteins*

S-glutathionylated proteins in *P. falciparum* were detected by a specific and sensitive method based on enzymatic deglutathionylation of mixed protein-SSG disulfides by recombinant glutaredoxin in cell extracts, as first described by Lind *et al.* (32). Initially, all free thiols in the *P. falciparum* cell extracts were alkylated by N-ethylmaleimide (NEM), and then *S*-glutathionylated proteins were reduced with recombinant PfGrx1 using PfGR, GSH, and NADPH as back-up system. The emerging sulfhydryl groups of initially *S*-glutathionylated proteins were subsequently tagged with biotin-maleimide and purified by avidin affinity chromatography (Fig. 1). Isolated proteins were trypsin-digested and identified using an linear trap quadrupole (LTQ)-Orbitrap mass spectrometer. Three experimental repetitions were conducted to ensure high specificity. We reproducibly identified 321 targets for protein *S*-glutathionylation (listed in Supplementary Table S1; Supplementary Data are available online at www.liebertonline.com/ars). In addition, we identified 172 *S*-glutathionylated hypothetical proteins (listed separately in Supplementary Table S2). Only proteins that were identified in at least two experiments were included in the target lists. A functional profile of the identified proteins was generated according to their contribution to cellular metabolic pathways and their catalytic activity using the program PatternLab for Proteomics (9). Many *S*-glutathionylated proteins are involved in nitrogen compound and protein metabolism (Fig. 2A), and according to their catalytic activity most of the proteins have hydrolase, transferase, or oxidoreductase activity (Fig. 2B).

Control experiments

To ensure specificity of the method, we performed two control experiments. In the first control experiment only the blocking of free thiols with NEM was carried out, aiming at identifying proteins that interact unspecifically with the avidin resin. In this approach we identified 13 proteins (Supplementary Table S1, column "w/o Grx1"; proteins bind unspecifically).

Besides reducing protein-SSG *via* a monothiol-mechanism, PfGrx1 can also reduce disulfides *via* a dithiol-mechanism and the emerging free sulfhydryl moieties can subsequently bind biotin-maleimide. To elucidate this aspect, we performed a parallel experiment using a mutant of PfGrx1 for the deglutathionylation step, where the resolving cysteine in the active site was mutated to serine ($PfGrx1^{C32S}$). In contrast to the wild type, this mutant can only catalyze a deglutathionylation but not a disulfide reduction reaction and is therefore very specific for deglutathionylation (Supplementary Table S1, column "$Grx1^{C32S}$").

Verification of S*-glutathionylation on selected proteins by protein immunoblotting*

We confirmed *S*-glutathionylation for some of the captured proteins by incubating the recombinant proteins with different GSSG concentrations and performing antiglutathione

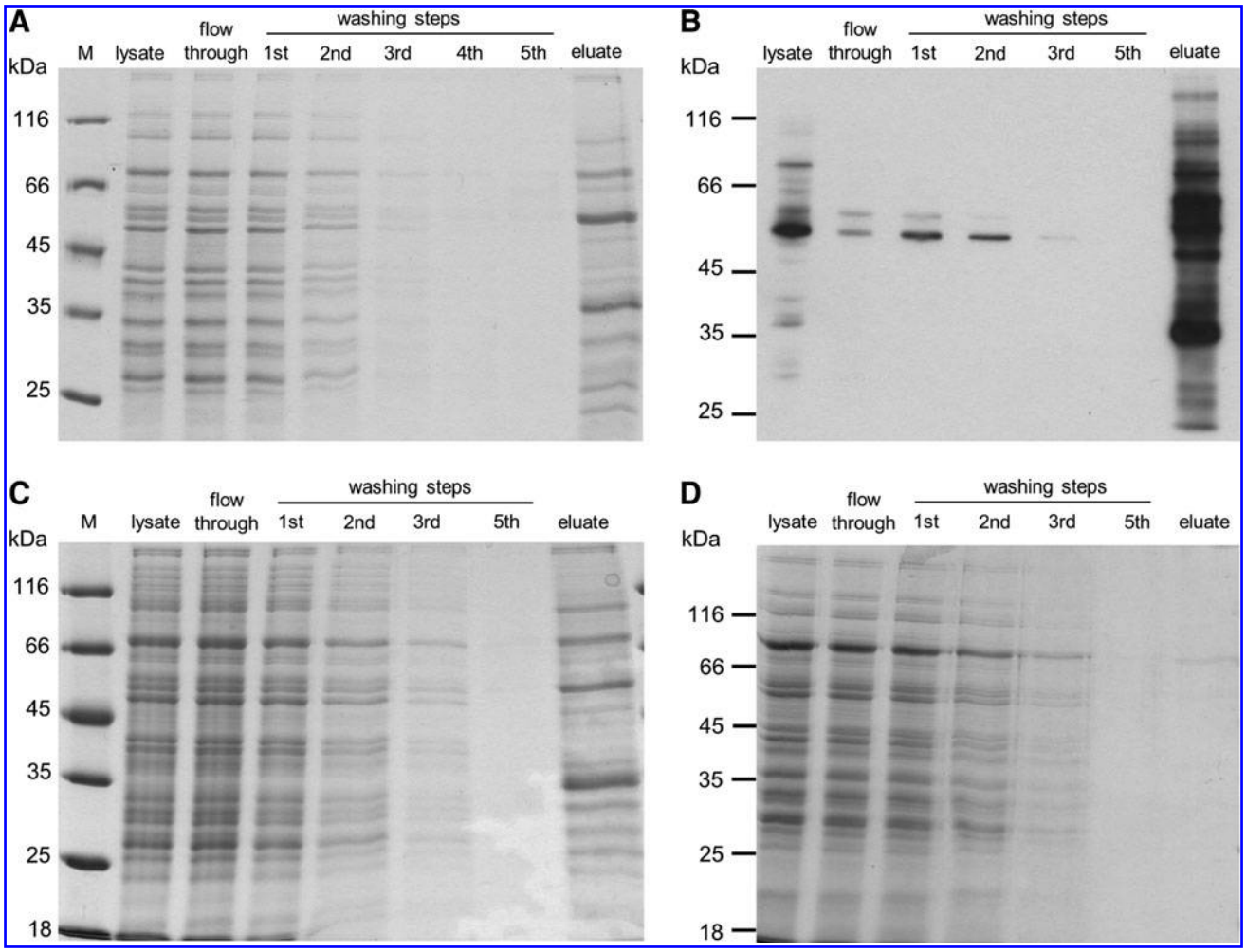

FIG. 1. *S*-glutathionylated proteins in *Plasmodium falciparum* cell extract. (A) Sodium dodecyl sulfate–polyacrylamide gel electrophoresis profile and **(B)** western blot using an antibiotin antibody (Santa Cruz Biotechnology; 1:200) of *S*-glutathionylated proteins isolated *via* glutaredoxin-dependent deglutathionylation and affinity purification of biotin-maleimide-tagged proteins. **(C)** Control experiment using the mutant PfGrx1^{C32S} for the deglutathionylation step. **(D)** Control experiment without both deglutathionylation and biotin-maleimide shows hardly any protein binding to the avidin resin. PfGrx1, glutaredoxin.

(Virogen, Watertown, MA) Western blots under nonreducing conditions. Proteins were tested for antibody cross-reactivity, and reversibility of *S*-glutathionylation was examined by adding dithiothreitol (DTT). We confirmed that Pf thioredoxin peroxidase 1 (TPx1), Pf thioredoxin 1 (Trx1), Pf thioredoxin reductase (TrxR), PfGR, Pf plasmoredoxin (Plrx), PfGST, Pf mitochondrial dihydrolipoamide dehydrogenase (mLipDH), Pf glutamate dehydrogenase 1 GluDH1, Pf cytosolic glyoxalase I (cGloI), Pf cytosolic glyoxalase II (cGloII), Pf lactate dehydrogenase (LDH), PfGAPDH, and Pf phosphoglycerate mutase (PGM) can be reversibly modified by *S*-glutathionylation (Supplementary Fig. S1). Cross-reactivity of the antibody with examined proteins could either not be observed or was negligible when compared with the signal of the respective glutathionylated protein sample.

In case of PfGST, we tested *S*-glutathionylation of its two cysteine residues by analyzing mutants lacking either one or both cysteine residues (PfGST^{C86A}, PfGST^{C101A}, PfGST$^{C86/101A}$). Glutathione was found to bind to both cysteine residues, since only the double mutant was not glutathionylated in western blots (Supplementary Fig. S1).

Adenylate kinase 1 (PfAK1) was identified in all *S*-glutathionylation experiments and found to bind unspecifically to the avidin material. As a specificity control, we tested whether PfAK1 can be *S*-glutathionylated *in vitro*. After incubation of PfAK1 with 10 mM GSSG, we performed nonreducing western blots, which confirmed that PfAK1 cannot be glutathionylated (Supplementary Fig. S1). These experiments verify the results from the identification approach and thus support the quality and sensitivity of the applied method. Protein deglutathionylation by dithiol oxidoreductases such as PfGrx1, PfTrx1, and PfPlrx was also studied exemplarily both by antiglutathione western blot and enzymatically (described below).

Regulation of PfOAT by GSH and GSSG

P. falciparum ornithine δ-aminotransferase (OAT) has been identified reproducibly as a target of *S*-glutathionylation (Supplementary Table S1). Incubation of PfOAT with GSSG results in a concentration- and time-dependent inhibition of

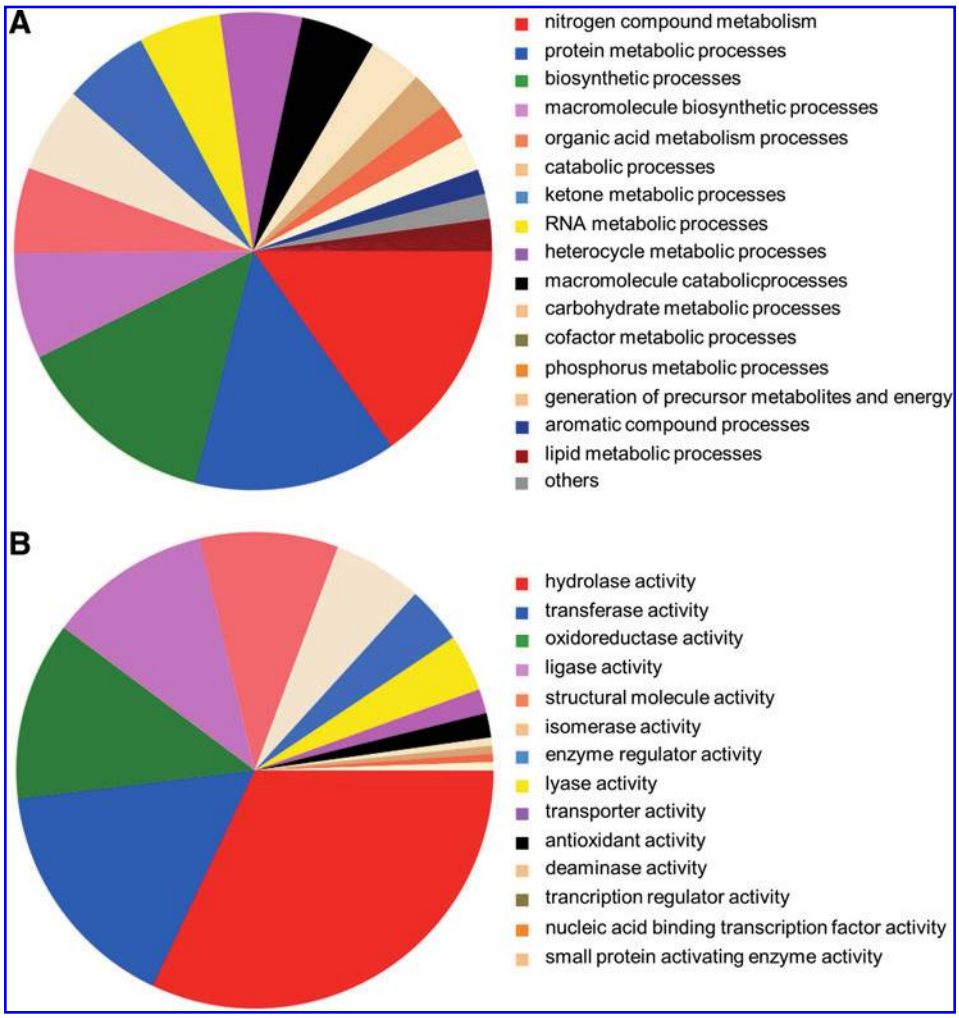

FIG. 2. Functional classification of the *S*-glutathionylated proteins identified with wild-type glutaredoxin 1 (dithiol-plus monothiol-mechanism). Proteins are clustered according to cellular metabolic processes **(A)** and according to their catalytic activity **(B)**. Only annotated proteins were used for analysis and classified using go annotations (downloaded from www.geneontology.org, accessed March 23, 2011) and the program PatternLab for Proteomics (24, 25). (To see this illustration in color the reader is referred to the web version of this article at www.liebertonline.com/ars).

PfOAT activity (Fig. 3A, C). The activity of PfOAT decreases rapidly, leaving <20% residual activity after 10 min of incubation with 1 mM GSSG. The effect is highly specific; already 10 μM GSSG inhibited PfOAT to 76% of its initial activity. Cysteine residues 154 and 163 can form a disulfide and are known to interfere with substrate binding of PfOAT (29). Thus, most likely *S*-glutathionylation of these cysteine residues inhibits PfOAT activity. The opposite effect can be observed after incubation of PfOAT with GSH, which leads to an increase in PfOAT activity (Fig. 3B, D). Incubation with 10 mM GSH enhances PfOAT activity to 700%, most likely due to the reduction of the Cys154-Cys163 disulfide. Reduction of this disulfide has previously been shown to mediate PfOAT activation by Trx (29).

Inactivation of PfGAPDH by GSSG

Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) is one of the best studied enzymes regulated by *S*-glutathionylation (28, 49), but *Plasmodium* GAPDH has not been reported to be redox-regulated before. Enzymatic assays demonstrate that PfGAPDH is nearly completely inhibited by GSSG, but not influenced by GSH. The degree of inhibition depends both on GSSG concentration and incubation time (Fig. 4A, B). Previous studies on GAPDH from other organisms reported that a highly conserved cysteine residue located in the active site is a target of thiol modifications that causes inhibition of the enzymatic activity (44, 49).

The substrate glyceraldehyde 3-phosphate (GAP) forms a covalent intermediate with the active site cysteine of GAPDH, and protects it from oxidation (49). To test if the observed inhibition of PfGAPDH is mediated by the binding of GSSG to the catalytic cysteine residue (Cys153), we added either the substrate (GAP) or the cofactor (NAD^+) to the incubation of PfGAPDH with GSSG. Both GAP and NAD^+ efficiently protect PfGAPDH from inactivation by GSSG, thereby indicating that the activity decrease observed with only GSSG in the incubation is caused by *S*-glutathionylation of the catalytic cysteine (Fig. 4C).

In general, deglutathionylation can be efficiently catalyzed by glutaredoxins (20, 23) and Trxs (24). We incubated glutathionylated PfGAPDH with 1–10 μM of prereduced PfGrx1, PfTrx1, or PfPlrx and monitored the activity before and after the incubation. All three redoxins are able to reactivate PfGAPDH and thus efficiently catalyze PfGAPDH deglutathionylation (Fig. 5).

Influence of S*-glutathionylation on the activity of PfPK*

The activity of *P. falciparum* pyruvate kinase (PfPK) is inhibited by GSSG, but not influenced by GSH. The

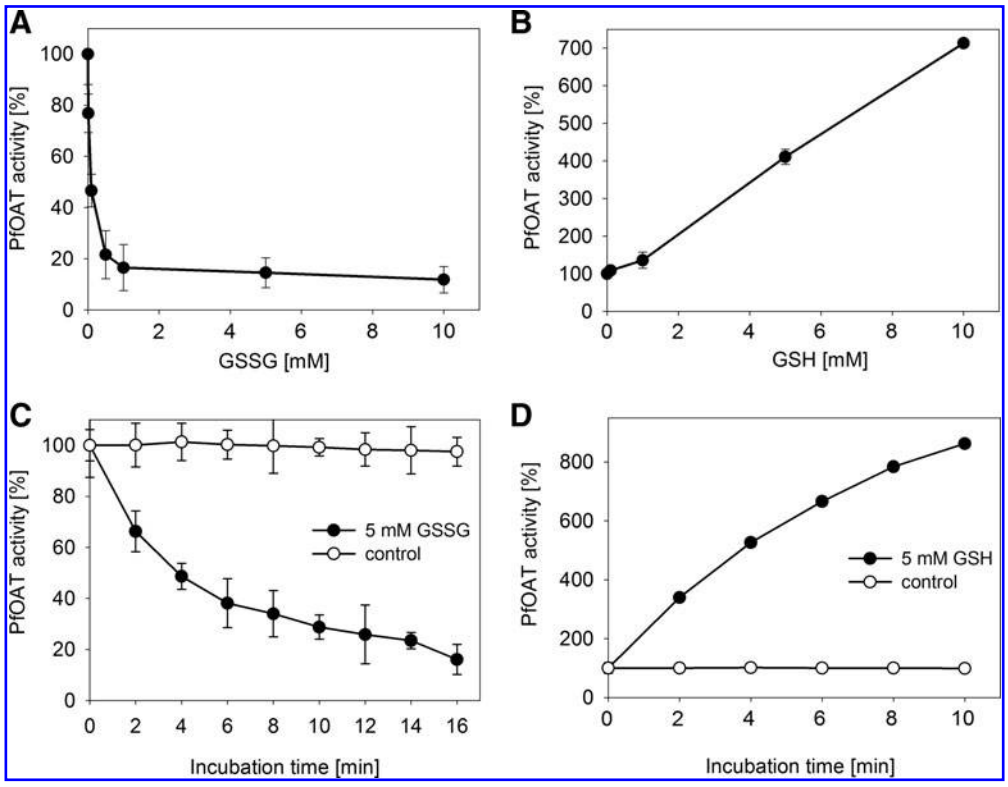

FIG. 3. Regulation of PfOAT activity by glutathione. (A) PfOAT was incubated with different concentrations of GSSG for 10 min at 37°C and **(B)** with different concentrations of GSH for 5 min at 37°C. **(C)** Time-dependent regulation of PfOAT with 5 mM GSSG and **(D)** 5 mM GSH. The samples were incubated at 37°C and aliquots were taken every 2 min to monitor the activity. Each value is a mean value from three independent determinations each including 6–8 measurements. Data are represented as mean ± standard deviation. GSH, reduced glutathione; GSSG, oxidized glutathione disulfide; OAT, ornithine δ-aminotransferase.

inhibitory effect of GSSG requires higher concentrations of GSSG compared with PfGAPDH and PfOAT, but shows a clearly concentration-dependent inhibition. Less than 20% residual activity of PfPK remains in the presence of 10 mM GSSG. This inhibition can be partially reversed to 70% of its initial activity by DTT (Fig. 6A, B). Further, PfPK can be enzymatically deglutathionylated by PfTrx1, PfGrx1, and PfPlrx as shown by protein immunoblotting (Fig. 7B).

S-*glutathionylation of PfTPx1*

We could not detect any effect of PfTPx1 *S*-glutathionylation on the Trx-dependent hydroperoxide reducing activity of the enzyme. However, this is most likely due to the presence of Trx in the assay, which can efficiently deglutathionylate PfTPx1 at low concentrations, as shown by anti-GSH immunoblotting of glutathionylated PfTPx1 with prereduced PfTrx1 (Fig. 7A). Under nonreducing conditions or conditions where only a limited amount of reducing agents is added, PfTPx1 runs as a dimer (Fig. 7A). Further, it has been hypothesized previously that the second (lower) band of PfTPx1 corresponds to the oxidized conformation of PfTPx1 (39). This putative redox-dependent conformational switch might represent a regulatory process that deserves to be studied in further detail (39).

S-*glutathionylation has no major effect on the enzymatic activities of PfLDH, PfcGloI, PfcGloII, and PfTrxR*

Although we could confirm that PfLDH can be reversibly *S*-glutathionylated (Supplementary Fig. S1), incubation of PfLDH with GSSG or GSH influenced neither the reduction of pyruvate to lactate nor the oxidation of lactate to pyruvate. Similar results were obtained for two *Plasmodium* glyoxalases, cGloI and cGloII. The enzymes can be reversibly *S*-glutathionylated (Supplementary Fig. S1), but the modification has no detectable effect on the activity of the enzymes.

Western blot analysis clearly confirms that PfTrxR can be *S*-glutathionylated (Supplementary Fig. S1), but in the Trx-reducing assay no activity change of *S*-glutathionylated PfTrxR compared with nonglutathionylated PfTrxR could be observed. To exclude that the missing effect is due to the deglutathionylating properties of Trx1, we measured the activity of *S*-glutathionylated PfTrxR by using the artificial substrate DTNB. However, no major change in activity (< 10%) was detected in the DTNB assay, either. One possible explanation for these results is that while Trx is reduced by the C-terminal redox center alone, DTNB can also be reduced by the N-terminal redox center of PfTrxR (31). Since *S*-glutathionylation might predominantly affect the highly accessible C-terminal redox-center, no inhibitory effect is necessarily determined with the substrate DTNB.

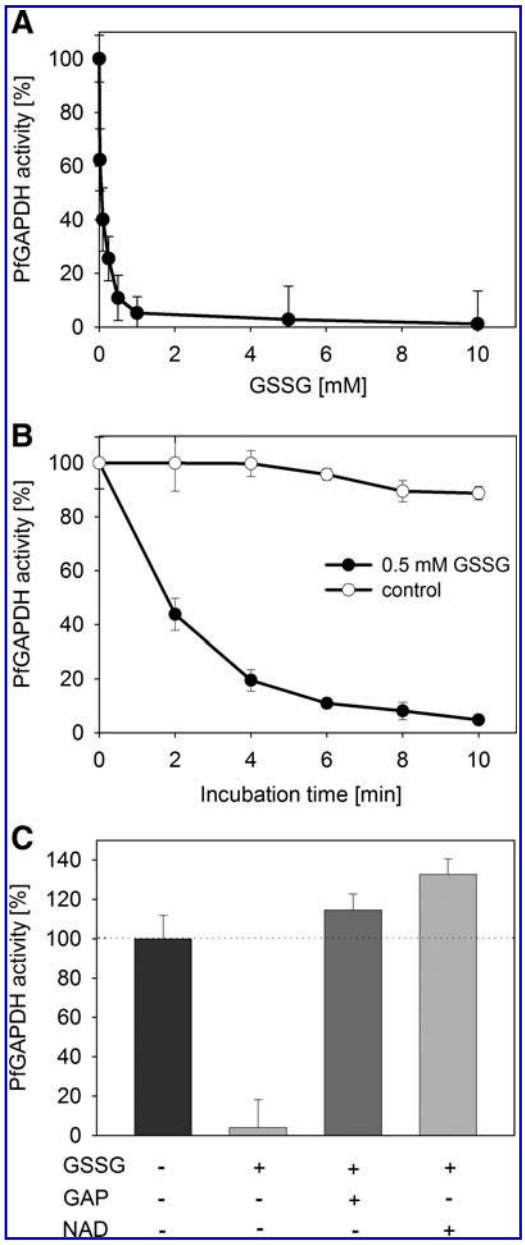

FIG. 4. Regulation of PfGAPDH activity by glutathione. **(A)** Concentration-dependent regulation of PfGAPDH when incubated with different GSSG concentrations for 5 min at 37°C. **(B)** Time-dependent regulation of PfGAPDH when incubated with 0.5 mM GSSG or with buffer (control) for 10 min at 37°C. Activity was monitored every 2 min. **(C)** Protection of PfGAPDH from GSSG-mediated inhibition by the substrates GAP and NAD^+. PfGAPDH was incubated with 0.5 mM GAP or 1 mM NAD^+ together with 10 mM GSSG for 5 min at 37°C. Each value is a mean value from at least three independent determinations each including five measurements. Data are represented as mean ± standard deviation. GAP, glyceraldehyde 3-phosphate; PfGAPDH, *P. falciparum* glyceraldehyde 3-phosphate dehydrogenase.

Discussion

Protein *S*-glutathionylation is gaining more and more scientific attention as a redox switch. Here, we examined the glutathionylome of the malaria parasite *P. falciparum* by a highly specific and sensitive method based on enzymatic deglutathionylation of mixed protein-SSG disulfides by glutaredoxin in cell extracts. Several other methods have been developed for identifying substrates of protein *S*-glutathionylation. Radiolabeling of the cellular glutathione pool by ^{35}S-cysteine followed by oxidative treatment to enhance *S*-thiolation is the most common method (19, 35). However, this method requires pretreatment of cells with protein synthesis inhibitors, potentially causing disturbances of cellular processes. Further, the approach does not distinguish between different types of *S*-thiolation and identifies proteins that are *S*-thiolated during treatment with oxidants, not reflecting *S*-glutathionylation under basal conditions [reviewed in (22)]. Another method, based on biotinylated glutathione, can be used in combination with affinity purification both *in vivo* and *in vitro*, but the biotin-tag can disturb the interaction between glutathione and the protein (22). Studies using antiglutathione antibodies in combination with immunoprecipitation are limited by their low sensitivity and thus a low number of detected proteins in cellular extracts (7). The identification of glutathionylated proteins by glutaredoxin reduction applied here allows the detection of proteins that are *S*-glutathionylated under physiological conditions, since the cells are not treated with protein synthesis inhibitors or oxidants (2, 32, 41). However, substantial knowledge on the specificity of deglutathionylation reactions by different dithiol oxidoreductases is missing. Further, it has to be considered that only proteins deglutathionylated by PfGrx1 are identified; proteins possibly deglutathionylated by, for example, *Plasmodium* Trx1, are not identified.

Using this strategy, we reproducibly identified 321 *S*-glutathionylated proteins with a predicted function in *P. falciparum* (Supplementary Table S1). An earlier study identified only 43 *S*-glutathionylated proteins in human ECV304 cells (32). Most likely the difference is due to the identification method used to detect isolated *S*-glutathionylated proteins in the elution fraction. In our case a highly sensitive LTQ-Orbitrap-based mass spectrometric analysis was used to detect target proteins directly. In contrast, Lind *et al.* separated the eluted proteins by two-dimensional gel electrophoresis and used MALDI-TOF to identify excised protein spots (32). This leads to a substantial decrease in sensitivity, because only highly abundant proteins in the range of a certain *pI* and molecular weight are recovered. In our approach, we identified proteins with a molecular weight ranging from below 10 kDa up to >200 kDa (Supplementary Table S1). Moreover, low abundance proteins are unlikely to be detected by excising protein spots from a gel, but are covered by our approach. High-resolution high-accuracy mass spectrometry also allowed the identification of more than 1000 proteins that undergo lysine acetylation (12), another post-translational modification.

To follow up our results on the glutathionylome of *Plasmodium* and prove the accuracy of the method, we validated the *S*-glutathionylation for 15 target proteins by western blot using recombinant proteins and an antiglutathione antibody. For the glutathionylated proteins, we could demonstrate that the modification is reversible, since glutathione could be removed by DTT (Supplementary Fig. S1). The antiglutathione antibody appears to be highly specific for glutathione, since we observe almost no cross-reactivity with the unglutathionylated proteins (discussed in Results section).

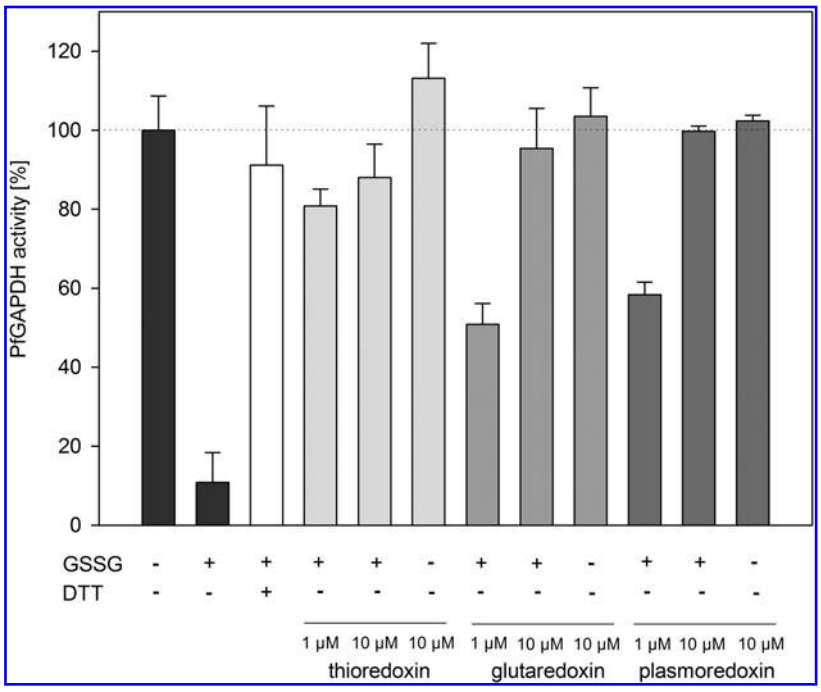

FIG. 5. Deglutathionylation of PfGAPDH by thioredoxin, glutaredoxin, and plasmoredoxin. PfGAPDH was incubated with 0.5 mM GSSG for 5 min at 37°C, excess GSSG was removed, and the *S*-glutathionylated PfGAPDH was incubated with DTT, or pre-reduced PfTrx, PfGrx, and PfPlrx for 5 min at 37°C. Each value is a mean value from at least two independent determinations each including six measurements. Data are represented as mean ± standard deviation. Plrx, plasmoredoxin.

We studied the influence of the observed *S*-glutathionylation on the enzymatic activity of three proteins: PfGAPDH, PfOAT, and PfPK. We chose GAPDH as an intensely studied enzyme in mammals and plants, which is inactivated by *S*-glutathionylation of its highly conserved active site cysteine (28, 33, 49). *Plasmodium* GAPDH was also detected to be susceptible to glutathionylation of the active site cysteine, and is inactivated by low glutathione concentrations, which are likely to reflect physiological concentrations (Fig. 4). PfGAPDH can be efficiently deglutathionylated and thus activated by PfTrx1, PfGrx1, and PfPlrx (Fig. 5). PfTrx1, PfGrx1, and PfPlrx have been shown to be involved in redox regulation of several proteins before (45). Besides its function in glycolysis, the highly redox-sensitive GAPDH is involved in several cellular processes such as transcription (50), apoptosis (26), and calcium homeostasis (37). The importance of the enzyme is underlined by the finding that GAPDH is essential for the survival of human pathogens such as *Trypanosoma brucei* (8).

Malaria parasites require glycolysis, since the second most common human enzyme disorder, PK-deficiency, protects against *Plasmodium* infection in human erythrocytes (3). *P. falciparum* PK is reversibly inactivated by *S*-glutathionylation (Fig. 6), indicating a role of cysteine residues in the catalytic mechanism. This has been suggested for human PK from red blood cells before, which is highly susceptible to inactivation by increased oxidative stress, but its activity can be recovered by glutathione (36). Despite its enormous potential as an antimalarial drug target, *Plasmodium* PK is barely studied (10), but has been identified as a putative target of PfTrx1, PfGrx1, and PfPlrx (45). This is also the case for PfOAT, which is redox-regulated by PfTrx1 *via* two critical cysteine residues that interfere with substrate binding (29). This motivated us to test whether the activity of PfOAT is affected by *S*-glutathionylation. Interestingly, PfOAT is differentially regulated by GSH and GSSG presumably by different mechanisms (Fig. 3): GSSG inhibits PfOAT by *S*-glutathionylation of functionally critical cysteine residues (presumably Cys136 and Cys154), whereas GSH is likely to reduce the disulfide between these cysteines, leading to an activation of PfOAT comparable to that induced by PfTrx1.

Further, we could show very recently that both enzymatic activities of the bifunctional enzyme glucose 6-phosphate

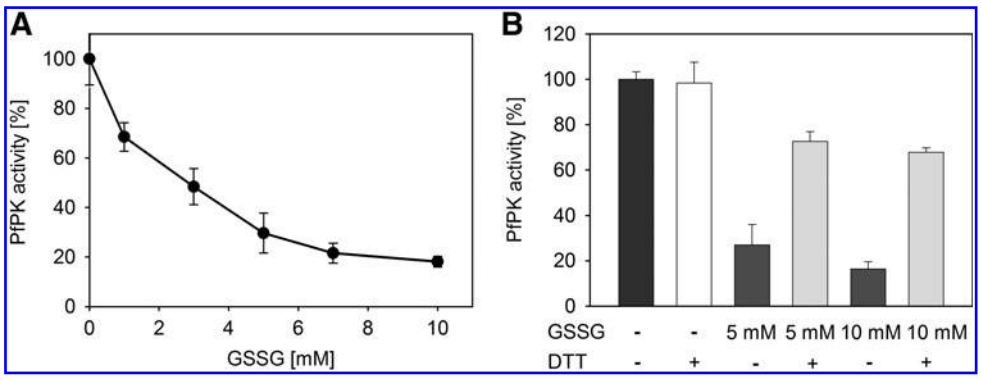

FIG. 6. Inhibition of PfPK by *S*-glutathionylation. (A) Concentration-dependent inhibition of PfPK by GSSG. **(B)** Reversibility of the GSSG-mediated inhibition of PfPK. Each value is a mean value from three independent determinations each including five measurements. Data are represented as mean ± standard deviation.

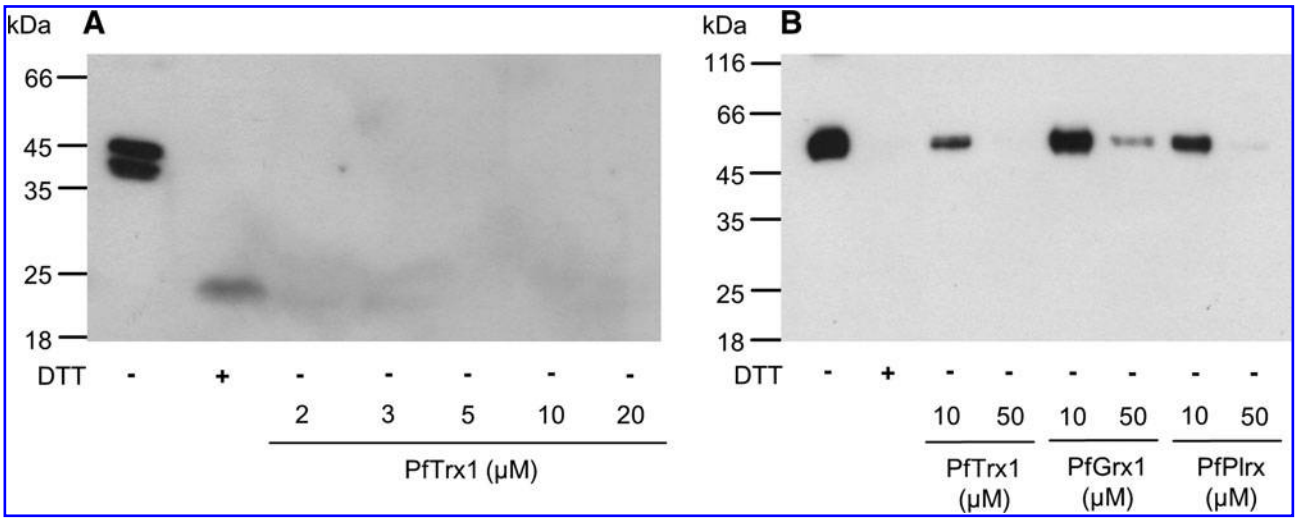

FIG. 7. Enzymatic deglutathionylation of (A) PfTPx1 and (B) PfPK by *P. falciparum* redoxins. PfTPx1 and PfPK were incubated with 10 mM GSSG over night at 23°C, and excess GSSG was removed by gel filtration chromatography. Glutathionylated PfTPx1 (4.5 μM) and PfPK (9 μM) were incubated with different concentrations of reduced PfTrx1, PfGrx1, and/or PfPlrx for 10 min at 37°C.

dehydrogenase 6-phosphogluconolactonase from *P. falciparum* are regulated by *S*-glutathionylation (30).

Not all enzymes examined here changed their activity as a consequence of *S*-glutathionylation: PfLDH as well as the glyoxalases PfcGloI and PfcGloII can be *S*-glutathionylated, but their enzymatic activity is not regulated by the modification (Supplementary Table S1 and Fig. S1). Interestingly, human cytosolic glyoxalase I is strongly inhibited by *S*-glutathionylation on cysteine 139 (corresponding to Cys311 in PfcGloI) (6). However, *S*-glutathionylation of PfLDH, PfcGloI, and PfcGloII might influence their oligomerization behavior or protein–protein interactions, which remains to be studied in detail.

The mechanism of protein (de)glutathionylation *in vivo* is still under investigation and not completely understood. The thiol disulfide oxidoreductase glutaredoxin is the best studied enzyme in terms of protein (de)glutathionylation and is supposed to catalyze the main part of protein deglutathionylation in mammalian cells (13), but also Trx has recently been reported to efficiently deglutathionylate protein targets in *Saccharomyces cerevisiae* (24). Here, we examined whether PfGrx1, PfTrx1, and/or PfPlrx can efficiently catalyze the deglutathionylation of PfGAPDH, PfTPx1, and PfPK using protein immunoblotting and enzymatic assays. Our results demonstrate a deglutathionylating activity not only for PfTrx and PfGrx, but also for PfPlrx, which is a new function attributed to this unique plasmodial protein.

Materials and Methods

Cultivation of P. falciparum *and preparation of parasite cell extract*

Intraerythrocytic stages of *P. falciparum* (3D7) were maintained in culture and prepared using standard procedures. Details are described in the Supplementary Materials and Methods.

Preparation of cell extracts, blocking of residual protein thiols, deglutathionylation, biotin-NEM tagging of deglutathionylated thiols, and purification of biotinylated proteins

Biotin-NEM-tagged proteins were obtained as previously described (2, 25, 32, 41), with slight modifications as described in Supplementary Materials and Methods.

Sample preparation for mass spectrometry

Protein fractions were prepared for MS-analysis using standard procedures as described in Supplementary Materials and Methods.

Multidimensional protein identification technology

The trypsin-digested protein samples were analyzed using multidimensional protein identification technology with slight variations depending on the protein amount of the sample as described in the Supplementary Materials and Methods.

Analysis of tandem mass spectra

MS/MS spectra were analyzed using the following software analysis protocol. Poor quality spectra were removed from the dataset using an automated spectral quality assessment algorithm (5). MS/MS spectra remaining after filtering were searched with the ProLuCID algorithm (48) against the NCBI-RefSeq *P. falciparum* database (8/24/2005) concatenated to a decoy database in which the sequence for each entry in the original database was reversed (38). All searches were parallelized and performed on a Beowulf computer cluster consisting of 100 1.2 GHz Athlon CPUs (42). Differential modifications of maleimide (+125.0477), biotin-maleimide (+451.1889), and iodoacetamide (+57.02146) were considered on cysteines. Peptides within 3 amu mass tolerance

of the precursor mass and have one or two tryptic ends were considered during the database searches. ProLuCID results were assembled and filtered using the DTASelect (version 2.0) program (15, 46). DTASelect 2.0 uses a linear discriminant analysis to dynamically set XCorr and DeltaCN thresholds for the entire dataset to achieve a user-specified false-positive rate (5% in this analysis). The false-positive rates are estimated by the program from the number and quality of spectral matches to the decoy database.

Construction of P. falciparum *expression plasmids and heterologous overexpression*

Recombinant proteins from *P. falciparum* (PfOAT, PfmLipDH, PfcGloI and II, PfGrx1 und PfGrx1^{C32S}, PfPlrx, PfTPx1, PfTrx1, PfTrxR, PfGR, PfGST, PfPK, PfGAPDH, PfLDH, PfPGM, and PfAK1) were produced and purified as described in the Supplementary Materials and Methods.

Protein immunoblotting analysis

Reduced proteins (0.4 mg/ml) were incubated with 0.1–10 mM GSSG in 50 mM Tris, 1 mM EDTA, pH 7.4 for 5 min at 37°C. Deglutathionylation experiments were performed by incubating *S*-glutathionylated proteins (0.4 mg/ml) with 2–50 μM reduced PfTrx1, PfGrx1, and/or PfPlrx for 10 min at 37°C. To detect recombinant *S*-glutathionylated proteins, western blots were performed under nonreducing conditions, using a monoclonal antiglutathione antibody (Virogen; diluted 1:500 in 5% nonfat milk with Tris-buffered saline Tween-20). Details are described in Supplementary Materials and Methods.

Enzyme activity assays and enzyme treatment

The enzymatic activity for PfGADPH, PfPK, PfLDH PfOAT, PfTPx1, PfcGloI, PfcGloII, and PfTrxR was measured with standard spectrophotometrically methods as described in the Supplementary Materials and Methods.

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Author Disclosure Statement

The authors have nothing to declare.

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List of Abbreviations

AK = adenylate kinase
cGloI = cytosolic glyoxalase I
cGloII = cytosolic glyoxalase II
GAP = glyceraldehyde 3-phosphate
GAPDH = glyceraldehyde 3-phosphate dehydrogenase
GluDH1 = glutamate dehydrogenase
GR = glutathione reductase
Grx = glutaredoxin
GSH = reduced glutathione
GSSG = oxidized glutathione disulfide
GST = glutathione *S*-transferase
LDH = lactate dehydrogenase
LTQ = linear trap quadrupole
mLipDH = mitochondrial dihydrolipoamide dehydrogenase
OAT = ornithine δ-aminotransferase
PGM = phosphoglycerate mutase
PK = pyruvate kinase
Plrx = plasmoredoxin
protein-SSG = mixed protein-glutathione disulfide
TPx = thioredoxin peroxidase
Trx = thioredoxin
TrxR = thioredoxin reductase 1

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