**Plasmodium falciparum** Possesses a Classical Glutaredoxin and a Second, Glutaredoxin-like Protein with a PICOT Homology Domain*

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The genes coding for two different proteins with homologies to glutaredoxins have been identified in the genome of the malarial parasite *Plasmodium falciparum*. Both genes were amplified from a gametocyte cDNA and over-expressed in *Escherichia coli*. The smaller protein (named PfGrx-1) with 12.4 kDa in size exhibits the typical glutaredoxin active site motif “CPYC,” shows glutathione-dependent glutaredoxin activity in the β-hydroxyethyl disulfide (HEDS) assay, and reduces *Trypanosoma brucei* ribonucleotide reductase. Glutathione:HEDS transhydrogenase activity (approximately 60 milliunits/mg of protein) was clearly detectable in trophozoite extracts from *Plasmodium falciparum* of the malarial parasite. Five different antimalarial drugs at 100 μM did not significantly influence isolated PfGrx-1 activity. In contrast, the second protein (deduced mass 19.9 kDa) with homology to glutaredoxins (31% identity to Schizosaccharomyces pombe in a 140-amino acid overlap) was not active in the HEDS assay; however, its general dithiol reducing activity was demonstrated in the insulin assay in the presence of dithiothreitol. Interestingly, the sequence contains a PICO'T (for protein kinase C-interacting cousin of thioredoxin) homology domain, which might suggest regulatory functions of the protein. We named this protein PfGLP-1, for *P. falciparum* 1-Cys-glutaredoxin-like protein-1. In contrast to glutaredoxin, PfGLP-1 could not be reduced by glutathione. This is the first report on glutaredoxin-like proteins in the family of *Plasmodia*.

Glutaredoxins (Grx), also called thioltransferases, are small heat-stable thiol-disulfide oxidoreductases that have been conserved throughout evolution. They are ubiquitously distributed and were found first in bacteria (1), then also in euarkia (2), and later in archaea (3, 4) and in plants (5, 6). Glutaredoxins belong to the thioredoxin superfamily, of which thioredoxins (Trx), tryaredoxin, and protein-disulfide isomerases are further members (7). They all show a similar structure, the “thioredoxin or glutaredoxin fold,” which consists of a central four-stranded β-sheet surrounded by α-helices. As shown for yeast, at least one out of four Grx and Trx genes must be present for viability (8).

Classical glutaredoxins have an active site with two conserved cysteine residues, Cys-Pro-Tyr-Cys, of which the N-terminal cysteine has been shown to be essential for both protein-disulfide reduction and reduction of mixed protein-glutathione disulfides (9). The C-terminal cysteine residue is required for protein-disulfide reduction but not for reduction of mixed protein disulfides. Glutaredoxin contributes to a range of important cellular processes. It is an important redox active protein protecting against oxidative damage (10), it serves as a hydrogen donor for ribonucleotide reductase (7, 11), and it is associated with transcriptional control (12). Furthermore, Grx is involved in vaccinia virion morphogenesis (13) and has even been detected in human immunodeficiency virus-1 (14). Many organisms including *Escherichia coli*, yeast, and mammals have more than one Grx. Even in the phage T4, where genetic space is limited, two glutaredoxins are encoded (15). However, as shown by genome sequencing, there are also organisms like *Mycoplasma*, which appear to lack a glutathione/glutaredoxin system (16). The transcription of genes contributing to Grx and Trx pathways and related enzymes is regulated in bacteria in response to oxidative stress (17). Recently, a number of Grx-like proteins including Grx3 to Grx5 of *Saccharomyces cerevisiae* and also the second glutaredoxin-like protein studied here have been grouped into one family of so-called PICO'T-HD (protein kinase C-interacting cousin of thioredoxin homology domain)-containing proteins of yet unknown function (18, 19).

The malarial parasite *Plasmodium falciparum* is known to be exposed to high fluxes of reactive oxygen species. The parasite multiplies in erythrocytes, an environment of high oxygen tension. Furthermore, toxic heme products, derived from heme degradation by the parasite, represent a continuous source of reactive oxygen species. Therefore, proteins involved in antioxidant defense are promising targets for antimalarial drug development (20).

*P. falciparum* has recently been shown to possess a functional thioredoxin system comprising NADPH, thioredoxin reductase (TrxR), and thioredoxin (21, 22). Furthermore, thioredoxin-dependent peroxidases have recently been characterized (23) as well as a 1-Cys-peroxiredoxin with yet unknown dependence (24). Glutathione peroxidase of *P. falciparum* (25) has also been shown to have a preference for thioredoxin and was therefore renamed thioredoxin-dependent peroxidase (26). In parallel, a functional glutathione system comprising NADPH, an FAD-dependent homodimeric glutathione reductase (PGR), and glutathione exists in *P. falciparum*. PGR has been studied in detail (27–29). Here we describe for the first time the presence of a functional glutaredoxin in *P. falciparum*, and demonstrate the presence of a...
Glutaredoxins of *P. falciparum*

**TABLE I**

| Property | PfGrx-1 | PGlp-L |
|----------|---------|---------|
| Accession no. | GenBank™ AF276083 | GenBank™ AY014839 |
| Location in *P. falciparum* genome | Chromosome 3 | Chromosome 3 |
| Genomic DNA numbering | bp 118703–117655 (according to GenBank™ AL034558), comprises 5 exons | bp 41725–42480 (according to GenBank™ AL034558, comprises 3 exons) |
| mRNA | 336 bp (with start and stop codon) | 384 bp (with start and stop codon) |
| Amino acids | 111 | 124.4 kDa |
| Molecular mass | 12.4 kDa | 37134 |
| Highest identity on amino acid level | 71% to a putative *P. berghei* Grx (GenBank™ accession no. AF288686) | 41.5% to *S. cerevisiae* Grx5 (acc. no. NP_015266) |
| Active site motif | ^CPYC$^\alpha$ (without MTS) | ^CGFS$^\alpha$ (without MTS) |
| Isoelectric point | 7.9 | 6.74 (10.02 with MTS) |
| $\varepsilon_{280}$ molecular extinction coefficient$^\theta$ | 9.62 mm$^{-1}$ cm$^{-1}$ | 18.52 mm$^{-1}$ cm$^{-1}$ |
| pH optimum | ~8.6 (8.5) | 6.74 (10.02 with MTS) |
| Specific activity$^\alpha$ | 47 units/mg | 18.52 mm$^{-1}$ cm$^{-1}$ |
| $K_m$ for HEDS$^\theta$ | 700 $\mu$M | 18.52 mm$^{-1}$ cm$^{-1}$ |
| $K_m$ for GSH$^\theta$ | >4 $\mu$M | 18.52 mm$^{-1}$ cm$^{-1}$ |

$^\alpha$ Deduced, without His tag

$^\theta$ Specific activity and $K_m$ values were determined in 100 mM Tris-HCl, 1 mM EDTA, pH 8.0, in the presence of 1 mM GSH, 1 unit/ml GR, and 735 $\mu$M HEDS at 25 °C. For $K_m$ values the respective substrate concentrations were systematically varied (300 $\mu$L to 4 mM for GSH and 150–735 $\mu$L for HEDS). In a reference cuvette without Grx, the spontaneous reaction between HEDS and GSH was subtracted.

**EXPERIMENTAL PROCEDURES**

**Materials**—All chemicals used were of the highest available purity and were obtained from Roth or Merck, HEDS was from Lancaster, and insulin and the inhibitors studied were obtained from Sigma/Aldrich. The cloning vector pBluescript SK (Stratagene, La Jolla, CA) was used as a vector for cloning glutaredoxin-like genes. Competent cells were transformed with pBluescript SK (provided by Prof. David Kaslow, National Institutes of Health, Bethesda, MD) (30) and used as template to amplify the two glutaredoxin-like genes by PCR. The PCR was carried out with *P. falciparum* primers for the open reading frames of two different glutaredoxin-like genes were identified by online screening of the *P. falciparum* genome sequencing project on chromosome 3 (www.ncbi.nlm.nih.gov/Malaria/plasmodium-buscus.html). Four homologous primers were derived from these genes. For subsequent cloning procedures, restriction site sequences (underlined) were introduced for BanHI and HindIII at the 5' end of the respective primers (primer for PfGrx-1, N-terminal (Opfglx-1); 5' - CGCCGGAATCCTGCTATACATTCGACATTTATTTTTTTTTTTTACCC-3') and C-terminal (Opfglx-1r; 5' - GGCGGAGTTCATGGCTGATCTAATTTGTTTTATTTATTATTATTTTTTACCC-3'); primer for Pfglp-L, N-terminal (Opfglp-lp; 5' - CGCGGGATCATGGCAGCTGCTATACATTCGACATTTATTTTTTTTTTTTACCC-3'). A gametocyte cDNA library from the *P. falciparum* strain 3D7 was kindly provided by Prof. David Kaslow (National Institutes of Health, Bethesda, MD) (30) and used as template to amplify the two glutaredoxin genes by PCR. The PCR was carried out with Taq polymerase (2.5 min at 94 °C; 94 °C, 30 s; 66 °C, 30 s; 72 °C, 45 s; 25 cycles, 72 °C, 2 min) and the derived fragments of correct size were cloned into pBluescript SK for sequencing and for subcloning into the expression vector pQE30. Expression of the Genes and Purification of the Recombinant Proteins—The *E. coli* strain M15 was used for expression of the *P. falciparum* glutaredoxin-like genes. Competent cells were transformed with the respective pQE30/grx plasmid. Five ml of LB medium was inoculated with a single colony and used as a starter culture for 250- or 500-ml cultures. Cells were grown at 37 °C in LB medium containing ampicillin (100 $\mu$g/ml) and kanamycin (50 $\mu$g/ml) to an $A_{560}$ of 0.5; subsequently, the expression was induced by adding 1 mM isopropyl-$\beta$-D-thiogalactopyranoside. Cells were grown for an additional 4 h, harvested, and directly used for protein purification or frozen at −20 °C. For purification, the cells were disintegrated by sonication in the presence of protease-inhibitors. After centrifugation, the supernatant was loaded onto a Ni²⁺-NTA column equilibrated with 50 mM sodium phosphate, 300 mM NaCl, pH 8.0. After washing the column with increasing imidazole concentrations, the respective protein was eluted with 175–200 mM imidazole; collected fractions were tested for enzymatic activity and for purity by 15% SDS gel electrophoresis. Active fractions were pooled, concentrated via ultrafiltration, and dialyzed against 50 mM Hepes, 500 mM KCl, 1 mM EDTA, pH 8.0, prior to use. Protein concentrations were determined by the Bio-Rad dye assay with bovine serum albumin as a standard and on the basis of the calculated extinction coefficient of the protein ($\varepsilon_{280}$ $\mu$L = 9.62 mm$^{-1}$ cm$^{-1}$; PGlp-L, 19.80 mm$^{-1}$ cm$^{-1}$). Glutathione: HEDS Transhydrogenase Assay—One ml of assay mixture containing 100 $\mu$L NADPH, 1 unit of PFRG (as determined with GSH as substrate; it was taken care that the PFRG concentration did not become rate-limiting), and 1 mM GSH was equilibrated to 25 °C. After addition of glutaredoxin, the reaction was started with 735 $\mu$L HEDS. NADPH consumption ($\varepsilon_{280}$ $\mu$L = 6.22 mm$^{-1}$ cm$^{-1}$) was monitored spectrophotometrically at 340 nm (11). In this coupled assay system, constantly high concentrations of reduced glutathione were maintained by the NADPH/GR system. The activities were corrected for the rate of the spontaneous chemical reaction of HEDS with GSH by using a reference cuvette that did not contain glutaredoxin.

**Insulin Assay**—One ml of reaction mixture contained 0.17 mM porcine insulin in 50 mM Tris-HCl, 1 mM EDTA, pH 7.4. The reaction was started at 25 °C by adding 1 mM DTT in the absence and presence of 10 $\mu$L PfGrx-1, PfGlp-L, and PfTrx, respectively. The precipitation of insulin reduced to A and B chains was followed turbidimetrically at 600 nm (31). Ribonucleotide Reductase Assay—Ribonucleotide reductase activity was determined from the rate of conversion of [3H]GDP into [3H]dGDP essentially as described for CDP reduction (32). The assay mixture contained in a total volume of 200 $\mu$L of 50 mM Hepes, pH 7.6, 500 $\mu$L GDP (including 1.25 $\mu$mCi of [3H]GDP), 100 $\mu$L DTT, 100 mM KCl, 6.4 mM MgCl$_2$, 400 $\mu$L NADPH, 400 millimolars of human glutathione reductase, 4 mM reduced glutathione, and various concentrations of glutaredoxin (0.1–3 $\mu$L). In the assay 1.9 $\mu$L Trypanosoma brucei ribonucleotide reductase subunit R1 with an excess of R2 (26.9 $\mu$L) was used (1 unit corresponds to 1 nmol of GDP formation/min). The reaction mixture was incubated at 37 °C for 20 min, the reaction was stopped by boiling for 10 min, and the precipitated protein was removed by centrifugation. The reaction components were dephosphorylated by 30-min incubation with 10 units of alkaline phosphatase. Guanosine, deoxyguanosine, and guanidine were separated isocratically by high performance liquid chromatography on an Aminex A9 column (250 × 4 mm) in 100 mM ammonium borate, pH 8.3, and quantified by scintillation counting (33).

**Interaction of PfGrx-1 with Antimalarial Drugs**—The effects of the antimalarial drugs chloroquine, primaquine, quinine, mefloquine blue, and artemisinin on PfGrx-1 were tested in the glutathione-HEDS transhydrogenase assay described above in the presence of 200 millimolars/ml GR. The respective drug at a concentration of 100 $\mu$L was added to the assay before the reaction was started with HEDS at 25 °C.
Reduction of PfGLP-1 and PfGrx-1 by GSH—Since PfGLP-1 was not active in the glutathione:HEDS transhydrogenase assay (see "Results"), the GSH dependence of the protein was tested in a GR-coupled reaction: Two different concentrations (8 and 16 μM) of PfGLP-1 and PfGrx-1 were incubated for 5 min at 25 °C with 1 mM GSH and 100 μM NADPH in 100 mM Tris-HCl, 1 mM EDTA, pH 8.0. The total sample volume was 1 ml. The formation of GSSG was determined by adding 1 unit/ml PfGR and measuring total NADPH consumption in comparison with control samples.

Cultivation of P. falciparum—Intraerythrocytic stages of eight different P. falciparum strains (four chloroquine-sensitive strains (HB3, S106, D10, 3D7) and four chloroquine-resistant strains (Dd2, K1, 7G8, FCR3)) were cultured in vitro according to Trager and Jensen (34), and synchronized to ring stages by the sorbitol method (35). After approximately 30 h, parasites in the trophozoite stage were isolated by suspending the red cells in a 20-fold volume of buffer containing 7 mM K2HPO4, 1 mM NaH2PO4, 1 mM NaHCO3, 58 mM KCl, 56 mM NaCl, 1 mM MgCl2, 4 mM glucose, and 0.02% saponin for 10 min at 37 °C. The pellets were washed three times, and the parasites were diluted in 150 μl of the same buffer and disrupted by freezing and thawing three times. After centrifugation, the supernatant was used for the various analyses.

Determination of Protein and HEDS-reducing Activity in Parasite Extracts—The parasite extract was diluted in buffer (150 mM KH2PO4, pH 8.0), and on the basis of the absorbance at 280 nm and the Bio-Rad protein dye assay (with bovine serum albumin as standard) the protein concentration was calculated. Glutathione:HEDS transhydrogenase activity was determined with 50 μl of parasite extract as described above for the isolated PfGrx-1, however, in the presence of 0.5 unit/ml GR.

RESULTS

Cloning and Sequencing of the Two P. falciparum Glutaredoxin Genes—Screening the Plasmodium genome sequencing data base with different known glutaredoxin sequences resulted in the identification of an expressed sequence tag clone sequence of Plasmodium berghei (submitted as GenBank accession no. AF288686). A further search with this putative P. berghei glutaredoxin-sequence led to a P. falciparum grx sequence (see Table I) which consists of 5 exons (Fig. 1). Perfect match primers were designed and a PCR was performed with PfcDNA as template. A fragment of expected size was obtained, cloned into pSK+, and completely sequenced. The sequence was in full agreement with the respective exon-sequence found in the genomic data base. The Pfgrx-1 gene is located on chromosome 3 and consists of 336 base pairs (GenBank accession no. AP2670083). The amino acid sequence contains the typical active site motif CPYC, and an alignment with the putative protein sequence of PfGrx-1 is shown in Table I.
P. falciparum Grx amino acid sequence shows high identities of 71% (Fig. 2).

A second glutaredoxin-like sequence was also found on chromosome 3 of P. falciparum. This sequence does not contain the typical active site motif CPYC. We cloned this unusual 516-base pair glutaredoxin-like gene, resubmitted the sequence to GenBank\textsuperscript{9} under the accession number AF1014839, and named the respective protein P. falciparum 1-Cys-glutaredoxin-like protein-1 (PfGLP-1).

Overexpression and Purification of Recombinant P. falciparum Glutaredoxins—The two grx genes were each inserted into the expression vector pQE30. Freshly transformed E. coli M15 cells were used for production of the recombinant proteins with a yield of 15 mg of PfGrx-1/liter of cell culture. The N-terminal hexahistidyl tag contributed by the pQE vector allowed purification over Ni\textsuperscript{2+}-NTA-agarose columns. All assays described in this paper (except for the data obtained with P. falciparum extracts) were carried out with the His-tagged proteins, the characteristics of which might differ slightly from the wild type enzymes. According to silver stained SDS gels, the proteins were >99% pure. The calculated molecular masses (of the His-tagged proteins) are 13.5 kDa for PfGrx-1 and 21 kDa for PfGLP-1. These values correspond well to the data obtained by SDS-polyacrylamide gel electrophoresis (data not shown). The concentrated glutaredoxins were stable at 4 °C over weeks.

Properties of P. falciparum Glutaredoxin-1—PfGrx-1 comprises 111 amino acids and has a deduced molecular mass of 12.4 kDa. Further characteristics of PfGrx-1 are summarized in Table I.

PfGrx-1 was found to be highly active in the glutathione:HEDS transhydrogenase assay, which represents a typical feature of glutaredoxins. The specific activity of PfGrx-1 was determined to be 47 units/mg in 100 mM Tris-HCl, 1 mM EDTA, pH 8.0. The addition of KCl to final concentrations of 50, 100, and 200 mM, respectively, did not enhance the enzyme activity. For comparability with previous studies and to guarantee appropriate activity of PfGR, we decided to perform further assays at the more physiological pH of 8.0. The addition of KCl to final concentrations of 50, 100, and 200 mM, respectively, did not enhance the enzyme activity.

To test whether PfGrx-1 shares the remarkable temperature stability with other glutaredoxins (1), we preincubated PfGrx-1 in 100 mM Tris-HCl, 1 mM EDTA, pH 8.0, at different temperatures for 10 min before testing activity in the standard glutathione:HEDS transhydrogenase assay. As shown in Fig. 3, the protein was stable up to 60 °C; even after 10 min of incubation at 90 °C, it was 50% active. Antibodies for further investigations like immunolocalization and stage-specific expression studies are presently produced.

Properties of PfGLP-1—In contrast to other glutaredoxins, PfGLP-1 lacks detectable activity in the glutathione:HEDS transhydrogenase assay. A computer algorithm (36) predicted a mitochondrial targeting sequence (MTS, with a probability of 68.5%) with a possible cleavage site (37) at position 43. The resulting protein has a size of 15 kDa. The MTS sequence was deleted from the Pfglp-1 gene by site-directed mutagenesis. The resulting gene was cloned as described above, and the respective recombinantly produced protein was named PfGLP-1-N. However, this new gene product was not active in the HEDS assay either.

We therefore tested the GSH dependence of PfGLP-1-N and PfGrx-1 (as control) in a GR-coupled reaction; PfGLP-1-N and PfGrx-1, respectively, were incubated for 5 min at 25 °C with 1 mM GSH and 100 μM NADPH. Then, the formation of GSSG...
was determined by adding 1 unit/ml PfGR and measuring total NADPH (ε_{440 nm} = 6.22 mM⁻¹ cm⁻¹) consumption. For the samples containing 16.5 μM PfGrx-1, an immediate decrease in absorbance of 0.106 was determined, which corresponds to an almost stoichiometric reduction of PfGrx-1. This result clearly supports the data from the HEDS assay and shows that PfGrx-1 is a typical glutaredoxin. For 8 and 16 μM PfGLP-1-N, however, the resulting change in absorbance did not significantly differ from control samples. This indicates that PfGLP-1-N cannot be reduced by GSH or, which is rather unlikely, is present in reduced state after purification and storage.

PfGLP-1-N and PfGrx-1 were tested as substrates of PfTrxR. In the presence of 50 milliunits/ml PfTrxR (as determined in the DTNB assay), 100 μM NADPH, and 40 μM amount of the respective substrate (in 100 mM potassium phosphate, 2 mM EDTA, pH 7.4, at 25 °C), no significant activity (ΔA/min < 0.001) was detected for PfGLP-1-N. PfGrx-1 was reduced with a ΔA/min of 0.010; the control with 40 μM PfTrx showed an immediate and complete reduction.

**PfGrx-1 and PfGLP-1-N Are Active in the Insulin Assay**—To test PfGLP-1-N and PfGrx-1 for their general thiol-disulfide oxidoreductase activity, the insulin assay was applied. In this assay the putative redox-active proteins are reduced by DTT and in turn reduce disulfide bonds in insulin. The following precipitation of insulin is followed turbidimetrically. Although this assay was originally designed for thioredoxins (31, 38), activity of glutaredoxins has also been described (39). As demonstrated in Fig. 4, 10 μM amounts of both PfGrx1 and PfGLP-1-N were able to reduce insulin with comparable activities, although not as efficiently as PfTrx. This insulin reduction was also determined at 5 and 20 μM protein and was found to be dose-dependent (data not shown).

**Activity of PfGrx-1 in the Ribonucleotide Reductase Assay**—PfGrx-1 was studied in the ribonucleotide reductase assay by determining the activity of T. brucei ribonucleotide reductase in the presence of varying PfGrx-1 concentrations. Indeed, PfGrx-1 was found to be a substrate of T. brucei ribonucleotide reductase, the Kₘ being 0.2–0.5 μM. This value is comparable to E. coli glutaredoxin-1 (Kₘ = 0.4 μM as determined in a parallel experiment). The turnover rates were, however, by a factor of 3–4 lower with PfGrx-1 than with E. coli Grx-1.

**Inhibitor Studies on PfGrx-1**—The effects of the antimalarial drugs chloroquine, primaquine, quinine, methylene blue, and artemisinin on PfGrx-1 were tested in the glutathione:HEDS transhydrogenase assay. Neither of the drugs (at a concentration of 100 μM) inhibited the PfGrx-1-catalyzed reaction by more than 20%. The only exception was methylene blue, which inhibited the reaction to more than 90%. This effect is, however, most likely due to specific inhibition of PfGR (28).

In a parallel experiment, the effects of the physiological NO-carrier S-nitroso(glutathione (GSNO; Ref. 40) on PfGrx-1 were studied. For GSNO an IC₅₀ value of 600 μM was determined. This inhibition did not increase after preincubation of PfGrx-1 and GSNO in the presence or absence of GSH. The addition of 1 mM *cis*-platinum(II)-diammine dichloride (cisplatin) and *platin*(II)diammine cyclobutane-1,1-dicarboxylate (carboplatin) to the assay system (or 2-h preincubation with 100 μM drug) did not significantly reduce PfGrx-1 activity. In contrast, 1 mM iodoacetamide fully inhibited PfGrx-1 when preincubated with the protein for 1 h. A 15-min preincubation of PfGrx-1 with 100, 200, and 500 μM iodoacetamide resulted in 25, 34, and 92% inhibition, respectively.

**Glutaredoxin Activity in P. falciparum**—Glutathione:HEDS transhydrogenase activity was determined in extracts from isolated trophozoites of eight different *P. falciparum* strains. Protein content of the extracts was determined in parallel. High activity was detected in all parasites: chloroquine-sensitive strains (HB3, 65 milliunits/mg; S106, 54 milliunits/mg; D10, 55 milliunits/mg; 3D7, 75 milliunits/mg) and chloroquine-resistant strains (Dd2, 63 milliunits/mg; K1, 93 milliunits/mg; 7G8, 40 milliunits/mg; FCR3, 64 milliunits/mg). The activities did not significantly vary between chloroquine-sensitive (mean = 62 milliunits/mg) and chloroquine-resistant strains (mean = 65 milliunits/mg).
Glutaredoxins of *P. falciparum*

**DISCUSSION**

The presence of both thioredoxins and glutaredoxins in different organisms, together with the conservation of their active sites through evolution, points to the importance of these antioxidative and regulatory proteins for central cellular functions. In this work we demonstrate the presence of a functional glutaredoxin as well as a glutaredoxin-like protein in the malarial parasite *P. falciparum*.

In the genomes of *P. berghei* and *P. falciparum*, respectively, a putative *P. berghei* glutaredoxin was identified as well as a *P. falciparum* glutaredoxin sequence (grx-1). The latter sequence consists of 5 exons and 336 base pairs and is located on chromosome 3. The gene was amplified by PCR, sequenced, cloned, and overexpressed in *E. coli*. The deduced amino acid sequence (PfGrx-1) comprises 111 residues and contains the active site motif CPYC both being typical for glutaredoxins (7, 11). An alignment with the putative *P. berghei* Grx shows high identities of 71.0%; to other glutaredoxins; for example, from the respective hosts of the two parasite species, man and mouse, identities of 39.0 and 38.0%, respectively, were determined (see Fig. 2). Molecular modeling (Cn3D version 3.00 of NCBI) revealed a typical overall globular fold of PfGrx-1 consisting of a central four-stranded β-sheet flanked by three helices in the order α2-β1-α1 (41).

Like most other glutaredoxins, purified PfGrx-1 was active in the glutathione:HEDS transhydrogenase assay, which represents a typical feature of glutaredoxins (11). The protein was clearly glutathione-dependent, the specific activity being 47 units/mg in the presence of 1 mM GSH and 735 μM HEDS at 25 °C. *Kₐ* values were, however, by a factor of 3–4 lower with PfGrx-1 than with *E. coli* glutaredoxin-1. The turnover rates were, however, by a factor of 3–4 lower with PfGrx-1 than with *E. coli* glutaredoxin-1.

Glutathione:HEDS transhydrogenase activity (40–93 milliunits/mg of protein) was shown to be present in extracts from isolated trophozoites of eight different *P. falciparum* strains and did not significantly differ between chloroquine-sensitive (mean = 62 milliunits/mg) and chloroquine-resistant parasites (mean = 65 milliunits/mg). None of the antimalarial drugs chloroquine, primaquine, quinine, methylene blue, and artemisinin significantly inhibited the Grx-1-catalyzed reaction at a concentration of 100 μM. Similar results were obtained for the cytostatic agents cisplatin and carboplatin. However, the physiological NO carrier GSNO, which had previously been shown to inhibit other glutathione-dependent proteins like glutathione reductase and glutathione S-transferase (40), reversibly inhibited PfGrx-1 with an IC₅₀ value of 600 μM. One molar iodoacetamide time and dose dependently inhibited PfGrx-1. These data indicate that detectable PfGrx-1 activity is present in blood stage parasites. PfGrx-1 is unlikely to be directly linked to chloroquine resistance or to be a target of antimalarial drugs.

A second glutaredoxin-like sequence was also identified on chromosome 3 of *P. falciparum*. This second gene was also PCR-amplified, cloned, and overexpressed in *E. coli*. The deduced amino acid sequence of 171 residues contains, instead of the typical active site motif CPYC, the sequence CGFS, which is comparable to *E. coli* glutaredoxin-1. This value is comparable to *E. coli* glutaredoxin-1. The turnover rates were, however, by a factor of 3–4 lower with PfGrx-1 than with *E. coli* glutaredoxin-1.
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68.5% probability a mitochondrial targeting sequence was predicted for the N-terminal 43 residues of PfGLP-1. PfGLP-1 lacking this MTS sequence (PfGLP-1-N) was also recombinantly produced. Together with ScGrx3 to ScGrx5, PfGLP-1 can be placed into a novel family of PICOT-homology domain-containing proteins (18, 19). The function of these proteins related to glutaredoxins and thioredoxins is not yet known; however, a negative regulatory role in cellular stress responses associated with activation of transcription factors has been postulated. According to data base searches (18, 19), PICOT-HD-containing proteins are expressed with a broad taxonomic distribution including mammals, yeast, bacteria, and plants and are likely to have been derived from a single gene that existed before the divergence of bacteria and eukaryotes.

Interestingly, in contrast to typical glutaredoxins, purified PfGLP-1 and PfGLP-1-N lacked activity in the glutathione: HEDS transhydrogenase assay. We therefore tested the GSH dependence of PfGLP-1-N in a GR-coupled reaction. In contrast to PfGrx-1, which was stoichiometrically reduced, PfGLP-1-N could not be reduced by GSH. A general thiol-disulfide oxidoreductase activity could, however, be demonstrated for PfGLP-1-N in the insulin assay. In this assay PfGLP-1-N significantly and dose dependently accelerated the reduction of insulin disulfide bonds by DTT with an activity comparable to PfGrx-1. This result is remarkable since the active site of PfGLP-1 only comprises 1 cysteine residue and since another member of the PICOT-HD family, ScGrx-3, was demonstrated not to reduce insulin disulfides (42). Apart from this observation, a possible in vivo function of PfGLP-1 represents the reduction of mixed protein disulfides, which are formed during exposure to reactive oxygen species, a reaction that proceeds via a monothiol mechanism (9, 43).

PfGLP-1 shares certain sequence similarities with thioredoxin-like proteins. Furthermore, as demonstrated for the Grx of the phage T4 (44), some glutaredoxins might also be directly reduced by TrxR. In addition, other recent observations, like the physiologically relevant reduction of GSSG by *P. falciparum* thioredoxin (21), the absence of a genuine glutathione reductase in *Drosophila melanogaster* (45), and the discovery of a thioredoxin reductase with a glutaredoxin domain in mouse (46), point to various possible interactions between thioredoxin and glutaredoxin systems. We therefore tested PfGrx-1 and PfGLP-1-N as substrates of PfTrxR. Whereas for PfGLP-1-N no significant activity was detected, PfGrx-1 was reduced with a ΔA/min of 0.010 under the experimental conditions chosen; the control with equimolar concentrations of PfTrx showed an immediate and complete reduction. This reduction of PfGrx-1 by PfTrxR will be studied in further detail.

As shown recently, a *S. cerevisiae* triple mutant lacking ScGrx3 to ScGrx5 is not viable (47) and a single mutant of ScGrx5 shows a significantly altered phenotype, which is more sensitive to oxidative stress. In analogy to yeast, one might therefore postulate an important role for PfGLP-1 in *P. falciparum*, a parasite that particularly depends on its antioxidant defense lines. In yeast, the novel family of glutaredoxin-like proteins (ScGrx3 to ScGrx5) appears to be unable to substitute for the classical glutaredoxin or thioredoxin systems as demonstrated by the lethality of a mutant lacking Trx1, Trx2, Grx1, and Grx2 (8). This phenomenon is likely to be based on the different architecture of the respective active sites (1-Cys versus 2-Cys), which favor monothiol and di thiol redox mechanisms, respectively.

In analogy to peroxiredoxins (48), glutaredoxins may be divided into 1-Cys and 2-Cys glutaredoxins or further, as we should like to propose here, into at least five subclasses. Class Ia glutaredoxins represent the classical Grx with a CPYC (or similar) active site and are GSH-dependent. Clearly, PfGrx-1 is a member of this group. Class Ib glutaredoxins are similar to the members of class Ia but are larger in size; they are GSH-dependent but do not serve as hydrogen donors for ribonucleotide reductase. A member of this class could be Pf col Grx2 (42). Class Ic glutaredoxins comprise NrdH-like proteins, which are glutaredoxin-like but have a thioredoxin-like activity profile; they have the active site sequence CXQG, are GSH-independent and, instead, reduced by TrxR. Members of this family are found in *E. coli*, *Salmonella typhimurium*, and *Lactobacillus lactis* (39). Class IIa glutaredoxins have only 1 cysteine residue at their active site, which shows the general structure PXC(GA)/F(S)/P (47), they contain a PICOT-HD element, and they are likely to be GSH-dependent although biochemical data are still missing. Class IIb glutaredoxins share sequence similarities with class IIA, but are GSH-independent. According to our data, PfGLP-1 described here is a member of class IIb glutaredoxins.

The malaria parasite *P. falciparum* is responsible for more than 2 million malaria deaths per year. The role of glutaredoxin-like proteins in this organism and its host cells will be studied in further detail. This will enhance our understanding of redox metabolism in malaria, which currently represents one of the most promising targets for new chemotherapy strategies.

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