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Plasmodium falciparum glutaredoxin-like proteins

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Abstract

Glutaredoxin-like proteins form a new subgroup of glutaredoxins with a serine replacing the second cysteine in the CxxC-motif of the active site. Yeast Grx5 is the only glutaredoxin-like protein studied biochemically so far. We identified and cloned three genes encoding glutaredoxin-like proteins from the malaria parasite *Plasmodium falciparum* (*Pf*Glp1, *Pf*Glp2, and *Pf*Glp3) containing a conserved cysteine in the CGFS-, CKFS-, and CKYS-motif, respectively. Here, we describe biochemical properties of *Pf*Glp1 and *Pf*Glp2. Cys^{99}, the only cysteine residue in *Pf*Glp1, has a pK_a value as low as 5.5 and is able to mediate covalent homodimerization. Monomeric and dimeric *Pf*Glp1 react with GSSG and GSH, respectively. *Pf*Glp2 is monomeric and both of its cysteine residues can be glutathionylated. Molecular models reveal a thioredoxin fold for the putative C-terminal domain of *Pf*Glp1, *Pf*Glp2, and *Pf*Glp3, as well as conserved residues presumably required for glutathione binding. However, *Pf*Glp1 and *Pf*Glp2 neither possess activity in a classical glutaredoxin assay nor display activity as glutathione peroxidase or glutathione S-transferase. Mutation of Ser^{102} in the CGFS-motif of *Pf*Glp1 to cysteine did not generate glutaredoxin activity either. We conclude that, despite their ability to react with glutathione, glutaredoxin-like proteins are a mechanistically and functionally heterogeneous group with only little similarities to canonical glutaredoxins.

Keywords: CxxS-motif; cysteine thiolate; glutaredoxin; glutathionylation; thioredoxin fold.

Introduction

Classical glutaredoxins (Grx) are small heat-stable glutathione:disulfide-oxidoreductases that transfer electrons from one or two molecules of glutathione (GSH) to an inter- or intramolecular disulfide (Vlamis-Gardikas and Holmgren, 2002; Johansson et al., 2004). As a group of the thioredoxin superfamily, Grx possess a "thioredoxin fold", which is highly conserved throughout evolution and comprises four or five central β-strands surrounded by α-helices (Martin, 1995). Structures of thioredoxin folds have been solved by X-ray crystallography or NMR for thioredoxins, classical Grx (thioltransferases), glutathione S-transferases, glutathione peroxidases (Martin, 1995), peroxiredoxins (Hofmann et al., 2002; Wood et al., 2003), tryparedoxin, and tryparedoxin peroxidase (Hofmann et al., 2002), as well as prokaryotic and eukaryotic protein disulfide isomerases (Hiniker and Bardwell, 2003). Many members of the thioredoxin superfamily share a conserved CxxC-motif in the active site, e.g., the sequence CP(Y/F)C is specific for classical Grx (Holmgren and Aslund, 1995). Reduced classical Grx transfer electrons to ribonucleotide reductase, protein-glutathionyl mixed disulfides, or disulfides of GSH with other low molecular weight thiols (Vlamis-Gardikas and Holmgren, 2002; Johansson et al., 2004). The first but not the second cysteine in the CP(Y/F)C-motif is necessary for the reduction of glutathionylated proteins (Yang and Wells, 1991) which accumulate under oxidative stress and could have buffering effects, preventing irreversible 'overoxidation' of protein thiols (Seres et al., 1996; Shenton et al., 2002). Grx-dependent deglutathionylation of proteins also influences the activity of transcription factors (Bandyopadhyay et al., 2002).

Over the last years a group of proteins with great sequence similarities to classical Grx has been identified. However, these glutaredoxin-like proteins possess a motif with serine replacing the second cysteine (CxxS) (Rahlfs et al., 2001; Belli et al., 2002; Rodriguez-Manzaneque et al., 2002). Grx5 of *Saccharomyces cerevisiae* is the only glutaredoxin-like protein that has been characterized in detail so far: an N-terminal sequence of 29 amino acids targets the mature protein to the mitochondrial matrix, where it is involved in synthesis, assembly or repair of Fe/S clusters, as shown by gene disruption *in vivo* (Rodriguez-Manzaneque et al., 2002). Deglutathionylation of glyceraldehyde-3-phosphate-dehydrogenase isoenzyme Tdh3 of cells exposed to H_2O_2 is delayed in the absence of Grx5 (Shenton et al., 2002). The redox potential of Grx5 determined by direct protein-protein redox equilibration with *E. coli* Grx1 is -175 mV, and the pK_a of Cys^{60} of Grx5 is 5.0 (Tamarit et al., 2003). Site-directed mutagenesis studies suggest that Cys^{60} and Gly^{61} of the CGFS-motif, but not necessarily Cys^{117} at the C-terminus, are essential for the biological activity of Grx5 (Belli et al., 2002). However, Cys^{60} and Cys^{117} are able to either form mixed disulfides with glutathione or an intramolecular disulfide *in vitro* (Tamarit et al., 2003). Although Grx5 reduces the mixed disulfide of glutathionylated carbonic anhydrase *in vitro*, it is inactive in the glutathione:bis-(2-hydroxyethyl)-disulfide transhydrogenase assay (HEDS assay), presumably as a result of the slow reduction of Grx5 by glutathione (Tamarit et al., 2003). Yeast Grx4 was identified as an interactor of the atypical Ser/Thr protein kinase Bud32, and was found to be phosphorylated by the recombinant kinase at Ser^{134} *in vitro*. Grx4 and Bud32 are both predominantly located in the nucleus (Lopreiato et al., 2004).

The glutaredoxin-like protein 1 from the malaria parasite *Plasmodium falciparum* contains a putative mito-

Figure 1 Multiple sequence alignment of glutaredoxin-like proteins from *Plasmodium falciparum* and *Saccharomyces cerevisiae*. The sequence of pig liver thioltransferase and *E. coli* Grx3 used for the molecular models (see below) are included for comparison. Conserved basic residues that might be involved in glutathione binding are indicated by a dot, and the conserved cysteine residue of the putative active site is indicated by a star. A repetitive element in Glp3 containing a cysteine residue flanked by charged amino acids is boxed. The starting point of recombinant Glp1 (Asn^{44}) is labeled with an arrow.

chondrial targeting sequence (mtsGlp1), and the recombinant protein, starting with Asn^{44} (Glp1), is also inactive in the HEDS assay, but demonstrates slight dithiol-reducing activity with dithiothreitol (DTT) in the insulin assay (Rahlfs et al., 2001). Since it is difficult to manipulate *P. falciparum* genetically, our approach to characterize purified recombinant Glps remains the most promising approach to analyze glutaredoxin-like proteins from this important human parasite. Here, we report the identification and cloning of two additional genes encoding glutaredoxin-like proteins from *P. falciparum* (*GLP2* and *GLP3*) and describe biochemical properties of Glp1 and Glp2 *in vitro*. Comparison of our results and results for yeast Grx4 and Grx5 support the hypothesis that glutaredoxin-like proteins are a mechanistically and functionally heterogeneous group. Although they probably share a thioredoxin fold, a deprotonated cysteine residue at physiological pH, and the ability to form a mixed disulfide with glutathione, they have only small similarities to classical glutaredoxins.

Results

Sequence comparison between glutaredoxin-like proteins

From a *P. falciparum* cDNA library we identified and cloned three genes encoding glutaredoxin-like proteins (Glp1, Glp2, and Glp3). In addition, we generated an artificial Glp1 with a CGFC-motif ($Glp1^{S102C}$) by mutation of Ser^{102} to cysteine in order to test potential thioltransferase activity. Glp1, Glp2, and Glp3 all contain the PICOT homology domain (Isakov et al., 2000) and share high sequence similarities with yeast glutaredoxins from the middle of the protein to the C-terminus (Figure 1). Glp1 and yeast Grx3, Grx4, and Grx5 possess a CGFS-motif, whereas the putative active site of Glp2 and Glp3 comprises CKFS- and CKYS-motifs, respectively. Cys^{99} is the only cysteine residue in Glp1, whereas the other glutaredoxin-like proteins contain additional cysteine residues. Glp2 possesses a second C-terminally located cysteine residue, and two cysteines are part of a repetitive sequence motif (NDKÇIK) in Glp3. This motif is followed by a highly charged sequence that is also present in other glutaredoxin-like proteins and precedes the putative thioredoxin fold.

Molecular modeling of a thioredoxin fold of Glps

The model of glutathionylated and unmodified Glp1 contains a thioredoxin fold similar to pig liver thioltransferase (Figure 2). In contrast to the C-terminus, the N-termini of glutaredoxin-like proteins from *P. falciparum* and yeast are divergent, and no suitable target structure has been identified to date. Comparison of sequence alignments and molecular models of yeast Grx5 (Belli et al., 2002) or Glps with classical glutaredoxins suggests that insertions and deletions are located in (flexible) loops, leading to a conserved structure of the central core. The second β-strand of modeled Glp1, connecting α-helices 2 and 3 (residues Gly^{100}–Asn^{110} and Asn^{126}–Tyr^{135}, respectively), is shorter in comparison to the templates, and a loop preceding the CGFS-motif is elongated in Glp1. Cys^{99} of this motif is located N-terminal to α-helix 2 and points to the edge of a dividing wall composed of Trp^{138} and Tyr^{140} at the protein surface. Modeling a mixed disulfide, glutathione fits snuggly around the edge of this wall, and the carboxylate group of the glycine residue can be electrostatically bound to the side chains of Arg^{129} and Lys^{91}. The corresponding homologous residues to Trp^{138}, Arg^{129} and Lys^{91}, are conserved in all other aligned glutaredoxin-like proteins (Figure 1). Ser^{102} is located close to Cys^{99} in analogy to the second cysteine residue of classical glutaredoxins and thioredoxins, and a hydrogen bond between SγH and OγH can be modeled without causing

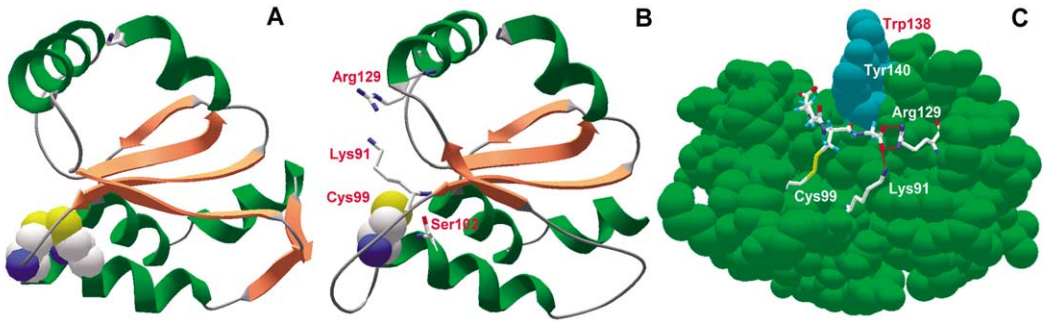

Figure 2 Model for Glp1 containing a thioredoxin fold.
(A) Thioredoxin fold of oxidized pig liver thioltransferase (Katti et al., 1995). (B) Model of Glp1 starting with residue Leu^{73} based on the crystal structure of pig liver thioltransferase. (C) Model of glutathionylated Glp1. Grx3 from *E. coli* served as a template for the glutathionylation (Aslund et al., 1996). The calculated force field energies of the models (without further optimization) and the template are -3.2 and -3.5 MJ/mol, respectively.

clashes. Modeling the C-terminal part of Glp2 and Glp3 resulted in very similar stable structures and force field energies. According to the model of Glp2, the distance between Cys^{142} and Cys^{216} is at least 2 nm.

Expression, purification and enzymatic assays of recombinant Glps

Expression and purification of recombinant Glps is summarized in Table 1. As a result of ineffective expression, mtsGlp1 was not characterized in more detail. Glp1 and $Glp1^{S102C}$ – both missing the putative N-terminal mitochondrial targeting sequence – are soluble, stable proteins starting with Asn^{44} after the N-terminal $MRGS(H)_6GS$-tag. During the purification process of $Glp1^{S102C}$ the nickel-nitrilo-triacetic acid (Ni-NTA) resin turned completely brown. This side observation indicates the reduction of Ni^{2+}. Slight changes in color, which can sometimes occur during purification of tagged proteins – even in the absence of DTT or other additional reducing agents – are usually reversed when the resin is washed with aerobic buffer. However, in the case of $Glp1^{S102C}$ the turquoise color of the resin was not recovered until elution with 150 mM imidazole. Application of approximately 10 mg of Glp1 to 1 ml of Ni-NTA resin led to similar observations, although the intensity of the brown color was lower than for the mutant protein. $Glp1^{S102C}$ can be rereduced with $NaBH_4$, recovering reactivity of approximately two cysteines per protein molecule in the 5,5′-dithio-bis-(2-nitrobenzoic acid) (DTNB) assay. Glp1 is relatively stable in solution but precipitates slowly when stored for weeks, independently of the presence or absence of DTT.

Recombinant Glp2 is poorly soluble and starts to elute from the Ni-NTA column at 40 mM imidazole. After gel filtration chromatography, purified Glp2 elutes as a monomer with an apparent molecular mass of 30 kDa (Figure 3). Under native (defined as non-denaturing) conditions, 1.3 cysteine residues per molecule were detected in the DTNB assay, whereas in the presence of 2% sodium dodecyl sulfate (SDS) 2.0 cysteine residues per molecule were detectable, indicating that both residues are reduced but one of them is less accessible or less reactive. Recombinant Glp3 is completely localized in the pellet after sonication.

Glp1 is inactive in the HEDS assay (Rahlfs et al., 2001). This is also the case for Glp2 and $Glp1^{S102C}$ (data not shown). Potential GSH-dependent activity of Glp1 and

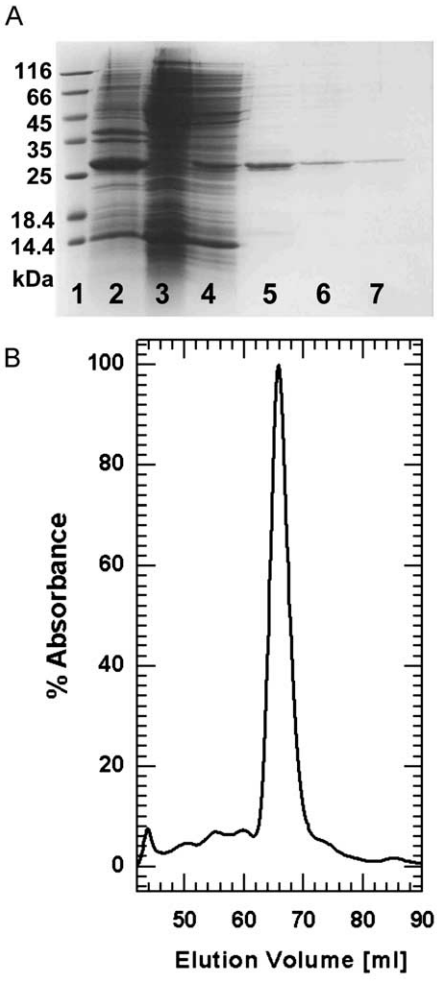

Figure 3 Purification of monomeric Glp2.
(A) Purification of recombinant Glp2 by Ni-NTA affinity chromatography. Eluted fractions were analyzed by reducing SDS-PAGE (15%). Lane 1, marker; lane 2, pellet; lane 3, flow-through; lane 4, 20 mM imidazole; lanes 5–7, elution with 55 mM imidazole. (B) Representative gel filtration chromatogram of native recombinant Glp2, eluting with an apparent molecular mass of 30 kDa. Glp2-containing fractions were pooled and analyzed using mass spectrometry and enzymatic assays.

Table 1 Properties of Glps.

	MtsGlp1	Glp1	Glp2	Glp3
PlasmoDB annotation	PFC0205c	PFC0205c	MAL6P1.72	PF07_0036
Accession number	AY014839	AY014839	CAG25239	CAD50844
Theoretical molecular mass	(21318)	(16365)	(27203)	(33736)
(with and without His-tag) (Da)	19920	14966	26035	32053
Number of amino acids (without His-tag)	171	128	219	272
Theoretical extinction coefficient $\varepsilon_{280\ nm}$ ($mM^{-1}\ cm^{-1}$)	19.8	18.5	26.2	23.6
Theoretical isoelectric point	(9.32)	(7.83)	(6.32)	(6.97)
(with and without His-tag)	9.22	6.53	5.55	6.80
Yield of pure protein (mg/l culture)	–	6–9	1.5–3	–
HEDS-assay activity	–	Negative	Negative	–
Solubility	Insoluble	Soluble	Poorly soluble	Insoluble

Glp2 as glutathione peroxidase or as glutathione S-transferase was assayed with *tert*-butyl hydroperoxide and 1-chloro-2,4-dinitrobenzene (CDNB) as substrate, respectively. In contrast to the positive controls, no significant activity was detectable in either assay system (data not shown), suggesting that Glp1 and Glp2 do not detoxify smaller molecules at the expense of GSH.

Determination of the thiol pK_a value of Cys^{99} in Glp1

Glp1 is relatively stable, even at extreme pH values. To determine the pK_a value of the sulfhydryl group of Cys^{99} at the putative active site, the apparent rate constant of alkylation of Cys^{99} with iodoacetamide was measured from six independent protein purifications at six different pH values by DTNB assay (Figure 4). Plotting the reciprocal concentration of reduced unmodified Glp1 versus time yielded straight lines, indicating that the kinetic data can be fitted according to a second-order reaction (Tamarit et al., 2003). The slope of each plot is the apparent second-order rate constant (k_{app}) at the particular pH value. A plot of k_{app} versus pH yields a sigmoidal curve with a pK_a for Cys^{99} at the inflection point at approximately pH 5.5.

Glp1 can form an intermolecular disulfide bond under native conditions

Freshly purified Glp1 was analyzed by SDS polyacrylamide gel electrophoresis (PAGE) and gel filtration chromatography after incubation with or without oxidizing or reducing agents (Figure 5, Table 2). Approximately 10% of native Glp1, purified under aerobic conditions without additional reducing agents, formed a dimer. This proportion, evaluated from the peak area in the chromatogram, was confirmed by DTNB assays, showing that 85–90% of Cys^{99} in freshly purified Glp1 was reduced and accessible. Incubation with H_2O_2 or long exposure to air increased the percentage of the dimer; whereas in the presence of excess GSH or DTT only monomeric Glp1 was detectable under native conditions. Furthermore, incubation of pooled fractions of the dimer (peak 1) from the gel filtration with GSH or DTT converted the dimer into the monomer (peak 2). Thus, the dimer is covalently linked as a disulfide between Cys^{99} and Cys$^{99'}$ under denaturing and native conditions. In contrast, both aged and freshly purified Glp1^{S102C} contained only monomeric protein without detectable cysteine residues in the DTNB assay, indicating that formation of an intramolecular disulfide was favored over an intermolecular disulfide.

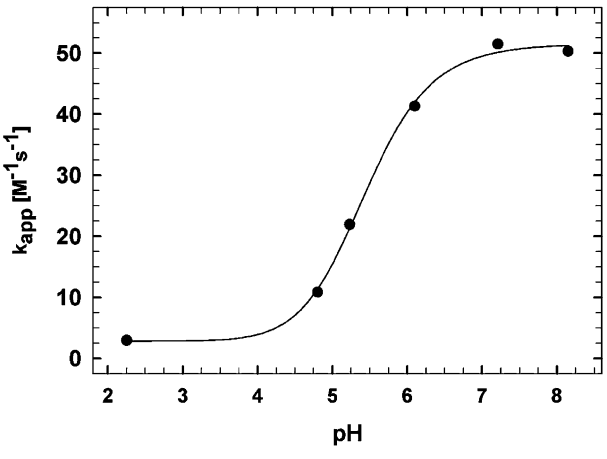

Figure 4 Determination of the thiol pK_a value for Cys^{99} at the putative active site of Glp1.

GSSG and GSH react with Glp1 and Glp2

Surprisingly, not only GSH and DTT, but also addition of excess GSSG to freshly purified Glp1 caused monomerization under native conditions (Table 2). Analysis of samples of Glp1 containing GSH or GSSG by gel filtration chromatography and HPLC/MS-ESI revealed that the monomeric protein was unmodified or completely glutathionylated, respectively (Table 2): with excess GSH, complete reduction of Glp1 occurs [Reactions (1a) and (1b)]. In the presence of a 33-fold excess of GSSG, no dimerization of Glp1 was observed. However, oxidation of reduced Glp1 (85%) by GSSG leads to the liberation of GSH. Liberated GSH subsequently cleaves oxidized Glp1 (15%) leading to glutathionylated Glp1 as the only product [Reactions (2a)–(2c)]:

$$\text{Glp1(}\mathbf{SS}\text{)Glp1} + \text{GSH} \rightarrow \text{Glp1(}\mathbf{SS}\text{G)} + \text{Glp1(SH)} \quad (1a)$$

$$\text{Glp1(}\mathbf{SS}\text{G)} + \text{GSH} \rightarrow \text{Glp1(SH)} + \text{GSSG} \quad (1b)$$

$$\text{Glp1(SH)} + \text{GSSG} \rightarrow \text{Glp1(}\mathbf{SS}\text{G)} + \text{GSH} \quad (2a)$$

$$\text{Glp1(}\mathbf{SS}\text{)Glp1} + \text{GSH} \rightarrow \text{Glp1(}\mathbf{SS}\text{G)} + \text{Glp1(SH)} \quad (2b)$$

$$\text{Glp1(SH)} + \text{GSSG} \rightarrow \text{Glp1(}\mathbf{SS}\text{G)} + \text{GSH} \quad (2c)$$

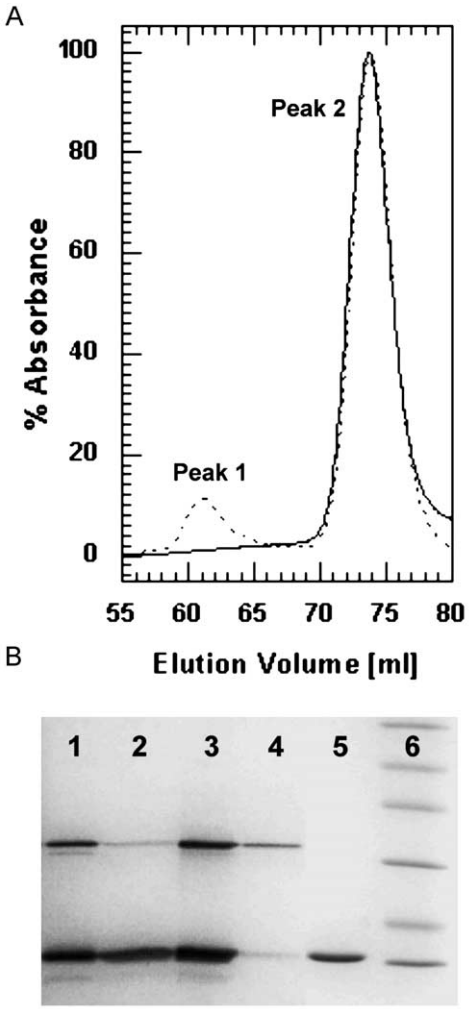

Figure 5 Monomerization of dimeric Glp1 is coupled to reduction of an intermolecular disulfide bond.
(A) Representative gel filtration chromatogram of native recombinant Glp1 under aerobic non-reducing (dashed line) and reducing (solid line) conditions. Samples were reduced by incubation with 2 mM GSH or 2 mM DTT for 3 h at 4°C. (B) Denatured Glp1 separated by SDS-PAGE on a 15% gel with or without additional reducing agents. Lane 1, negative control without additional reducing agents; lane 2, Glp1 with 2 mM GSH; lane 3, pooled fractions of peak 2 exposed to air for several days after gel filtration; lane 4, pooled fractions of peak 1; lane 5, Glp1 with 2 mM DTT; lane 6, marker (molecular mass in kDa: 66, 45, 35, 25, 18.4, and 14.4).

Incubation of Glp2 with GSSG led to glutathionylation of one cysteine residue, whereas incubation with GSH for 1 day under aerobic conditions yielded a mixture of unmodified, mono- and diglutathionylated protein, as confirmed by mass spectrometry. Thus, both Cys^{142} and Cys^{216} of Glp2 are accessible and are able to react with glutathione.

Discussion

Grx5 from yeast is involved in synthesis, assembly or repair of iron-sulfur clusters (Rodriguez-Manzaneque et al., 2002). One question arising is therefore whether yeast Grx5 reacts directly or indirectly with iron-sulfur clusters. Although possessing a CxxC-motif, classical thioredoxins or glutaredoxins purified in our laboratory usually do not reduce Ni^{2+} complexed in Ni-NTA. The observed reduction of complexed Ni^{2+} by wild-type and mutant Glp1 might be a first indication that the putative active site of Glp1 has increased affinity for metal ions complexed by bulky ligands, in contrast to classical glutaredoxins and thioredoxins. Thus, Glp1 might be an ortholog of Grx5 from yeast. Interestingly, sequences similar to the repetitive motif in the putative N-terminal domain of Glp3 (Figure 1) are also present in many archaeal and bacterial iron-sulfur cluster binding proteins, such as ferredoxins (pfam00037, alignments not shown).

Based on molecular models, glutaredoxin-like proteins most likely possess a thioredoxin fold similar to classical glutaredoxins (Figure 2). Beside the possible structural similarities, the putative N-terminal domain of glutaredoxin-like proteins differs significantly in size, sequence, and cysteine content (Figure 1), possibly leading to completely different protein structures and cellular functions. For example, yeast Grx5 is located at the mitochondria, whereas Grx4 is phosphorylated at Ser^{134} by the kinase Bud32 in the nucleus (Lopreiato et al., 2003). Interestingly, Ser^{134} is located at the end of the putative N-terminal domain, and the introduction of negative charges by the phosphate is likely to have a significant structural influence on the following acidic residues connecting both domains.

The reactivity of proteins containing a cysteine residue at the active site depends on the pK_a value of the sulfhydryl group, because a thiolate is a much stronger nucleophile than a sulfhydryl group. The cysteine residues in the CxxS-motif of Glp1-3 and yeast Grx3-5 are presumably located N-terminal to an α-helix and are neighbored by a positively charged residue. Thus, formation of the cysteine thiolate could be facilitated by: (i) the dipole of the C-terminally flanked α-helix, which is caused by partial dipoles of peptide bonds; (ii) the additional positive charge at the N-terminus of the α-helix caused by a basic residue; and (iii) by residues forming hydrogen bonds with the sulfhydryl group (e.g., serine in CxxS). These conditions (partially) explain the low pK_a values of 5.0 for yeast Grx5 – containing a FP*K*C̲GFS*R*-motif (Tamarit et al., 2003) – and 5.5 for Glp1 containing

Table 2 Glutathionylation and dimerization of 60 μM Glp1.

Additional redox reagent	Peak area[a] (%)	Molecular mass	
		Apparent[a] (kDa)	Actual[b] (Da)
None	90; 10	16.5; 35.5	
2.0 mM DTT	100	16.5	
2.0 mM GSH	100	16.7	16 368
2.0 mM GSSG	100	16.5	16 672
60 μM H_2O_2[c]	57; 43	16.5; 35.5	
None, aged[d]	14; 86	16.5; 35.5	

[a]Determined by gel filtration chromatography. The first and second values correspond to peak 2 and peak 1, respectively.
[b]Determined by HPLC/ESI-MS. The calculated molecular mass of Glp1 and glutathionylated Glp1 is 16 365 and 16 672 Da, respectively.
[c]One equivalent of H_2O_2 was added to reduced enzyme and incubated for 1 h at room temperature. The elution buffer did not contain H_2O_2. This is in contrast to the other redox compounds that were also present in the elution buffer.
[d]The fraction was stored aerobically without reducing agents for 2 weeks at 4°C.

a *K*PLÇGFSA-motif. However, it cannot be concluded that all glutaredoxin-like proteins (and other members of the thioredoxin superfamily) have cysteine residues with similar pK_a values. For example the N-terminal cysteine residues of *E. coli* and human thioredoxin, both sharing a CGPC-motif, have pK_a values of 7.5 and 6.4, respectively (Chivers et al., 1997).

Glp2 is the third recombinant glutaredoxin-like protein inactive in the HEDS assay investigated so far, but, in contrast to Glp1 and yeast Grx5 (Belli et al., 2002), Glp2 has a CKFS-motif instead of a CGFS-motif at the putative active site. The question arises as to why glutaredoxin-like proteins are inactive in the HEDS assay. The lack of the second cysteine residue in the CxxC-motif might be a reason. However, in the case of Glp1^{S102C}, replacement of serine in the CGFS-motif by cysteine does not generate an artificial glutaredoxin with activity in the HEDS assay. In addition, successful reaction between HEDS and GSH catalyzed by a protein with only one cysteine residue at the active site could occur from a mechanistic point of view. Such a reaction could theoretically proceed by a ping-pong mechanism with a protein-glutathionyl mixed disulfide as an intermediate. The one and only cysteine residue of Glp1, as well as both cysteine residues in yeast Grx5 (Tamarit et al., 2003), can be glutathionylated. Substitution of glycine in the CGFS-motif by valine or serine (but not by alanine) seems to inhibit the formation of the glutathione mixed disulfide in the case of yeast Grx5 *in vivo* (Belli et al., 2003). However, although glycine is replaced by lysine in the CGFS-motif, both cysteine residues present in Glp2 can be glutathionylated *in vitro*. All these aspects considered, a kinetic and not a thermodynamic factor might be responsible for the missing activity of glutaredoxin-like proteins in the HEDS assay. Reduction of oxidized Grx5 from yeast by GSH is at least 20-fold slower than that of Grx1 from *E. coli* (Tamarit et al., 2003), supporting this hypothesis. Thus, for rapid reaction with HEDS and/or GSH there are additional structural requirements – besides the second cysteine residue in the CxxC-motif – that are missing in yeast Grx5, Glp1, and Glp2.

Oxidized dimeric Glp1 is unlikely to be a predominant form of the protein under reducing conditions *in vivo*. Since Cys^{99} is accessible to a bulky sulfhydryl-carrying molecule and oxidized dimeric Glp1 is stable in solution, Glp1 could, however transiently, interact with cysteines of other proteins. The accessibility of Cys^{99} or Cys^{142} in monomeric Glp1 or Glp2, respectively, could also enable covalent interaction with other proteins during glutathionylation or deglutathionylation. This hypothesis is supported by the glutathionylation of Glp1 and Glp2 *in vitro*. In addition, an arginine and a lysine residue are conserved among the glutaredoxin-like proteins from yeast and *P. falciparum* and could be involved in binding of glutathione according to molecular models.

To subdivide the heterogeneous group of glutaredoxin-like proteins we suggest four potential different mechanistic classes, depending on the sulfhydryl group cleaving the intermediate mixed disulfide between the glutaredoxin-like protein and the substrate. The source of this resolving sulfhydryl group could be: (i) the substrate (e.g., a protein containing two reactive cysteine residues); (ii) a second substrate molecule (e.g., GSH); (iii) a cysteine residue of a second subunit, forming an intermolecular disulfide (as is possible for Glp1); or (iv) a cysteine residue forming an intramolecular disulfide (e.g., Grx5; see Tamarit et al., 2003). Based on the molecular model of Glp2, Cys^{142} and Cys^{216} are too far away to form an intramolecular disulfide, as is the case for Cys^{60} and Cys^{117} of yeast Grx5 (Tamarit et al., 2003). If Cys^{216} is involved in the formation of a disulfide (e.g., during catalysis) it has to form an intermolecular disulfide. The mechanistic classification suggested is comparable to peroxiredoxins, which share a PxxxTxxC-motif, and which are subdivided into 1-Cys peroxiredoxins, and typical and atypical 2-Cys peroxiredoxins (Hofmann et al., 2002; Wood et al., 2003). Interestingly, these possible alternative mechanisms are not restricted to members of the thioredoxin superfamily. Members of the peptide methionine sulfoxide reductase family also have an $\alpha\beta$ core and a CxxC/S-motif at the N-terminus of an α-helix. However, the thioredoxin family and the peptide methionine sulfoxide reductase family exhibit different topologies and folds, suggesting convergent evolution of alternative sequence motifs and reaction mechanisms (Gladyshev, 2002; Fomenko and Gladyshev, 2003).

Materials and methods

Materials

All chemicals used were of the highest purity available and were obtained from Roth (Karlsruhe, Germany), Merck (Darmstadt, Germany) or Sigma (Steinheim, Germany). HEDS was from Lancaster, and GSSG was obtained from Roche (Mannheim, Germany). The cloning vector pBluescriptSK$^+$ was obtained from Stratagene (Heidelberg, Germany), PCR primers were obtained from MWG-Biotech (Ebersberg, Germany), and the expression system QIAexpress, comprising vector pQE30, *Escherichia coli* host strain M15, and Ni-NTA agarose resin, was purchased from Qiagen (Hilden, Germany).

PCR amplification, sequencing, cloning and site-directed mutagenesis

Cloning of *GLP1* has been described previously (Rahlfs et al., 2001). A mutation coding for Ser^{102}Cys in the CGFS motif of Glp1 was introduced by PCR with *Pfu* polymerase (Promega) using mutated primers and the pQE30/*GLP1-N* plasmid as a template. Methylated non-mutated template plasmids were digested with DpnI (Promega, Mannheim, Germany), and competent XL1-blue cells were subsequently transformed. Sequencing both strands confirmed the correct mutation of *GLP1^{S102C}*.

Using the database of the malaria parasite genome project (http://plasmodb.org/) two complete open reading frames encoding two further glutaredoxin-like proteins on chromosomes 6 and 7 were identified *in silico* (PlasmoDB annotations MAL6P1.72 and PF07_0036, respectively). No further genes with obvious similarities to *GLP*s could be found. Restriction sites (underlined) were introduced for BamHI or SacI, and HindIII at the 5′ end of the respective primers for subsequent PCR amplification and cloning of the two genes: primer for *GLP2*, N-terminal (OPfglp2v; 5′-CGCGGGATCCGATTTTATTAAGGTTGAGGACCAACG-3′), and C-terminal (OPfglp2h; 5′-GCGCAAGCTTTTATTCTTCGAAACAGTCATCTGG-3′); primer for *GLP3*, N-terminal (OPfcg6v; 5′-CGCGGAGCTCAACAAATACATAAGA-

GCGCC-3′), and C-terminal (OPfcg6h; 5′-GCGCAAGCTTTTA-TATAATATCTTTAATTTTATTTTGG-3′).

The according genes were amplified by PCR using a gametocyte cDNA library from *P. falciparum* strain 3D7 as a template. The fragments of correct size derived were cloned into p-BluescriptSK$^+$, followed by sequencing and subcloning into pQE30.

Protein purification

The genes *mtsGLP1*, *GLP1*, *GLP1*^{S102C}, and *GLP3* were expressed in *E. coli* M15 cells, and the recombinant His-tagged proteins were purified by affinity chromatography using Ni-NTA as described previously (Rahlfs et al., 2001). *GLP2* was expressed overnight at 30°C with 0.5 mM isopropyl-β-D-1-thio-galactopyranoside. The proteins were eluted with Glp buffer containing 50 mM sodium phosphate, 300 mM NaCl, 55–150 mM imidazole, pH 8.0. Protein concentrations were determined using the Bradford (1976) assay with bovine serum albumin as a standard, and measuring the absorbance at 280 nm with the according buffer as a blank. Glutathione reductase (*Pf*GR) and *Pf*Grx-1, as well as glutathione S-transferase (*Pf*GST) from *P. falciparum* were produced and purified as previously described (Rahlfs et al., 2001; Harwaldt et al., 2002).

DTNB assays

Accessible, reduced sulfhydryl groups were determined with 600 μM 5,5′-dithio-bis-(2-nitrobenzoic acid) (DTNB, Ellman's reagent), detecting the liberation of 5-thio-2-nitrobenzoate at 412 nm (Ellman, 1959). In parallel, 2-mercaptoethanol (0–80 μM) and DTT (0–40 μM) were used for calibration curves with 600 μM DTNB in Glp buffer. In the case of oxidized Glp1^{S102C}, $NaBH_4$ was added at a ca. 50-fold molar excess over Glp1^{S102C}. Accessible sulfhydryl groups were detected after excess $NaBH_4$ was degraded (Davis and Swain, 1960). $NaBH_4$ was dissolved in NaOH, pH 10 prior to the experiment. Salt concentrations and pH of the buffer did not change significantly after addition of the $NaBH_4$-containing solution (<1% v/v).

Determination of the thiol pK_a value of Cys^{99} in Glp1

The rate of carboxymethylation of freshly purified Glp1 by iodoacetamide at pH 2–8 was used to determine the pK_a value of Cys^{99}. Freshly purified protein (50–100 μM) was completely reduced with 1 mM DTT before each measurement. DTT was removed by washing repeatedly in a Centriprep YM-10 (Millipore, Schwalbach, Germany) with buffer containing 200 mM KCl, 1 mM EDTA and 10 mM of the buffering agent [glycine, acetate, 2-(*N*-morpholino)ethane sulfonic acid (MES), morpholinopropane sulfonic acid (MOPS), or Tris at pH values between 2.2 and 8.2]. Subsequently, 50 μM Glp1 was incubated with 500 μM iodoacetamide at 25°C and aliquots of 100 μl were removed between 1 and 120 min (pH 2.2) or 0.5 and 15 min (pH 8.2). The reaction was stopped with 2 ml of Glp buffer containing 10 mM DTT. Aliquots were washed seven times in a Centricon YM-10 (Millipore) with Glp buffer. DTNB assays were subsequently carried out in microcuvettes as described above.

Enzymatic assays

Glutathione:HEDS transhydrogenase assays were carried out as previously described (Rahlfs et al., 2001). Glp1, Glp1^{S102C}, and Glp2 were dialyzed versus assay buffer before each experiment. Final protein concentrations in the assay were up to 12 μM, and *Pf*Grx-1 (final concentration ≤0.1 μM) served as a positive control.

Glutathione peroxidase assays were carried out at 25°C in buffer containing 100 mM Tris, 1 mM EDTA, pH 8.0, 2 mM GSH, 1.0 U/ml glutathione reductase from *P. falciparum*, and 100 μM NADPH. Glp1 was added at a final concentration of up to 3 μM. After incubation for 10 min, the assay was started by adding 70 μM *tert*-butyl hydroperoxide. A reference cuvette containing all components without Glp1 was used as a control, and its absorbance at 340 nm was subtracted from the sample cuvette.

Glutathione S-transferase assays were carried out at 25°C in two different buffers containing either 100 mM potassium phosphate, 1 mM EDTA, pH 7.0, or 100 mM HEPES, 1 mM EDTA, pH 6.5. Then 500 μM 1-chloro-2,4-dinitrobenzol (CDNB), 1 mM GSH, and up to 6 μM Glp1 were added to the buffer. A reference cuvette containing all components without Glp1 was used as a control, and its absorbance at 340 nm was subtracted from the sample cuvette. *Pf*GST served as a positive control (final concentration ≤0.6 μM).

Dimerization and glutathionylation experiments

Aliquots of freshly purified Glp1 (61 μM, 1.0 mg/ml) were incubated for 3 h at 4°C in Glp-buffer containing 2 mM GSH, or 2 mM GSSG, or no additional oxidizing or reducing reagents. The batches incubated were analyzed by gel filtration chromatography, SDS-PAGE, and HPLC/electrospray ionization-mass spectrometry (ESI-MS): 3 μl of each batch incubated was mixed with 20 μl of non-reducing Laemmli (1970) buffer, and 1 μl of each batch incubated was diluted 1:10 with 1% formic acid (see below). The remainder of each batch was separated by gel filtration chromatography on a HiLoad 16/60 Superdex 75 prep grade column, which was connected to an ÄKTA FPLC system (Amersham Pharmacia Biotech, Freiburg, Germany). The column was calibrated with a gel filtration standard (Amersham Pharmacia Biotech) and equilibrated with buffer containing 50 mM sodium phosphate, 300 mM NaCl, pH 7.4, and the same concentration of oxidizing or reducing reagent as the corresponding sample. FPLC fractions of Glp1 were detected photometrically, and peak areas and k_{AV} values were evaluated using the software UNICORN 4.11 (Amersham Pharmacia Biotech). Protein-containing FPLC fractions and the denatured samples collected prior to the gel filtration were analyzed by SDS-PAGE. In addition, in order to analyze the molecular weight, 10 μl of 6.1 μM Glp1 of each batch in 1% formic acid (see above) was injected in a Surveyor HPLC system (ThermoFinnigan, Dreieich, Germany) with a 10×1 mm BioBasic C18 column (ThermoHypersil) coupled to a LCQ deca ESI-MS (ThermoFinnigan).

Molecular modeling

The Protein Data Bank was searched for protein structures containing a thioredoxin fold, and the sequences of the potential templates identified were aligned with Glps using the program ClustalW (Thompson et al., 1994). The same templates as for yeast Grx5 (Belli et al., 2002) were identified as best suited for modeling Glps: models of monomeric Glps with and without covalently bound glutathione were generated based on the NMR structures of glutaredoxin 3 from *E. coli* (Aslund et al., 1996) and the crystal structure of recombinant pig liver thioltransferase (Katti et al., 1995) (Protein Data Bank accession numbers 3GRX and 1KTE). Alignments between Glps and the templates were optimized manually in the Swiss-PDB Viewer (spdbv). Computations of the models were carried out at the Swiss-Model server using the optimize (project) mode (Guex and Peitsch, 1997; Schwede et al., 2003). The force field energy of the models was calculated with the GROMOS96 implementation of spdbv.

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