Interference with Redox-Active Enzymes as a Basis for the Design of Antimalarial Drugs

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Abstract: Antimalarial drugs are urgently and continuously required. Parasite enzymes involved in antioxidant defence represent interesting target molecules for rational drug development. Here we summarize the currently available data on structural, biochemical, and functional properties of these proteins in an attempt to evaluate and compare their potential as drug targets.

Keywords: *Plasmodium*, drug development, redox metabolism, thioredoxin, glutathione, structure, target validation, malaria.

I. THE URGENT NEED FOR NOVEL ANTIMALARIAL DRUGS

The protozoon *Plasmodium falciparum*, an apicomplexan parasite, is the causative agent of tropical malaria. Malaria is transmitted by the bite of female mosquitoes, *Anopheles gambiae* being the most prominent example. After an initial liver stage the parasites live and multiply in red blood cells. Red cell lysis releases fresh merozoites into the blood stream every two days and leads to the fever characterizing malaria (for a review on the biology of malarial parasites please see [1]). Each year, up to three million deaths due to malaria and up to 500 million episodes of clinical illness occur throughout the world, with Africa having more than 90% of this burden. Almost 3% of disability adjusted life years are due to malaria morbidity and mortality globally, 10% in Africa [2]. The onset of chloroquine resistance marked the beginning of a new chapter in the history of malaria in Southeast Asia and by 1973 chloroquine had to be replaced by the combination of sulphadoxine and pyrimethamine (SP). By 1985, SP was replaced by mefloquine. The rapid development of resistance to this new drug has led to the introduction of artemisinin-derivatives as drugs in the mid-1990s. Therapeutic regimens for prevention and treatment of chloroquine-resistant *P. falciparum* are associated with higher costs and side-effects compared to chloroquine. Urgent efforts are needed to identify effective, affordable, alternative antimalarial regimens and considerable work is still required to achieve a better understanding of the processes involved in the pathogenesis of severe malaria [3-5].

The progress made over the last years in the fields of genomics, proteomics, and clinical medicine is essential to support drug development approaches. A review on the current proteomic analyses employed to elucidate global protein expression profiles, subcellular localization of gene products, and host-pathogen interactions that are central to disease pathogenesis and treatment is given by Johnson *et al.* [6]. The high-throughput nature of these techniques is in accord with the pace of drug and vaccine development that have the potential to directly reduce the morbidity and mortality of the disease. In parallel, genomic approaches facilitate the access to and the validation of novel drug targets [7-9]. Coupling the *Plasmodium* sequence data with bioinformatics, proteomics and RNA transcript expression profiling opens unprecedented opportunities for exploring new malaria control strategies [10]. Ideally, proteomic and genomic approaches are complemented by crystallographic studies characterizing target proteins and protein-inhibitor or protein-substrate complexes. A recent review by Brady and Cameron [11] summarises the current involvement of crystallography within the frame of anti-malarial drug development programmes.

II. REDOX METABOLISM AS A TARGET FOR ANTIMALARIAL DRUG DEVELOPMENT

Experimenta naturae, like human glucose 6-phosphate dehydrogenase deficiency, indicate that limited availability of reducing equivalents in the form of NADPH confers protection from malaria [12, 13]. This underlines the fact that malarial parasites are susceptible to oxidative stress and to alterations in the redox equilibrium of the host-parasite-unit.

The detoxification of reactive oxygen and nitrogen species (ROS and RNS) is a challenge for erythrocytes infected with *Plasmodium*. As a result of (i) the high metabolic rate of the rapidly growing and multiplying parasite, (ii) the host immune response to the parasite infection, and (iii) the degradation of hemoglobin by the parasite, large quantities of toxic redox-active by-products are generated. At the same time the multiple biochemical pathways involved in the generation of oxidative stress and in its detoxification represent a range of potential targets for interfering with redox balance (see Table **1**).

Over the last years *Plasmodium* has been shown to possess a whole range of antioxidant defence mechanisms, namely a complete glutathione system comprising NADPH, highly active glutathione reductase (GR), glutathione (in reduced form GSH), and different glutaredoxin-like proteins as well as a functional glutathione dependent glyoxalase system and a glutathione *S*-transferase with peroxidase activity [see 1, 13-15 for reviews]. In addition, a complete

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Table 1. Potential Drug Targets in the *Plasmodium falciparum* Redox Metabolism

	Name	Abbreviation	Synonyms	Chromosomal/ subcellular localization	Accession numbers[1]	Biochemical characterization/ structural data/target verification
Plasmodial enzymes which do not occur in the human host	Glucose 6-phosphate dehydrogenase/6-phosphoglucono-lactonase fusion protein	G6PD/6PGL		Chr14	NP_702400 NC_004317	Catalyzes the first two steps of the pentose phosphate shunt
	Plasmoredoxin	PfPlrx	FRED, thioredoxin-like redox-active protein	Chr3/cytosolic	chr3.phat_63 AAF87222 AF234633	Transfers electrons to ribonucleotide reductase, reacts with PfTPx1 and PF AOP
	Glutathione *S*-transferase E.C. 2.5.1.18	PfGST		Chr14/ cytosolic	PF14_0187 AAK00582 AY014840	CDNB turnover, ligandin binding heme, peroxidase activity, homodimer, structure solved at 1.9 Å resolution
	Cytosolic Glyoxalase I E.C. 4.4.1.5	cPfGloI	Lactoylgluta-thione lyase, methylglyoxalase	Chr11/cytosolic	PF11_0145 AAQ05975 AF486284	Evolved *via* gene duplication and fusion events; glyoxalase I family glutathione + methylglyoxal ↔ (R)-S-lactoylglutathione Zn^{2+}-dependent, gene duplication
	Glyoxalase I like protein E.C. 4.4.1.5	PfGILP		Chr6/ts, apicoplast transit peptide predicted	MAL6P1.50 NP_703709	Glyoxalase I family similarity; function still unknown
	Selenoproteins	PfSel1 PfSel2 PfSel3 PfSel4		Chr4, 8, 14, 14 Two proteins have a putative apicoplast import sequence	PF14_0033 PFI1515w MAL8P1.86 PF14_0251 but misannotated	Function unknown
Plasmodial enzymes with marked structural differences to their human counterpart	Glutathione reductase E.C. 1.8.1.7 formerly 1.6.4.2	PfGR		Chr14/cytosolic N-terminally extended version putatively mitochondrial	PF14_0192 CAA63747 X93462	2 GSH + $NADP^+$<=> GSSG + NADPH + H^+ FAD-dependent, homodimeric, structure solved at 2.6 Å resolution
	Thioredoxin reductase E.C. 1.8.1.9 formerly 1.6.4.5	PfTrxR		Chr9/ cytosolic, putatively N-terminally extended version	PFI1170c CAA60574 X87095	NADPH + H^+ + $Trx(S_2)$ ↔ $Trx(SH)_2$ + $NADP^+$, FAD-dependent, homodimeric. Validated as drug target by gene knock out
	Glutaredoxin like protein 1	PfGlp1	1-Cys-glutaredoxin-like protein 1	Chr3/targeting sequence, putatively mitochondrial	PFC0205c AAK00581 AY014839	Monothiol glutaredoxin 1, negative in HEDS-assay, reacts with glutathione, reduces insulin
	Glutaredoxin like protein 2	PfGlp2	1-Cys-glutaredoxin-like protein 2	Chr6/putatively cytosolic	MAL6P1.72 CAG25239 CR382398	Monothiol glutaredoxin 2, negative in HEDS-assay, reacts with glutathione
	Glutaredoxin like protein 3	PfGlp3	1-Cys-glutaredoxin-like protein 3, CG6	Chr7	Pf07_0036 CAD50844 AAC47843	Monothiol glutaredoxin 3
	Ferredoxin	PfFd		Chr 13/putative signal sequence	NP_705089 NC_004331	Structure solved by Kimata-Ariga *et al*. (1.7 Å; 1IUE)

(Table 1) contd…..

	Name	Abbreviation	Synonyms	Chromosomal/ subcellular localization	Accession numbers[1]	Biochemical characterization/ structural data/target verification
Plasmodial enzymes/biochemical pathways that might be interesting as drug target	Cytosolic glyoxalase II E.C. 3.1.2.6	cPfGloII	Hydroxyacyl-glutathione hydrolase	Chr 4/cytosolic	chr4.gen_37 AY494055	(S)-(2-Hydroxyacyl)glutathione + <=>glutathione + α 2-hydroxy acid anion, Fe^{2+}- or Zn^{2+}-dependent?
	Targeted glyoxalase II E.C. 3.1.2.6	tPfGloII	Hydroxyacyl-glutathione hydrolase	Chr 12/ts	PFL0285w AAQ05976 AF486285	See cytosolic glyoxalase II, Zn^{2+}-dependent
	Dihydrolipoamide dehydrogenase E.C. 1.8.1.4	PfLipDH1	Lipoamide dehydrogenase	Chr12/mito-chondrial	PFL1550w CAF34426 AJ630268	Protein N^6-(dihydrolipoyl)lysine + NAD^+ <=> protein N^6-(lipoyl)lysine + NADH; FAD-dependent, homodimeric
	Dihydrolipoamide dehydrogenase E.C. 1.8.1.4	PfLipDH2	Lipoamide dehydrogenase	Chr8/ts, apicoplast transit peptide predicted	PF08_0066 CAD51214 AL844507	See above
	Superoxide dismutase 1 E.C. 1.15.1.1	PfSOD1 PfFeSOD1		Chr8/cytosolic	PF08_0071 CAA89971 Z49819	$2\ O_2^{\bullet-} + 2\ H^+ <=> O_2 + H_2O_2$ iron dependent, homodimeric
	Superoxide dismutase 2 E.C. 1.15.1.1	PfSOD2 PfFeSOD2		Chr6/mitochon-drial	PFF1130c AAT11554 AY586514	$2\ O_2^{\bullet-} + 2\ H^+ <=> O_2 + H_2O_2$ likely to be iron-dependent, homodimeric
	GPx-like TPx E.C. 1.11.1.-	$PfTPx_{Gl}$ PfGPx	Glutathione peroxidase like TPx	Chr12/- apicoplast transit peptide predicted	PF00255 CAA92396 Z68200	Thioredoxin-dependent detoxification of peroxides
	2-Cys peroxiredoxin 1 E.C. 1.11.1.-	PfTPx1 Pf 2-CysPrx1 PfPrx1 PfPx1 PfTrx-Px1	Thioredoxin peroxidase 1	Chr14/cytosolic	PF14_0368 AF225977 BAA97121	Detoxification of peroxides with Trx1 and Grx1 and Plrx as reductants; involved in $ONOO^-$ detoxification, knock out was not lethal but increased sensitivity to oxidative stress
	2-Cys peroxiredoxin 2 E.C. 1.11.1.-	Pf 2-CysPrx2 PfTPx2 PfPrx2 PfPx2 PfTrx-Px2	Thioredoxin peroxidase 2	Chr12/mito-chondrial	PFL0725w AF225978 AAK20024	Detoxification of peroxides, source of reducing equivalent needs to be established
	1-Cys peroxiredoxin E.C. 1.11.1.-	Pf 1-Cys Prx Pf 1-Cys TPx Pf 1-Cys Trx-Px Pf 1-Cys Px PfPx1	1-Cys peroxiredoxin	Chr8/cytosolic	PF08_0131 BAA78369 AB020595	Detoxification of peroxides, slight activity with thioredoxin system measured, detoxification of peroxides with GSH, DTT. Structure of the *P. yoelii* enzyme solved by Vedadi *et al.* (2.3 Å; 1XCC)
	PfAOP E.C. 1.11.1.-	Pf antioxidant protein	Grx/Trx-dependent peroxidase	Chr7	MAL7P1.159 CAD51033 AL844506	Detoxification of peroxides with PfGrx1, PfTrx1 and Plrx, structure solved at 1.8 Å resolution by Sarma *et al.*, 1X1Y
	Thioredoxin 1	PfTrx1		Chr14/cytosolic	PF14_0545 AAF34541 AF202664	Active with PfTrxR, PfTPx1 and AOP, reduces insulin disulfides and transfers electrons to ribonucleotide reductase, structure solved according to Robien and Hol (2.95 Å; 1SYR)

Due to the high number of publications in this field no references are given here (please see text).

[1]PlasmoDB Accession number from http://www.plasmodb.org; protein and nucleotide accession numbers from http://www.ncbi.nlm.nih.gov/entrez. The protein entries in the Entrez search and retrieval system have been compiled from a variety of sources, including SwissProt, PIR, PRF, PDB, and translations from annotated coding regions in GenBank and RefSeq. The Entrez Nucleotides database is a collection of sequences from several sources, including GenBank, RefSeq, and PDB.

thioredoxin system comprising NADPH, thioredoxin reductase (TrxR), different thioredoxin-like proteins and the most potent hydrogen peroxide detoxifying enzymes, namely thioredoxin-dependent peroxidases (TPx) has been characterized and two functional superoxide dismutases as well as two lipoamide dehydrogenases are present [1, 13, 15]. Interestingly, catalase (detoxifying hydrogen peroxide) and glutathione peroxidase (reducing peroxides glutathione-dependently) are absent in the parasite. This suggests that the thioredoxin system plays a prominent role in *P. falciparum* – a hypothesis that is further supported by PfTrxR knock out studies proving that this enzyme is essential for erythrocytic stages of the parasite [16]. Many other of the enzymes mentioned here have recently been studied in functional and structural detail and their potential as promising targets for the development of novel antimalarial drugs is presently being evaluated.

A number of in-depth review articles on redox metabolism in malaria has been published over the last years. These articles include overviews on the thioredoxin systems in different parasites [17], function and metabolism of glutathione in *P. falciparum* [14], redox and antioxidant systems of *P. falciparum* [15], as well as on oxidative stress in malaria parasite-infected erythrocytes and host-parasite interactions [13]. Recent transcriptome analyses [18-20] serve as a data mine for many malaria researchers. Bozdech and Ginsburg, 2004 [21], have analyzed the transcription of genes coding for redox-active proteins and viewed them in the time-frame of the intraerythrocytic cycle of *P. falciparum* (DeRisi transcriptome database http://malaria.ucsf.edu/, further data on http://carrier.gnf.org/publications/CellCycle and http://www.PlasmoDB.org). In addition, there are very recent articles on parasite disulfide reductases as targets for chemotherapy [22], on redox reactions in malarial parasites [23], and on glutathione *S*-transferase of *P. falciparum* [24]. Metabolic pathways in *P. falciparum* are summarized under http://sites.huji.ac.il/malaria. In order to avoid redundancies we thus should like to focus in this article on other antioxidant enzymes of *P. falciparum* and compare their potential value as drug targets.

III. REDOX-ACTIVE PROTEINS UNIQUE FOR MALARIAL PARASITES

There are a number of structural and functional features which define a protein as a promising drug target. Ideally the respective parasite pathway or protein does not occur in the (human) host and can thus be specifically targeted – a strategy decreasing the risk of side effects. An example in *P. falciparum* would be the non-mevalonate pathway of isoprenoid biosynthesis in the apicoplast [25, 26]. Also in the redox metabolism of *P. falciparum* there are some proteins which do not occur in the human host. These include (i) a chimeric enzyme exhibiting both glucose 6-phosphate dehydrogenase and 6-phosphogluconolactonase activity, (ii) plasmoredoxin, a redox-active protein with similarities to thioredoxins, (iii) a novel class of glutathione *S*-transferases, (iv) a glyoxalase I which has evolved *via* a gene duplication and fusion event, (v) a glyoxalase I-like protein of yet unknown function, and (vi) at least four very recently identified putatively redox-active selenoproteins.

The first two steps of the pentose phosphate pathway are catalysed by a single bifunctional enzyme. The glutathione and the thioredoxin system depend on reducing equivalents provided by NADPH, which is at least in part produced by the enzymes glucose 6-phosphate dehydrogenase (G6PD) and 6-phosphogluconate dehydrogenase (6PGD) in the hexose-monophosphate shunt (HMS). *P. falciparum* G6PD, compared to other G6PDs has a 300 amino acids extension at the N-terminus [27, 28]. This sequence is not related to G6PD but is similar to a family of proteins (devb) of unknown function, some of which are encoded next to G6PD in certain bacteria. The human devb homologue has been shown to have 6-phosphogluconolactonase (6PGL) activity. This suggested that PfG6PD may be a bifunctional enzyme, the evolution of which has involved the fusion of adjacent genes [28]. This hypothesis was verified using *P. berghei*. In spite of solubility problems, conditions were found where the full-length *P. berghei* enzyme was produced in soluble form and purified. This enzyme was shown to have both G6PD and 6PGL activities. Thus the first two steps of the HMS are catalysed by a single novel bifunctional enzyme in malarial parasites. In addition to the HMS, glutamate dehydrogenase and isocitrate dehydrogenase activities have been postulated as alternative mechanisms of $NADP^+$ reduction in malarial parasites. The elucidation of the three-dimensional structure of *P. falciparum* G6PH/6PGL would be essential for judging whether this bifunctional enzyme can be specifically targeted.

Plasmoredoxin is a thioredoxin-like protein unique for malarial parasites. Plasmoredoxin (Plrx) is a 22 kDa redox-active protein which does – based on the currently available database information – only occur in malarial parasites [29]. Plasmoredoxins of comparable size were identified in the genomes of *P. vivax*, *P. berghei*, *P. yoelii*, and *P. knowlesi* showing amino acid sequence identities of 67.4%, 66.9%, 72.6%, and 67.2% with *P. falciparum* Plrx. Plrx are members of the thioredoxin superfamily, however, they cluster separately from other members in a phylogenetic comparison and harbours the unique active site motif WCKYC. Thioredoxins represent a group of small (appr. 12 kDa) redox-active proteins involved in cellular redox regulatory processes as well as in deoxyribonucleotide synthesis and antioxidant defense [17, 30]. Thioredoxin, glutaredoxin, and trypanosomal tryparedoxin are members of the thioredoxin superfamily and share many structural and functional features. The identities of Plrx with other members of this family, for example PfTrx (31.4%) or PfGrx (27.5%) are comparatively low. Apart from members of this superfamily, the highest degree of identity was determined as 31.3% with ResA (P35160), a respiration regulating protein of *Bacillus subtilis*, and 32.6% with HelX (M96013), a putative periplasmic disulfide oxidoreductase of the photosynthetic bacterium *Rhodobacter capsulatus*. Homology modelling resulted in a partial three-dimensional structure of *P. falciparum* Plrx. Residues 43 to 94, representing 28% of the complete amino acid sequence were modelled and indicated a characteristic thioredoxin fold including the active site motif.

Recombinantly produced Plrx can be reduced by glutathione but much faster by dithiols like thioredoxin,

glutaredoxin, and the trypanosomal dithiols trypanothione, and tryparedoxin. Most interestingly, Plrx is not reduced by thioredoxin reductase or glutathione reductase. Reduced Plrx on the other hand reduces glutathione disulfide and provides electrons for ribonucleotide reduction, thioredoxin peroxidase 1, and antioxidant protein (see below). Its capability of reducing H_2O_2 and hydroxyethyl disulfide GSH-dependently is negligible. As demonstrated by Western blotting, the protein is present in blood stage forms of malarial parasites. Neither the full three-dimensional structure nor the exact function of the protein in malarial parasites is known. In spite of its unique active site motif WCKYC, a specific targeting of Plrx by potential antimalarial drugs is most likely to be hampered by the close relationship to other members of the thioredoxin superfamily. However, the specificity of plasmoredoxin offers the opportunity to improve diagnostic tools based on PCR or immunological reactions.

Apart from Plrx, one typical thioredoxin (PfTrx1), with CGPC as active site motif, was described in *P. falciparum* [31]. It is reduced by PfTrxR and provides reducing equivalents to peroxidases (PfTPx1, $PfTPx_{Gl}$, and antioxidant protein, see below) as well as to ribonucleotide reductase. Furthermore, non-enzymatic reduction of glutathione disulfide and different peroxides by PfTrx1 was observed [31, 32]. A PDB (protein database) entry with structural data for PfTrx1 (1SYR) exists, a complete threedimensional structure has, however, not yet been reported. Additional thioredoxin-like proteins (Tlps) have been identified in the genome of *P. falciparum*. Four annotations may represent thioredoxins [32] and are presently under investigation. Like for plasmoredoxin, a specific targeting of these proteins by drugs will – due to the close structural relationship between them - be difficult. However, the potential of human thioredoxin inhibitors which are presently tested for their anticancer activity [33, 34], remains to be investigated also as a antimalarial approach.

P. falciparum *glutathione S-transferase cannot be assigned to any of the known GST classes.* The development of drug resistance often encountered in cancer therapy and the therapy of parasitic diseases has been related to the function of glutathione *S*-transferase (GST), which can contribute to drug clearance [35]. There are reports indicating that in chloroquine resistant parasites GST activity is related to drug pressure [36, 37]. This topic is, however, controversially discussed [38, 39]. In cases where GSTs are thought to play a role in drug resistance, chemomodulation might be achieved by using inhibitors of glutathione synthesis or by using GST inhibitors.

Inhibition of PfGST is expected to act at different vulnerable metabolic sites of the parasite in parallel, which further supports its promising potential as drug target. PfGST inhibition is likely to disturb GSH-dependent detoxification processes, to enhance the levels of cytotoxic peroxides - since the protein has peroxidase activity - , and possibly to increase the concentration of toxic hemin. It has been shown that in the presence of GSH the parasitotoxic hemin inhibits PfGST in the lower micromolar range indicating that free hemin might be buffered by the highly abundant enzyme *in vivo* [38, 40]. It has furthermore been shown that chloroquine inhibits hemin catabolism leading to intracellular hemin accumulation [41]. It might thus be speculated that PfGST inhibitors act synergistically with chloroquine.

Recently it became evident from the genome sequence that *P. falciparum* possesses only one GST. The x-ray structure of PfGST at 1.9 Å resolution [42, 43] cannot be assigned to any of the GST classes described so far and thus represents a novel GST-isoform. Whereas the glutathione binding site of PfGST is highly conserved, its H-site, the binding site for the second substrate - differs significantly from its human counterparts (Fig. **1**) [42, 43]. In contrast to all other GSTs, PfGST contains only five residues following helix α8, which is too short to form a wall (mu- or pi-class) or an α-helix (alpha-class) as it is the case in human GSTs. This leads to a more solvent accessible H-site in PfGST than in the other classes which suggests that the substrate spectrum of PfGST is broader, includes amphiphilic compounds, and is accessible to amphiphilic inhibitors which are not able to enter the H-site of the human isoenzymes.

In addition to peptide inhibitors, hemin and protoporphyrin IX (K_i = 10 μM), cibacron blue (K_i = 0.5 μM), ethacrynic acid (IC_{50} = 30 μM), and *S*-hexylglutathione (K_i = 35 μM) as well as a whole range of additional H-site and G-site interacting compounds have been characterized as PfGST inhibitors [38; see Table **2** and 24 for review]. Ellagic acid, e.g., is an anticarcinogen inducing the transcription of GST genes thus enhancing the anticarcinogenic capacity of cells. Furthermore ellagic acid is a known inhibitor of GSTs and has been previously shown to possess antimalarial activity in the upper nanomolar range (Table **2**). The x-ray structure of PfGST as a complex with *S*-hexyl-glutathione has been reported by Perbandt *et al.* [43]. The elucidation of further PfGST-inhibitor interactions using steady-state kinetics, site-directed mutagenesis, and x-ray crystallography will allow for optimisation of G-site and H-site inhibitors. These compounds could then be even coupled resulting in highly specific double-headed drugs.

Glyoxalase I has evolved from a gene duplication event and has twice the size of the human enzyme. The glyoxalase system consists of glyoxalase I (GloI), glyoxalase II (GloII), and the coenzyme glutathione. It is a cyclic metabolic pathway removing toxic 2-oxoaldehydes like methylglyoxal by converting them to the corresponding non-toxic 2-hydroxycarboxylic acids like D-lactate. Glyoxalase I catalyzes the formation of S-2-hydroxyacylglutathione (a thiol ester of GSH), and glyoxalase II then hydrolyses the ester thus producing GSH and a free 2-hydroxycarboxylic acid. Thus the glyoxalase system catalyzes an intramolecular redox reaction. As a first contribution to the elucidation of their system in malarial parasites, recombinant *P. falciparum* cytosolic glyoxalase I (PfGloI) was characterized as a monomeric Zn^{2+}-containing enzyme of 44 kDa [44]. Most interestingly, PfGloI was found to consist of two very similar halves each of which is homologous to the small 2-domain glyoxalase I of man. Both parts of the *pfgloI* gene were overexpressed separately; the C-terminal half of PfGloI was found to be a stable protein and formed an enzymatically active dimer. These results support the hypothesis of domain-swapping and subunit fusion as mechanisms in *P.*

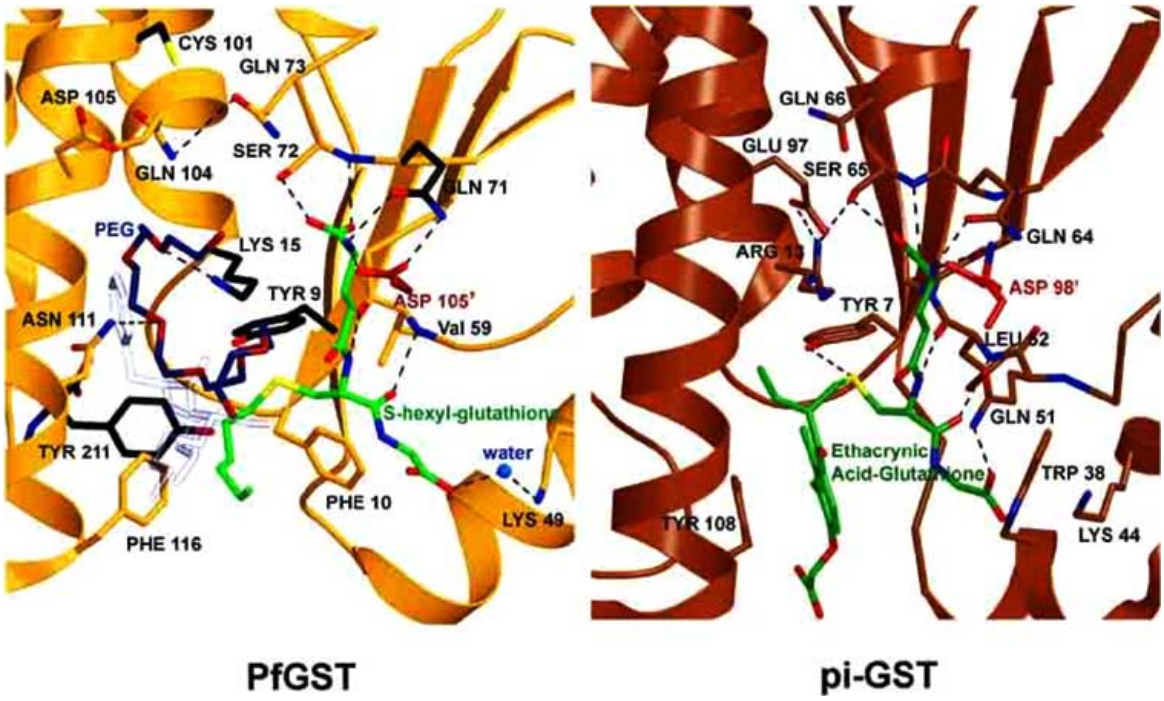


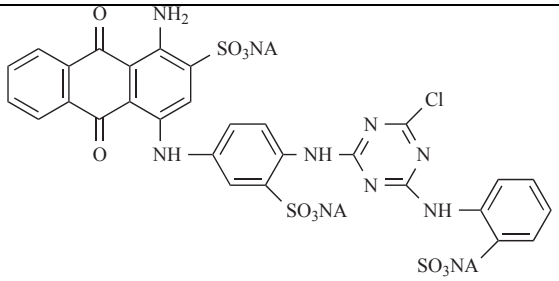
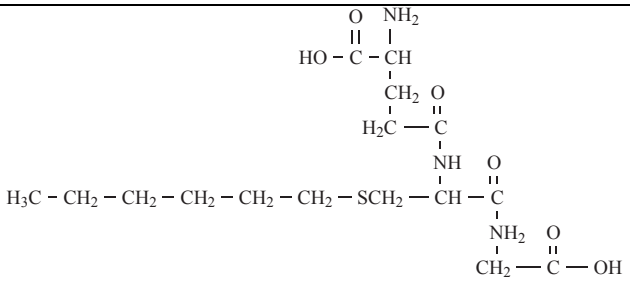
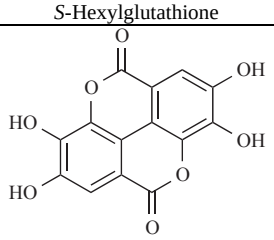
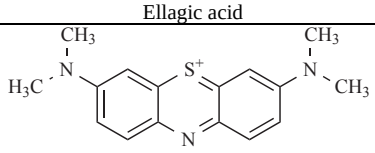
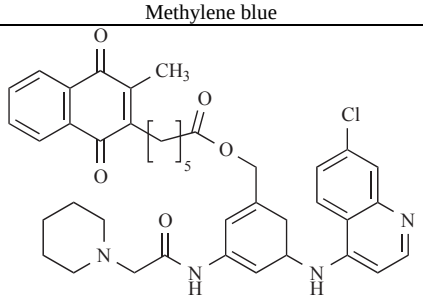
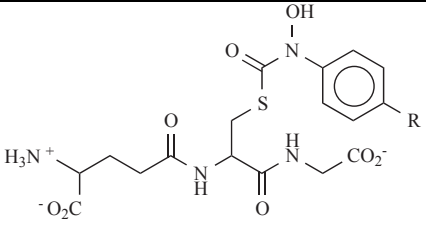
Fig. (1). Equivalent views of the PfGST H-site. Close up of PfGST (a, gold) and a human pi-GST (b, brown, 11gs), focusing on the H-site. H-site residues and glutathione conjugate moieties (green) are shown opaque. In PfGST, the H-site region contains a polyethylene glycol molecule (blue). Important active site residues are shown in black. The peptide segment (Leu115-Phe116-Lys117) of the crystallographically related neighbouring monomer, which occupies the H-site in the native structure, is shown transparent [42]. The figure was kindly provided by Karin Fritz-Wolf, Max-Planck Institute for Medical Research, Heidelberg.

falciparum glyoxalase I evolution (see Fig. **2**). The monomeric parasite cytosolic GloI comprises two independent potential active sites which are presumably very similar to the active sites of the human homodimer and coordinate a zinc ion each. Also most of the residues involved in the formation of a hydrophobic binding pocket at the active site of human GloI [45] are conserved in cytosolic GloI. Despite the sequence similarities with the human enzyme, a complete structural model of cytosolic GloI could not be generated which indicates substantial differences between the two proteins [46]. The fact that the parasite enzyme is monomeric with four domains and appr. twice the size of human GloI, whereas the human enzyme is dimeric with 2 domains per subunit, might offer the opportunity to selectively target the parasite enzyme.

Glyoxalase I-like protein is also unique for malarial parasites. In addition to cytosolic GloI, a second protein which has highest similarities to GloI, was identified in *P. falciparum* and recombinantly produced [46]. The protein named glyoxalase-like protein (GILP) was found to be unique for malarial parasites. The GILP gene from chromosome 6 consists of 924 bp and its gene product of 307 amino acids contains an N-terminal apicoplast targeting sequence (with a probability of >98%, PATS 1.2.1N) [47]. High sequence identities were obtained with the respective proteins from other *Plasmodium* species (e.g. 67% identity with *P. yoelii*) but only low identities were found with proteins from other organisms, the highest value being 25% identity with a protein from *Arabidopsis thaliana*. GILP has a calculated molecular mass of 35.8 kDa and is not active as glyoxalase under standard assay conditions. It was not possible to generate a model of GILP based on human GloI due to insufficient sequence similarity [46]. The yet undefined substrate specificity of GILP as well as the localization of the protein, its putative function in the apicoplast, and its potential as drug target will have to be studied in further detail.

There are at least four unique potentially redox-active selenoproteins in P. falciparum. Four putative selenoproteins (located on chromosomes 4, 8, and 14) as well as a complete selenoprotein translation machinery have recently been identified in the genome of *P. falciparum* [48, 49]. All four proteins are unique for malarial parasites and two of them, a 13 kDa and a 28 kDa protein, have a putatively redox-active cysteine-selenocysteine containing active site. Two of them, the 28 kDa and a 42 kDa protein are preceded by a putative signal peptide sequence and may be located to the apicoplast. Since none of these proteins occurs in the human host and selenocysteine residues show high reactivity towards electrophilic compounds, the selenoproteins of malarial parasites represent potential drug targets of highest interest. Although the Sec-insertion machinery in *P. falciparum* has not yet been characterized, phylogenetic trees suggest that the plasmodial selenocysteine insertion system is perhaps the most divergent among previously characterized eukaryotic systems [48]. The biochemical properties of the four putative selenoproteins and the selenocysteine insertion system as well as their suitability as drug targets is presently being studied.

Table 2.

<table>
<tr><th>Inhibitor structure</th><th>Comment</th></tr>
<tr><td>Asn-Asn-Thr-Asn-Leu-Phe-Lys-Asn-Asn-Ala-Thr</td><td>This undecapeptide was deduced from a peptide loop of a neighbouring GST monomer which interacts with the active site of PfGST in the crystal structure [42]. The peptide inhibits PfGST with a K_i of 115 μM and serves as lead for peptidomimetic inhibitors.</td></tr>
<tr><td> 
Cibacron blue 3GA</td><td>Cibacron blue 3GA is a competitive inhibitor of PfGST with a K_i of 0.5 μM [38].</td></tr>
<tr><td> 
S-Hexylglutathione</td><td>*S*-Hexylglutathione is a competitive inhibitor of PfGST with a K_i of 35 μM. An x-ray structure of the *S*-hexylglutathione-PfGST complex is available [38, 43].</td></tr>
<tr><td> 
Ellagic acid</td><td>Ellagic acid ([1]benzopyrano[5,4,3-cde][1]benzopyran-5,10-dione,2,3,7,8-tetrahydroxy-(7Cl,8Cl,9Cl)) is a competitive inhibitor of PfGST (K_i ca. 3 μM) and inhibits the growth of *Plasmodium falciparum* in the upper nM range [95].</td></tr>
<tr><td> 
Methylene blue</td><td>Non-competitive inhibitor and subversive substrate of GR and TrxR [22].</td></tr>
<tr><td> 
M5Q ester, a double prodrug</td><td>The prodrug consists of a menadione-derived GR-inhibitor linked to a 4-anilinoquinoline *via* an ester bond. The prodrug is most likely to be hydrolyzed in the parasite's food vacuole and releases both active components [65].</td></tr>
<tr><td>$ONOO^-$
Peroxynitrite</td><td>Inactivating nitration of Tyr86 and Tyr94 of PfGR [94]; in addition $ONOO^-$ is a substrate of GR and a moderate inhibitor of PfGST.</td></tr>
<tr><td> 
R = H in HPC-GSH
Cl in HCPC-GSH
Br in HBPC-GSH
Glutathione derivatives</td><td>HPC-GSH: S-(*N*-hydroxy-*N*-phenylcarbamoyl) glutathione. HCPC-GSH: S-(*N*-hydroxy-*N*-chlorophenylcarbamoyl)glutathione. HBPC-GSH: S-(*N*-hydroxy-*N*-bromophenylcarbamoyl)glutathione.
These GSH-derivatives are potent inhibitors of *P. falciparum* glyoxalases (Glo); c, cytosolic; t, targeted. The following table gives K_m/K_i values in μM [46].
<table>
<tr><th></th><th>cGloI</th><th>tGloII</th><th>cGloII</th></tr>
<tr><td>HPC-GSH</td><td>10/ND</td><td>185/ND</td><td>6/6</td></tr>
<tr><td>HCPC-GSH</td><td>0.11/0.08</td><td>30/50</td><td>2/0.7</td></tr>
<tr><td>HBPC-GSH</td><td>0.06/0.06</td><td>20/30</td><td>1.6/0.5</td></tr>
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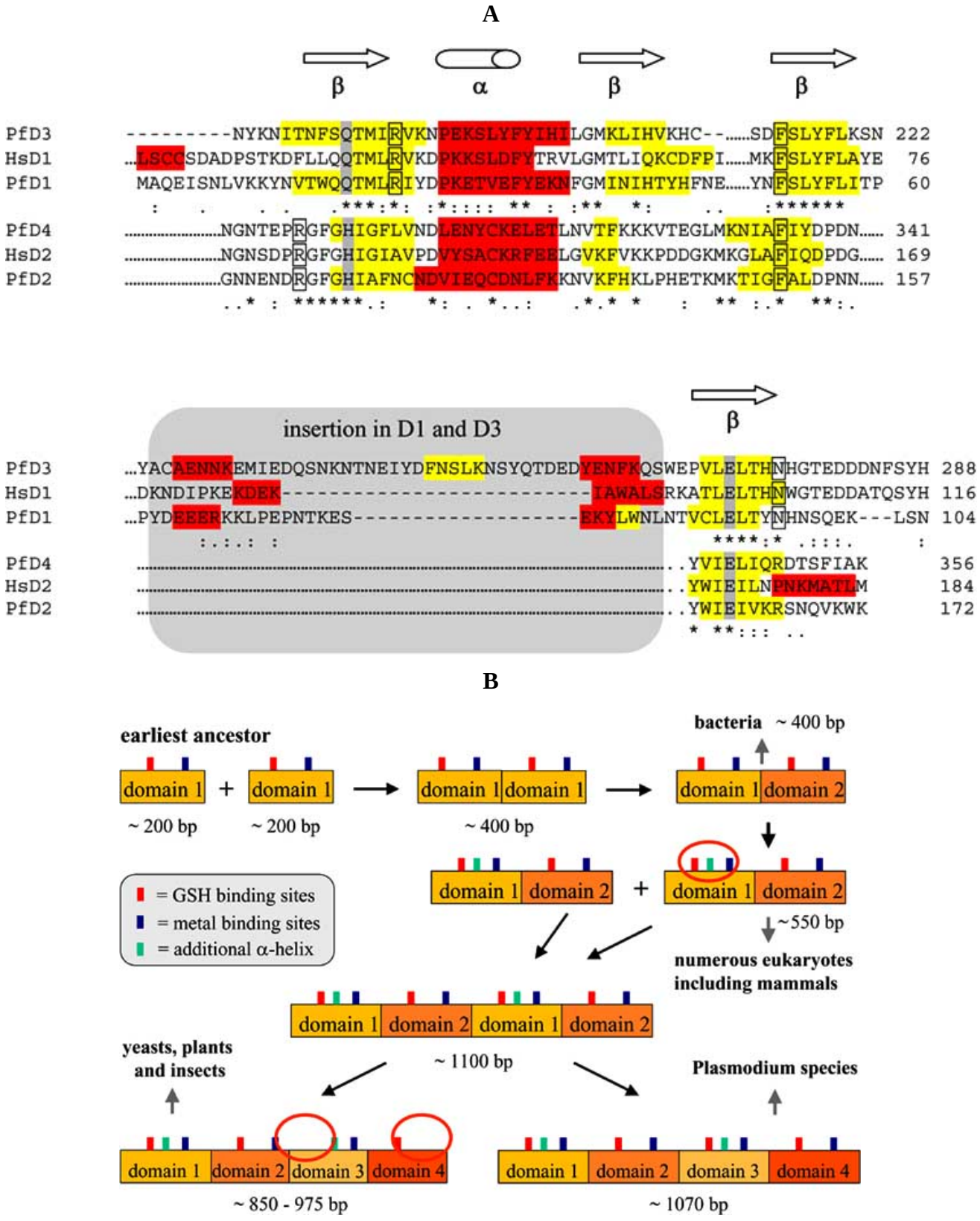

Fig. (2). a) Alignment of the individual domains from human glyoxalase I (HsD1 and HsD2) and *P. falciparum* glyoxalase I (PfD1 to PfD4). Residues involved in GSH-binding (as shown for the human enzyme by Cameron *et al.*, 1997 [92]) are boxed, residues involved in metal binding are shadowed. The secondary structure of the βαβββ motif was derived from the crystal structure of the human enzyme [92], and deduced from a secondary structure prediction program [93] for *P. falciparum* glyoxalase I. red = α-helix, yellow = β-sheet. **b) Scheme of a possible evolution of *gloI*-genes**. The red circles indicate the acquisition of an additional helix or the partial loss of binding site motifs.

IV. REDOX-ACTIVE PROTEINS WITH STRUCTURAL AND FUNCTIONAL DIFFERENCES IN COMPARISON TO THEIR HUMAN COUNTERPARTS

In malarial parasites there is a number of redox-active proteins which have isofunctional human counterparts but differ significantly in their structure from the host enzymes. Particularly when employing structure-based drug design, these proteins might be successfully targeted by potential antimalarial drugs. For substantiating this approach a target validation is essential. This validation might be achieved e.g. by gene disruption (knockout) or gene silencing *via* RNA interference. Due to the haploid nature of blood stage parasites, their intraerythrocytic localization, and their morphology these approaches are very complex and the experiments are difficult to conduct [50-52]. The respective technologies are gradually but with much effort established in the field. Also cloning and heterologous overexpression of *P. falciparum* genes is - due to the high AT content of the genes and the specific codon usage of the parasites - not always trivial. For specific inhibitor development, the elucidation of the three-dimensional structure of the target protein is of great advantage. Ideally the architecture of a target molecule is compared with the respective host enzyme in atomic detail. Until now, only about 50 structures of proteins from malarial parasites have been deposited in the NCBI or PDB database (http://www.ncbi.nlm.nih.gov/; http://www.rcsb.org/pdb/). The overall sum of appr. 80 listed structures is higher due to doublets generated by depositing different subunit-entries, different crystal forms of the same protein, and additional structures of enzyme-inhibitor complexes. Considering a total number of ~5500 genes in *Plasmodium* [53], below 1% of the proteins have been structurally characterized. The crystal structures available include important proteins like dihydrofolate reductase-thymidylate synthase, lactate dehydrogenase, major surface protein 1, and plasmepsin [54-60]. However, also the structures of three major players in the redox metabolism of *P. falciparum* have been solved, namely glutathione reductase [61], glutathione *S*-transferase (see above) [42, 43], and a peroxiredoxin named antioxidant protein (AOP) [62]. Furthermore, there are entries for structural data on PfTrx1, ferredoxin, and a 1-Cys peroxiredoxin from *P. yoelii*, which have, however, not yet been published in detail (see also Table **1**).

The redox-active parasite proteins described in the following paragraphs represent enzymes which do have an (isofunctional) counterpart in man but which exhibit structural features that might be exploited for drug development.

Glutathione reductase is known in atomic detail. High levels of GSH in malarial parasites are NADPH-dependently maintained by the homodimeric FAD-containing enzyme GR [63]. *P. falciparum* GR has been characterized biochemically and kinetically [14, 22 for reviews]. A number of reports indicate that a lack of FAD, the prosthetic group of GR, or of the cosubstrate NADPH, as observed in glucose-6-phosphate dehydrogenase deficiency, lead to substantial protection from severe malaria. These clinical and epidemiological observations suggest that both human GR and PfGR are potential targets for the development of antimalarial drugs. The three dimensional structure of PfGR was solved at a resolution of 2.6 Å [61]. Although the overall architecture of PfGR is very similar to human GR, specific features of PfGR have been identified that might serve as starting point for the development of specific inhibitors. These include the intersubunit cavity, two major insertions in *P. falciparum* GR, and the pair of interface helices. GR is constitutively transcribed throughout the intraerythrocytic life cycle. Relative transcript levels of GR are low in the ring stage and rise in the early trophozoite stages. The highest level is reached in the trophozoite and early schizont stage with a maximum at 22 hours post invasion (HPI) [21]. A number of inhibitors have already been tested and are presently being developed (see Table **2**). Since a comprehensive review article on parasite disulfide reductases as drug targets has been published very recently [22], this topic shall not be addressed in more detail here.

Thioredoxin reductase fuels the thioredoxin system using its accessible C-terminal redox center. The *P. falciparum* thioredoxin reductase [for reviews see 13, 15, 17, 22] is a homodimeric, FAD-dependent oxidoreductase. As is the case for mammalian TrxRs, the role of an additional C-terminal redox center in catalysis has been proven and its interaction with the active site disulfide-dithiol has been studied in detail [64]. The fact that PfTrxR is non-selenium dependent and differs in its C-terminal active site motif (CGGGKC) from human TrxR (Cys-Sec) represents a good starting point for the development of antiparasitic drugs. As recently demonstrated by knock out studies, PfTrxR is indeed essential for erythrocytic stages of *P. falciparum* [16]. Until now no crystals suitable for x-ray crystallography could be produced. However, several lines of drug development are presently being followed [22, 65, 66].

Glutaredoxin-like proteins represent a heterogeneous group of proteins with unknown functions. As mentioned above, also glutaredoxins (Grx) belong to the thioredoxin superfamily. Like the other members, Grx possess a "thioredoxin or glutaredoxin fold" which consists of central β-sheets surrounded by α-helices. Grx are involved in cellular redox reactions and have also been shown to reduce ribonucleotide reductase. Apart from one classical glutaredoxin [67] a group of proteins with great sequence similarities to classical Grx has been identified in *P. falciparum* and other organisms. However, these *glutaredoxin-like proteins* (Glp) possess a CxxS-active site motif with serine replacing the second cysteine [67-69]. Besides possible structural similarities - modelling of yeast and *Plasmodium* proteins revealed a thioredoxin-fold - the putative N-terminal domains of glutaredoxin-like proteins differ significantly in size, sequence, and cysteine content, possibly leading to completely different cellular functions. Most knowledge on these new proteins has so far been obtained using yeast as a model organism. Grx5 of *Saccharomyces cerevisiae* possesses an N-terminal sequence of 29 amino acids which targets the mature protein to the mitochondrial matrix, there it is involved in synthesis, assembly or repair of Fe/S clusters as has been shown by gene disruption *in vivo* [68]. Deglutathionylation of glyceraldehyde-3-phosphate dehydrogenase isoenzyme Tdh3 of cells exposed to H_2O_2 is delayed in the absence of Grx5 [70]. Yeast Grx5 is inactive in the HEDS-assay, presumably as a result of the slow reduction of Grx5 by glutathione [71].

Yeast Grx4 can be phosphorylated at Ser134 by the kinase Bud32 in the nucleus [72].

Three genes encoding glutaredoxin-like proteins (PfGlp1, PfGlp2, and PfGlp3) were detected in *P. falciparum.* The respective proteins contain the conserved active site cysteine in a CGFS-, CKFS-, and CKYS-motif, respectively [67]. Biochemical properties of recombinantly produced PfGlp1 and PfGlp2 have been described by Deponte *et al.* [73]. Cys99, the only cysteine residue in PfGlp1, has a pK_a of 5.5 and is able to mediate covalent homodimerization. Monomeric and dimeric PfGlp1 react with GSSG and GSH, respectively. PfGlp2 is monomeric and both of its cysteine residues can be glutathionylated although glycine is replaced by lysine in the CGFS-motif. Molecular models reveal a thioredoxin fold for the potential C-terminal domains of all three PfGlps as well as conserved residues presumably required for glutathione binding. However, PfGlp1 and PfGlp2 neither possess activity in the classical glutaredoxin assay nor as glutathione peroxidase or glutathione *S*-transferase. Mutation of Ser102 in the CGFS-motif of PfGlp1 to cysteine to recover a CxxC-motiv did not generate glutaredoxin activity either. It is concluded that, despite the ability to form a mixed disulfide with glutathione, Glps are a mechanistically and functionally heterogeneous group with only little similarities to classical glutaredoxins. PfGlp3 carries a putative apicoplast targeting sequence. The physiological function of PfGlps as well as their potential value for drug development remains to be studied in more detail.

Ferredoxin. Ferredoxin is one of the so-called ISC(iron-sulfur cluster)-proteins found in many organisms from bacteria to mammals. Ferredoxin - being a [2Fe-2S]-protein - is reduced by NADPH in a reaction catalyzed by ferredoxin $NADP^+$-reductase (FNR). Both proteins have been identified in *Plasmodium* and are supposed to be located in the apicoplast [74, 75]. Data available support a mechanism by which ferredoxin accepts electrons during Fe-S cluster biosynthesis on IscU (a scaffold protein for Fe-S synthesis) to form a stable oxidized cluster [for review see ref. 76]. Vollmer *et al*., 2001, [74] showed that apicomplexa possess a plant-type FNR/Ferredoxin redox system. The exact biochemical role of this prototype of a plant electron transport system in protists needs to be determined. Nevertheless, the authors suggest, that its main task lies in the provision of reduced ferredoxin, whose most obvious function might be - besides involvement in FeS-assembly - its involvement in fatty acid desaturation by ferredoxin-dependent desaturases [77, 78]. This is most likely to be an essential function in the parasite, given the various membranes it has to build up and to maintain under different environmental conditions (vacuolar membrane, three-laminar cell membrane, various organellar membranes, plus the four plastid membranes). Because of its absence in the host, the apicomplexan FNR/Ferredoxin system offers an attractive target for developing specific drugs. This goal is facilitated by the three-dimensional structures which have been elucidated for several plant FNRs and ferredoxins [79, 80 and references therein]. Furthermore, there exists a PDB entry with structural data obtained for *P. falciparum* ferredoxin (PDB: 1IUE).

V. REDOX-RELATED PROTEINS WHICH HAVE CLOSELY RELATED COUNTERPARTS IN THE HOST BUT MAY STILL BE VALUABLE TARGETS

As mentioned above, malarial parasites seem to be susceptible to alterations in redox metabolism. Thus, also redox-linked enzymes which are neither unique for *Plasmodium* nor have any structural peculiarities should be considered as drug targets. In many cases a parallel inhibition of the isofunctional host enzyme can even support antimalarial therapy – glutathione reductase and glucose 6-phosphate dehydrogenase depletion being prominent examples [13, 14, 22]. In these cases it is of course of particular importance to be aware of potential side effects. Here we should like to briefly discuss glyoxalases II, lipoamide dehydrogenases, superoxide dismutases, and peroxiredoxins. Of course there is a number of other potential redox-associated targets for antimalarial drug development. These proteins include e.g. the enzymes of glutathione biosynthesis (glutathione synthetase and γ-glutamylcysteine synthetase, see 15, 81) which are not described here since they are but indirectly associated with redox reactions.

The glyoxalase system as drug target. In addition to PfGloI and GILP which have been described above, two *P. falciparum* GloIIs (cytosolic GloII named cGloII, and tGloII preceded by a targeting sequence) have been studied and were directly compared with the respective isofunctional host enzymes [46]. Molecular models suggest very similar active sites/metal binding sites of parasite and host cell enzymes. Structures and calculated force field energies of tGloII and cGloII are very similar to the used human template. The residues coordinating two zinc ions at the active site are conserved and the resulting metal binding site is also very similar for the three glyoxalases II. Only a few residues contributing to the glutathione-binding site differ between targeted GloII, cytosolic GloII, and human GloII. Various S-(*N*-hydroxy-*N*-arylcarbamoyl)glutathiones tested as *P. falciparum* GloI and GloII inhibitors were active in the lower nanomolar range (see Table **2**) [46]. Unexpectedly, S-p-bromobenzylglutathione, the diethyl ester prodrug form of which has been demonstrated to inhibit the growth of *P. falciparum in vitro* [82], is but a weak cytosolic GloI inhibitor with a K_i of 20 μM. A much stronger competitive inhibition is observed for human GloI, the K_i value being in the range of 0.17 μM [46, 83, 84]. This strongly indicates that the antiparasitic effects of the compound are not primarily based on the inhibition of the parasite's glyoxalase system but of other enzymes, or on interference with the human methylglyoxal detoxification in red cells. The glyoxalase system of *Plasmodium* will be further evaluated as a target for the development of antimalarial drugs.

Lipoamide dehydrogenases differ in their intracellular localization from host enzymes. Two genes coding for lipoamide dehydrogenases (LipDH), which belong to the same disulfide reductase family as GR and TrxR, have been identified in the genome of *P. falciparum* [85]. LipDHs are constituents of alpha-ketoacid dehydrogenase multienzyme complexes (KADHs). The genes encoding subunits of three different KADHs were identified in the genome and shown to be transcribed during the erythrocytic development of *P.*

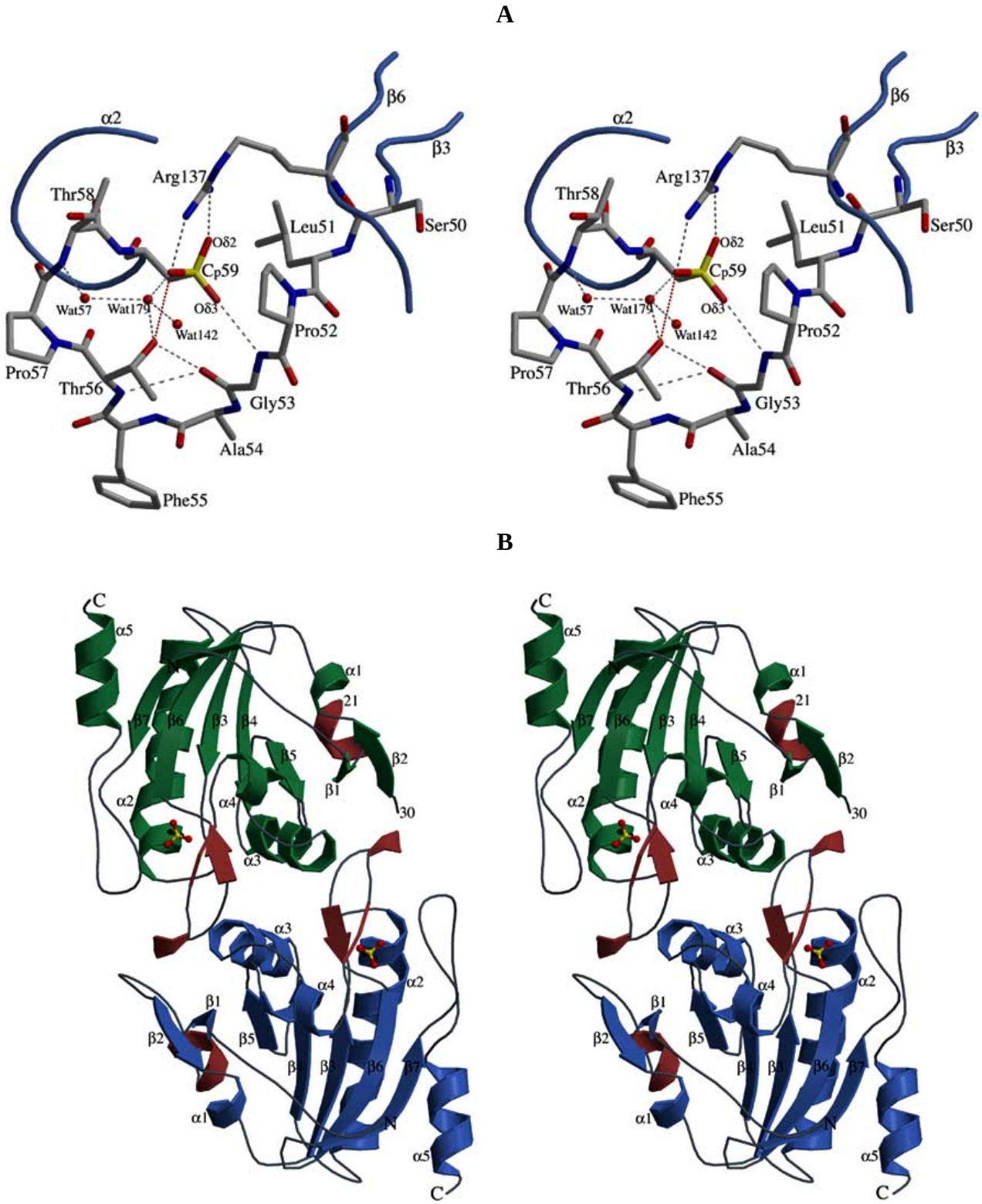

Fig. (3). a) Active site structure of *P. falciparum* antioxidant protein (AOP) [62]. A stereoview shows clear density for the active site cysteine which is present as sulfonic acid (Ocys59) occuring spontaneously under non-reducing conditions during the crystallization process. Water binding sites and active site residues are labeled. Hydrogen bonds are indicated by broken gray lines and the close approach of Thr56 to OCys59 by red dotted lines. The figure was prepared using Bobscript and Raster3D. **b) The structure and the mobility of the PfAOP homodimer.** A ribbon diagram of PfAOP is shown looking down the 2-fold axis. The core secondary structure elements common to all Prxs (β1–β7 and α1–α5) are colored blue. The helix α1 may be an α- or a 3_{10}-helix in various Prxs. Additional PfAOP β-strands and helices (α- and 3_{10}-) are colored red. The active site cysteines, seen here as cysteic acids, are represented by ball-and-stick models. The figure, prepared using Molscript and Raster3D, was kindly provided by G. Sarma and P. A. Karplus, Oregon State University.

falciparum. The localization of the two LipDHs to the parasite's apicoplast and mitochondrion, respectively, was demonstrated by immunofluorescence. McMillan *et al*., 2005, [85] propose that the mitochondrial LipDH is part of the mitochondrial alpha-ketoglutarate dehydrogenase and branched chain alpha-ketoacid dehydrogenase complexes and that the apicoplast LipDH is an integral part of the pyruvate dehydrogenase complex which occurs only in the apicoplast in *P. falciparum.*

Superoxide dismutases will have to be further evaluated. Superoxide dismutase (SOD) is the major enzyme involved in catabolizing the superoxide anion, resulting in production of molecular oxygen and hydrogen peroxide ($2O_2^{\bullet -} + 2H^+ \rightarrow O_2 + H_2O_2$). The superoxide anion promotes cellular oxidative stress by leading to the production of hydroxyl radicals in the Haber-Weiss-reaction sequence, interacting with iron-sulfur clusters and being the reaction partner of NO in the production of peroxynitrite [86]. Endogenously superoxide is produced by the enzyme dihydroorotate dehydrogenase, putatively by the endoplasmic reticulum-resident oxidoreductin [21] and during hemoglobin digestion in the food vacuole [86]. Exogenous superoxide anions result e.g. from hemoglobin autoxidation and from dietary oxidants. However, also the product of SOD, hydrogen peroxide, is toxic. So far, two plasmodial SODs have been identified, namely PfFeSOD1 and PfFeSOD2 [87]. PfFeSOD1 has been shown to be a homodimeric, iron dependent cytosolic enzyme. It is highly expressed in Pf blood cycle stages, especially in trophozoites and early schizonts [21]. The second enzyme, PfFeSOD2, is a mitochondrial homodimeric enzyme expressed more weakly than the PfFeSOD1. The PfFeSOD2 has not yet been functionally characterized and predicted features need to be proven, especially since often the mitochondrial enzymes are Mn-dependent. So far no structure of a parasite SODs has been reported and the potential of the enzymes as drug targets remains to be established.

Inhibition of antioxidant protein and other peroxidases might be valuable for enhancing peroxidative stress. P. falciparum possesses at least five proteins with homologies to peroxiredoxins and other peroxidases. Peroxiredoxins form a family of antioxidant enzymes that act as peroxidases by reducing hydrogen peroxide and other hydroperoxides to water or the corresponding alcohol at the expense of reduced thioredoxin ($Trx(SH)_2$). The protein previously described as glutathione peroxidase (GPx) [88] was shown to be thioredoxin rather than GSH-dependent; it is, however, not a member of the peroxiredoxin family [89]. As a non-selenocysteine GPx homologue, its reactions with hydroperoxides and GSH are 3 orders of magnitudes lower than those of typical selenoperoxidases in other organisms. The majority of the peroxide-detoxifying capacity in malarial parasites seems to be provided by peroxiredoxins. Two peroxiredoxins of the 2-Cys family, one 1-Cys Prx with homologies to atypical 2-Cys Prx, named PfAOP (antioxidant protein), and one typical 1-Cys peroxiredoxin have been identified (see 13, 15, 17, 23 for reviews). One of the 2-Cys Prx, namely PfTPx-1, has been shown to be thioredoxin dependent and seems to be the major cytosolic peroxidase in *P. falciparum.* As shown by gene disruption, the protein is not essential for *P. falciparum*, but it contributes to defence against ROS and RNS (reactive nitrogen species) [90]. Recent kinetic studies on isolated recombinant PfTPx-1 have furthermore shown that the enzyme turns over peroxynitrite and can – apart from thioredoxin – be reduced by glutaredoxin and plasmoredoxin [91].

As shown by titration experiments and the x-ray structure [62], PfAOP represents mechanistically a 1-Cys Prx which can be assigned to the PrxV class [91]. The 1.8 Å resolution crystal structure of homodimeric PfAOP was recently reported. Although characteristic features predisposing the protein for a drug target were not obvious – the active site of PfAOP is given in Fig. **3a** – the structural features of PfAOP – particularly at the subunit interface (Fig. **3b**) – may be used as markers to better classify Prxs and study their evolution [62].

According to transcriptome analyses, cytosolic TPx1 seems to represent the major defence line against peroxides. The transcript level peaks at 11 HPI but stays high until 30 HPI. AOP has a similar profile at a lower intensity whereas 1-Cys Prx is transcribed in higher amounts from ~20-30 HPI with a very high average median intensity. The putatively mitochondrial TPx2 (2-Cys Prx2) and the $TPx_{Gl,}$ which has a predicted apicoplast transit peptide, have maxima at 36 and 34 HPI, respectively. The transcription levels of Trx1 and the genes of some Trx1-dependent enzymes (TrxR, AOP, TPx_{Gl}) seem to be coordinated [21, 23].

Proteins associated with the redox metabolism of malaria parasitized red blood cells do certainly range among the most interesting potential targets for drug development. The functions of and the interactions between most of these proteins remain to be studied in more detail. The same holds true for intracellular localization, transcriptome analyses and knock out studies validating a drug target. Essential for a rational approach to drug development is to enhance information on the three-dimensional structure of the proteins and of protein-ligand complexes.

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