The glutathione redox couple as a thiol switch operator in the malaria parasite  
*Plasmodium falciparum*

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**Summary**

Malaria parasites have stage-dependent high proliferation rates resulting in an increased demand for reducing equivalents. In addition, the high metabolic fluxes of proliferating parasites, the digestion of hemoglobin, and their lifestyle in prooxidant environments lead to an increased endogenous formation of reactive oxygen and nitrogen species. Antioxidant defense, redox regulation, and signaling are thus expected to play crucial roles in *Plasmodium*, represent major targets for chemotherapeutic interventions, and have been shown to be involved in drug resistance. *Plasmodium* has been demonstrated to possess a complex redox system comprising glutathione and thioredoxin-based components. Unique features include specialized selenoproteins and peroxiredoxins, the thioredoxin-like protein plasmoredoxin, and the absence of catalase and a classical glutathione peroxidase. Over the last years we characterized more than 20 redox-active proteins of the parasites structurally and functionally; we elucidated the subcellular compartmentation of redox metabolism; unraveled binding partners of thioredoxin, glutaredoxin, and plasmoredoxin; and identified targets of proteins S-glutathionylation and protein S-nitrosylation.

Within the SPP 1710 project proposed here, we aim to further elucidate the function and regulation of redox switches operated via the glutathione redox couple in *P. falciparum* by focusing on (1) dynamics of the glutathione redox potential under drug-induced stress and on (2) changes of the S-glutathionylation patterns as a regulatory modification under different stress conditions.

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**State of the art and preliminary work**

Malaria is a serious health concern, particularly in Africa and Asia. In its most severe form, malaria is caused by the apicomplexan parasite *Plasmodium falciparum* which is responsible for about 1 million human deaths annually. The development of parasite resistance to clinically used drugs has triggered the search for new targets and further substantiates the need for novel therapeutics [1]. Malaria parasites have stage-dependent high proliferation rates resulting in an increased demand for reducing equivalents. Additionally, the high metabolic fluxes of proliferating parasites lead to an increased endogenous formation of reactive oxygen and nitrogen species as well as redox-active byproducts such as electrophilic 2-oxoaldehydes. Furthermore, the parasite lives in a prooxidant environment, and the immune system of the host increases the oxidative burden on the parasite. Redox regulation and signaling is expected to be a major regulatory mechanism in cellular and metabolic functions of *Plasmodium*. Due to its crucial functions, the redox metabolism of many protozoan parasites including
*Plasmodium* is considered as a major target for chemotherapeutic interventions (for review see [2]). Several antimalarial drugs are supposed to mediate their effects at least partially by increasing oxidative stress in the parasite [3,4]. During recent years *Plasmodium* has been shown to possess an unusually complex system of antioxidant defense mechanisms, namely a glutathione system comprising NADPH, a dual-targeted glutathione reductase (GR), glutathione (GSH), different monothiol glutaredoxins, a functional glutathione-dependent glyoxalase system, and a glutathione S-transferase with peroxidase activity. Additionally, a complete thioredoxin system comprising NADPH, thioredoxin reductase (TrxR), three thioredoxins (Trx), different thioredoxin-like proteins, and several thioredoxin-dependent peroxidases (TPx) has been characterized (see [5] for review). Two major antioxidant enzymes, catalase and glutathione peroxidase, do not occur in the parasite. The redox system of the malaria parasite is distributed in all major subcellular compartments (Figure 1) [6], indicating a strict control of the antioxidant balance and redox-regulatory processes in the parasite.

The glutathione system in *P. falciparum*

Similar to many other organisms, the cysteine-containing tripeptide glutathione is the most abundant low molecular weight thiol in malaria parasites [7]. A high ratio of reduced and oxidized glutathione (GSH/GSSG) is maintained by the flavoenzyme GR, which has been characterized biochemically and kinetically (see [2,5] for reviews). PfGR is a dimeric flavoenzyme that specifically catalyzes the NADPH-dependent regeneration of GSH from GSSG. The GSSG-reducing activity depends on two redox-active cysteine residues (Cys40 and Cys45) within the active site of the enzyme [8].

*P. falciparum* possesses a GR gene with two alternative starts, the first 5’ methionine leads to transcription of GR localized in the apicoplast, while the previously predicted start without the signal sequence leads to a cytosolic GR [6]. Thus, the parasite has an efficient GSSG reduction capacity both in the cytosol and in the apicoplast, indicating a strict regulation of the glutathione redox potential. When comparing *P. falciparum* GR to its human host cell enzyme, several structural differences such as large insertions and differences in the active site architecture become evident; these variations represent potential drug targets within PfGR [9]. We have shown that a GR knock-out in the rodent malaria parasite *P. berghei* blood stages leads to a reduction in total GSH concentrations but does not affect the growth of the rodent parasite [10]. In contrast, the enzyme is essential for oocyst development in the mosquito stage [11]. This indicates that, at least in the rodent model, GSSG can also be partially reduced in the absence of GR, which is likely to be accompanied by a GSSG export from the parasite into the host compartment [7]. In *P. falciparum*, GSSG has been shown to be reduced by PfTrx [12] and also by plasmoredoxin, a Trx-like protein unique for malaria parasites [13]. Multiple reduction pathways of GSSG as one of the central molecules operating thiol switches in the parasite underline the importance of GSH-related regulatory pathways.

To elucidate the role of glutathione for parasite biology and the mechanisms of drug action, we recently established the glutathione biosensor hGrx1-roGFP2, consisting of human glutaredoxin 1 coupled to a redox-sensitive green fluorescent protein, for real-time monitoring of the cytosolic...
glutathione redox potential \( E_{GSH} \) in \( P. falciparum \) blood stages. The cytosolic basal \( E_{GSH} \) of the \( P. falciparum \) strains 3D7 (chloroquine-sensitive) and Dd2 (chloroquine-resistant) are -314 mV and -313 mV, respectively, which is indicative of a highly reducing environment. We furthermore monitored changes in \( E_{GSH} \) after treating parasitized red blood cells with antimalarial drugs. Interestingly, the redox cycler methylene blue depleted GSH in the cytosol of \( P. falciparum \) within minutes. In contrast, quinoline and artemisinin-based compounds influenced cytosolic \( E_{GSH} \) after longer incubations (24 h). Principally, all effects of drugs and oxidants on \( E_{GSH} \) were more pronounced in the chloroquine-sensitive strain compared to the resistant one. Depletion of GSH may cause disruption of vital cellular processes in \( P. falciparum \) and is a possible but not necessary sign of incipient parasite death.

Glutathione itself can regulate the activity of proteins by forming a disulfide bond with sulfhydryl groups of proteins, a reversible process that can occur non-enzymatically. This posttranslational thiol modification is called protein S-glutathionylation and leads to protein-specific structural changes that can modulate enzymatic functions. Furthermore, S-glutathionylation can protect redox-sensitive cysteine residues from irreversible overoxidation under increased oxidative stress [14]. We identified nearly 500 S-glutathionylated proteins in \( P. falciparum \) blood stage parasites, a number that indicates a wide regulatory usage of this modification [15].

Among other proteins, GSH reduces glutaredoxins (Grx), which belong to the thioredoxin superfamily and are thiol-disulfide oxidoreductases. Grx have a broad range of functions including the reduction of ribonucleotide reductase. Based on the presence of one or two cysteines in their active site, Grx can be divided into monothiol and dithiol forms with different reaction mechanisms. While dithiol Grx act on the basis of a classic dithiol-disulfide exchange mechanism, monothiol Grx can attack a disulfide bridge and form a stable intermediate complex but require GSH (or a third cysteine in the target protein) to dissociate the intermediate, thereby releasing a Grx with bound glutathione and a reduced target protein [16]. \( P. falciparum \) is equipped with a single dithiol Grx and three monothiol glutaredoxins (glutaredoxin-like proteins, Glp), which are located in cytosol and mitochondria [6]. PfGrx contains the typical active site motif CPYC, while cytosolic PfGlp1 contains a CGFS motif [17]. PfGlp2 and mitochondrial PfGlp3 carry a CKFS and a CKYS motif, respectively [18]. The function of monothiol glutaredoxins in \( P. falciparum \) still remains to be elucidated.

The thioredoxin system in \( P. falciparum \)

To complement the glutathione system, malaria parasites employ a thioredoxin system formed by NADPH, thioredoxin reductase (TrxR), thioredoxin (Trx), and thioredoxin-like proteins (Tlp) (reviewed in [5]). Furthermore, \( Plasmodium \) codes for plasmoredoxin (Plrx), a CxxC-containing protein with largely unknown functions uniquely found in malaria parasites [13]. \( P. falciparum \) contains two isoforms, a cytosolic and a mitochondrial version of PfTrxR encoded by one gene [6]. Recently, we could solve the crystal structure of PfTrxR in complex with its substrate PfTrx, which revealed differences in Trx binding when compared to the human counterparts [19]. TrxR is essential for the survival of \( P. falciparum \) blood stages and therefore a highly interesting target for specific inhibitors [20]. However, we showed that the rodent malaria
parasite *P. berghei* can survive in the absence of TrxR, indicating functional differences in the redox systems of the two parasites species [10]. The main substrate of TrxR, Trx, is a general cellular redox messenger. Trx possesses a Cys-xx-Cys active site motif, which is kept in a reduced state as a dithiol by TrxR. Reduced thioredoxin transfers electrons to cysteine residues in target proteins such as thioredoxin-dependent peroxidases, ribonucleotide reductase or glutathione disulfide [12]. Thioredoxins have broad cellular functions in most organisms by regulating cell growth and transcription factor activity and inhibiting apoptosis (reviewed in [21], for example). *P. falciparum* expresses three thioredoxins (PfTrx1-3) and two thioredoxin-like proteins (PtItpl and 2). Cytosolic PfTrx1 can directly detoxify peroxides and reduces glutathione disulfide and S-nitrosoglutathion, therefore contributing to the antioxidant defense of *P. falciparum* [12]. It furthermore regulates a complex range of target proteins as discussed below. PfTrx2 is located in the parasitophorous vacuole, that separates the parasite from its hosts cell [6]. PfTrx2 is a component of the translocon of exported proteins (PTEX), which exports proteins to the host cell and most likely assists in unfolding proteins before secretion [22]. PfTrx3 seems to contribute to the redox balance in the endoplasmic reticulum but so far has unknown functions. Additionally, two proteins with high sequence similarity to thioredoxin were identified and named thioredoxin-like proteins 1 and 2; also their function is so far unknown [23].

**The peroxiredoxin system in *P. falciparum***

Eukaryotic peroxiredoxins (Prx) are important components of redox regulation and redox signaling in cells, since they regulate H$_2$O$_2$-mediated signal transduction. H$_2$O$_2$ is an effective signaling molecule that can act either as a destructive oxidant or a positive signal. The cellular switch between these two functions is supposed to be mediated by 2-Cys Prx via a mechanism that is still a matter of debate [24,25]. *P. falciparum* is equipped with five peroxiredoxins: two 2-Cys Prx located in the cytosol and mitochondria (PfPdx1a and PfPdx1m), two 1-Cys Prx in the apicoplast and cytosol (PfPdx5 and PfPdx6) and a nuclear Prx (PfPdxQ). Additionally, the parasite imports human peroxiredoxin 2 from the host erythrocyte [26]. Compared to other organisms, the Prx system in malaria parasites is unusually complex. The cytosolic forms PfPdx1a and PfPdx6 both reduce a broad range of peroxides by accepting PfTrx1 as a reducing partner. Both can be knocked out without affecting the viability of the parasite under unstressed conditions and can thus most likely compensate for each other [27]. Nuclear PfPdxQ is essential for the parasite and most likely protects DNA from oxidative damage [28]. Unraveling the signaling functions of *Plasmodium* Prx will be of high interest for understanding redox regulation and signaling processes.

**Redox-regulatory thiol modifications in *P. falciparum***

Many redox-regulated proteins use cysteine residues as nano switches to change their activity in response to changes in the cellular redox state. Trx, Grx, and Plrx from *P. falciparum* are involved in different cellular processes by interacting with diverse proteins, and thereby play integral roles in redox signaling and redox regulation [6,29]. They typically modify the redox status of cysteine residues that are important for catalytic activity or protein folding. Well-studied enzymes regulated by Trx in different organisms are peroxiredoxins, ribonucleotide reductase, and transcription factors such as NFκB, AP-1, and p53 [21]. Similarly, glutaredoxins can not only reduce ribonucleotide reductase and transcription factors but also directly detoxify hydrogen peroxide [16]. We could show before that Trx, Grx, and Plrx operate a complex set of thiol switches in the malaria parasite; we identified around 30 target proteins of the redoxins in blood stage parasites that interact with the redoxins. Using plasmon resonance and activity assays, we confirmed for several
of the target proteins an interaction, demonstrating that Trx, Grx, and Plrx appear to regulate a complex network of proteins by changing their redox state (unpublished results and [29,30]).

Moreover, *P. falciparum* Trx, Grx, and Plrx have the potential to regulate protein S-glutathionylation by catalyzing the removal of the glutathione moiety from the protein, a process called deglutathionylation [14]. Another oxidative thiol modification is protein S-nitrosylation, a posttranslational modification based on the covalent incorporation of a nitrosol group (-NO) into a protein thiol resulting in an S-nitrosothiolo. Similar to S-glutathionylation, S-nitrosylation can regulate protein functions and is a key mediator of the biological effects of NO [31]. Very recently, we analyzed protein S-nitrosylation as a regulatory thiol switch in *P. falciparum* and demonstrated that PfTrx1 possesses both denitrosylating and transnitrosylating activities.

Deglutathionylation processes seem to depend mainly on NADPH as source of reducing equivalents. NADPH in *Plasmodium* is mainly produced by the pentose phosphate shunt [32], possessing the unique bifunctional enzyme glucose-6-phosphate dehydrogenase 6-phosphogluconolactonase, rather than by glutamate dehydrogenases [33]. Thus, our previous work on thiol-based redox regulation in *P. falciparum* is a starting point of systematically analyzing its function, mechanism, and role in drug resistance and development in malaria parasites.

**Project-related publications**

Figure 5. Real-time imaging of the glutathione redox potential in *P. falciparum*. (A) Confocal live cell images of 3D7 parasites showing expression of hGrx1-roGFP2 localized in the cytosol. (B) Live cell imaging of trophozoite stages of 3D7 hGrx1-roGFP2. After 60s, 3D7 parasites were treated with 1 mM diamide followed 4 min later by the addition of 10 mM dithiothreitol (DTT). 405 nm, 488 nm, merge (405/488 nm), and false-color ratio images at different time points are shown. (C) The ratio of emissions (ratio 405/488 nm) after excitation at 405 and 488 nm was computed for both strains and plotted against time. (D) Fluorescence ratio of untreated cells as a function of time. The ratio 405/488 nm remained stable over a period of at least 10 min. Data from 5 trophozoites for each strain were analyzed. Mean and standard errors of the mean are shown.

Objectives

The project aims to elucidate the role and function of redox switches mediated by the glutathione redox couple in the malaria parasite *P. falciparum*. Here we will focus on two major research questions in glutathione-based regulation.

(1) What is the glutathione concentrations and the glutathione redox potential in *P. falciparum* and how is it affected or does mediated the effects of antimalarial drugs?

**Patents**


Keese MA, Saffrich R, Dandekar T, **Becker K** & Schirmer RH (1999) Microinjected glutathione reductase crystals as indicators of the redox status in living cells. DE # 44 30 719-5


(2) How do levels of protein S-glutathionylation as a thiol-based regulatory switch in *P. falciparum* change in response to different stress conditions and drug treatment? How is protein S-glutathionylation regulated in *P. falciparum*?

![Figure 6. Quantification of S-glutathionylated proteins.](image)

S-glutathionylated proteins are isolated and analysed by MudPIT.

**Cooperation with other researchers**

The following scientists not participating in the SPP “Thiol Switches” will be involved in the proposed project:

Prof. John Yates III, The Scripps Research Institute, La Jolla, CA, USA: mass spectrometry; Prof. P. Andrew Karplus, Oregon State University, Corvallis, USA: crystallography, structural biology; Dr. Jude Przyborski, Philipps University Marburg, Germany: confocal laser microscopy; Prof. Kai Matuschewski, Max Planck Institute for Infection Biology, Berlin: *P. berghei* knock-out models; Prof. Bernd Zechmann, Graz University: glutathione measurements via electron microscopy.

**Scientific equipment**

Our laboratories at the Interdisciplinary Research Center, Giessen University, are equipped according to the present standards of modern molecular biology, cell biology, and biochemistry techniques. This includes centrifuges, chromatography units for protein purification, different thermostatted spectrophotometers, and a multi-well reader, microscopes for crystal chemistry and cell biology including fluorescence microscopy, sterile hoods, incubators, PCR-cyclers including a gradient cyclcer and a real-time PCR cyclcer, HPLC, FPLC, crystallization facility ‘Honey Bee,’ liquid handling robot ‘Lissy,’ and a fully equipped laboratory for cultivating malarial parasites (S2 level). Directly accessible are a DNA-sequencer, confocal laser scanning microscopy, mass spectrometry, and a X-ray diffractometer. For the crystallization studies the X-ray source of the Max-Planck-Institute for Medical Research in Heidelberg as well as the Swiss Light Source synchrotron (Paul Scherrer Institute; Villingen) will be available.
Bibliography


