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1 **Glutathione and thioredoxin systems of the malaria parasite *Plasmodium falciparum*:**
2 **partners in crime?**

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23 **Abstract**

24 In *P. falciparum*, antioxidant proteins of the glutathione and thioredoxin systems are
25 compartmentalized. Some subcellular compartments have only a partial complement of these
26 proteins. This lack of key anti-oxidant proteins in certain sub-cellular compartments might be
27 compensated by functional complementation between these systems. By assessing the cross-
28 talk between these systems, we show for the first time, that the glutathione system can reduce
29 thioredoxins that are poor substrates for thioredoxin reductase (Thioredoxin-like protein 1 and
30 Thioredoxin 2) and thioredoxins that lack access to thioredoxin reductase (Thioredoxin 2). Our
31 data suggests that crosstalk between the glutathione and thioredoxin systems does exist; this
32 could compensate for the absence of certain antioxidant proteins from key subcellular
33 compartments.

34 **Introduction**

35 The malaria parasite *P. falciparum* completes its life cycle in two different organisms:
36 the *Anopheles* mosquito and the vertebrate host. In both hosts, the parasite is subjected to
37 constant oxidative insults. In humans, parasites replicating within red blood cells (RBCs) are
38 bombarded with a high flux of oxygen, resulting in the generation of reactive oxygen species
39 (ROS) [1]. ROS are also generated by metabolic reactions during rapid parasite multiplication,
40 and when the parasite degrades hemoglobin to heme and amino acids [1,2]. Additionally, the
41 apicoplast and the mitochondrion share the heme biosynthetic pathway and electron transport
42 chain, both of which also contribute to ROS production significantly [3-6]. Indeed, parasite-
43 infected RBCs generate twice the amount of ROS compared to uninfected RBCs [1].
44 Given the susceptibility of parasites to oxidative challenge, efficient ROS detoxification
45 systems are vital. The antioxidant network in the cell is compartmentalized into several key sub-
46 cellular systems within the cytosol, the apicoplast and the mitochondrion [7,8]. These systems
47 achieve detoxification through thiol-disulphide exchange reactions and are comprised of two
48 major branches: the glutathione and the thioredoxin systems. The glutathione system includes
49 glutathione (γ-L-glutamyl-L-cysteinylglycine) as a major regulator of redox homeostasis and
50 other thiol-disulphide oxidoreductases: glutathione reductase (GR), thioredoxin peroxidase-like

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51 glutathione peroxidase (TPxGl), glutathione S-transferase (GST), glutaredoxins (Grx) and
52 glutaredoxin-like proteins (GrxlP). The thioredoxin system consists of the small redox-active
53 proteins thioredoxins (Trx), thioredoxin like proteins (Tlp), thioredoxin reductase (TrxR),
54 thioredoxin peroxidase (TPx), peroxiredoxins (Prx) and *Plasmodium*-specific plasmoredoxin
55 (Plrx) [1,2,8].

56 In accordance with the different subcellular locations of oxidative stress generation, the
57 proteins belonging to each of these systems are also compartmentalized in the parasite.
58 Importantly, some subcellular compartments have only a partial complement of proteins
59 required for thiol-disulphide reactions [7,8]. It is, therefore, likely that functional
60 complementation between the glutathione and thioredoxin systems handles the lack of key
61 anti-oxidant proteins in certain sub-cellular compartments.

62 To test this hypothesis, we first defined parasite antioxidant pathways and their sub-
63 cellular localizations. This was done by making a comprehensive list of these proteins and their
64 localizations by compiling data from published literature (immunofluorescence and stable
65 transfection of GFP fusion proteins) and data from predictions using online algorithms available
66 on the *Plasmodium* genome database PlasmoDB [9]. The resulting model of the parasite
67 antioxidant network suggested that key sub-cellular locations in the parasite were lacking a few
68 components of the thioredoxin and glutathione systems.

69 Next, we assessed the possibility of crosstalk between the glutathione and thioredoxin
70 systems by studying the interaction between glutathione and several different thioredoxins
71 using enzymatic assays. For the first time, we show that the glutathione system could play an
72 important role in reducing those thioredoxins that are poor substrates for thioredoxin
73 reductase (Thioredoxin-like protein 1 and Thioredoxin 2) and those thioredoxins that lack
74 access to thioredoxin reductase (Thioredoxin 2). These reactions also could be relevant when
75 thioredoxin reductase is inactivated by electrophilic attack under oxidative stress. Similarly, we
76 show that thioredoxins from the parasite can, in turn, reduce glutathione and act as a backup
77 for the lack of glutathione reductase in a particular subcellular compartment. However, this
78 reaction seems to be of physiological relevance only for Thioredoxin1 and Thioredoxin-like
79 protein 2 as other thioredoxins reduce glutathione disulphide at negligible rates. The results

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80 presented in this report suggest that crosstalk between the glutathione and thioredoxin
81 systems exists and that key antioxidant proteins might exhibit flexibility with respect to their
82 reducing partners and their functions.
83
84 **Materials and Methods**
85 **Cloning and purification of thioredoxins and thioredoxin reductase from *P. falciparum***
86 The *P. falciparum* genome contains genes for three classical thioredoxins (Trx1, Trx2 and Trx3),
87 two thioredoxin-like proteins (Tlp1 and Tlp2) and thiordoxin reductase (TrxR). The cDNA for
88 these were generated using parasite RNA, amplified, and cloned in the bacterial expression
89 vector pET28a (cloning strategy detailed in Supplementary Table 3). All thioredoxins and the
90 thioredoxin reductase were expressed in *E.coli* BL21-DE3 with His-tags. Expressed proteins were
91 purified by Ni^{2+}-NTA column chromatography according to the protocol specified by the
92 manufacturer (Ni^{2+}-NTA superflow cartridge, Qiagen). Trx2, Trx3 and Tlp2 were always
93 associated with the pellet fraction consistent with the presence of targeting sequences in these
94 proteins. Extensive attempts to improve the solubility of these proteins were not successful. To
95 overcome this problem, we re-cloned these genes lacking the putative organellar targeting
96 signals and found that only Trx2 and Tlp2 could be purified from the soluble fraction
97 (Supplementary Figure 1). The variants of Trx3 were hardly soluble and extensive attempts to
98 improve the solubility of these variants were not successful. After purifying four thioredoxin
99 proteins (Trx1, Trx2, Tlp1 and Tlp2), we systematically tested the activities of these thioredoxins
100 as described in the next section.
101
102 **Insulin reduction assay for thioredoxin activity**
103 The activity of each thioredoxin was assayed in an insulin reduction assay that works on the
104 principle of reduction of disulphide bonds in insulin leading to the precipitation of the β-chain
105 [10]. This precipitation can be quantified spectrophotometrically at 600 nm and directly reflects
106 the extent of protein disulphide oxidoreductase activity. Each 500 µl reaction contained 50 mM
107 potassium phosphate buffer (pH 7.4), 1 mM EDTA, 170 µM bovine insulin (in 50mM Tris/HCl, 1
108 mM EDTA at pH 7.4) and 5 or 10 µM of respective thioredoxin. The reaction was initiated at 25

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109 °C by adding dithiothreitol (DTT) at 1 mM final concentration. 1 mM DTT with 5 µM bovine
110 serum albumin (BSA) served as a negative control. An increase in absorbance at 600 nm was
111 monitored every 5 seconds for 30 minutes. Activity for each thioredoxin at different
112 concentrations was expressed as a slope of a linear part of a turbidity curve to the lag time
113 (reported as $\Delta 600 \cdot min^{-2} \times 10^{-3}$) as described by Martinez-Galisteo *et al* [11]. In additional assays,
114 to identify the functional *Plasmodium falciparum* thioredoxin reductase (PfTR)-thioredoxin
115 pairs, DTT was replaced by 200 µM NADPH and 1 µM PfTR at pH 7.4. In these assays, reaction
116 was initiated by addition of NADPH and increase in absorbance at 600 nm was monitored every
117 5 seconds for 60 minutes. Activity of each PfTR-Trx pair was expressed as described earlier.
118

119 **Thioredoxin reduction by glutathione system**

120 Thioredoxin reduction by the glutathione system was tested using insulin reduction assays.
121 Here, DTT was replaced with glutathione as a reducing agent. Thus, protein disulphide
122 reductase activity of thioredoxins would be observed only if thioredoxins were reduced by
123 glutathione. Each 500 µl reaction contained 50 mM potassium phosphate buffer (pH 7.4), 1 mM
124 EDTA, 170 µM bovine insulin (in 50 mM Tris/HCl, 1 mM EDTA at pH 7.4), 10 mM GSH, 1 $U \cdot ml^{-1}$ *S.*
125 *cerevisiae* glutathione reductase (Sigma) and 10 µM of the respective thioredoxin. The reaction
126 was initiated at 25 °C by adding NADPH at 200 µM final concentration. An increase in
127 absorbance at 600 nm was monitored every 5 seconds for 60 minutes. The reaction without
128 glutathione did not show insulin reduction by any of the thioredoxins, thereby ruling out the
129 possibility of direct reduction of thioredoxins by glutathione reductase. Additionally, assays
130 carried out without thioredoxins confirmed that glutathione alone does not result in insulin
131 reduction. The activity for each thioredoxin at different concentrations was expressed as a
132 slope of a linear part of a turbidity curve to the lag time reported as $\Delta 600 \cdot min^{-2} \times 10^{-3}$.
133

134 ***In vitro* reduction of GS-SG by purified thioredoxins and thioredoxin reductase**

135 Thioredoxins were tested for their ability to reduce GS-SG. A typical reaction mixture contained
136 50 mM potassium phosphate buffer (pH 7.4), 1mM EDTA, 5 µM respective thioredoxin, 1 µM

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137 PfTR, 250 µM NADPH. Reactions were started at 25 °C by the addition of GS-SG at 1 mM final
138 concentration and the formation of GSH from GS-SG was estimated by following NADPH
139 oxidation at 340 nm. Positive control for the reaction system contained 50 mM potassium
140 phosphate buffer (pH 7.4), 1mM EDTA, 250 µM NADPH, 1mM GS-SG and 0.1 nM GR.

141 **Results**

142 **A model of anti-oxidant networks in *Plasmodium falciparum***

143 The subcellular localizations of antioxidant proteins were predicted by bioinformatics
144 tools and these predictions were compared with experimental evidence as shown in
145 Supplementary Table 1. Most predictions were consistent with the experimental data and
146 enabled us to propose a model network of antioxidant proteins in different compartments of
147 the parasite cell (Figure 1). Although most bioinformatics predictions were consistent with
148 experimental data, a few discrepancies were reported and are listed in Supplementary Table 2a
149 and 2b. After analysis of the discrepancies between predictions and experimental data, we
150 placed nine proteins (Trx2, Trx3, 1-cys-Glrx, AOP, GlP3, TPx_{Gl}, GR, TrxR and glyoxalase 2) in all
151 predicted and experimentally defined subcellular compartments. These are shown in a model
152 network of antioxidant proteins (Figure 1) with question marks indicating the discrepancy
153 between predicted localization and experimental localization.

154 This proposed model of antioxidant proteins suggests that a few subcellular locations in
155 the parasite cell lack key components of the antioxidant defense systems. For example, the
156 mitochondrion lacks glutathione reductase. Similarly, the parasitophorous vacuole contains
157 Trx2 as a part of the protein export pump; however, it does not contain thioredoxin reductase
158 required for reducing thioredoxins. Strikingly, the thioredoxin system is completely absent in
159 the apicoplast. The organelle contains neither thioredoxins nor thioredoxin reductase.

160 The absence of key enzymes like thioredoxin reductase and glutathione reductase in a
161 few subcellular locations suggests that crosstalk between the glutathione and thioredoxin
162 systems might exist to ensure better redox regulation. This led us to ask whether the
163 glutathione and thioredoxin systems can serve as backups for each other.

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166 **Biochemical properties of thioredoxins**

167 Thioredoxins and thioredoxin reductase were purified as described in the materials and
168 methods section. All of these proteins (Trx1, Trx2, Tlp1, Tlp2 and TR), each with a His-tag, were
169 purified to electrophoretic homogeneity as shown by SDS-PAGE (Supplementary Figure 1).
170 Next, the activity of each thioredoxin was assayed in an insulin reduction assay (detailed in the
171 Materials and Methods section). All thioredoxins were found to be active in the dithiothreitol-
172 dependent insulin reduction assays (Figure 2) with varying levels of protein disulphide
173 reductase activity.

174 The highest DTT-dependent protein disulphide reductase activity among all thioredoxins
175 was exhibited by Trx1. Trx2 (active site sequence: WCQAC) and Tlp2 (active site sequence:
176 WCAPC) which show partial conservation of the consensus thioredoxin active site motif
177 (WCGPC) showed 12 fold and 75 fold lower activities than Trx1 (active site sequence: WCGPC)
178 (Figure 1 inset). Surprisingly, despite having a consensus thioredoxin active site motif, Tlp1
179 (active site sequence: WCGPC) at twice the concentration of Trx1, showed 120 fold lower
180 protein disulphide reductase activity than Trx1. The results presented here suggest that
181 thioredoxins might show differential activities under *in vivo* conditions as well.

182 Next, we examined the ability of *Plasmodium* thioredoxin reductase to serve as an
183 electron donor to these thioredoxins. This was important to analyse as we expected that
184 thioredoxins which are poor substrates of thioredoxin reductase might be linked with other
185 reducing partners *in vivo*. This was tested using insulin reduction assay where TR and NADPH
186 were used as a reducing system. Of the four thioredoxins tested, Trx1, Tlp2 and Trx2 were
187 capable of reducing insulin when incubated with TR. The highest activity was reported for Trx1
188 followed by Tlp2 (~4 fold lower activity than Trx1), suggesting that these thioredoxins are linked
189 with TR *in vivo* (Figure 3). Trx2, which demonstrated substantial protein disulphide reductase
190 activity using DTT as a reductant, exhibited far less activity (63 fold and 16 fold lower than Trx1)
191 with TR (Figure 3). Not surprisingly, Trx2 showed less activity with TR. This is expected because
192 Trx2 is located in the parasitophorous vacuole where there is no TR. Additionally, Tlp1 did not
193 show any activity with TR as a reducing partner. These results suggested that Trx2 and Tlp1
194 might be associated with other reducing partners *in vivo*.

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195

196 **Only Trx1 and Tlp2 support GS-SG reduction in the parasite**

197 In a possible interaction between glutathione and thioredoxin systems, *P. falciparum*
198 Trx1 has been shown to reduce GS-SG *in vitro*. This reaction seems to be relevant for a
199 particular subcellular location that lacks glutathione reductase or under conditions of enzyme
200 insufficiency. Therefore, we decided to test whether any of the other thioredoxins could reduce
201 GS-SG *in vitro*. This was investigated by assessing the reduction of GS-SG in presence of purified
202 TR, NADPH and each of the four different thioredoxins. In this assay, *E. coli* glutathione
203 reductase was used as a standard for GS-SG reduction.

204 The data from Figure 4 indicates that TR cannot reduce GS-SG in the absence of Trx1 or
205 Tlp2. The Trx1-TR and Tlp2-TR combinations reduced GS-SG with different efficacies. Other
206 thioredoxins, viz. Trx2 and Tlp1, showed GS-SG reduction at negligible rates. These results
207 suggest that, in the malaria parasite, GS-SG reduction by thioredoxins might be physiologically
208 feasible only in the cases of Trx1 and Tlp2.

209

210 **Glutathione can reduce *Plasmodium* thioredoxins which are poor substrates of TR**

211 *Plasmodium* thioredoxins are localized to different subcellular compartments (Figure 1).
212 Some of these compartments lack thioredoxin reductase, thereby questioning the presence of a
213 complete functional thioredoxin system at those locations. Additionally, when we checked for
214 functional TR-thioredoxin pairs, we found that Trx2 was a poor substrate for TR and that Tlp1
215 could not serve as a substrate at all.

216 In order to understand alternative mechanisms for the reduction of these thioredoxins,
217 we performed biochemical assays using Trx-dependent insulin reduction. In these assays, DTT
218 was replaced with glutathione as a reducing agent. Therefore, protein disulphide reductase
219 activity of thioredoxins would only be observed if the thioredoxins were reduced by
220 glutathione. This was verified by performing the assay without glutathione, in whose absence
221 insulin reduction by any of the thioredoxins did not take place. This ruled out the possibility of
222 the direct reduction of these thioredoxins by glutathione reductase, and not by glutathione

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223 itself. Additionally, assays carried out without thioredoxins confirmed that glutathione alone
224 cannot reduce insulin.
225 The data presented in Figure 5 show that all thioredoxins from the parasite can reduce
226 disulphide bridges in insulin, using glutathione as the reducing power. Of the four thioredoxins
227 tested, the highest activity was reported for Trx2 followed by Trx1, Tlp2 and Tlp1. Of note is the
228 fact that Trx2, which exhibits far less activity with TR and NADPH as a reducing system, was
229 efficiently reduced by glutathione. Using glutathione as a reducing system, it showed 4 fold and
230 5 fold higher activities than Trx1 and Tlp2 respectively. Similarly, Tlp1 which does not serve as a
231 substrate for TR, was effectively reduced by glutathione. Together, these observations lead us
232 to propose that the glutathione and the thioredoxin systems closely interact with each other,
233 and that the glutathione system might reduce those thioredoxins that are poor substrates for
234 thioredoxin reductase (such as Tlp1 and Trx2) or those thioredoxins located at a subcellular
235 compartment lacking thioredoxin reductase (such as Trx2).
236

237 **Discussion**

238 Antioxidant proteins in the parasite are localized to various subcellular compartments.
239 However, our proposed model network of antioxidant proteins in different compartments of
240 the parasite cell suggested that not all of these compartments are equipped with a complete
241 glutathione or thioredoxin system. These results suggest either that each organelle utilizes only
242 one of the glutathione or thioredoxin systems, or alternatively that the two systems interact
243 with each other. Therefore, we decided to assess the interdependence of the glutathione and
244 thioredoxin systems. We speculated that the absence of a few critical antioxidant proteins from
245 these compartments should have been compensated either by novel proteins or by additional
246 roles played by the existing antioxidant proteins. In this study, we have shown that both
247 glutathione and thioredoxin systems closely interact with each other for the antioxidant
248 defense of the parasite.
249 The interaction between thioredoxin and glutathione systems has been reported
250 previously in *Plasmodium*. This was, however, with a small subset of proteins. It has been

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251 shown that Trx1 from the parasite can support glutathione reduction in a variety of
252 physiological conditions [12]. We performed extensive analyses with multiple thioredoxins that
253 span a majority of the compartments in the parasite. We found that only Trx1 and Tlp2 could
254 support glutathione reduction. This reaction seems to be relevant for those subcellular
255 compartments lacking glutathione reductase, such as the mitochondrion. In support of this
256 hypothesis, our enzymatic analyses using GHOST assays indicate that Tlp2, which is localized to
257 the mitochondrion, can support the reduction of GS-SG, possibly compensating *in vivo* for the
258 lack of glutathione reductase [7]. This phenomenon might be important during the loss of
259 glutathione reductase at a particular stage of the parasite life cycle. For example, *Plasmodium*
260 *falciparum* merozoites contain glutathione but lack glutathione reductase [13]. Furthermore, a
261 glutathione reductase null mutant of *Plasmodium* is viable in the intra-erythrocytic stage of the
262 life cycle [14]. Therefore, it is necessary that select thioredoxins from the parasite (Trx1 and
263 Tlp2) compensate for this loss of GR function.

264 An interesting question arises regarding the capacity of the glutathione system to act as
265 a backup in the absence of thioredoxin reductase in a particular subcellular compartment. For
266 instance, the parasitophorous vacuole contains Trx2 as a component of the multimeric PETEX
267 translocon which is responsible for exporting proteins into the host RBCs [15]. Here, Trx2 is
268 speculated to reduce the disulphide bridges thereby unfolding the proteins to facilitate their
269 export through the translocon [15,16]. Therefore, in order to have a functional translocon, Trx2
270 must be maintained in its reduced state. The parasitophorous vacuole, however, lacks the
271 thioredoxin reductase required for the regeneration of oxidized Trx2, thus suggesting an
272 alternative mechanism of Trx2 reduction [7]. On the other hand, Tlp1 and TR are both localized
273 to the cytosol in the parasite [7]. Although both of these proteins reside in the same
274 compartment, Tlp1 does not get reduced by TR, suggesting the involvement of a different
275 reducing partner. When we evaluated the capacity of *Plasmodium* thioredoxin reductase to
276 serve as an electron donor to Trx2 and Tlp1, we observed that Trx2 exhibited very low activity
277 and Tlp1 did not show any activity. Collectively, these data suggest that there might be
278 alternative mechanisms of thioredoxin reduction in the cell.

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279 It has been recently demonstrated that the glutathione system, particularly glutaredoxin
280 1 and glutaredoxin 2, can act as backups for the thioredoxin reductase, and have a role in
281 thioredoxin reduction in cells that have lost TrxR activity [17,18]. Recently, it has been shown
282 that, thioredoxin from the parasitic flatworm *Fasciola gigantica* is preferentially reduced by the
283 glutathione system and could be acting as a glutaredoxin [19]. A direct reduction of
284 thioredoxins by glutathione in our biochemical analyses suggests that glutathione might serve
285 as a backup for thioredoxin reductase. In addition to this role, it might be the sole reductant for
286 the thioredoxins that are not exposed to TR (such as Trx2 in parasitophorous vacuole), or for
287 thioredoxins that are not substrates of TR (such as Tlp1 in the cytosol). Based on our results, we
288 speculate that Trx2 and Tlp1 might be reduced by glutathione *in vivo*. Similarly, reduction of
289 thioredoxin by glutathione might also occur at other subcellular compartments where
290 thioredoxin reductase is absent or inactivated by an electrophilic attack.
291 In our enzymatic analysis, Trx1 and Tlp2 were efficiently reduced by thioredoxin
292 reductase and the localization of these proteins overlaps (Trx1 and TR in cytosol, Tlp2 and TR in
293 mitochondrion). In contrast, Trx2 was found to be a poor substrate for thioredoxin reductase
294 and of note is the fact that, localization of these proteins does not overlap (Trx2 in PV
295 compartment and TR in cytosol). These results show that thioredoxin-thioredoxin reductase
296 pairs can evolve both in function and substrate specificity with respect to their localization. The
297 observed flexibility and adaptations of thioredoxins for their reducing partners due to
298 differential localization makes these proteins particularly suitable for novel redox reactions.
299 In conclusion, our data suggest that the absence of certain antioxidant proteins in the
300 key subcellular compartments is compensated by crosstalk between the glutathione and
301 thioredoxin systems of the cell. This mutual interaction might strengthen the antioxidant
302 network, thereby ensuring cell survival under constant oxidative stress.
303

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309

310 **Conflict of interest**

311 The authors declare no conflict of interest.

312

313 **References**

314

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364 **Figure Legends**

365 Figure 1. Schematic of the putative model of antioxidant networks in *P. falciparum*. Question
366 marks indicate that the localization is ambiguous or that the reaction proposed is not
367 experimentally verified yet. Abbreviations: HbO-Fe^{3+}; oxyhemoglobin with ferriprotoporphyrin,
368 HbO-Fe^{3+}; oxyhemoglobin containing ferroprotoporphyrin, O_2; molecular oxygen, $O_2^{\cdot-}$;
369 superoxide anion, SOD1; superoxide dismutase 1, SOD2; superoxide dismutase 2, H_2O_2;
370 hydrogen peroxide, TPx_{Gl}; glutathione peroxidase-like thioredoxin peroxidase, 1-cys-Prx; 1-
371 cysteine peroxiredoxin, AOP; anti-oxidant protein, TPx1; thioredoxin peroxidase 1, TPx 2;
372 thioredoxin peorxidase 2, Trx1; thioredoxin 1, Trx2; thioredoxin 2, Trx3, thioredoxin 3, Tlp1;
373 thioredoxin like protein 1, Tlp2; thioredoxin like protein 2, Glrx1; glutaredoxin 1, Plrx;
374 plasmoredoxin, TR; thioredoxin reductase, GR; glutathione reductase, GSH; reduced
375 glutathione, GS-SG; oxidized glutathione, Trx-S_2; oxidized thioredoxin, Trx-SH_2; reduced
376 thioredoxin, GloI; glyoxalase I, tGloII; targeted glyoxalase II, ER; endoplasmic reticulum, PC;
377 parasite cytosol, PV; parasitophorous vacuole.
378

379 Figure 2. Dithiothreitol dependent activities of different thioredoxins from *P. falciparum* in
380 insulin reduction assay. Turbidity due to thioredoxin mediated insulin precipitation by DTT was
381 measured at 600nm and plotted as function of time. The assay contained 50 mM potassium
382 phosphate buffer (pH 7.4), 1mM EDTA, 170 µM bovine insulin (in 50mM Tris/HCl, 1mM EDTA at
383 pH 7.4), 5/10 µM of respective thioredoxin and DTT at 1mM final concentration. 1mM DTT with
384 5 µM bovine serum albumin (BSA) served as a negative control. Trx1; thioredoxin 1, Trx2;
385 thioredoxin 2, Trx3, thioredoxin 3, Tlp1; thioredoxin like protein 1, Tlp2; thioredoxin like protein
386 2. The specific activity of each thioredoxin is shown in inset (expressed as $\Delta600\cdot min^{-2} \times 10^{-3}\ mg^{-1}$
387). The data are the mean ± SD of triplicate reactions.
388

389 Figure 3. Comparison of Insulin reduction by *P. falciparum* thioredoxin system components.
390 Turbidity due to insulin precipitation by thioredoxin system components was measured at
391 600nm and plotted as function of time. The assay contained 50 mM potassium phosphate
392 buffer (pH 7.4), 1mM EDTA, 170 µM bovine insulin (in 50mM Tris/HCl, 1mM EDTA at pH 7.4), 10

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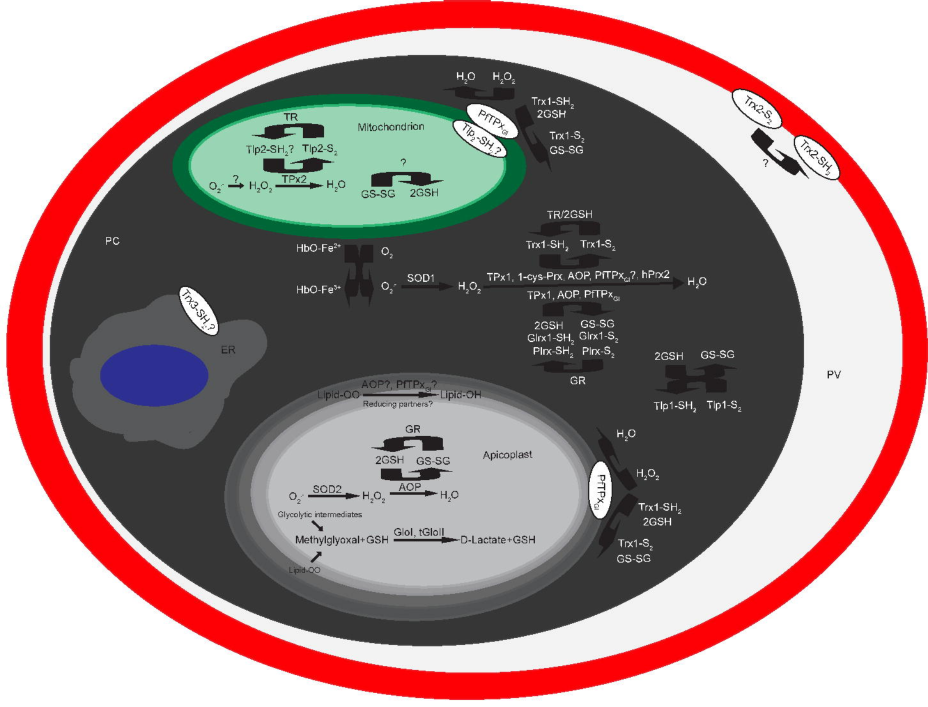
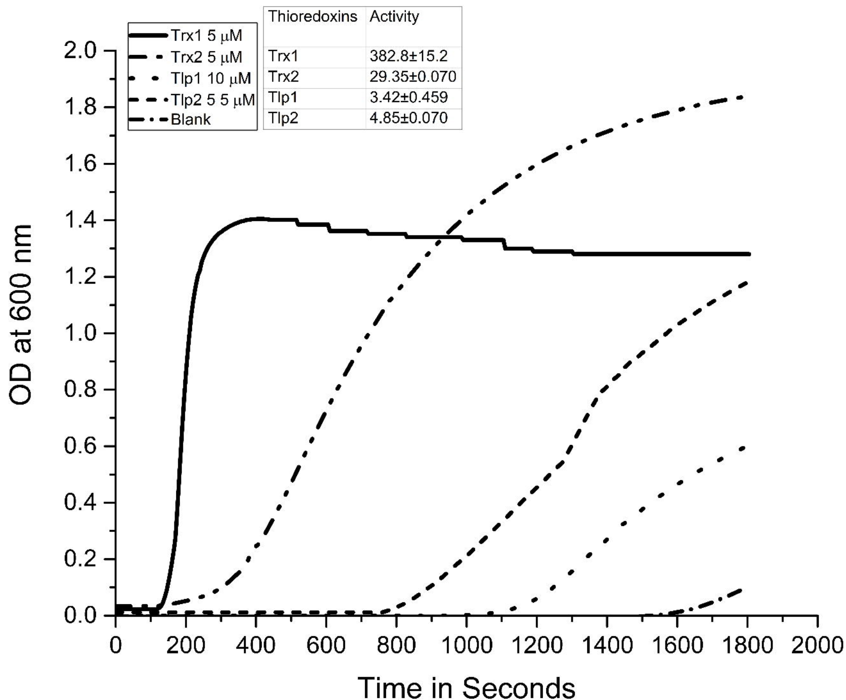
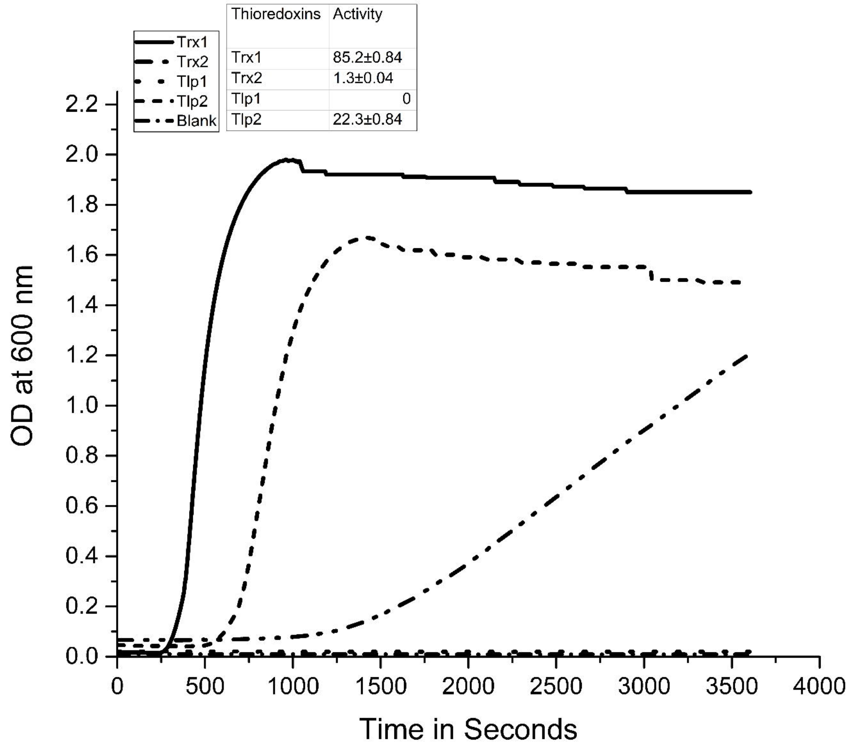
393 µM of respective thioredoxin, 200 µM NADPH and 1 µMPfTR. The reaction without any of the
394 thioredoxins served as a negative control. Trx1; thioredoxin 1, Trx2; thioredoxin 2, Trx3,
395 thioredoxin 3, Tlp1; thioredoxin like protein 1, Tlp2; thioredoxin like protein 2. The specific
396 activity of each thioredoxin is shown in inset (expressed as $\Delta600 \cdot min^{-2}$ x 10^{-3} mg^{-1}). The data are
397 the mean ± SD of triplicate reactions.
398
399 Figure 4. *In vitro* GS-SG reduction by *Plasmodium* thioredoxin system components. Only Trx1
400 and Tlp2 support reduction of GS-SG *in vitro*. Purified thioredoxins were reconstituted with the
401 indicated components and the NADPH consumption was monitored at 340 nm. Error bars
402 indicate S.D. of three replicates.
403
404 Figure 5. Reduction of *Plasmodium* thioredoxins by glutathione. Turbidity due to thioredoxin
405 mediated insulin precipitation by glutathione was measured at 600nm and plotted as function
406 of time. In this assay protein disulphide reductase activity of thioredoxins should be observed
407 only if thioredoxins are reduced by glutathione. The assay contained 50 mM potassium
408 phosphate buffer (pH 7.4), 1mM EDTA, 170 µM bovine insulin (in 50mM Tris/HCl, 1mM EDTA at
409 pH 7.4), 10mM GSH, 1 $U \cdot ml^{-1}$ S. cerevisiae glutathione reductase (Sigma), 200 µM NADPH and
410 10µM of respective thioredoxin. The reaction without glutathione served as a negative control.
411 Trx1; thioredoxin 1, Trx2; thioredoxin 2, Trx3, thioredoxin 3, Tlp1; thioredoxin like protein 1,
412 Tlp2; thioredoxin like protein 2. The specific activity of each thioredoxin is shown in inset
413 (expressed as $\Delta600 \cdot min^{-2}$ x 10^{-3} mg^{-1}). The data are the mean ± SD of triplicate reactions.
414
415



H2O H2O2
Trx1-SH2
2GSH
PfTPxGl
Tlp2-SH2?
Trx1-S2
GS-SG
Trx2-S2
?
Trx2-SH2
TR
Mitochondrion
Tlp2-SH2? Tlp2-S2
?
O2- ? H2O2 TPx2 H2O
GS-SG 2GSH
TR/2GSH
Trx1-SH2 Trx1-S2
PC
HbO-Fe2+ O2
HbO-Fe3+ O2- SOD1 H2O2
TPx1, 1-cys-Prx, AOP, PfTPxGl?, hPrx2
H2O
TPx1, AOP, PfTPxGl
Trx3-SH2?
2GSH GS-SG
Glrx1-SH2 Glrx1-S2
Plrx-SH2 Plrx-S2
GR
2GSH GS-SG
Tlp1-SH2 Tlp1-S2
ER
PV
Lipid-OO AOP?, PfTPxGl? Lipid-OH
Reducing partners?
GR
Apicoplast
2GSH GS-SG
H2O
H2O2
PfTPxGl
Trx1-SH2
2GSH
O2- SOD2 H2O2 AOP H2O
Glycolytic intermediates
Methylglyoxal+GSH GloI, tGloII D-Lactate+GSH
Lipid-OO
Trx1-S2
GS-SG



Trx1 5 μM
Trx2 5 μM
Tlp1 10 μM
Tlp2 5 5 μM
Blank
Thioredoxins Activity
Trx1 382.8±15.2
Trx2 29.35±0.070
Tlp1 3.42±0.459
Tlp2 4.85±0.070
OD at 600 nm
0.0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
1.6
1.8
2.0
0
200
400
600
800
1000
1200
1400
1600
1800
2000
Time in Seconds

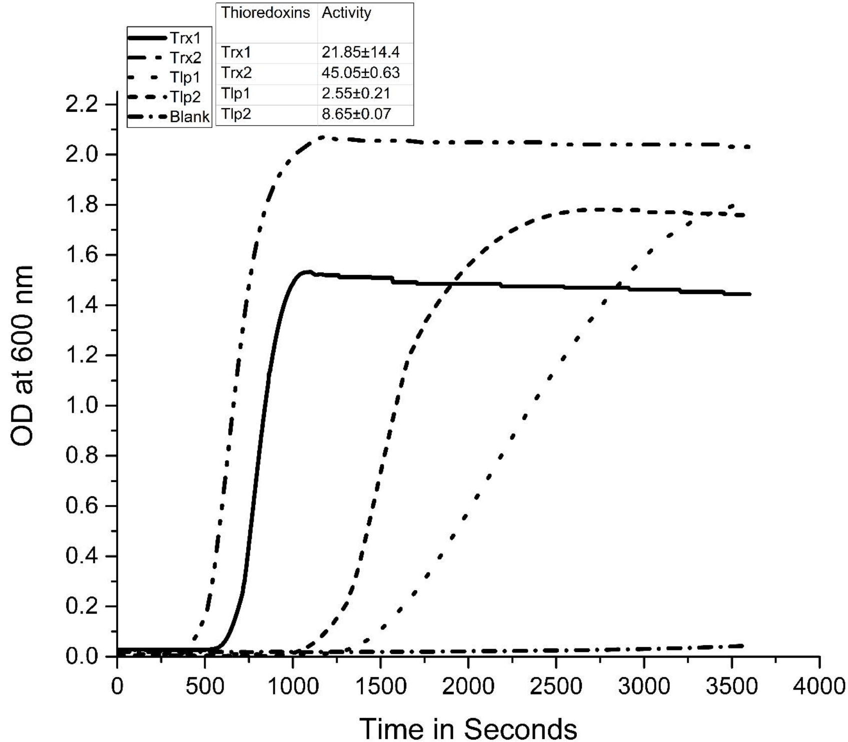


Thioredoxins	Activity
Trx1	85.2±0.84
Trx2	1.3±0.04
Tlp1	0
Tlp2	22.3±0.84

NADPH oxidation
µMoles/min

32
30
28
26
24
22
20
18
16
14
12
10
8
6
4
2
0

Trx1	+	-	-	-	+	-	-	-	-
Trx2	-	+	-	-	-	+	-	-	-
Tlp1	-	-	+	-	-	-	+	-	-
Tlp2	-	-	-	+	-	-	-	+	-
TR	+	+	+	+	+	+	+	+	-
GS-SG	-	-	-	-	+	+	+	+	+
GR	-	-	-	-	-	-	-	-	+



Thioredoxins	Activity
Trx1	21.85±14.4
Trx2	45.05±0.63
Tlp1	2.55±0.21
Tlp2	8.65±0.07