1   **Glutathione and thioredoxin systems of the malaria parasite *Plasmodium falciparum*:**

2   **partners in crime?**

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Abstract

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

Introduction

The malaria parasite *P. falciparum* completes its life cycle in two different organisms: the *Anopheles* mosquito and the vertebrate host. In both hosts, the parasite is subjected to constant oxidative insults. In humans, parasites replicating within red blood cells (RBCs) are bombarded with a high flux of oxygen, resulting in the generation of reactive oxygen species (ROS) [1]. ROS are also generated by metabolic reactions during rapid parasite multiplication, and when the parasite degrades hemoglobin to heme and amino acids [1,2]. Additionally, the apicoplast and the mitochondrion share the heme biosynthetic pathway and electron transport chain, both of which also contribute to ROS production significantly [3-6]. Indeed, parasite-infected RBCs generate twice the amount of ROS compared to uninfected RBCs [1].

Given the susceptibility of parasites to oxidative challenge, efficient ROS detoxification systems are vital. The antioxidant network in the cell is compartmentalized into several key sub-cellular systems within the cytosol, the apicoplast and the mitochondrion [7,8]. These systems achieve detoxification through thiol-disulphide exchange reactions and are comprised of two major branches: the glutathione and the thioredoxin systems. The glutathione system includes glutathione (γ-L-glutamyl-L-cysteinylglycine) as a major regulator of redox homeostasis and other thiol-disulphide oxidoreductases: glutathione reductase (GR), thioredoxin peroxidase-like

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

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

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 $\Delta600 \cdot min^{-2} \times 10^{-3}$) 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 $\Delta600 \cdot min^{-2} \times 10^{-3}$.

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

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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165

Biochemical properties of thioredoxins

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

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

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

195

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

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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

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

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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

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

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.

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^{-};
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^{-}
387 ^{1}). 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

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 $\Delta 600 \cdot min^{-2}$ 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 $\Delta 600 \cdot min^{-2}$ x 10^{-3} mg^{-1}). The data are the mean ± SD of triplicate reactions.

414

415

Thioredoxins	Activity
Trx1	382.8±15.2
Trx2	29.35±0.070
Tlp1	3.42±0.459
Tlp2	4.85±0.070

Legend:
- Trx1 5 µM
- Trx2 5 µM
- Tlp1 10 µM
- Tlp2 5 5 µM
- Blank

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

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