Identification and Characterization of Heme-interacting Proteins in the Malaria Parasite, Plasmodium falciparum*

Naomi Campanale‡§, Christine Nickelt*, Claudia A. Daubenberger†, Dean A. Wehlal**, Jeff J. Gorman**, Nectarios Klonis‡§, Katja Becke†, and Leann Tilley‡§‡‡

From the ‡Department of Biochemistry and §Co-operative Research Centre for Diagnostics, La Trobe University, Melbourne 3086, Victoria, Australia, the ¶Interdisciplinary Research Center, Giessen University, Giessen D-35392, Germany, the ¶§Molecular Immunology, Swiss Tropical Institute, 4002 Basel, Switzerland, and **CSIRO Division of Health Sciences and Nutrition, Parkville 3052, Victoria, Australia

The degradation of hemoglobin by the malaria parasite, Plasmodium falciparum, produces free ferrisprotoporphyrin IX (FP) as a toxic by-product. In the presence of FP-binding drugs such as chloroquine, FP detoxification is inhibited, and the build-up of free FP is thought to be a key mechanism in parasite killing. In an effort to identify parasite proteins that might interact preferentially with FP, we have used a mass spectrometry approach. Proteins that bind to FP immobilized on agarose include P. falciparum fumarate hydratase (PfFHB), P. falciparum glutathione reductase (PfGR), and P. falciparum protein disulfide isomerase. To examine the potential consequences of FP binding, we have examined the ability of FP to inhibit the activities of GAPDH and GR from P. falciparum and other sources. FP inhibits the enzymatic activity of Pf-GAPDH with a Ki value of 0.2 μM, whereas red blood cell GAPDH is much less sensitive. By contrast, PfGR is more resistant to FP inhibition (Ki > 25 μM) than its human counterpart. We also examined the ability of FP to inhibit the activities of the additional antioxidant enzymes, P. falciparum thioredoxin reductase, which exhibits a Ki value of 1 μM, and P. falciparum glutaredoxin, which shows more moderate sensitivity to FP. The exquisitely sensitive of PfGAPDH to FP may indicate that the glycolytic pathway of the parasite is particularly susceptible to modulation by FP stress. Inhibition of this pathway may drive flux through the pentose phosphate pathway ensuring sufficient production of reducing equivalents to counteract the oxidative stress induced by FP build-up.

The malaria parasite feeds by degrading hemoglobin in an acidic food vacuole, producing as by-products, free ferrisprotoporphyrin IX (FP)1 moiety and reactive oxygen species. If the released FP were allowed to accumulate within this compartment, the intravacuolar FP level could reach 300–500 mM (1). The parasite is thought to detoxify most of the FP by sequestration into a pigment known as hemozoin (2). Degradation of FP has been proposed as an alternative mechanism for detoxification (3, 4). However, recent chemical analyses of parasitized erythrocytes indicate that most of the iron is located within the food vacuole and that at least 90% of it is in the form of hemozoin (5). Nonetheless, it is likely that a part of the population of FP escapes the crystallization process and diffuses down the concentration and pH gradient into the parasite cytosol. Indeed, the cellular “free” (i.e., detergent-soluble) FP in Plasmodium falciparum-infected erythrocytes has been estimated to be about 0.1 mM (4, 6). Free FP is toxic because of its detergent-like properties and its ability to undergo redox chemistry.

Thus, the degradation of hemoglobin and the consequent production of FP and reactive oxygen species represent an “Achilles heel” that leaves the parasite susceptible to attack by drugs that interfere with detoxification or enhance the toxicity of the waste products (see Refs. 1 and 7 for reviews). For example, the 4-aminoquinoline, chloroquine (CQ), has been shown to accumulate in the food vacuole and to form a complex with FP (8, 9). As a result CQ inhibits FP detoxification processes and thereby increases free FP levels (3, 4, 10). Similarly, the endoperoxide antimalarial artemisinin is thought to react with reduced FP to form a cytotoxic carbon-centered radical intermediate that oxidizes susceptible groups within parasite enzymes and lipids (11–13).

The parasite protects itself against oxidative stress utilizing small molecular weight antioxidants and a series of antioxidant enzymes. The tripeptide glutathione (GSH) represents the most important low molecular weight antioxidant in P. falciparum. It is involved in redox regulation and the detoxification of reactive oxygen species by direct chemical reactions and functions as an electron donor for glutathione S-transferase and glutaredoxins (14–16). The regeneration of GSH from oxidized glutathione (GSSG) is achieved mainly by the NADPH-dependent flavoenzyme glutathione reductase (GR) (17).

In addition, the thioredoxin system, consisting of thioredoxin (Trx), thioredoxin reductase (TrxR) (18), and Trx-dependent peroxidases (19), represents an important line of antioxidant defense in malaria parasites (20). Apart from its redox-regulatory functions, Trx serves as an electron donor for ribonucleotide reductase, thereby supporting DNA synthesis (21, 22). Trx is reduced by TrxR, a flavoenzyme with high structural and

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†† To whom correspondence should be addressed. Tel: 61-3-94791375; Fax: 61-3-94792467; E-mail: L.Tilley@LaTrobe.edu.au.

‡‡ The abbreviations used are: FP, hemeFerrisprotoporphyrin IX; CQ, chloroquine; RBC, red blood cell; Pf, Plasmodium falciparum; GAP3, glyceraldehyde-3-phosphate; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GR, glutathione reductase; HRP2, histidine-rich protein 2; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight; MOPS, 4-morpholinepropanesulfonic acid; PBS, phosphate-buffered saline; PDI, protein disulfide isomerase; Trx, thioredoxin; TrxR, thioredoxin reductase; Grx, glutaredoxin; GST, glutathione S-transferase; HEDS, bis(2-hydroxyethyl)disulfide; HEDS, bis(2-hydroxyethyl)disulfide; HEDS, bis(2-hydroxyethyl)disulfide; HEDS, bis(2-hydroxyethyl)disulfide; HEDS, bis(2-hydroxyethyl)disulfide; HEDS, bis(2-hydroxyethyl)disulfide; HEDS, bis(2-hydroxyethyl)disulfide; HEDS, bis(2-hydroxyethyl)disulfide; HEDS, bis(2-hydroxyethyl)disulfide; HEDS, bis(2-hydroxyethyl)disulfide; HEDS, bis(2-hydroxyethyl)disulfide; HEDS, bis(2-hydroxyethyl)disulfide; HEDS, bis(2-hydroxyethyl)disulfide; HEDS, bis(2-hydroxyethyl)disulfide; HEDS, bis(2-hydroxyethyl)disulfide; HEDS, bis(2-hydroxyethyl)disulfide; HEDS, bis(2-hydroxyethyl)disulfide; HEDS, bis(2-hydroxyethyl)disulfide; HEDS, bis(2-hydroxyethyl)disulfide; HEDS, bis(2-hydroxyethyl)disulfide; HEDS, bis(2-hydroxyethyl)disulfide; HEDS, bis(2-hydroxyethyl)disulfide; HEDS, bis(2-hydroxyethyl)disulfide; HEDS, bis(2-hydroxyethyl)disulfide; HEDS, bis(2-hydroxyethyl)disulfide; HEDS, bis(2-hydroxyethyl)disulfide; HEDS, bis(2-hydroxyethyl)disulfide; HEDS, bis(2-hydroxyethyl)disulfide; HEDS, bis(2-hydroxyethyl)disulfide; HEDS, bis(2-hydroxyethyl)disulfide; HEDS, bis(2-hydroxyethyl)disulfide; HEDS, bis(2-hydroxyethyl)disulfide; HEDS, bis(2-hydroxyethyl)disulfide; HEDS, bis(2-hydroxyethyl)disulfide; HEDS, bis(2-hydroxyethyl)disulfide; HEDS, bis(2-hydroxyethyl)disulfide; HEDS, bis(2-hydroxyethyl)disulfide; HEDS, bis(2-hydroxyethyl)disulfide; HEDS, bis(2-hydroxyethyl)disulfide; HEDS, bis(2-hydroxyethyl)disulfide.

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mechanistic homology to GR. A glutathione-dependent peroxidase
disease seems to be absent in P. falciparum, whereas at least four
Trx-dependent peroxidases appear to be present (19, 23, 24).
Both the glutathione system, as indicated by drug studies (25),
and the Trx system, as shown by TrxR knock-outs (26), are essential for parasite viability.

The work described here employed a protein profiling approach
to identify FP-binding proteins within the parasite. Some of the enzymes that were identified by this approach were further characterized by examining the activity of FP to inhibit their enzymic activities. These studies allow us to speculate on the possible regulatory role of free FP in controlling parasite metabolism.

**EXPERIMENTAL PROCEDURES**

**Materials**—Recombinant human and P. falciparum GR were pro-
duced as described by Nordhoff et al. (27) and Färber et al. (17) and
were kindly provided by Prof. Heiner Schirmer, Biochemiezentrum, Heidel-
berg University. Recombinant PfGrx1 was produced as described by
Rahlf et al. (15), PfTrxR and PfTrx according to Kanzok et al. (18),

PigAPDH as described by Daubenberger et al. (28), and histidine-rich protein 2
(HRP2) as described by Papalexis et al. (29). FP was pur-
chased as hemin chloride from Sigma or Porphyrin Products and was

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**Fractionation of P. falciparum-infected Erythrocytes**—Parasites
(D10, HB3 strains) were cultured and harvested as described previously
(29). Harvested parasites were mixed with 0.15% saponin (v/v) for 10
min at room temperature to release the erythrocyte cytosol and para-
sitophorous vacuolar contents. The parasite pellet was washed twice
with ice-cold PBS and extracted with 20 ml of 50 mM MgCl₂, 50 mM
MOPS, 50 mM Tris, 25 mg/ml DNase I, 0.25 mg/ml RNase I, and Roche
de complete protease inhibitor mixture, pH 7.2. The sample was triturated
using a 27-gauge needle and further disrupted in a bath sonicator for 10
min. The insoluble material was pelleted at 400,000 × g for 30 min and the soluble proteins concentrated by ultrafiltration.

**Binding to Immobilized FP**—Typically, 0.2–0.5 ml of a sample con-
taining 150–400 μg of protein was mixed with FP to a final concentra-
tion of 0.5 μM or with an equivalent volume of PBS and incubated for
5 min at room temperature. Agarose-immobilized FP (50 μl of 6.7 μmol
FP/ml beads from Sigma) was added and incubated at room tempera-
ture for 2 min. The beads were pelleted and washed twice with PBS, 1.5
M MgCl₂, 0.5 mM NaCl. Bound proteins were eluted by boiling in SDS
sample buffer and analyzed by SDS-PAGE. Alternatively, samples
responding to the applied sample and the flow-through were analyzed by
SDS-PAGE, and FP-binding proteins were identified from the dif-
ference between the two protein profiles.

**Tryptic Digestion of FP-binding Proteins**—Comassie Blue-stained
peptides excised from SDS-polyacrylamide gels and destained with 200 μl of 200 mM NH₄HCO₃, 10% acetonitrile for 45 min at 37 °C.
The supernatants were replaced with 100 μl of 0.5 mM NAD⁺, 10% acetonitrile for 5 min at room temperature. A further 50 μl of NH₄HCO₃, 10% acetonitrile was added, and digestion of protein was continued for 18 h at 37 °C with agitation. The supernatant was collected and the gel pieces collected, washed three times with 200 μl of 25 mM NH₄HCO₃ for 15 min at 37 °C, and then dried in vacuo. The gel pieces were rehydrated with a 1.5× volume (~20 μl) of 0.2 μg/ml sequencing grade modified trypsin (Promega) in 40 mM
NH₄HCO₃, 10% acetonitrile, pH 8.1, and incubated for 60 min at room temperature. A further 50 μl of NH₄HCO₃, 10% acetonitrile was added, and digestion of protein was continued for 18 h at 37 °C with agitation. The supernatant was collected and the gel pieces collected, washed four times for 40 min at 37 °C with successive 50-μl aliquots of 0.1% trifluoroacetic acid and 0.1% trifluoroacetic acid, 50% acetonitrile. The combined extracts were purified and concentrated using C₁₈ Reversed-Phase Zip-Tips™(
Milli-
pose). The samples were eluted with saturating substrate concentrations of 0.1–20 nM and 20 nM FP for 10 min and 30 min, respectively.

**MALDI-TOF and Electrospray Ionization Mass Spectrometry**—For measurement of specific activity (method adapted from Ferdinand (34)), GAPDH (~1 μg) was mixed with saturating substrate concentrations (0.5 mM NAD, 2 mM GAP3) in 40 mM triethanolamine, 50 mM sodium phosphate, pH 7.6, at 25 °C. The reaction was measured over the initial 1–2 min period during which the kinetics were linear (~10% of NAD⁺ converted to NADH). To examine the effect of FP on GAPDH kinetics, the GAPDH enzymes were incubated with either buffer (control) or FP for 10 min at room temperature prior to being added to a 200 mM NH₄HCO₃ (0.3 ml final volume, containing 0.5 mM NAD⁺, 0–200 μM GAP3, and 0–10 μM FP in assay buffer). GAPDH concentrations greater than 1 μM contribute significantly to the absorbance measured and can exhibit a temporal dependence that can interfere with the analysis of the kinetics.

**RESULTS**

**Binding of Parastate Proteins to Immobilized FP**—We have attempted to identify parasite proteins that might interact with FP that diffuses out of the parasite food vacuole. Soluble P. falciparum proteins were extracted by sonication of saponin-
treated infected erythrocytes. Analysis of [35S]methionine-labeled aliquots indicated that most of the proteins in this fraction are parasite-encoded (data not shown). Western blot analysis revealed that a parasite protein located within the
endoplasmic reticulum, PfERC (36), was efficiently released by these protocols (data not shown), indicating that soluble proteins from both the parasite cytosol and at least some of the organelles were present in this sample. By contrast, the exported protein, HRP2, was released during saponin lysis of the host cell and was largely absent from this fraction (data not shown).

In an effort to identify the major FP-interacting proteins, we have examined the abilities of parasite proteins to interact with FP-linked agarose beads (linked via one of the carboxylic acid groups (37)). To validate the binding assay, we examined the binding of a known FP-interacting protein, HRP2 (29, 38, 39). Recombinant HRP2 bound efficiently to the FP-agarose beads equilibrated with PBS (Fig. 1, lane a). Subsequent addition of excess FP only partly eluted the bound FP (data not shown). However, preincubation of the HRP2 solution in the presence of an excess of free FP efficiently inhibited binding of this protein to the immobilized FP beads (Fig. 1, lane b). We therefore examined the ability of soluble extracts of D10 strain parasite proteins to bind to the FP-agarose beads in the presence and absence of an excess of free FP.

As shown in Fig. 1 (lane d), a number of parasite proteins bound to the immobilized FP. The profile of bound proteins is quite distinct from that for the supernatant fraction (Fig. 1, lane c). The binding of a subset of proteins was inhibited in the presence of an excess of free FP (Fig. 1, lane e, asterisks). As an alternative approach, samples corresponding to the applied protein preparation and the flow-through fraction were analyzed, and potential FP-binding proteins were identified from the difference between the two protein profiles. A similar set of proteins was identified using this approach (not shown). Similar profiles were also obtained when extracts of HB3 strain parasites were examined for FP-binding proteins (data not shown). In an effort to determine the identities of some of these FP-binding proteins, the regions containing some of the Coomassie Blue-stained bands were excised from larger format gels and subjected to trypsin digestion. The peptide fragments were eluted from the gel pieces using acetonitrile and examined by MALDI-TOF mass spectrometry.

Mass Spectrometry—The results of the mass spectrometric analysis are given in Table I. Nine of the excised regions of the gels gave data that could be used to identify proteins. The known proteins that were identified with a good degree of confidence include P. falciparum glyceraldehyde-3-phosphate dehydrogenase (PfGAPDH), P. falciparum protein disulfide isomerase (PfPDI), P. falciparum glutathione reductase (PfGR), and a protein with a Tas domain, which is found in aryl alcohol dehydrogenases. The metalloenzyme falcisyn was also detected but the Z-value is lower. The predicted molecular masses of each of these proteins were similar to the apparent molecular masses estimated from SDS-PAGE. The number of matched peptides, the degree of coverage by peptide sequencing and the Z-scores give some estimate of the degree of confidence for the identification of proteins in the different hands.

For example, the peptides identified from band 5 covered 34% of the PfPDI sequence and gave a Z-score of 2.31 when a mass accuracy of ±0.1 Da was employed. The identification of PfGAPDH was confirmed by tandem mass spectrometry fragmentation sequencing using an electrospray ionization mass spectrometer and by N-terminal sequencing (data not shown), giving further confidence in this assignment. In some cases more than one protein was identified in a particular band. For example, two polypeptide species were apparently present in band 9. Surprisingly, some of the apparently more abundant proteins, for example, the protein with an apparent molecular weight of 50 kDa, did not yield useful matches. This may be because of the specific physicochemical properties of some of the polypeptides such that they are inefficiently eluted from the gels or to the complexity of the samples, leading to weak matches to a mixture of hypothetical proteins. Given the relative complexity of the protein mixtures analyzed on these one-dimensional gels, there is some ambiguity in the protein identification; it is therefore important to emphasize that the ability of each of these proteins to bind FP needs to be confirmed in independent assays.

Inhibition of GAPDH—In an effort to confirm an interaction of some of these proteins with FP and to study the likely consequences of FP binding, we investigated the effects of FP on their enzymatic activities. For example, GAPDH is an important regulatory enzyme in the glycolytic pathway. As the parasite lacks the complete pathway for oxidative phosphorylation, glycolysis is the major source of ATP equivalents. The activities of recombinant PfGAPDH (28) and human red blood cell (RBC) GAPDH were examined under saturating substrate concentrations (0.5 mM NAD, 2 mM GAP3). The specific activities of recombinant PfGAPDH and human RBC GAPDH were 90–120 and 155 units/mg, respectively, in good agreement with the literature (28). In microplate-based assays, PfGAPDH showed a $K_M$ for GAP3 of 1028 $\mu$M and a $V_{max}$ value of 3.57 nmol/min (Fig. 2D; no FP curve). For RBC GAPDH, the $K_M$ for GAP3 was 540 $\mu$M and the $V_{max}$ value was 4.78 nmol/min (Fig. 2C; no FP curve).

Initial studies indicated that the addition of FP resulted in a rapid initial decrease in both RBC and plasmoidal GAPDH activities followed by a much slower time-dependent inactivation of the enzyme (data not shown). For a more extensive analysis of the effect of FP on GAPDH activity, we preincubated the enzyme preparations with FP for a period of 5 min and then examined its effect on the reaction kinetics. As shown in Fig. 2A, the presence of 2 $\mu$M FP had a small effect on the kinetics of RBC GAPDH. This small apparent decrease in RBC GAPDH activity may reflect a real inhibitory effect or may be due to a time-dependent change in FP absorption. The absorbance of FP at higher concentrations interfered with the spectrophotometric assay of NADH production, which precluded a...
detailed kinetic analysis for FP inhibition of RBC GADPH. Nonetheless, it is clear that even at a concentration of 5 μM, FP had only a limited effect on RBC GAPDH activity (Fig. 2C). In contrast, the presence of 2 μM FP completely ablated the Pf-GADPH activity (Fig. 2B). The magnitude of this decrease cannot be explained by changes in FP absorption. Moreover, lower FP concentrations, which did not interfere with absorption measurements, produced dramatic effects on the kinetics of reaction.

### Table I

Proteins identified by mass spectrometric analysis of tryptic digests of putative FP-binding proteins

| Band | Protein                                          | Observed mol. mass | Calculated mol. mass | Min. sequence coverage | Matched peptides/Z-score |
|------|--------------------------------------------------|--------------------|----------------------|------------------------|--------------------------|
| 1    | PfGADPHa                                         | 36                 | 36.63                | 28                     | 6/2.32                   |
| 2    | *P. falciparum* Gap protein (gi:8250569)b        | 38                 | 39.39                | 21                     | 5/1.48                   |
| 3    | Hypothetical protein (gi:23509307)               | 40                 | 40.95                | 22                     | 6/1.05                   |
| 4    | Merozoite surface antigen 3                      | 44                 | 43.38                | 15                     | 5/0.64                   |
| 5    | PfPDI                                            | 56                 | 55.83                | 34                     | 16/2.31                  |
| 6    | PfGR                                             | 57                 | 56.89                | 23                     | 12/1.97                  |
| 7    | Ribosomal processing protein, putative (gi:6562730) | 57                 | 57.21                | 11                     | 6/0.78                   |
| 8    | Hypothetical protein (gi:23508084)               | 62                 | 61.27                | 8                      | 4/1.28                   |
| 9a   | falcilysin                                       | 130                | 137.02               | 14                     | 13/0.44                  |
| 9b   | Hypothetical protein (gi:23509309) (Tas domain; related to ary1-alcohol dehydrogenases) | 130 | 136.5 | 15 | 14/1.03 |

* a Identified by Edman degradation/N-terminal sequencing and by tandem mass spectrometry using an electrospray ionization mass spectrometer.

* b NCBI gene identifier.

Fig. 2. Analysis of reaction kinetics of RBC and plasmodial GAPDH. RBC GAPDH (0.17 μg) (A) or PfGAPDH (0.11 μg) (B) was incubated with either buffer (●) or 2 μM FP (●) for 5 min prior to being added (in duplicate) to samples containing 0.5 mM NAD⁺ and GAP3 (150–2000 μM). C and D, Lineweaver-Burk analyses of RBC (C) and PfGAPDH (D) kinetic data. The $K_m$ and $V_{max}$ values for the substrate GAP3 in the presence of increasing concentrations of FP were estimated from the $x$- and $y$-intercepts. Insets, determination of the inhibition constant ($K_i$, $K'_i$) for the inhibition of GAPDH by FP.
of PfGAPDH (Fig. 2D). The FP appears to increase the $K_m$ and decrease the $V_{\text{max}}$ values suggesting a mixed type of inhibition of PfGAPDH by FP. The $K_i$ and $K'_i$ values determined by constructing secondary plots from the Lineweaver-Burk plots (Fig. 2D, insets) are similar (0.2 and 0.3 $\mu M$, respectively), indicating that FP binds equally well to PfGAPDH and the PfGAPDH substrate complex. These data suggest that PfGAPDH could be a primary target for FP inhibitory activity in the parasite.

Inhibition of GR—Aft and Müller (35) reported that yeast GR is inactivated by FP in the micromolar range in a time-dependent manner. When we used the experimental protocol described by Aft and Müller, similar data were obtained. That is, incubation with NADPH and 25 $\mu M$ FP resulted in an almost linear decrease in activity, reaching 10–15% residual activity after 50 min. However, when we carried out a control, which contained NADPH only (in the absence of FP), a strong GR inactivation was detected that was almost identical to the inhibition by FP plus NADPH at 60 min (data not shown). A significant inhibitory effect of FP (~40%) in comparison with the NADPH only (in the absence of FP) was detectable upon the addition of 25 $\mu M$ FP. Upon further incubation an almost linear decrease in activity was measured, until after 40 min only 20% residual activity was left (Fig. 3A). Incubation with NADPH in the absence of FP also had a pronounced effect, reaching 70% inhibition after 40 min. Yeast GR is obviously very susceptible to destabilization by FP. Because the effects of NADPH and FP, respectively, might be synergistic but could also lead to partial neutralization of the individual effects, it is difficult to quantify FP-dependent inhibition. The 45% inhibition at $t = 0$, however, supports quite a high inhibitory potential of FP on yeast GR. For human GR the immediate inhibitory effect of FP (60%) was even more obvious than for yeast GR. However, in contrast to yeast GR, this inhibition increased by only 10% over the 40-min incubation time. Also the destabilizing effect of NADPH alone was not as marked (Fig. 3B).

For PfGR two additional samples (GR ± FP in the absence of NADPH) were included. The addition of FP led to an immediate loss of GR activity of only 20% (Fig. 3C). This effect did not depend on NADPH. However, both NADPH-containing samples (±FP) lost their activity markedly over time, reaching 20% residual activity at $t = 40$ min. Incubation with FP alone resulted in 40% inhibition after 40 min, which corresponded to a higher activity than the control sample without FP. However, similar values were obtained when we accounted for the spontaneous reaction between FP and NADPH. This reaction of FP with NADPH might, in itself, contribute to the cytotoxicity of FP. Overall these data indicate that PfGR is particularly sensitive to the destabilizing effects of NADPH but that the immediate inhibitory effect of 25 $\mu M$ FP on PfGR is only ~20%.

Although the interplay between components within the GR enzyme reactions is clearly complex, we can conclude that PfGR is the most sensitive of the three enzymes to the destabilizing effect of NADPH (plus dilution), whereas human GR is least sensitive. The immediate inhibitory effect of FP is strongest for the human enzyme and weakest for PfGR. Thus PfGR appears to be relatively insensitive to the effects of FP compared with the host enzyme. This is consistent with the proposed role of GR as a part of the defense against the deleterious effects of FP-induced oxidative stress.

**Inhibition of TrxR and Glutaredoxin—**An NADPH-dependent TrxR has recently been described in *Plasmodium* (18, 20). In the presence of the natural substrate Trx, FP was found to inhibit PfTrxR with an IC$_{50}$ value of 1.25 $\mu M$ (Fig. 4A). These assays were initiated with Trx and did not involve preincubation with FP. Although the spontaneous reaction between 25 $\mu M$ FP and NADPH (0.013 min$^{-1}$) was a concern in the studies on GR, it was negligible at the FP concentrations (~5 $\mu M$) used in these assays. Analogous assays initiated with NADPH revealed a much weaker inhibitory effect of FP (44% at 25 $\mu M$ FP), indicating that the effect of FP was dependent on the pre-reduction of the enzyme.

Varying the Trx concentrations in the presence of different FP concentrations resulted in a competitive inhibition pattern with a $K_i$ of ~1 $\mu M$. However, apart from this clearly competitive component, a time-dependent inhibitory effect was observed (Fig. 4B) when PfTrxR was incubated with FP in the presence of NADPH. For example, after a 30-min incubation period with 1.25 $\mu M$ FP, the enzyme activity was reduced to
20% of controls (Fig. 4B, diamonds). The destabilizing effect of NADPH alone was much weaker, and incubation with FP alone revealed no marked time dependence (Fig. 4B, crosses). From these data it can be concluded that PfTrxR is very sensitive to inhibition by FP. In addition to the competition between FP and Trx, there is a time-dependent and probably irreversible inhibitory component.

Two different proteins with homology to glutaredoxins have recently been described in plasmodia (15). A 12.4-kDa protein (referred to as PfGrx-1) exhibits the typical glutaredoxin active site motif, CPYC, and shows glutathione-dependent glutaredoxin activity in the HEDS assay. For these assays it was necessary to correct for the spontaneous reactions between HEDS, GSH, NADPH, and FP. Having made these corrections, we were able to estimate that the HEDS reducing activity of PfGrx-1 was inhibited by FP in a concentration-dependent manner. Five, 10, and 25 μM FP, respectively, led to 27, 51, and 70% inhibition (data not shown).

**DISCUSSION**

The malaria parasite consumes most of the hemoglobin content of its host cell in order to obtain a ready source of protein components in order to preserve osmotic stability within the infected erythrocyte and to create space for growth (41). The consumption of this blood meal creates logistical problems for the parasite because of the release of the free FP moieties and reactive oxygen species that are by-products of hemoglobin breakdown. The parasite is likely to have a range of strategies to protect itself against the deleterious effects of FP. However, if FP levels are raised, they may reach levels that overwhelm the defense systems of the parasite. In this work, we have attempted to identify FP-binding proteins in order to determine proteins that form part of the system for protection against FP or proteins that represent likely first-line targets for FP inhibition.

The putative FP-binding proteins that we identified range from the glycolytic enzyme, PGAPDH, through the anti-oxidant enzyme PfGR, the endoplasmic reticulum luminal protein PIPDI, and the metalloprotease falcilysin. Interestingly, a number of these proteins have active site thiol moieties. For example, GAPDH has an active site cysteine residue that is susceptible to S-nitrosylation by NO. NO-induced inhibition of GAPDH can be reversed by low molecular weight thiols such as glutathione (42). The flavoprotein glutathione reductase contains two cysteine residues that comprise its redox-active disulfide bridge. PfGR is inhibited by S-nitrosogluthathione, which induces oxidation of one of the catalytically essential cysteines (43). Protein disulfide isomerases are members of the thiol/disulfide oxidoreductase superfamily (44). Two Cys residues form part of the Trx-like catalytic sites (-CGHC-), and are essential for activity. Falcilysin activity is inhibited by the sulfhydryl modifying reagent N-ethylmaleimide, indicating that a free thiol is required for the activity of the enzyme (45). FP is known to form both liganded and covalent thioether linkages to cysteine residues (46–48). This suggests that at least some of the captured proteins might bind to the FP column via very tight interactions with the active site cysteines. Despite some similarities between the identified proteins, it is important to point out that these data are merely indicative of FP-binding potential. It remains necessary to confirm the FP interaction using individual proteins.

GAPDH is a pivotal enzyme in the glycolytic pathway. It uses NAD\(^+\) to catalyze the reversible oxidative phosphoryla-

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**Fig. 4. Inhibition of P. falciparum TrxR by FP.** PtTrx (20 μM) was added to PtTrxR (2–10 milliunits/ml) in the presence of 100 μM NADPH and different concentrations of FP. NADPH consumption was followed spectrophotometrically at 340 nm to determine the IC\(_50\) (A) and the time dependence (B) of inhibition by 1.25 μM FP. All values are the means of four independent determinations that differed by less than 10%.

**Fig. 5. Working model showing the effects of increased FP concentrations on central metabolic pathways of P. falciparum.** The uptake and consumption of glucose by infected erythrocytes is increased by a factor of ~100 when compared with normal RBCs (56). The two major glucose-utilizing pathways are glycolysis and HMPS. Inhibition of GAPDH by FP will lead to increased HMPS activity and NADPH production. The parallel inhibition of TrxR and glutaredoxin (not shown) by FP is likely to reduce DNA synthesis. GR remains comparatively unaffected by FP (only 20% inhibition by 25 μM FP). This constellation is likely to support glutathione reduction. GSH acts directly and indirectly as an antioxidant and fuels GST, which can destroy peroxides. GST binds FP noncompetitively (in the presence of GSH) and is highly concentrated in malarial parasites (up to 100 μM). This liganding function of GST may serve as an intracellular buffer for FP (16, 57).
tion of glyceraldehyde-3-phosphate to 1,3-diphosphoglycerate, thereby generating reducing equivalents. In addition, GAPDH is thought to be involved in a variety of other cellular activities (49). These include putative roles in membrane fusion, tubulin bundling, phosphotransferase activity, nuclear RNA transport, and DNA replication and repair (49). Given the central role of this protein, it is interesting that plasmodial GAPDH appears to be particularly sensitive to inhibition by FP. The $K_e$ value for inhibition of GAP3 consumption by PfGAPDH by FP was estimated to be 0.2 mM.

The sensitivity of PfGAPDH to FP could represent a vulnerable target within the metabolism of the parasite. Inhibition of glycolysis could represent the immediate downstream target of increasing FP levels consequent upon CQ treatment. This would suggest that a specific PfGAPDH inhibitor might act synergistically with the 4-aminoquinoline antimalarials. However, it is intriguing that the plasmodial enzyme is so much more sensitive to FP than the host cell enzyme. Given the nature of the cell that is parasitized by P. falciparum, it is surprising that evolution has not fitted the parasite with a more robust enzyme. We therefore considered the possibility that the particular sensitivity of PfGAPDH to FP might be an important control mechanism. It is possible that it is advantageous to the parasite to inhibit GAPDH activity when it is under FP stress. If glycolysis is inhibited, glucose may be preferentially metabolized by the hexose monophosphate shunt (HMPS) yielding NADPH. NADPH could be used to fuel antioxidant systems including glutathione and TrxRs (see Fig. 5 for a diagrammatic analysis of the potential effects of FP on glycolysis and the redox metabolism of the parasite). In this context, it is interesting to note that there is a 24-fold up-regulation of the red cell HMPS upon malaria infection (50).

PfGR was also identified as one of the proteins captured by the immobilized FP column. However, in this case, preincubation with free FP did not appear to prevent binding, suggesting that the presentation of the bound FP might favor interaction with PfGR. This is consistent with our finding that recombinant PfGR was not particularly sensitive to inhibition by free FP. The maintenance of PfGR activity in the presence of FP would enhance replenishment of the reduced GSH, which could be used to detoxify reactive oxygen species and lipid peroxides produced as a result of FP-induced damage (see Fig. 5).

In view of our findings for PfGAPDH and PfGR, we were interested to examine the effect of FP on other available plasmodial antioxidant enzymes. Plasmodial Grx-1 and TrxR represent additional arms to the antioxidant defense system of the parasite. TrxRs possess, in addition to their redox-active NT-terminal pair of cysteines, a pair of cysteine residues or a selenenylsulfide motif at their C termini (18). Similarly, classical glutaredoxins have an active site with two conserved cysteine residues, CPyC, of which the N-terminal cysteine has been shown to be essential for both protein-disulfide reduction and reduction of mixed protein-glutathione disulfides (15).

In this work, we found that the activity of TrxR is quite sensitive to FP ($K_e = 1 \mu M$). This suggests that the Trx pathway might be an early target as FP levels rise within the parasite. Although comparatively less sensitive, PfGrx-1 is also inhibited by FP ($I_C_{50} = 10 \mu M$ FP), which may, together with the inhibition of TrxR and the impaired reduction of Trx, result in decreased production of deoxyribonucleotides. In a previous study, the effect of FP on glutathione S-transferase (GST) has been examined (16). Human GST was inhibited by FP with a $K_e$ of 4 mM (uncompetitive inhibition). PfGST was also sensitive to FP; however, the $K_e$ was slightly higher (6.5 mM). In the absence of a classical glutathione peroxidase in plasmodia, GST is likely to play a role in the destruction of lipid peroxides (51) and the conjugation of GSH to various secondary substrates of lipid peroxidation (52, 53). These activities might be important in coping with the downstream effect of FP and reactive oxygen species toxicity. Thus the parasite GST may be able to withstand low levels of FP, but this pathway for the detoxification of lipid peroxidation metabolites may be inhibited as the FP levels increase.

While this manuscript was in preparation, a report appeared describing another approach to the identification of FP-binding proteins in parasites (54). These authors identified a number of FP-binding proteins including 6-phosphogluconate dehydrogenase, aldolase, lactate dehydrogenase, and GAPDH. These authors suggested that CQ-induced binding of FP to parasite glycolytic enzymes could undergo the previously reported inhibition of glycolysis by CQ (55), which is in agreement with our findings. The authors examined the activity of 6-phosphogluconate dehydrogenase in some detail and found that a commercially available enzyme was inhibited by 50% by 5 $\mu M$ FP (54). Treatment of parasitized cells with 10 $\mu M$ CQ inhibited the activity in parasite extracts by 40%. The ability of higher level of FP produced during CQ treatment to inhibit an enzyme of the HMPS is also consistent with our suggestion that increased FP levels may eventually overwhelm the parasite’s defense systems.

Overall our data provide insights into the effects of the accumulation of FP in the parasite. We have identified some proteins of the glycolytic and redox pathways and examined their interactions with FP in detail. However, we have also identified proteins such as the food vacuole-located protease, falcilysin, and the endoplasmic reticulum-located protein, PDI, that may be sensitive to the effects of FP. Moreover we have identified a number of FP-binding proteins that are hitherto listed only as hypothetical proteins. The data are consistent with the suggestion that low micromolar concentrations of FP may have a regulatory function in malaria parasites. The potent inhibition of GAPDH suggests a metabolic constellation in which glycolysis and DNA synthesis (requiring reduced Trx or glutaredoxin for deoxyribonucleotide synthesis) are suppressed, whereas the glutathione system is favored. The maintenance of GSH levels is consistent with the known uncompetitive binding of FP to PfGST, which may serve as an intracellular ligand. Clearly further work is required to follow up the FP sensitivity of P. falciparum proteins.

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Identification and Characterization of Heme-interacting Proteins in the Malaria Parasite, *Plasmodium falciparum*

Naomi Campanale, Christine Nickel, Claudia A. Daubenberger, Dean A. Wehlan, Jeff J. Gorman, Nectarios Klonis, Katja Becker and Leann Tilley

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