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Glucose 6-phosphate dehydrogenase 6-phosphogluconolactonase: a unique bifunctional enzyme from *Plasmodium falciparum*

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Synopsis

The survival of malaria parasites in human erythrocytes depends on the pentose phosphate pathway both in *Plasmodium falciparum* and its human host. Glucose 6-phosphate dehydrogenase (G6PD) deficiency, the most common human enzyme deficiency, leads to a lack of NADPH in erythrocytes, and protects from malaria. In *Plasmodium falciparum*, G6PD is combined with the second enzyme of the pentose phosphate pathway to a unique bifunctional enzyme named glucose 6-phosphate dehydrogenase 6-phosphogluconolactonase (GluPho). Here, we report for the first time the cloning, heterologous overexpression, purification, and kinetic characterization of both enzymatic activities of full length PfGluPho, and demonstrate striking structural and functional differences to the human enzymes. Detailed kinetic analyses indicate that PfGluPho functions on the basis of a rapid equilibrium random bi bi mechanism, where the binding of the second substrate depends on the first substrate. We furthermore show that PfGluPho is inhibited by *S*-glutathionylation. The availability of recombinant PfGluPho and the major differences to human G6PD facilitate studies on PfGluPho as an excellent drug target candidate in the search for new antimalarial drugs.

Abbreviations: G6PD, glucose 6-phosphate dehydrogenase; GluPho, glucose 6-phosphate dehydrogenase 6-phosphogluconolactonase; PPP, pentose phosphate pathway; 6PGL, 6-phosphogluconolactonase; RBC, red blood cell; $6PGL_{PfGluPho}$, 6PGL part of GluPho; $G6PD_{PfGluPho}$, G6PD part of GluPho; IPTG, isopropyl-β-D-1-thiogalactopyranoside; dithiothreitol, DTT; G6P, glucose 6-phosphate; 6PGγL, 6-phosphoglucono-γ-lactone; 6PGD, 6-phosphogluconate dehydrogenase

Introduction

Glucose 6-phosphate dehydrogenase (G6PD) deficiency is the most common human enzyme defect and associated with resistance to malaria, most likely due to decreased levels of reducing equivalents in form of NADPH [1]. Malaria is one of the most severe infectious diseases with 240 million cases in 2009 (WHO, 2009). The increasing resistance of *Plasmodium falciparum* to antimalarial drugs, including the artemisinin-based combination treatments, intensifies the search for new antimalarial drug targets [2, 3].

The pentose phosphate pathway (PPP) is the major source of NADPH and pentose sugars, which are crucial for oxidative stress defense and nucleotide synthesis. Enhanced oxidative stress induced by G6PD deficiency or prooxidant antimalarial compounds such as 4-aminoquinolines (e.g. chloroquine), 8-aminoquinolines (e.g. primaquine), as well as artemisinins suppresses parasite growth and enhances the elimination of parasitized red blood cells (RBC) by the immune system (for review see [4]).

The first steps of the PPP are catalyzed by G6PD (EC 1.1.1.49), 6-phosphogluconolactonase (6PGL, EC 3.1.1.31), and 6-phosphogluconate dehydrogenase (6PGD, EC 1.1.1.44). Previous studies suggest that the PPP of both the human host and the malaria parasite are important during the infection of RBC with *Plasmodium*: (i) Human G6PD deficiency and thus a restricted PPP limits NADPH production and protects from malaria [5]. (ii) Malaria parasites require NADPH produced in the PPP. (iii) The PPP in *Plasmodium* is highly regulated in different developmental stages and by the level of oxidative stress [6].

The existence of a G6PD in malaria parasites has been controversially discussed, until the enzyme could be partially purified from infected RBCs [7, 8]. In contrast to the PPP enzymes in vertebrates and bacteria, the first two enzymes of the *Plasmodium* PPP are combined in a unique bifunctional enzyme of 910 amino acids called glucose 6-phosphate dehydrogenase 6-phosphogluconolactonase (GluPho) [9, 10] (Figure S1). Its C-terminal part (aa 311-911) is homologous to other G6PDs, although interrupted by an insertion of 62 amino acids [9], while the N-terminal 310 amino acids show a high similarity to 6PGL [11]. The insertion in the G6PD part is highly conserved among *Plasmodium*, and is essential for the G6PD activity of *P. berghei* GluPho [12].

The importance of PfGluPho is further substantiated by the finding that RNA-mediated gene silencing results in arrest at the trophozoite stage and enhanced gametocyte formation [13]. However, this result has to be handled with care, since the existence of RNAi in *Plasmodium* is questionable [14, 15]. Nonetheless immediate transcript enhancement of thioredoxin reductase accompanies PfGluPho knock down and suggests a central role of PfGluPho in the response towards oxidative stress [13].

The outmost importance of the parasite's PPP in the parasite-host unit suggests that PfGluPho is a most attractive drug target [16]. In a recent screening approach 172 out of around 300,000 chemical compounds were identified to be active against *P. falciparum in vitro*. Interestingly, two compounds with antimalarial activity (C276-1187 and D052-0147, Chemdiv) bound to the separately cloned 6PGL part of PfGluPho (referred to as $6PGL_{PfGluPho}$) in thermal melt shift assays, thereby underlining the potential of PfGluPho as an antimalarial drug target [17]. Until now, analyses of *P. falciparum* GluPho were restricted because the full length gene could not be cloned [10]. Previous functional studies focused on the corresponding enzyme of the rodent parasite *P. berghei* [10, 12]. Here we report for the first time the successful production of the complete, active, recombinant GluPho from *P. falciparum*. Biochemical analyses reveal unique structural and functional features of PfGluPho, which clearly distinguish the bifunctional enzyme from the monofunctional human enzymes and provide a basis for the development of new therapeutic agents.

Materials and Methods

PCR amplification, sequencing, and cloning of PfGluPho, G6PD$_{PfGluPho}$, hG6PD, and h6PGL

PfGluPho: The gene of PfGluPho (PlasmoDB accession number PF14_0511) was identified on chromosome 14 and amplified by PCR on a *P. falciparum* 3D7 gametocyte cDNA library. Perfect match primers (forward: 5′-CGCGGGATCCGATTATGAGAATTTTGTAAAAAGTGCAG-3′; reverse: 5′-GCGCAAGCTTTCAATTAATATCTAACAAATCGTCTTC-3′; MWG-Biotech) introduced BamHI and *Hin*dIII restriction sites (underlined). Cloning of the complete PfGluPho gene was not successful; therefore we cloned the gene in two separate parts. A silent mutation was introduced to create a *Sac*I restriction site within the PfGluPho gene. The first part was amplified using the same *Bam*HI forward restriction site primer and a reverse primer containing a *Sac*I restriction site (5′-GCGCGAGCTCTTCTTTATTCAAACTATTAGAATAAAGAG-3′); the second part was amplified using a *Sac*I restriction site primer (5′-GCGCGAGCTCTTAACTATAATAATTTTTGGCTGTTCAG-3′) and the reverse primer containing a *Hin*dIII restriction site. Both constructs were separately cloned into a pSK vector (Stratagene). A triple ligation using the vector pET28a and the two parts of PfGluPho was performed to successfully combine the PfGluPho parts in the vector pET28a. For optimization of heterologous overexpression, the complete PfGluPho construct was also cloned into the vector pQE-30 (Qiagen). Both constructs contain an N-terminal His-Tag.
G6PD$_{PfGluPho}$: The G6PD part of PfGluPho ranging from aa 339-910 was cloned as described for PfGluPho (forward: 5′-CGGGATCCACTATAATAATTTTTGGCTGTTCAG-3', reverse: 5′-GCAAGCTTTCAATTAATATCTAACAACTCGTC-3').
hG6PD: A clone of hG6PD (Gen bank accession number NP_001035810) from the NIH Mammalian Gene Collection (clone ID 282264) was purchased from Invitrogen. The gene was amplified by PCR using primers designed to introduce restriction sites for *Nde*I and *Xho*I (underlined) (forward: 5′-GCGCCATATGGCAGAGCAGGTGGCCCT-3'; reverse: 5′-CGCGCTCGAGGAGCTTGTGGGGGTTCACC-3') and the construct was cloned into the expression vector pET24a (Novagen) with a C-terminal His-Tag.
h6PGL: A clone of h6PGL (Gen bank accession number NP_036220.1) from the NIH Mammalian Gene Collection (clone ID 4053022) was purchased from Invitrogen. The gene was amplified by PCR using primers that introduce *Nde*I and *Bam*HI restriction sites (forward: 5′-CGCGCATATGGCCGCGCCGGCCCCG-3'; reverse: 5′-GCGCGGATCCCTACAAAGTGGAATGCTTCTCGAA-3') and cloned into the expression vector pET28a (Novagen) with a N-terminal His-Tag.

Heterologous overexpression and purification

PfGluPho: The highest yield of recombinant PfGluPho was obtained by heterologous overexpression of PfGluPho in the vector pQE-30 in *E. coli* M15 cells (Qiagen) with pRAREII (Novagen). The cells were grown in Terrific Broth medium supplemented with carbenicillin, kanamycin (both 50 µg/mL), and chloramphenicol (12.5 µg/ml) at 23 ℃ to an optical density at 600 nm (OD_{600}) of 0.6, and the expression was induced by 1 mM isopropyl-β-D-1-thiogalactopyranoside (IPTG). After 24 hours, the cells were harvested by centrifugation (15 min at 6,000 g and 4 ℃), resuspended in 5 mL 0.1 M Tris, pH 7.8, and 0.5 M NaCl per g cell pellet, and mixed with protease inhibitors (50 µM phenylmethylsulfonyl fluoride, 150 nM pepstatin, and 40 nM cystatin). The cells were lysed by lysozyme and DNAse for 2 hours at 4 ℃, sonicated, and centrifuged (30 min at 30,600 g and 4 ℃). The supernatant was applied to a Ni-NTA column (Qiagen) and recombinant proteins were eluted with 0.1 M Tris, pH 7.8,

0.5 M NaCl containing 250 mM imidazole. Purity of protein samples was controlled by SDS-PAGE. The protein was further purified by gel filtration chromatography.
$G6PD_{PfGluPho}$: *E. coli* M15 cells containing the plasmids pQE30 and pRAREII (Novagen) were grown in Terrific Broth medium supplemented with carbenicillin, kanamycin (both 50 µg/mL), and chloramphenicol (12.5 µg/ml) at 23 ℃, and the expression was induced at OD_{600} of 0.6 with 0.5 mM IPTG. The cells were harvested after 20 hours, and resuspended as described for PfGluPho. The cells were lysed for 1 hour at 4 ℃ by lysozyme and DNase, sonicated, and centrifuged (30 min at 30,600 g and 4 ℃). Rec ombinant proteins were eluted from a Ni-NTA column with 0.1 M Tris, pH 7.8, 0.5 M NaCl containing 0.3 M imidazole yielding 1.5 mg pure protein per liter *E. coli* culture.
hG6PD: Overexpression of hG6PD was performed in *E. coli* BL21 cells (Invitrogen) containing pRAREII in 2xYT medium with kanamycin (50 µg/mL) and chloramphenicol (12.5 µg/mL) at 23 ℃. At an OD_{600} of 1, the expression was induced by 0.1 mM IPTG and continued for 24 hours. Harvest, cell lysis, and purification in 50 mM Tris, pH 8.0, 300 mM NaCl, 0.1 mM $NADP^+$ were performed as described for $G6PD_{PfGluPho}$. hG6PD can be eluted from the Ni-NTA column with buffer containing 150 and 200 mM imidazole.
h6PGL: h6PGL was overexpressed in *E. coli* BL21/pRAREII in Terrific Broth medium supplemented with kanamycin (50 µg/mL), and chloramphenicol (12.5 µg/ml) at 37 ℃. The expression was induced at OD_{600} of 0.6 with 1 mM IPTG, and the cells were harvested 4 hours after induction as described above. The cells were resuspended in 100 mM triethanolamine, 350 mM NaCl, pH 7.4. h6PGL was purified as described for $G6PD_{PfGluPho}$ and eluted from the Ni-NTA column at 50 – 200 mM imidazole yielding 30 mg pure protein per liter *E. coli* culture.

Protein immunoblotting analyses

For the recombinant PfGluPho, semi dry Western Blots using a anti-$(His)_6$-Tag antibody (Dianova) and a phosphatase-conjugated goat anti-mouse antibody (Dianova) were performed. Specific antibodies for PfGluPho were obtained from rabbits that had been immunized with synthetic peptides of PfGluPho: a N-terminal peptide composed of aa 118-133 (KEQLYKPDTTKSIVDC, anti-Pho) and a C-terminal peptide composed of aa 896-910 (CVRKSSFYEDDLLDIN, anti-Glu) (Eurogentec, diluted 1:5,000). Peroxidase-conjugated anti-rabbit antibody (Dianova, 1:50,000) was used as secondary antibody.

Gel filtration chromatography

Gel filtration chromatography was used to enhance the purity of the protein samples as well as to study the oligomerization behavior of PfGluPho. The experiments were performed on a HiLoad 16/60 Superdex 200 prep grade column connected to an ÄKTA FPLC system (Amersham Pharmacia Biotech). The column was calibrated with a gel filtration standard (Amersham Pharmacia Biotech) and equilibrated with the respective buffer. Protein-containing fractions were detected at 280 nm, and peak areas and k_{AV} values were evaluated using the software UNICORN 4.11. Protein-containing fractions were analyzed by SDS-PAGE and enzymatic assays. To study the oligomerization behavior, PfGluPho in 50 mM Tris, 0.3 M NaCl, pH 7.8 was incubated for 12 h at 4 ℃ with 2 mM $NADP^+$, 2 mM NADPH, or 2 mM dithiothreitol, respectively. To study the oligomerization behavior at high pH and ionic strength, PfGluPho was diluted in 0.5 M NaCl, 0.25 M Tris, pH 9.0.

Measurement of kinetic parameters for the G6PD reaction

The G6PD activities of PfGluPho, PfGluPho's G6PD part, and hG6PD were measured at 25 ℃ by monitoring the reduction of $NADP^+$ to NADPH at 340 nm according to Beutler [18]. The reaction mixture contained 0.1 M Tris, pH 8.0, 10 mM $MgCl_2$, 0.5 mM EDTA with varying

amounts of enzyme, 200 µM $NADP^+$, and 200 µM G6P, which was added to initiate the reaction. For the determination of the K_m for $NADP^+$ the concentration of $NADP^+$ was varied from 1 – 200 µM and determined at different fixed G6P concentrations, while the K_m for G6P was measured by varying the G6P concentration from 1–200 µM at different $NADP^+$ concentrations. The steady state kinetics of the G6PD activity for G6P were performed by fixing the concentration of $NADP^+$ at the K_m while varying the concentration of G6P (1–200 µM). Likewise the steady state kinetics for $NADP^+$ were performed by holding the concentration of G6P at K_m value with different concentrations of $NADP^+$ (1–200 µM). Enzyme activity was assayed with a U-2001 spectrophotometer (Hitachi).
Kinetic parameters in this study where calculated by non-linear regression using the program GraphPadPrism, as well as from Dalziel parameters. Dalziel's method was applied as an independent method to analyze and identify the kinetic mechanism of PfGluPho, since it allows to distinguish rival bisubstrate kinetic mechanisms. The initial-rate equation for the G6PD-catalyzed reaction according to Dalziel is of the form [19]:

$$\frac{e}{v} = \phi_0 + \frac{\phi_{S1}}{[S1]} + \frac{\phi_{S2}}{[S2]} + \frac{\phi_{S1S2}}{[S1][S2]} \quad (1)$$

S1 and S2 are the coenzyme ($NADP^+$) and the sugar phosphate (G6P), respectively, while e is the total enzyme concentration, v represents the initial rate of the enzymatic reaction, and Φ_0 equals $1/k_{cat}$. The Φ parameters are calculated from initial-rate measurements at varying concentrations of S2 for several fixed concentrations of S1. Rearrangement of the equation shows that the intercepts of primary double reciprocal plots with 1/[S2] as the variable are given by $\Phi_0 + \Phi_{S1}/[S1]$ and the slopes by $\Phi_{S2} + \Phi_{S2S1}/[S1]$. The secondary plots of the intercepts and slopes against 1/[S1] provide estimates for the individual-rate parameters [19]. Analogous kinetic experiments were carried out with hG6PD to allow direct comparison with the *Plasmodium* enzyme.

Alternative substrate and inhibition studies

The steady state kinetics of PfGluPho's G6PD activity as well as for hG6PD using the G6P analogue 2deoxyG6P (Sigma Aldrich) were performed by varying the concentration of 2deoxyG6P from 1 to 10 mM in the presence of $NADP^+$ at the K_m, while $NADP^+$ was varied from 1–200 µM with the concentration of 2deoxyG6P at K_m.
In product inhibition studies with PfGluPho and hG6PD, the initial rates were measured for a series of NADPH concentrations (0–30 µM) with 60 µM G6P and $NADP^+$ concentrations from 2–200 µM. Likewise the experiment was carried out by varying the G6P concentrations from 5–200 µM and NADPH (0–20 µM) while fixing the concentration of $NADP^+$ at 10 µM. In analogous fashion, 0–30 mM of glucosamine 6-phosphate was used as an inhibitor covering the same combinations and ranges of substrate concentration as used in the experiments with NADPH.
The inhibition studies with compound C276-1187 (4-(4-bromophenyl)-7-(3,4-dimethoxyphenyl)-3,4,7,8-tetrahydroquinoline-2,5(1H,6H)-dione, Chemdiv) were performed using 40-300 µM C276-1187 both in the G6PD and the 6PGL assay. Higher concentrations could not be used due to solubility problems.

Measurement of kinetic parameters for the 6PGL reaction

The 6PGL assays were carried out using the stable 6-phosphoglucono-γ-lactone (6PGγL) instead of the unstable natural substrate 6-phosphoglucono-δ-lactone. 6PGγL was produced according to Beutler *et al.* 1986, and the 6PGL-activity was determined spectrophotometrically at 340 nm and 25 ℃ as descr ibed before [20]. The assay mixture contained 0.1 M Tris, pH 7.0, 10 mM $MgCl_2$, 0.5 mM EDTA, 0.6 mM $NADP^+$, 3 U/ml 6-phosphogluconate dehydrogenase (yeast, Sigma Aldrich), and 1 mM 6PGγL. For K_m measurement, the concentration 6PGγL was varied from 20–3000 µM.

S-glutathionylation studies

MALDI-ToF mass spectrometry
For investigating the susceptibility of PfGluPho to *S*-glutathionylation the recombinant enzyme (1 mg/ml) was incubated with 10 mM GSSG in 50 mM Tris, 0.5 M NaCl, pH 7.8 for 5 min at 37 ℃. 2 mM iodoacetamide was added to block residual cysteines. An analogous experiment was performed for untreated PfGluPho. The samples were digested with trypsin for 12 h at 37 ℃. The tryptic peptides were analyz ed by MALDI-ToF MS on an Ultraflex I TOF/TOF mass spectrometer (Bruker, Daltonics, Germany). A comparison of the data with the theoretical molecular weight of the tryptic peptides revealed cysteine residues with attached glutathione.
Enzymatic assays
Enzymatic assays on *S*-glutathionylated G6PD were performed after incubation of recombinant PfGluPho and hG6PD with 0-10 mM GSSG or GSH for 5 min at 37 ℃. The reversibility of the modification was studied by incubating PfGluPho with the reducing agent dithiothreitol for 30 min at 23 ℃ after pre-incuba tion with GSSG.

Results

Cloning, heterologous overexpression, and purification of PfGluPho

Previous publications reported major difficulties in cloning PfGluPho, making it impossible to heterologously overexpress and characterize the enzyme [9, 10]. As reported here, we were able to clone, overexpress, and purify the recombinant full length PfGluPho (Figure 1). Cloning, overexpression and purification of PfGluPho was extremely challenging, dealing with major problems of insolubility, low yield, and degradation products. We used different *E. coli* strains (KRX, Rosetta, C41, M15, and the G6PD deficient cells PD2000), combined with different helper plasmids (pRARE, pRARE II, pRIG), a variety of growth, induction, and expression conditions, different purification strategies (Ni-NTA, 2'5' ADP-sepharose 4B, gel filtration), as well as several buffer systems. The best condition yields 2-3 mg pure and active PfGluPho per liter *E. coli* culture. In addition to the full length PfGluPho, we cloned the G6PD domain (aa 339 – 910, named $G6PD_{PfGluPho}$) separately in order to test whether it functions independently of the 6PGL part.

Oligomerization studies of PfGluPho

PfGluPho purified from parasite lysate has a tetrameric structure [8, 9, 21], while hG6PD exists as a monomer, dimer, and tetramer depending on ionic strength, pH, and the presence of its substrates [22]. According to our studies, PfGluPho exists as a tetramer with a molecular weight of 443 kDa under native conditions (Figure S2). The tetrameric state is stable under reducing conditions (2 mM DTT), indicating that the oligomerization is not based on intermolecular disulfide bonds. Oligomerization studies after incubation with $NADP^+$ (2 mM) and NADPH (2 mM) revealed that PfGluPho maintains its tetrameric structure in the presence of its substrate and product inhibitor as well as at alkaline pH.

Western Blot analyses

We obtained peptide specific antibodies against the G6PD and the 6PGL part of PfGluPho (Glu and Pho antibody), which are highly specific for PfGluPho since they do not react with human G6PD or 6PGL (Figures 1 C, D). In addition, we detected full length PfGluPho in *P. falciparum* parasite lysate using the peptide antibodies (Figures 1 E, F).
Besides the signal with a molecular mass of 107 kDa, the expected molecular mass of recombinant PfGluPho deduced from the amino acid sequence, we obtained a second signal of approximately 80 kDa. MALDI-ToF peptide mass fingerprinting of the two bands of recombinant PfGluPho identified peptides from all parts of the full-length protein (aa 9 to aa 911, coverage 55.8 % (80 kDa band) and 48.6 % (107 kDa band)), indicating that both protein bands represent the full-length protein and thus exclude a proteolytic truncation of PfGluPho. The two bands were also detected with the peptide antibodies in parasite lysate and thus occur *in vivo* (Figure 1). This phenomenon might be due to posttranslational modifications; however, it remains to be studied in detail by high accuracy mass spectrometry.

PfGluPho's G6PD reaction

The G6PD activity of PfGluPho as well as the activity of the separately cloned G6PD part of PfGluPho ($G6PD_{PfGluPho}$) were characterized in detail and in direct comparison to hG6PD. The kinetic studies were performed in the standard G6PD assay buffer (0.1 M Tris, 10 mM $MgCl_2$, 0.5 mM EDTA, pH 8.0) to allow a direct comparison to hG6PD from our own studies as well as from previously published data. A pH profile of the G6PD activity of PfGluPho in direct comparison to hG6PD shows that both enzymes catalyze the reaction with a maximum activity at pH 8.0 (Figure S3 A, B). EDTA stabilizes the dimeric form of hG6PD [23], but does not influence the activity of PfGluPho (Figure S3 C).
hG6PD is stable for several months when stored at a concentration of 30 mg/ml in 50 mM Tris, 300 mM NaCl, pH 8.0, 0.1 mM $NADP^+$ at 4 °C. $NADP^+$ binding to a second, "structural" $NADP^+$ binding site is required for the long-term structural integrity of the enzyme [24]. This phenomenon is strongly conserved in eukaryotic organisms [25]. However, $NADP^+$ does not affect the stability or oligomerization state of PfGluPho, suggesting that a putative second $NADP^+$ binding site does not have a structural function in PfGluPho. PfGluPho is stable for several months when stored at -80 °C in 50 mM Tris, 500 mM NaCl, pH 7.8, and 50 % glycerol.
The G6PD activity of PfGluPho was directly compared to hG6PD. The apparent K_m of PfGluPho for $NADP^+$ is 6.5 ± 2.2 µM, while we calculated a K_m of 8.1 µM from the Dalziel parameters (according to Dalziel [19]). The apparent K_m for G6P is 19.2 ± 3.9 µM, and 15.9 µM was calculated from the Dalziel equations (Tables 1 and 2). The K_m values differ slightly due to differences in the methodology such as concentration of the co-substrate. hG6PD has a comparatively lower affinity for G6P and $NADP^+$, but a higher specific activity (Table 1).
The kinetic data were analyzed by non-linear regression using GraphPad Prism yielding kinetic constants and Dalziel parameters (Table 1 and 2). The data are displayed using linear transformation to simplify interpretation of the results: the studies of PfGluPho's G6PD activity using different combinations of G6P and $NADP^+$ yielded linear converging double reciprocal plots (Figure 2 A). An intersecting pattern was also observed with G6P as the independent variable.
To study the G6PD part of PfGluPho independently from the 6PGL part, we also cloned and overexpressed the G6PD part separately ($G6PD_{PfGluPho}$). Interestingly, $G6PD_{PfGluPho}$ catalyzes the reaction with the same specific activity as PfGluPho, but slightly higher K_m values for both substrates (Table 1), indicating that the G6PD activity is not largely influenced by the 6PGL part.

PfGluPho's 6PGL reaction

Studies on the activity of 6PGL are limited since the natural substrate 6-phosphoglucono-δ-lactone (6PGδL) is highly unstable. Thus, Beutler *et al.*, 1986 optimized an assay system using the more stable γ-lactone (6PGγL) as a substrate for 6PGL, which contains a five-membered heterocyclic ring whereas the δ-lactone contains a six-membered heterocyclic ring [20]. We successfully employed 6PGγL as a substrate to study PfGluPho in direct comparison with h6PGL in an assay system coupled to the reaction of 6PGD, the succeeding step of the PPP. PfGluPho catalyses the hydrolysis of 6PGγL with a specific activity of 46 U/mg (Table 1). Despite the use of the synthetic 6PGγL, this is a range comparable with a NMR-based study where the 6PGL part of PfGluPho catalyzes the hydrolysis of 6PGδL with 60 U/mg [26]. The specific activity of PfGluPho is remarkably lower when compared to h6PGL with 1065 U/mg, although PfGluPho shows a slightly higher substrate affinity (Table 1).

Alternative substrate studies

Since alternative substrates can be used to differentiate kinetic models, we used 2deoxyG6P as an alternative substrate for G6P in the G6PD reaction as shown in Figure S4 and Table 1. As expected, PfGluPho has a higher affinity for its natural substrate G6P than for the substrate analogue 2deoxyG6P. The difference between natural and alternative substrate is emphasized especially at low sugar phosphate concentrations, which is in good agreement with the Dalziel parameters, since $\Phi_{2deoxyG6P}$ and $\Phi_{NADP^+2deoxyG6P}$ are much higher than those for G6P (Table 2).

Inhibition studies

Inhibition studies with the product inhibitor NADPH and the dead-end inhibitor glucosamine 6-phosphate were performed to investigate the kinetic mechanism of PfGluPho's G6PD activity (Figure 3). Inhibition assays can be used to differentiate between a sequential and a random order reaction, and have been performed for hG6PD and the inhibitors NADPH and glucosamine 6-phosphate before [27, 28]. Glucosamine 6-phosphate is an analogue of glucose 6-phosphate and acts as a dead-end inhibitor, meaning that the enzyme is completely inactive after binding of the inhibitor [27, 28]. NADPH was found to be a competitive inhibitor with respect to $NADP^+$, as indicated by the intersection on the x-axis in Figure 3 A and increasing K_m values with increasing inhibitor concentrations (Figures 3 C, D). Towards G6P, NADPH acts as a mixed type inhibitor (Figure 3 B). Glucosamine 6-phosphate acts as a mixed type inhibitor with respect to $NADP^+$ and shows a competitive inhibition pattern towards G6P (Figures 3 E-F), which is sometimes rather mixed-type.
Analogous experiments with hG6PD demonstrate that NAPDH acts as a competitive inhibitor towards $NADP^+$ and non-competitively with respect to G6P, while glucosamine 6-phosphate showed a competitive inhibition towards G6P and a non-competitive inhibition towards $NADP^+$.
The recently identified compound C276-1187 with antimalarial activity does bind to $6PGL_{PfGluPho}$ [17], but we could not detect an inhibitory effect on the 6PGL-activity of PfGluPho or h6PGL at concentrations up to 300 µM. However, the compound was found to inhibit the G6PD activity of PfGluPho with an IC_{50} of 127 ± 17 µM. Compound C276-1187 acts on PfGluPho's G6PD activity as a non-competitive inhibitor towards both G6P and $NADP^+$ (Figure S5). $G6PD_{PfGluPho}$ is inhibited with an IC_{50} of 130 ± 11 µM, while the inhibition of hG6PD is more efficient with an IC_{50} of 76 ± 16 µM.

S-glutathionylation studies on PfGluPho

A previous study suggests that PfGluPho contains a peptide with high similarity to the glutathione binding site of glutathione S-transferase [9]. This motivated us to test whether recombinant PfGluPho is *S*-glutathionylated using MALDI-ToF mass spectrometry and kinetic analyses. Recombinant PfGluPho incubated with glutathione disulfide (GSSG) was analysed by peptide mass fingerprinting with MALDI-ToF after trypsin digestion. The matched peptides covered 38 % of the protein. A clear mass increase of ~305 Da could be shown for the peptides Ser^{140}-Lys^{151}, Asn^{506}-Arg^{524}, Thr^{804}-Lys^{821}, and Lys^{824}-Lys^{840} containing Cys^{144}, Cys^{507}, Cys^{806}, and Cys^{838}, respectively. Except for Cys^{144}, all *S*-glutathionylated cysteines are located in the G6PD domain.
The G6PD assay was performed after incubating PfGluPho with GSSG or reduced glutathione (GSH) and demonstrated that PfGluPho's G6PD activity as well as its 6PGL-activity are down-regulated by *S*-glutathionylation in a concentration-dependent manner (Figures 4 A, C). In contrast, incubation with GSH as well as dithiothreitol increases PfGluPho's G6PD activity by 25 %, while the 6PGL-activity is not affected by reduction. Incubation of the *S*-glutathionylated PfGluPho with dithiothreitol showed that the inhibition of both activities could be partially reversed (~80 %) by dithiothreitol, showing that PfGluPho is inhibited by a reversible mechanism (Figures 4 B, D). In contrast, hG6PD was not found to be regulated by GSSG, while h6PGL shows a similar inhibition by *S*-glutathionylation when compared to PfGluPho (Figures 4 B, C).

Discussion

For the first time we describe the cloning, heterologous overexpression, purification, and kinetic characterization of PfGluPho, a bifunctional enzyme combining G6PD and 6PGL of the pentose phosphate pathway from *P. falciparum* with unique structural and functional characteristics. Our studies demonstrate that PfGluPho shows remarkable differences to the corresponding human enzymes regarding the primary structure, oligomerization behaviour, the substrate affinity, regulation, as well as the kinetic mechanism.
Previous attempts to clone the full length PfGluPho failed, most likely due to the high AT-content and the size of the gene [10]. Cloning and overexpression of the 6PGL part of PfGluPho yielded only 10 µg protein per liter *E. coli* culture, since most of the protein was insoluble [26]. Thus, studies using PfGluPho have so far been restricted to the enzyme purified from parasite extract [7, 8, 21], which is limiting in terms of the enzyme quantity required for biochemical characterisation, inhibitor tests, and crystallization screening, and might be contaminated with G6PD from human RBC. Cloning, overexpression, and purification of full length PfGluPho was challenging, but finally we were able to produce 2-3 mg pure PfGluPho per liter *E. coli* culture.
According to our studies, recombinant PfGluPho is a tetramer with 443 kDa (Figure S2), which is in agreement with the tetrameric structure of PfGluPho purified from parasite extract [8, 9, 21]. h6PGL is described as a monomer [29], while hG6PD exists in transition between dimer and tetramer, depending on ionic strength, pH, and substrate concentration, with the dimer most likely being the predominant form *in vivo* [23, 30]. In contrast, the quaternary structure of PfGluPho is not influenced by the presence of $NADP^+$, DTT, or NADPH, and is also stable at alkaline pH. A dimeric or monomeric form could not be detected in our experiments.

PfGluPho exhibits both G6PD and 6PGL activity, which we characterized in detail for the first time using the recombinant full length enzyme. Previous kinetic data on the G6PD activity of PfGluPho were obtained from parasite extracts, which explains the small discrepancies observed between previous and our current data. Nevertheless, our K_m values for the G6PD activity of PfGluPho obtained from the pure, recombinant enzyme are similar to those reported by Kurdi-Haidar and Luzzatto (Table 1) [8]. Analysis of the separately cloned G6PD part of PfGluPho ($G6PD_{PfGluPho}$) demonstrates that the fusion of G6PD and 6PGL does not enhance the efficiency of the first PPP reaction.

PfGluPho has higher substrate affinities for both $NADP^+$ and G6P. This implies that in infected RBC, where G6P and particularly $NADP^+$ concentrations are lower than the K_m values of hG6PD, PfGluPho utilizes most of the G6P and $NADP^+$ present for the generation of NADPH, although this effect might in part be compensated by the higher specific activity of hG6PD (63.9 U/mg compared to 4.5 U/mg).

The kinetic mechanism of hG6PD has been controversially discussed by several groups, which might be due to the heterogeneous origin of hG6PD used in the respective studies [23, 28]. The discrepancy was solved by Wang *et al*., who clearly documented a rapid equilibrium random order mechanism for recombinant hG6PD [27]. We applied product inhibition studies to elucidate the binding order of substrates and release order of products as an independent test to differentiate between an ordered and a random mechanism. The studies of PfGluPho's G6PD activity point to a rapid equilibrium random bi bi system, in which both substrates must bind before product formation can occur. In both cases the binding of the second substrate depends on the binding of the first substrate and indicates a sequential order mechanism of substrate binding – however, without a leading substrate in PfGluPho. Thus, in the parasite enzyme it is not important which substrate binds first, but the two substrates cannot bind simultaneously. Analogous experiments with hG6PD imply that hG6PD acts via a rapid-equilibrium random-order mechanism with both binding sites acting independently from each other, which has been proposed by Wang *et al* before [27].

An independent examination of the kinetic mechanism was applied using the Dalziel parameters to test whether PfGluPho's G6PD activity acts according to a compulsory-order mechanism where one substrate is the leading substrate. If substrate 1 ($NADP^+$) binds first to the enzyme, $\Phi_{NADP^+G6P}/\Phi_{G6P}$ is K_d, the dissociation constant for substrate 1 leaving the binary-enzyme complex. This value as well as the values for Φ_{NADP^+} and $\Phi_{NADP^+G6P}/\Phi_{G6P}\Phi_{NADP^+}$ should not change if an alternative substrate 2 is used, while the values of Φ_0 ($1/k_{cat}$), Φ_{G6P}, and Φ_{NADP^+G6P} can be different [19]. Whether $NADP^+$ is the leading substrate can be deduced from parameters that were obtained using 2deoxyG6P instead of G6P (Table 2): If $NADP^+$ is the leading substrate, $\Phi_{NADP^+G6P}/\Phi_{G6P}$ should be equal to $\Phi_{NADP^+2deoxyG6P}/\Phi_{2deoxyG6P}$. We obtained with 8.1 µM and 4.3 µM quite different values for these parameters. Φ_{NADP^+} with G6P as a substrate was 1.3 µM*s, while Φ_{NADP^+} with 2deoxyG6P as a substrate was 0.9 µM*s. In parallel, the mean values for $\Phi_{NADP^+G6P}/\Phi_{G6P}\Phi_{NADP^+}$ are with 6.2 s^{-1} (G6P) and 4.7 s^{-1} (2deoxyG6P) different. These results exclude a compulsory-order mechanism with $NADP^+$ as the leading substrate for PfGluPho and support the results of the inhibition studies.

Product inhibition studies and an alternative substrate imply that the reaction of PfGluPho's G6PD activity follows a rapid equilibrium random bi bi system without a leading substrate where both substrates cannot bind simultaneously (Figure 5). This kinetic mechanism clearly distinguishes PfGluPho from hG6PD, which follows a rapid equilibrium random order mechanism with both substrates binding independently from each other [27].

In addition to the G6PD activity we examined PfGluPho's 6PGL activity for the first time. Due to instability of the substrate, the enzymatic properties of 6PGL from various organisms including man are barely studied. Using the γ-lactone instead of the unstable δ-lactone as a substrate to study PfGluPho's 6PGL activity, we did not only confirm the specific activity using 6PGδL in a previous NMR-based study (Table 1), but also established a reproducible and reliable system to compare PfGluPho and h6PGL in future inhibitor screens. A previous study reported, that the γ-lactone can neither spontaneously nor enzymatically be hydrolyzed and forms a "dead-end" of the PPP [26]. However, according to our study both PfGluPho and h6PGL accept the γ-lactone as a substrate. Compared to the human enzyme, PfGluPho's

6PGL activity is rather low (Table 1). The relevance of 6PGL was questioned, since its substrate 6PGδL undergoes spontaneous hydrolysis. However, enzyme-catalyzed hydrolysis of 6PGδL is required to maintain a low $NADP^+$/NADPH ratio by avoiding that the uncatalyzed hydrolysis becomes rate-limiting for the dehydrogenase reactions of the PPP [29]. Regarding the dependence of the malaria parasite on the PPP and the tight regulation, the combination of G6PD and 6PGL in PfGluPho might improve the efficiency and the regulation of the PPP.
A recent chemical genetic screen identified two compounds that are active against drug-resistant *P. falciparum* strains and bind to $6PGL_{PfGluPho}$ in thermal melt shift experiments [17]. We were able to obtain one of the two compounds (C276-1187) and could show that it does not inhibit the 6PGL activity of PfGluPho, but acts as a non-competitive inhibitor towards the G6PD activity of PfGluPho. The weak inhibition of PfGluPho by C276-1187 suggests that its antimalarial activity is not mainly due to PfGluPho inhibition. Furthermore, the compound is not specific for PfGluPho, since it is more active on hG6PD.
Protein *S*-glutathionylation is a reversible process that regulates the activity of several proteins via functionally or structurally critical cysteine residues (reviewed in [31]). Mass spectrometric and enzymatic data clearly demonstrate that PfGluPho can undergo *S*-glutathionylation, which leads to a concentration-dependent and reversible inactivation of both the G6PD and the 6PGL activity (Figures 4), indicating that the intracellular GSH/GSSG ratio regulates PfGluPho activity. hG6PD activity did not respond to glutathione, while h6PGL was affected by high glutathione concentrations (Figures 4 B, D). A comparable glutathione-mediated inhibition has been shown for h6PGL before [32]. PfGluPho is completely inhibited by 8 mM GSSG, a concentration that might occur locally in the cell under high oxidative stress. This suggests that *S*-glutathionylation of PfGluPho might rather be important for protection of PfGluPho from irreversible oxidation under high oxidative stress than for regulation of enzyme activity.
The PPP of human erythrocytes is highly up-regulated after *Plasmodium* infection, with the PPP of malaria parasites accounting for about 80 % of the total PPP activity in infected erythrocytes. This up-regulation is attributed to high oxidative stress in infected erythrocytes, since the PPP provides NADPH for reducing reactions. Additionally, a high turnover in the parasitic PPP resulting in high concentrations of ribose 5-phosphate is required for the synthesis of nucleotides [6]. G6PD has been shown to be the rate limiting enzyme in the PPP, and is essential for the survival of various organisms such as mice [33] and trypanosomes [34]. Transient silencing of PfGluPho results in growth arrest at the trophozoite stage, suggesting that PfGluPho is crucial for malaria parasites [13]. PfGluPho as the first and presumably rate-limiting enzyme of the *Plasmodium* PPP must be tightly regulated, an aspect that has to be addressed in future studies.
Our study revealed major differences between hG6PD and PfGluPho: the bifunctional enzyme is not expressed in mammals, contains an insertion sequence with unknown but essential function, has a stable tetrameric structure, is inhibited by *S*-glutathionylation, and differs in substrate affinity as well as kinetic mechanism from the human counterparts. The unique characteristics of PfGluPho provide the starting point for developing new antimalarial drugs.

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References

1 Ruwende, C. and Hill, A. (1998) Glucose-6-phosphate dehydrogenase deficiency and malaria. J Mol Med. **76**, 581-588

2 Carrara, V. I., Zwang, J., Ashley, E. A., Price, R. N., Stepniewska, K., Barends, M., Brockman, A., Anderson, T., McGready, R., Phaiphun, L., Proux, S., van Vugt, M., Hutagalung, R., Lwin, K. M., Phyo, A. P., Preechapornkul, P., Imwong, M., Pukrittayakamee, S., Singhasivanon, P., White, N. J. and Nosten, F. (2009) Changes in the treatment responses to artesunate-mefloquine on the northwestern border of Thailand during 13 years of continuous deployment. PLoS One. **4**, e4551

3 Andriantsoanirina, V., Ratsimbasoa, A., Bouchier, C., Jahevitra, M., Rabearimanana, S., Radrianjafy, R., Andrianaranjaka, V., Randriantsoa, T., Rason, M. A., Tichit, M., Rabarijaona, L. P., Mercereau-Puijalon, O., Durand, R. and Menard, D. (2009) Plasmodium falciparum drug resistance in Madagascar: facing the spread of unusual pfdhfr and pfmdr-1 haplotypes and the decrease of dihydroartemisinin susceptibility. Antimicrob Agents Chemother. **53**, 4588-4597

4 Becker, K., Tilley, L., Vennerstrom, J. L., Roberts, D., Rogerson, S. and Ginsburg, H. (2004) Oxidative stress in malaria parasite-infected erythrocytes: host-parasite interactions. Int J Parasitol. **34**, 163-189

5 Min-Oo, G. and Gros, P. (2005) Erythrocyte variants and the nature of their malaria protective effect. Cell Microbiol. **7**, 753-763

6 Atamna, H., Pascarmona, G. and Ginsburg, H. (1994) Hexose-monophosphate shunt activity in intact Plasmodium falciparum-infected erythrocytes and in free parasites. Mol Biochem Parasitol. **67**, 79-89

7 Yoshida, A. and Roth, E. F., Jr. (1987) Glucose-6-phosphate dehydrogenase of malaria parasite Plasmodium falciparum. Blood. **69**, 1528-1530

8 Kurdi-Haidar, B. and Luzzatto, L. (1990) Expression and characterization of glucose-6-phosphate dehydrogenase of Plasmodium falciparum. Mol Biochem Parasitol. **41**, 83-91

9 O'Brien, E., Kurdi-Haidar, B., Wanachiwanawin, W., Carvajal, J. L., Vulliamy, T. J., Cappadoro, M., Mason, P. J. and Luzzatto, L. (1994) Cloning of the glucose 6-phosphate dehydrogenase gene from Plasmodium falciparum. Mol Biochem Parasitol. **64**, 313-326

10 Clarke, J. L., Scopes, D. A., Sodeinde, O. and Mason, P. J. (2001) Glucose-6-phosphate dehydrogenase-6-phosphogluconolactonase. A novel bifunctional enzyme in malaria parasites. Eur J Biochem. **268**, 2013-2019

11 Scopes, D. A., Bautista, J. M., Vulliamy, T. J. and Mason, P. J. (1997) Plasmodium falciparum glucose-6-phosphate dehydrogenase (G6PD)-the N-terminal portion is homologous to a predicted protein encoded near to G6PD in Haemophilus influenzae. Mol Microbiol. **23**, 847-848

12 Clarke, J. L., Sodeinde, O. and Mason, P. J. (2003) A unique insertion in Plasmodium berghei glucose-6-phosphate dehydrogenase-6-phosphogluconolactonase: evolutionary and functional studies. Mol Biochem Parasitol. **127**, 1-8

13 Crooke, A., Diez, A., Mason, P. J. and Bautista, J. M. (2006) Transient silencing of Plasmodium falciparum bifunctional glucose-6-phosphate dehydrogenase- 6-phosphogluconolactonase. Febs J. **273**, 1537-1546

14 Cerutti, H. and Casas-Mollano, J. A. (2006) On the origin and functions of RNA-mediated silencing: from protists to man. Curr Genet. **50**, 81-99

15 Xue, X., Zhang, Q., Huang, Y., Feng, L. and Pan, W. (2008) No miRNA were found in Plasmodium and the ones identified in erythrocytes could not be correlated with infection. Malar J. **7**, 47

16 Rahlfs, S. and Becker, K. (2006) Interference with redox-active enzymes as a basis for the design of antimalarial drugs. Mini Rev Med Chem. **6**, 163-176

17 Guiguemde, W. A., Shelat, A. A., Bouck, D., Duffy, S., Crowther, G. J., Davis, P. H., Smithson, D. C., Connelly, M., Clark, J., Zhu, F., Jimenez-Diaz, M. B., Martinez, M. S., Wilson, E. B., Tripathi, A. K., Gut, J., Sharlow, E. R., Bathurst, I., El Mazouni, F., Fowble, J. W., Forquer, I., McGinley, P. L., Castro, S., Angulo-Barturen, I., Ferrer, S., Rosenthal, P. J., Derisi, J. L., Sullivan, D. J., Lazo, J. S., Roos, D. S., Riscoe, M. K., Phillips, M. A., Rathod, P.

K., Van Voorhis, W. C., Avery, V. M. and Guy, R. K. (2010) Chemical genetics of Plasmodium falciparum. Nature. **465**, 311-315
18 Beutler, E. (1984) Red cell metabolism: A manual of biochemical methods. Grune and Stratton, New York
19 Dalziel, K. (1957) Initial steady state velocities in the evaluation of enzyme-coenzyme-substrate reaction mechanisms. Acta Chem Scand. **11**, 1706-1723
20 Beutler, E., Kuhl, W. and Gelbart, T. (1986) Blood cell phosphogluconolactonase: assay and properties. Br J Haematol. **62**, 577-586
21 Ling, I. T. and Wilson, R. J. (1988) Glucose-6-phosphate dehydrogenase activity of the malaria parasite Plasmodium falciparum. Mol Biochem Parasitol. **31**, 47-56
22 Wrigley, N. G., Heather, J. V., Bonsignore, A. and De Flora, A. (1972) Human erythrocyte glucose 6-phosphate dehydrogenase: electron microscope studies on structure and interconversion of tetramers, dimers and monomers. J Mol Biol. **68**, 483-499
23 Birke, S., Kim, H. W., Periclou, A., Schorsch, B., Grouse, D. and Craney, C. (1989) Kinetics of human erythrocyte glucose-6-phosphate dehydrogenase dimers. Biochim Biophys Acta. **999**, 243-247
24 Wang, X. T., Chan, T. F., Lam, V. M. and Engel, P. C. (2008) What is the role of the second "structural" NADP+-binding site in human glucose 6-phosphate dehydrogenase? Protein Sci. **17**, 1403-1411
25 Au, S. W., Gover, S., Lam, V. M. and Adams, M. J. (2000) Human glucose-6-phosphate dehydrogenase: the crystal structure reveals a structural NADP(+) molecule and provides insights into enzyme deficiency. Structure. **8**, 293-303
26 Miclet, E., Stoven, V., Michels, P. A., Opperdoes, F. R., Lallemand, J. Y. and Duffieux, F. (2001) NMR spectroscopic analysis of the first two steps of the pentose-phosphate pathway elucidates the role of 6-phosphogluconolactonase. J Biol Chem. **276**, 34840-34846
27 Wang, X. T., Au, S. W., Lam, V. M. and Engel, P. C. (2002) Recombinant human glucose-6-phosphate dehydrogenase. Evidence for a rapid-equilibrium random-order mechanism. Eur J Biochem. **269**, 3417-3424
28 Adediran, S. A. (1991) Kinetic properties of normal human erythrocyte glucose-6-phosphate dehydrogenase dimers. Biochimie. **73**, 1211-1218
29 Bauer, H. P., Srihari, T., Jochims, J. C. and Hofer, H. W. (1983) 6-phosphogluconolactonase. Purification, properties and activities in various tissues. Eur J Biochem. **133**, 163-168
30 Cohen, P. and Rosemeyer, M. A. (1969) Subunit interactions of glucose-6-phosphate dehydrogenase from human erythrocytes. Eur J Biochem. **8**, 8-15
31 Mieyal, J. J., Gallogly, M. M., Qanungo, S., Sabens, E. A. and Shelton, M. D. (2008) Molecular mechanisms and clinical implications of reversible protein S-glutathionylation. Antioxid Redox Signal. **10**, 1941-1988
32 Fratelli, M., Demol, H., Puype, M., Casagrande, S., Eberini, I., Salmona, M., Bonetto, V., Mengozzi, M., Duffieux, F., Miclet, E., Bachi, A., Vandekerckhove, J., Gianazza, E. and Ghezzi, P. (2002) Identification by redox proteomics of glutathionylated proteins in oxidatively stressed human T lymphocytes. Proc Natl Acad Sci U S A. **99**, 3505-3510
33 Pandolfi, P. P., Sonati, F., Rivi, R., Mason, P., Grosveld, F. and Luzzatto, L. (1995) Targeted disruption of the housekeeping gene encoding glucose 6-phosphate dehydrogenase (G6PD): G6PD is dispensable for pentose synthesis but essential for defense against oxidative stress. EMBO J. **14**, 5209-5215
34 Cordeiro, A. T., Thiemann, O. H. and Michels, P. A. (2009) Inhibition of Trypanosoma brucei glucose-6-phosphate dehydrogenase by human steroids and their effects on the viability of cultured parasites. Bioorg Med Chem. **17**, 2483-2489

Table 1. Kinetic characteristics of PfGluPho, $G6PD_{PfGluPho}$, hG6PD, and h6PGL.

Each value is a mean value from at least five independent determinations each including at least five measurements. Data are represented as mean +/- standard deviation.

	K_m (µM)	V_{max} (U/mg)	k_{cat} (s^{-1})	K_m (µM)	V_{max} (U/mg)
	PfGluPho's G6PD activity			**Literature values**	
G6P	19.2 ± 3.9	5.2 ± 1.6	8.6 ± 1.5	11[a], 27[b]	-
$NADP^+$	6.5 ± 2.2	4.6 ± 0.8	8.2 ± 1.2	0.8[a], 4.5[b]	-
2deoxyG6P	2670 ± 320	4.1 ± 0.8	7.3 ± 1.5	10000[a]	-
	$G6PD_{PfGluPho}$			**Literature values**	
G6P	33.2 ± 1.2	5.5 ± 0.2	6.3 ± 0.3	-	-
$NADP^+$	6.1 ± 0.7	5.5 ± 0.1	6.3 ± 0.1	-	-
	Human G6PD			**Literature values**	
G6P	116 ± 8.5	64.4 ± 11	64.6 ± 8.9	54[c], 43[d]	
$NADP^+$	17.5 ± 2.8	57.7± 15	56.9 ± 15	6.7[c], 11[d]	100[c], 170[d]
2deoxyG6P	nd	nd	nd	1674[c]	
	PfGluPho's 6PGL activity			**Literature values**	
6PGγL	172 ± 36	46.6 ± 10	106 ± 24	-	-
6PGδL	nd	nd	nd	-	60[e]
	Human 6PGL			**Literature values**	
6PGγL	242 ± 48	1065 ± 97	505215 ± 3083	-	-
6PGδL	nd	nd	nd	-	3330[e]

[a] Yoshida and Roth, 1987; [b] Kurdi-Haidar and Luzzatto, 1990; [c] Wang *et al*., 2002; [d] Birke *et al*., 1989, [e] Miclett *et al*., 2001
nd – not determined

Table 2. Dalziel parameters and ratios for the G6PD reaction of PfGluPho and human G6PD with the substrates $NADP^+$ and G6P as well as 2deoxyG6P as an alternative substrate.

The data were determined from the primary plots against the reciprocal of $NADP^+$ (1) and G6P (2). The mean value is shown in row (3). Data are represented as mean +/- standard deviation.

A) PfGluPho with the substrates $NADP^+$ and G6P

	Φ_0 (s)	Φ_{NADP^+} (μM*s)	Φ_{G6P} (μM*s)	$\Phi_{NADP^+ G6P}$ (μM^2*s)	$\Phi_{NADP^+ G6P}/\Phi_{NADP^+}$ (μM)	$\Phi_{NADP^+ G6P}/\Phi_{G6P}$ (μM)	$\Phi_{NADP^+ G6P}/\Phi_{G6P}\Phi_{NADP^+}$ (s^{-1})	k_{cat} (s^{-1})
1	0.16 ± 0.01	1.27 ± 0.17	2.49 ± 0.11	20.2 ± 2.29	15.9	8.1	6.4	6.4
2	0.17 ± 0.02	1.36 ± 0.13	2.67 ± 0.38	21.6 ± 2.22	15.9	8.1	5.9	5.9
3	0.16	1.31	2.58	20.9	15.9	8.1	6.2	6.2

B) PfGluPho with the substrates $NADP^+$ and 2deoxyG6P (2dG6P)

	Φ_0 (s)	Φ_{NADP^+} (μM*s)	Φ_{2dG6P} (μM*s)	$\Phi_{NADP^+ 2dG6P}$ (μM^2*s)	$\Phi_{NADP^+ 2dG6P}/\Phi_{NADP^+}$ (μM)	$\Phi_{NADP^+ 2dG6P}/\Phi_{2dG6P}$ (μM)	$\Phi_{NADP^+ 2dG6P}/\Phi_{2dG6P}\Phi_{NADP^+}$ (s^{-1})	k_{cat} (s^{-1})
1	0.19 ± 0.01	0.82 ± 0.19	425 ± 32.6	1806 ± 338	2200	4.25	5.2	5.18
2	0.24 ± 0.05	1.01 ± 0.44	524 ± 77.9	2228 ± 454	2200	4.25	4.2	4.2
3	0.22	0.92	474	2017	2200	4.25	4.69	4.69

C) hG6PD with the substrates $NADP^+$ and G6P

	Φ_0 (s)	Φ_{NADP^+} (μM*s)	Φ_{G6P} (μM*s)	$\Phi_{NADP^+ G6P}$ (μM^2*s)	$\Phi_{NADP+G6P}/\Phi_{NADP^+}$ (μM)	$\Phi_{NADP^+ G6P}/\Phi_{G6P}$ (μM)	$\Phi_{NADP^+ G6P}/\Phi_{G6P}\Phi_{NADP^+}$ (s^{-1})	k_{cat} (s^{-1})
1	0.02 ± 0.002	0.28 ± 0.05	1.77 ± 0.45	19.9 ± 0.47	111.9	17.3	55.0	55.0
2	0.02 ± 0.004	0.34 ± 0.09	2.15 ± 0.11	23.5 ± 0.92	111.9	17.3	59.0	59.9
3	0.02	0.31	1.96	21.7	111.9	17.3	57.5	57.5

Figure legends

Figure 1. SDS-PAGE and Western Blot of PfGluPho, hG6PDH, and h6PGL. (A) 12 % SDS-PAGE showing recombinant PfGluPho (6 µg). **(B)** Immunoblot with anti-His antibody showing recombinant PfGluPho and human G6PD (5 µg). **(C)** and **(D)** Immunoblot with peptide antibody against the C-terminal G6PD-part of PfGluPho (anti-Glu) (C) and the N-terminal 6PGL-part of PfGluPho (anti-Pho) (D) with recombinant PfGluPho (0.05 µg), h6PGL (5 µg), and hG6PDH (5 µg). **(E)** and **(F)** Immunoblot with anti-Glu (E) and anti-Pho antibody (F) with recombinant PfGluPho (0.05 µg) and *P. falciparum* lysate (100-200 µg).

Figure 2. PfGluPho's double reciprocal plots for the reaction with $NADP^+$ and glucose 6-phosphate (G6P) as substrates. (A) Primary plots of 1/v against 1/[$NADP^+$] at various concentrations of G6P. **(B)** Secondary plots of intercepts of primary plots against 1/[G6P]. **(C)** Secondary plots of slopes of primary plots against 1/[G6P]. The experiments were performed three times and the reproducibility was within 10 %. The results of one representative experiment are shown here.

Figure 3. Inhibition studies of PfGluPho by the product inhibitor NADPH (A-D) and the dead-end inhibitor glucosamine 6-phosphate (E-H) with varied $NADP^+$ and varied glucose 6-phosphate concentrations. Lineweaver-Burk plots were obtained at 60 µM G6P and different $NADP^+$ concentrations **(A, E)** or at 10 µM $NADP^+$ and different G6P concentrations **(B, F)** both in the presence of 0-30 µM NADPH or 0-30 mM glucosamine 6-phosphate, respectively. The apparent K_m values for $NADP^+$ **(C, G)** and G6P **(D, H)** are plotted against the concentration of NADPH or glucosamine 6-phosphate. Each experiment was performed three times and the reproducibility was within 15 %. The results of one representative experiment are shown here.

Figure 4. *S*-glutathionylation of PfGluPho and hG6PD. (A) and (C) Concentration-dependent inactivation of PfGluPho's G6PD (A) and PfGluPho's 6PGL (C) activity after incubation with different GSSG concentrations for 5 min at 37 ℃. Activity is given as percentage of initial activity. **(B)** Activity of PfGluPho's G6PD (grey) and hG6PD (black) after incubation with glutathione. **(D)** Activity of PfGluPho's 6PGL (grey) and h6PGL (black) after incubation with glutathione. Each value is a mean value from at least three independent determinations each including five measurements. Data are represented as mean +/- standard deviation.

Figure 5. PfGluPho's G6PD reaction follows a rapid equilibrium random bi bi mechanism. The binding of the second substrate depends on the binding of the first substrate. Both substrates must bind to PfGluPho, before product formation can occur.

Figure 1. SDS-PAGE and Western Blot of PfGluPho, hG6PDH, and h6PGL.

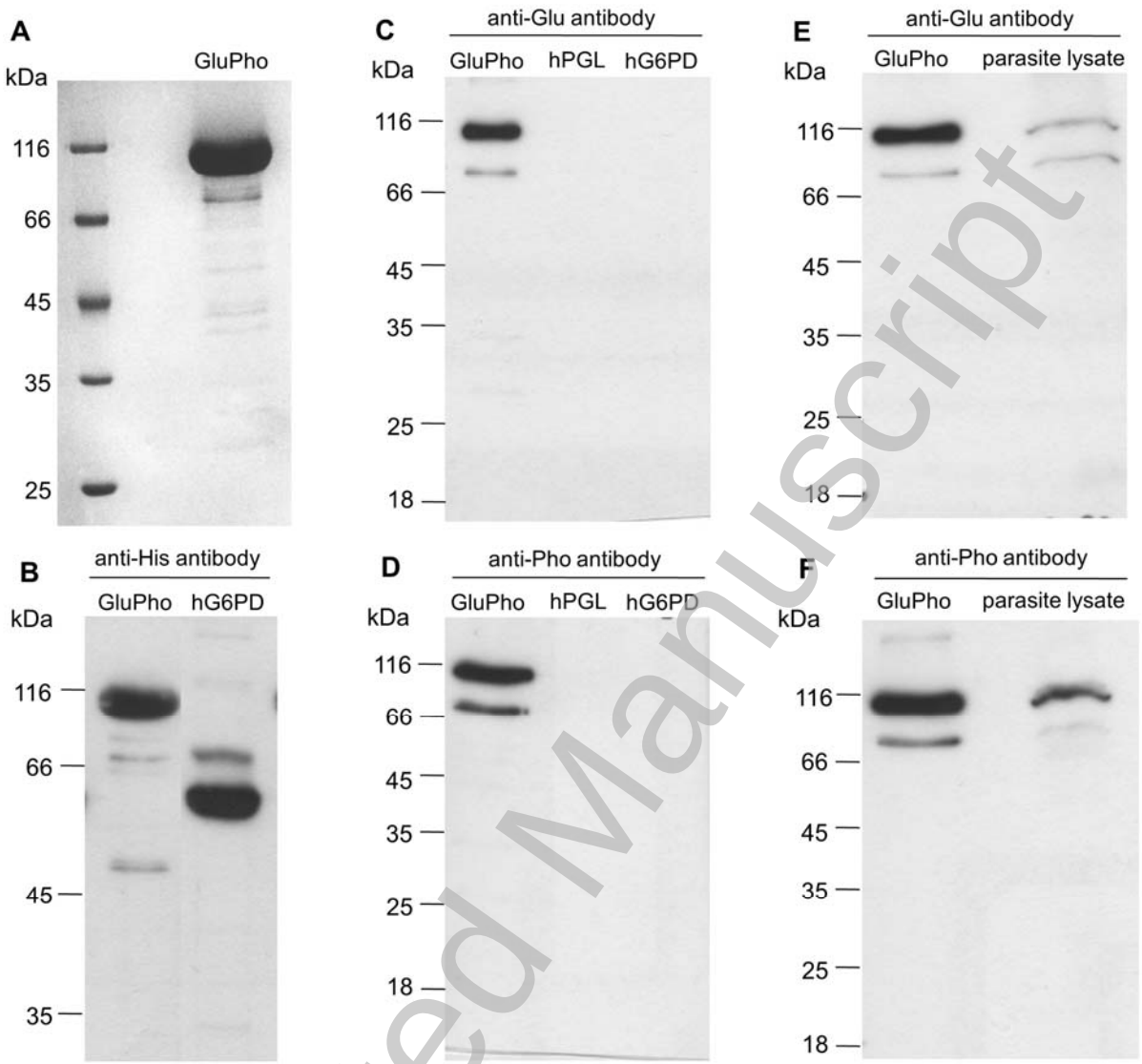

Figure 2. PfGluPho's double reciprocal plots for the reaction with $NADP^+$ and glucose 6-phosphate (G6P) as substrates.

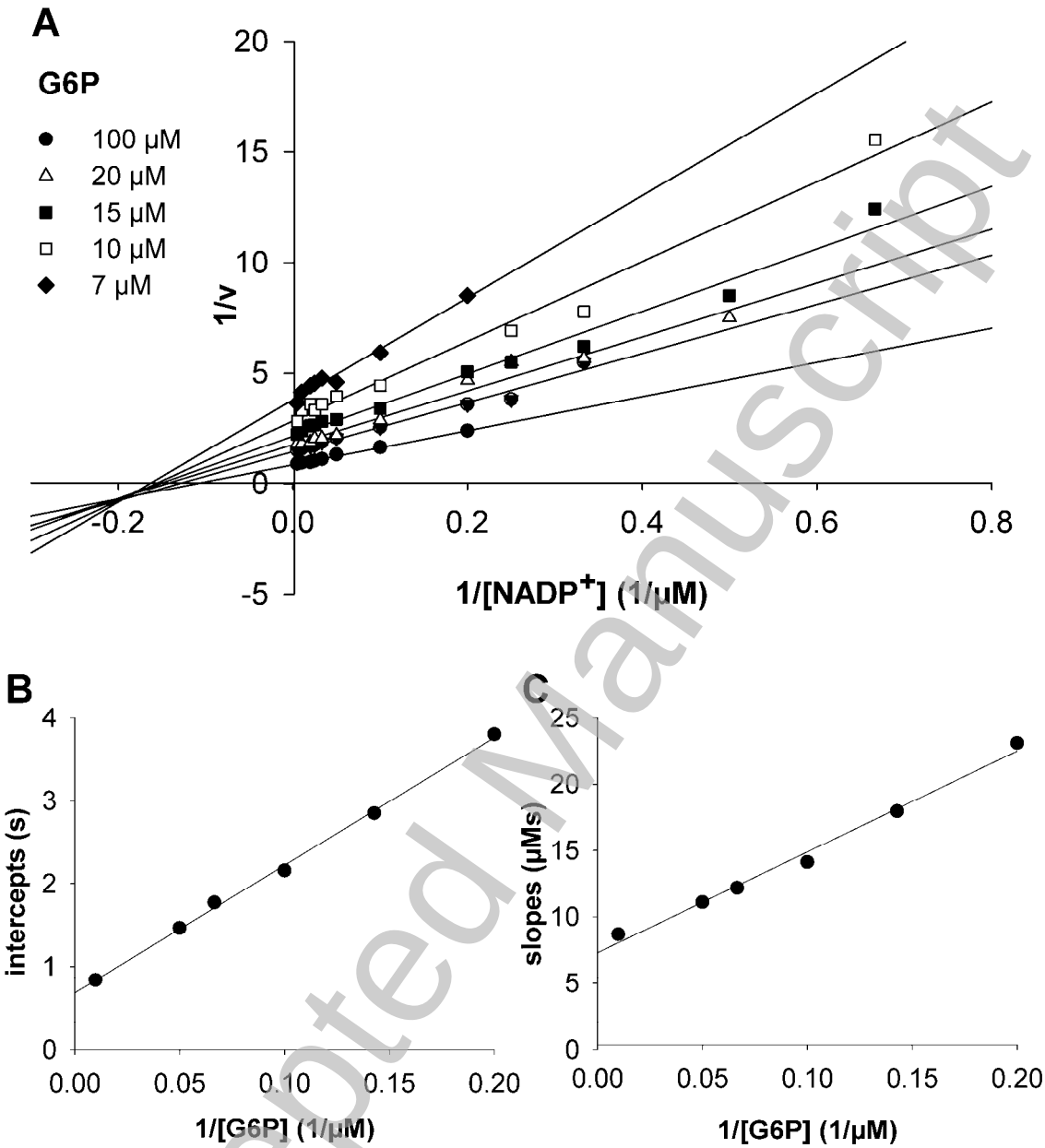

Figure 3. Inhibition studies of PfGluPho by the product inhibitor NADPH (A-D) and the dead-end inhibitor glucosamine 6-phosphate (E-H) with varied $NADP^+$ and varied glucose 6-phosphate concentrations.

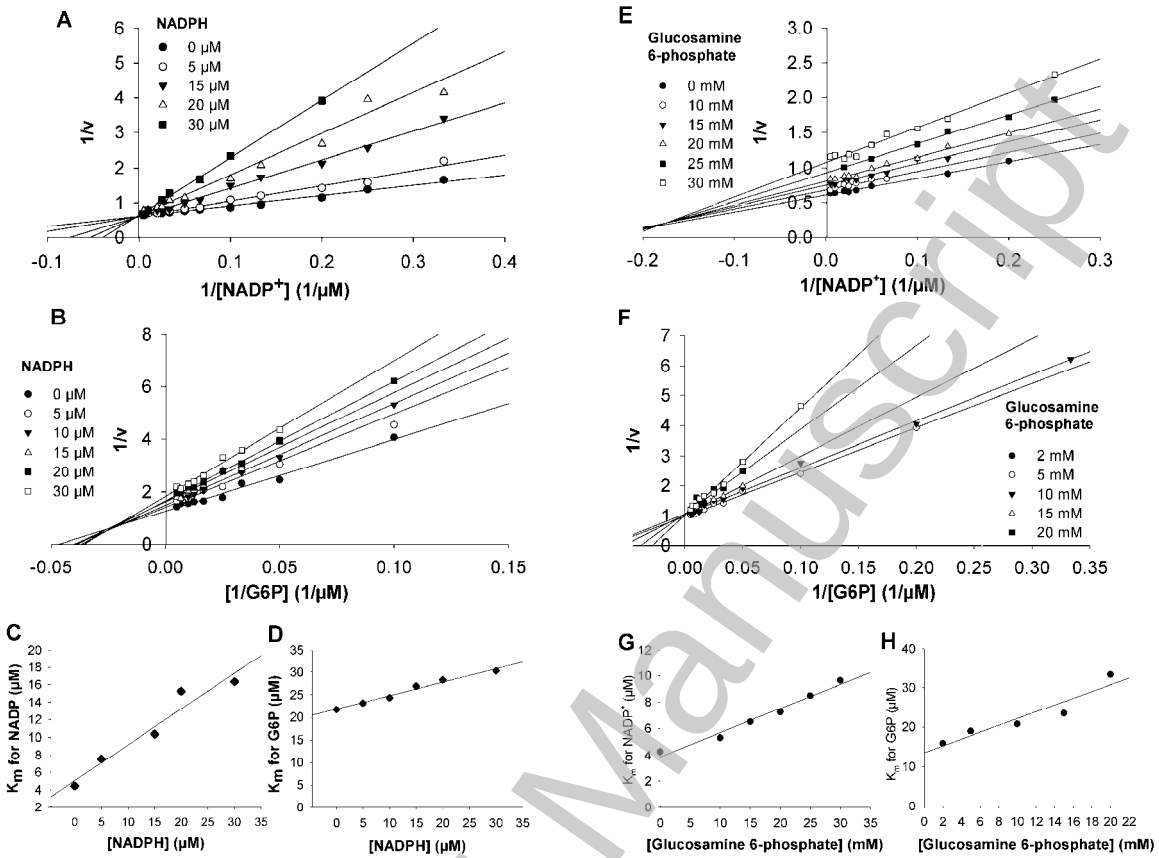

Figure 4. *S*-glutathionylation of PfGluPho and hG6PD.

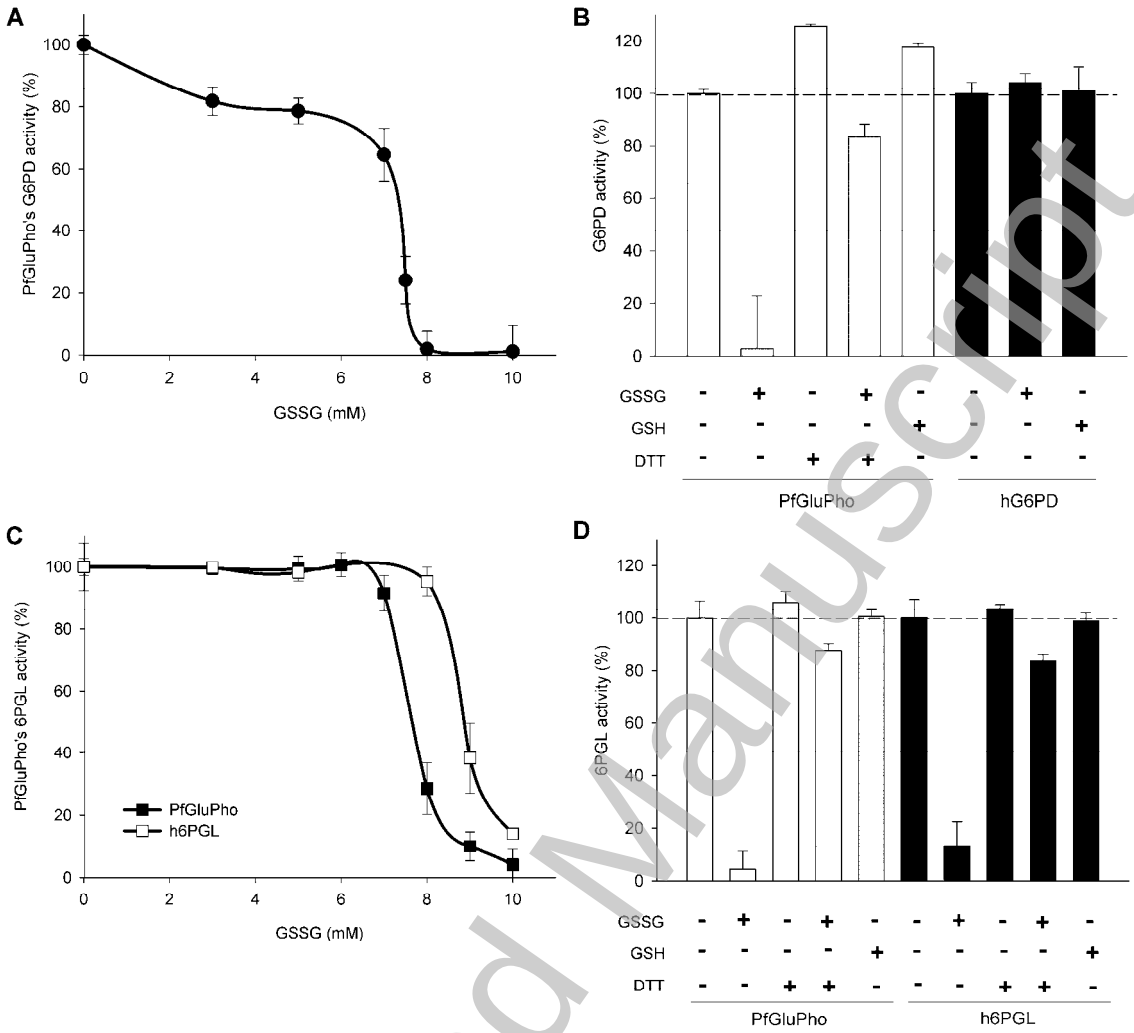

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Figure 5. PfGluPho's G6PD reaction follows a rapid equilibrium random bi bi mechanism.