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

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The survival of malaria parasites in human RBCs (red blood cells) depends on the pentose phosphate pathway, both in *Plasmodium falciparum* and its human host. G6PD (glucose-6-phosphate dehydrogenase) deficiency, the most common human enzyme deficiency, leads to a lack of NADPH in erythrocytes, and protects from malaria. In *P. falciparum*, G6PD is combined with the second enzyme of the pentose phosphate pathway to create a unique bifunctional enzyme named GluPho (glucose-6-phosphate dehydrogenase–6-phosphogluconolactonase). In the present paper, we report for the first time the cloning, heterologous overexpression, purification and kinetic characterization of both enzymatic activities of full-length PfGluPho (*P. falciparum* GluPho), and demonstrate striking structural and functional differences with 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 hG6PD (human G6PD) facilitate studies on PfGluPho as an excellent drug target candidate in the search for new antimalarial drugs.

Key words: glucose 6-phosphate dehydrogenase, pentose phosphate pathway, 6-phosphogluconolactonase, *Plasmodium*, malaria, redox.

INTRODUCTION

G6PD (glucose-6-phosphate dehydrogenase) deficiency is the most common human enzyme defect and is associated with resistance to malaria, most probably due to decreased levels of reducing equivalents in the form of NADPH [1]. Malaria is one of the most severe infectious diseases with 240 million cases in 2009 (http://www.who.int/malaria/world_malaria_report_2010/worldmalariareport2010.pdf). 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 PPP (pentose phosphate pathway) is the major source of NADPH and pentose sugars, which are crucial for oxidative stress defence and nucleotide synthesis. Enhanced oxidative stress induced by G6PD deficiency or pro-oxidant antimalarial compounds such as 4-aminoquinolines (e.g. chloroquine) and 8-aminoquinolines (e.g. primaquine), as well as artemisinins, suppresses parasite growth and enhances the elimination of parasitized RBCs (red blood cells) by the immune system (for a review see [4]).

The first steps of the PPP are catalysed by G6PD (EC 1.1.1.49), 6PGL (6-phosphogluconolactonase; EC 3.1.1.31), and 6PGD (6-phosphogluconate dehydrogenase; EC 1.1.1.44). Previous studies have suggested that the PPP of both the human host and the malaria parasite are important during the infection of RBCs with *Plasmodium*: (i) hG6PD (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; and (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 had been controversially discussed, until the enzyme could be partially purified from infected RBCs [7,8]. In contrast with 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 GluPho (glucose-6-phosphate dehydrogenase–6-phosphogluconolactonase) [9,10] (Supplementary Figure S1 at http://www.BiochemJ.org/bj/436/bj4360641add.htm). The C-terminal part (amino acids 311–911) of GluPho is homologous with other G6PDs, although interrupted by an insertion of 62 amino acids [9], whereas 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 *Plasmodium berghei* GluPho [12].

The importance of PfGluPho (*Plasmodium falciparum* GluPho) 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 (RNA interference) in *Plasmodium* is questionable [14,15]. Nonetheless immediate transcript enhancement of thioredoxin reductase accompanies PfGluPho knockdown 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

Abbreviations used: DTT, dithiothreitol; G6P, glucose 6-phosphate; GluPho, glucose-6-phosphate dehydrogenase–6-phosphogluconolactonase; G6PD, glucose-6-phosphate dehydrogenase; hG6PD, human G6PD; IPTG, isopropyl-β-D-thiogalactopyranoside; MALDI, matrix-assisted laser-desorption ionization; NIH, National Institutes of Health; PfGluPho, *Plasmodium falciparum* GluPho; 6PGD, 6-phosphogluconate dehydrogenase; 6PGL, 6-phosphogluconolactonase; h6PGL, human 6PGL; 6PGγL, 6-phosphoglucono-γ-lactone; 6PGδL, 6-phosphoglucono-δ-lactone; PPP, pentose phosphate pathway; RBC, red blood cell; TOF, time-of-flight.

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target [16]. In a recent screening approach, 172 out of around 300000 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 PfGluPho 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]. In the present paper 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

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 HindIII 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 SacI restriction site within the *PfGluPho* gene. The first part was amplified using the same BamHI forward restriction site primer and a reverse primer containing a SacI restriction site (underlined) (5′-GCGCGAGCTCTTCTTTATTCAAACTATTAGAATAAAGAG-3′); the second part was amplified using a SacI restriction site primer (5′-GCGCGAGCTCTTAACTATAATAATTTTTGGCTGTTCAG-3′) and the reverse primer containing a HindIII 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 pQE30 (Qiagen). Both constructs contain an N-terminal His tag.

The G6PD part of PfGluPho ranging from amino acid 339 to 910 was cloned as described for PfGluPho (forward: 5′-CGGGATCCACTATAATAATTTTTGGCTGTTCAG-3′, reverse: 5′-GCAAGCTTTCAATTAATATCTAACAACTCGTC-3′; BamHI and Hind III restriction sites underlined).

A clone of hG6PD (GenBank® accession number NP_001035810) from the NIH (National Institutes of Health) Mammalian Gene Collection (clone ID 282264) was purchased from Invitrogen. The gene was amplified by PCR using primers designed to introduce restriction sites for NdeI and XhoI (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.

A clone of h6PGL (GenBank® 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 NdeI and BamHI restriction sites (underlined) (forward: 5′-CGCGCATATGGCCGCGCCGGCCCCG-3′; reverse: 5′-GCGCGGATCCCTACAAAGTGGAATGCTTCTCGAA-3′) and cloned into the expression vector pET28a (Novagen) with an N-terminal His tag.

Heterologous overexpression and purification

The highest yield of recombinant PfGluPho was obtained by heterologous overexpression of PfGluPho in the vector pQE30 in *Escherichia coli* M15 cells (Qiagen) with pRAREII (Novagen). The cells were grown in Terrific Broth (12 g of tryptone, 24 g of yeast extract, 9.4 g of K_2HPO_4 and 4 ml of glycerol per litre of medium) medium supplemented with carbenicillin, kanamycin (both 50 μg/ml) and chloramphenicol (12.5 μg/ml) at 23 °C to an attenuance at 600 nm (D_{600}) of 0.6, and the expression was induced by 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside). After 24 h, the cells were harvested by centrifugation (15 min at 6000 ***g*** and 4 °C), resuspended in 5 ml of 0.1 M Tris/HCl (pH 7.8) and 0.5 M NaCl per g of 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 h at 4 °C, sonicated (three cycles of 20 s at 70 % and 4 °C), and centrifuged (30 min at 30600 ***g*** and 4 °C). The supernatant was applied to a Ni-NTA column (Qiagen) and recombinant proteins were eluted with 0.1 M Tris/HCl (pH 7.8) and 0.5 M NaCl containing 250 mM imidazole. The purity of protein samples was controlled by SDS/PAGE (12 % gel). The protein was further purified by gel-filtration chromatography.

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 °C, and the expression was induced at D_{600} of 0.6 with 0.5 mM IPTG. The cells were harvested after 20 h, and resuspended as described for PfGluPho. The cells were lysed for 1 h at 4 °C by lysozyme and DNase, sonicated (three cycles of 20 s at 70 % and 4 °C), and centrifuged (30 min at 30600 ***g*** and 4 °C). Recombinant proteins were eluted from a Ni-NTA column with 0.1 M Tris/HCl (pH 7.8) and 0.5 M NaCl containing 0.3 M imidazole yielding 1.5 mg of pure protein per litre of *E. coli* culture.

Overexpression of hG6PD was performed in *E. coli* BL21 cells (Invitrogen) containing pRAREII in 2×YT (16 g of tryptone, 10 g of yeast and 5 g of NaCl per litre of medium) medium with kanamycin (50 μg/ml) and chloramphenicol (12.5 μg/ml) at 23 °C. At a D_{600} of 1, the expression was induced by 0.1 mM IPTG and continued for 24 h. Harvesting, cell lysis and purification in 50 mM Tris/HCl (pH 8.0), 300 mM NaCl and 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 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 °C. The expression was induced at a D_{600} of 0.6 with 1 mM IPTG, and the cells were harvested 4 h after induction as described above. The cells were resuspended in 100 mM triethanolamine and 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 of pure protein per litre of *E. coli* culture.

Protein immunoblotting analyses

For the recombinant PfGluPho, semi-dry Western blots using an anti-His_6 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: an N-terminal peptide composed of amino acids 118–133 (KEQLYKPDTTKSIVDC; anti-Pho) and a C-terminal peptide composed of amino acids 896–910 (CVRKSSFYEDDLLDIN; anti-Glu) (Eurogentec, diluted 1:5000). Horseradish peroxidase-conjugated anti-rabbit antibody (Dianova, diluted 1:50000) was used as the 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 behaviour 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 analysed by SDS/PAGE (12 gel) and enzymatic assays. To study the oligomerization behaviour, PfGluPho in 50 mM Tris/HCl and 0.3 M NaCl (pH 7.8) was incubated for 12 h at 4 °C with 2 mM $NADP^+$, 2 mM NADPH or 2 mM DTT (dithiothreitol) respectively. To study the oligomerization behaviour at high pH and ionic strength, PfGluPho was diluted in 0.5 M NaCl and 0.25 M Tris/HCl (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 °C by monitoring the reduction of $NADP^+$ to NADPH at 340 nm using a method described by Beutler [18]. The reaction mixture contained 0.1 M Tris/HCl (pH 8.0), 10 mM $MgCl_2$ and 0.5 mM EDTA with varying amounts of enzyme, 200 μM $NADP^+$ and 200 μM G6P (glucose 6-phosphate), 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 with 200 μM G6P, whereas the K_m for G6P was measured by varying the G6P concentration from 1–200 μM at 200 μM $NADP^+$. 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 a K_m value with different concentrations of $NADP^+$ (1–200 μM). Enzyme activity was assayed with a U-2001 spectrophotometer (Hitachi).

The kinetic parameters in the present study were calculated by non-linear regression using the program GraphPad Prism, as well as from Dalziel parameters. Dalziel's method was applied as an independent method to analyse and identify the kinetic mechanism of PfGluPho, since it allows rival bisubstrate kinetic mechanisms to be distinguished. The initial-rate equation for the G6PD-catalysed reaction according to Dalziel is of the form (eqn 1) [19]:

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

where, $S1$ and $S2$ are the coenzyme ($NADP^+$) and the sugar phosphate (G6P) respectively, 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 1–10 mM in the presence of $NADP^+$ at the K_m, whereas $NADP^+$ 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), whereas 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 assays. 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 6PGγL (6-phosphoglucono-γ-lactone) instead of the unstable natural substrate 6PGδL (6-phosphoglucono-δ-lactone). 6PGγL was produced as described by Beutler et al. [20], and the 6PGL activity was determined spectrophotometrically at 340 nm and 25 °C as described previously [20]. The assay mixture contained 0.1 M Tris/HCl (pH 7.0), 10 mM $MgCl_2$, 0.5 mM EDTA, 0.6 mM $NADP^+$, 3 units/ml 6-phosphogluconate dehydrogenase (yeast, Sigma–Aldrich) and 1 mM 6PGγL. For K_m measurements, the concentration of 6PGγL was varied from 20–3000 μM.

S-glutathionylation studies

MALDI (matrix-assisted laser-desorption ionization)–TOF (time-of-flight)-MS

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/HCl and 0.5 M NaCl (pH 7.8) for 5 min at 37 °C. Iodoacetamide (2 mM) was added to block residual cysteine residues. An analogous experiment was performed for untreated PfGluPho. The samples were digested with trypsin for 12 h at 37 °C. The tryptic peptides were analysed by MALDI–TOF-MS on an Ultraflex I TOF/TOF mass spectrometer (Bruker, Daltonics). A comparison of the results with the theoretical molecular mass 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

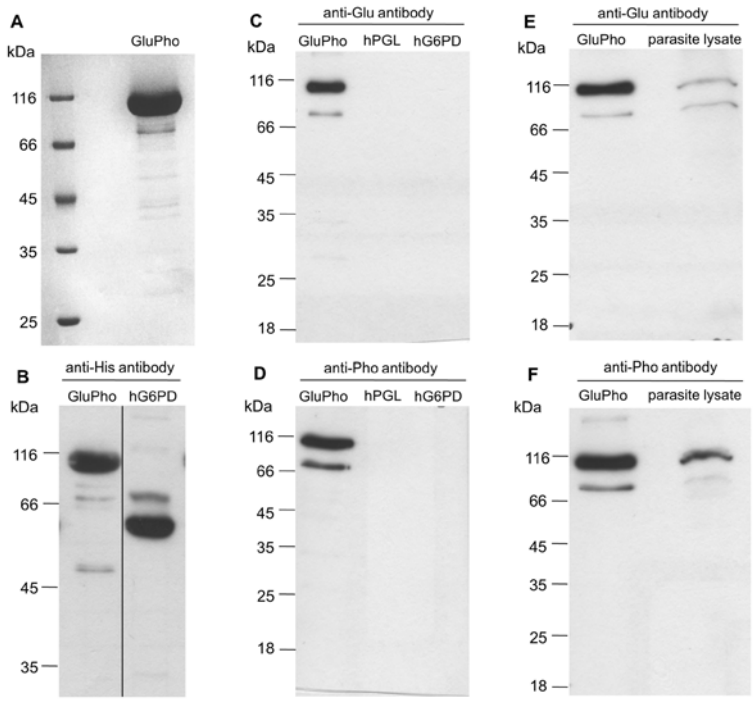

Figure 1 SDS/PAGE and Western blot analyses of PfGluPho, hG6PD and h6PGL

(**A**) SDS/PAGE (12 % gel) showing recombinant PfGluPho (6 μg). (**B**) Immunoblotting with anti-His_6 antibody showing recombinant PfGluPho and hG6PD (5 μg). Immunoblotting 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 hG6PD (5 μg). Immunoblotting with anti-Glu (**E**) and anti-Pho (**F**) antibody with recombinant PfGluPho (0.05 μg) and *P. falciparum* lysate (100–200 μg). Molecular masses are shown on the left-hand side.

0–10 mM GSSG or GSH for 5 min at 37 °C. The reversibility of the modification was studied by incubating PfGluPho with the reducing agent DTT for 30 min at 23 °C after pre-incubation with GSSG.

RESULTS

Cloning, heterologous overexpression and purification of PfGluPho

Previous publications have reported major difficulties in cloning PfGluPho, making it impossible to heterologously overexpress and characterize the enzyme [9,10]. As reported in the present paper, we were able to clone, overexpress and purify the recombinant full-length PfGluPho protein (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 G6PD-deficient PD2000 cells), combined with different helper plasmids (pRARE, pRARE II and pRIG), a variety of growth, induction and expression conditions, different purification strategies (Ni-NTA, 2′5′ ADP-Sepharose 4B and gel filtration), as well as several buffer systems. The best condition yielded 2–3 mg of pure and active PfGluPho per litre of *E. coli* culture. In addition to the full-length PfGluPho, we cloned the G6PD domain (amino acids 339–910, named $G6PD_{PfGluPho}$) separately in order to test whether it functions independently of the 6PGL part of the enzyme.

Oligomerization studies of PfGluPho

PfGluPho purified from parasite lysate has a tetrameric structure [8,9,21], whereas hG6PD exists as a monomer, dimer and tetramer depending on ionic strength, pH and the presence of its substrates [22]. According to the present study, PfGluPho exists as a tetramer with a molecular mass of 443 kDa under native conditions (Supplementary Figure S2 at http://www.BiochemJ.org/bj/436/bj4360641add.htm). 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 (anti-Glu and anti-Pho antibodies), which are highly specific for PfGluPho since they do not react with hG6PD or 6PGL (Figures 1C and 1D). In addition, we detected full-length PfGluPho in *P. falciparum* parasite lysate using the peptide antibodies (Figures 1E and 1F).

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 [amino acids 9–911, 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 post-translational modifications; however, it remains to be studied in detail by high-accuracy MS.

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

Each value is a mean ± S.D. from at least five independent determinations each including at least five measurements. nd, not determined. *Yoshida and Roth [7]; † O'Brien et al. [9]; ‡Wang et al. [27]; §Birke et al. [23]; ‖Miclet et al. [26].

	K_m (μM)	V_{max} (units/mg)	k_{cat} (s^{-1})	K_m (μM)	V_{max} (units/mg)
	PfGluPho's G6PD activity			Literature values	
G6P	19.2 ± 3.9	5.2 ± 1.6	8.6 ± 1.5	11*, 27†	-
$NADP^+$	6.5 ± 2.2	4.6 ± 0.8	8.2 ± 1.2	0.8*, 4.5†	-
2deoxyG6P	2670 ± 320	4.1 ± 0.8	7.3 ± 1.5	10000*	-
	$G6PD_{PfGluPho}$			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‡, 43§	100‡, 170§
$NADP^+$	17.5 ± 2.8	57.7 ± 15	56.9 ± 15	6.7‡, 11§	
2deoxyG6P	nd	nd	nd	1674‡	
	PfGluPho's 6PGL activity			Literature values	
6PGγL	172 ± 36	46.6 ± 10	106 ± 24	-	-
6PGδL	nd	nd	nd	-	60‖
	Human 6PGL			Literature values	
6PGγL	242 ± 48	1065 ± 97	505215 ± 3083	-	-
6PGδL	nd	nd	nd	-	3330‖

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 with hG6PD. The kinetic studies were performed in the standard G6PD assay buffer (0.1 M Tris/HCl, 10 mM $MgCl_2$ and 0.5 mM EDTA, pH 8.0) to allow a direct comparison with hG6PD from our own studies as well as from previously published data. A pH profile of the G6PD activity of PfGluPho in direct comparison with hG6PD showed that both enzymes catalysed the reaction with a maximum activity at pH 8.0 (Supplementary Figures S3A and S3B at http://www.BiochemJ.org/bj/436/bj4360641add.htm). EDTA stabilizes the dimeric form of hG6PD [23], but did not influence the activity of PfGluPho (Figure S3C).

hG6PD is stable for several months when stored at a concentration of 30 mg/ml in 50 mM Tris/HCl, 300 mM NaCl (pH 8.0) and 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/HCl, 500 mM NaCl (pH 7.8) and 50 % glycerol.

The G6PD activity of PfGluPho was directly compared with 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 cosubstrate. hG6PD has a comparatively lower affinity for G6P and $NADP^+$, but a higher specific activity (Table 1).

The kinetic data were analysed 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 2A). 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 $G6PD_{PfGluPho}$ catalyses the reaction with the same specific activity as PfGluPho, but with 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 6PGδL is highly unstable. Thus Beutler et al. [20] 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. 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 units/mg (Table 1). Despite the use of the synthetic 6PGγL, this is a range comparable with an NMR-based study where the 6PGL part of PfGluPho catalyses the hydrolysis of 6PGδL with 60 units/mg [26]. The specific activity of PfGluPho is remarkably lower when compared with h6PGL with 1065 units/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

Table 2 Dalziel parameters and ratios for the G6PD reaction of PfGluPho and hG6PD with the substrates NADP$^+$ and G6P as well as 2deoxyG6P as an alternative substrate

The results were determined from the primary plots against the reciprocal of NADP$^+$ (row 1) and G6P (row 2). The mean value is shown in row (3). Values are represented as means ± S.D.

(**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)	Φ_{NADP^+2dG6P} (μM^2*s)	$\Phi_{NADP^+2G6P}/\Phi_{NADP^+}$ (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

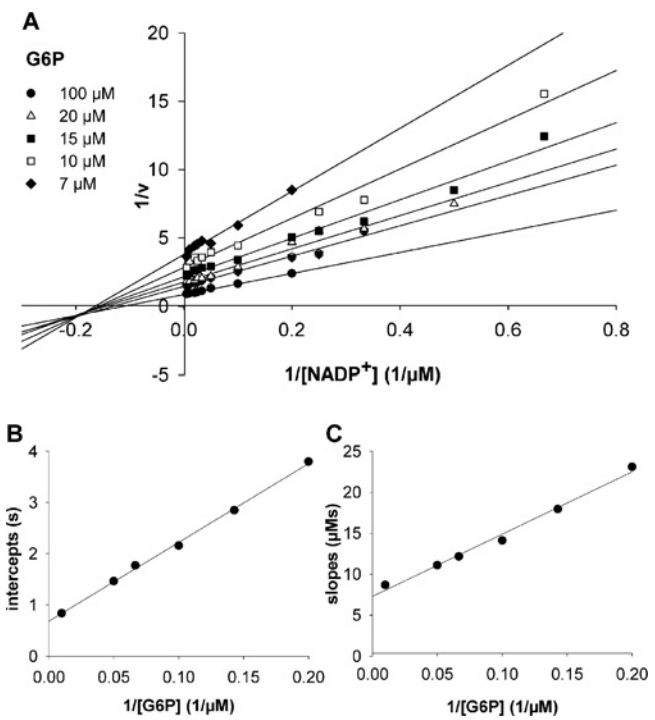

Figure 2 PfGluPho's double reciprocal plots for the reaction with NADP$^+$ and G6P as substrates

(**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.

in the G6PD reaction as shown in Supplementary Figure S4 (at http://www.BiochemJ.org/bj/436/bj4360641add.htm) 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 previously [27,28]. Glucosamine 6-phosphate is an analogue of G6P 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 3C and 3D). Towards G6P, NADPH acts as a mixed-type inhibitor (Figure 3B). Glucosamine 6-phosphate acts as a mixed-type inhibitor with respect to NADP$^+$ and shows a competitive inhibition pattern towards G6P (Figures 3E and 3F), which is sometimes a rather mixed type.

Analogous experiments with hG6PD demonstrate that NAPDH acts as a competitive inhibitor towards NADP$^+$ and non-competitively with respect to G6P, whereas glucosamine 6-phosphate showed a competitive inhibition towards G6P and a non-competitive inhibition towards NADP$^+$.

The recently identified compound C276-1187 that has 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

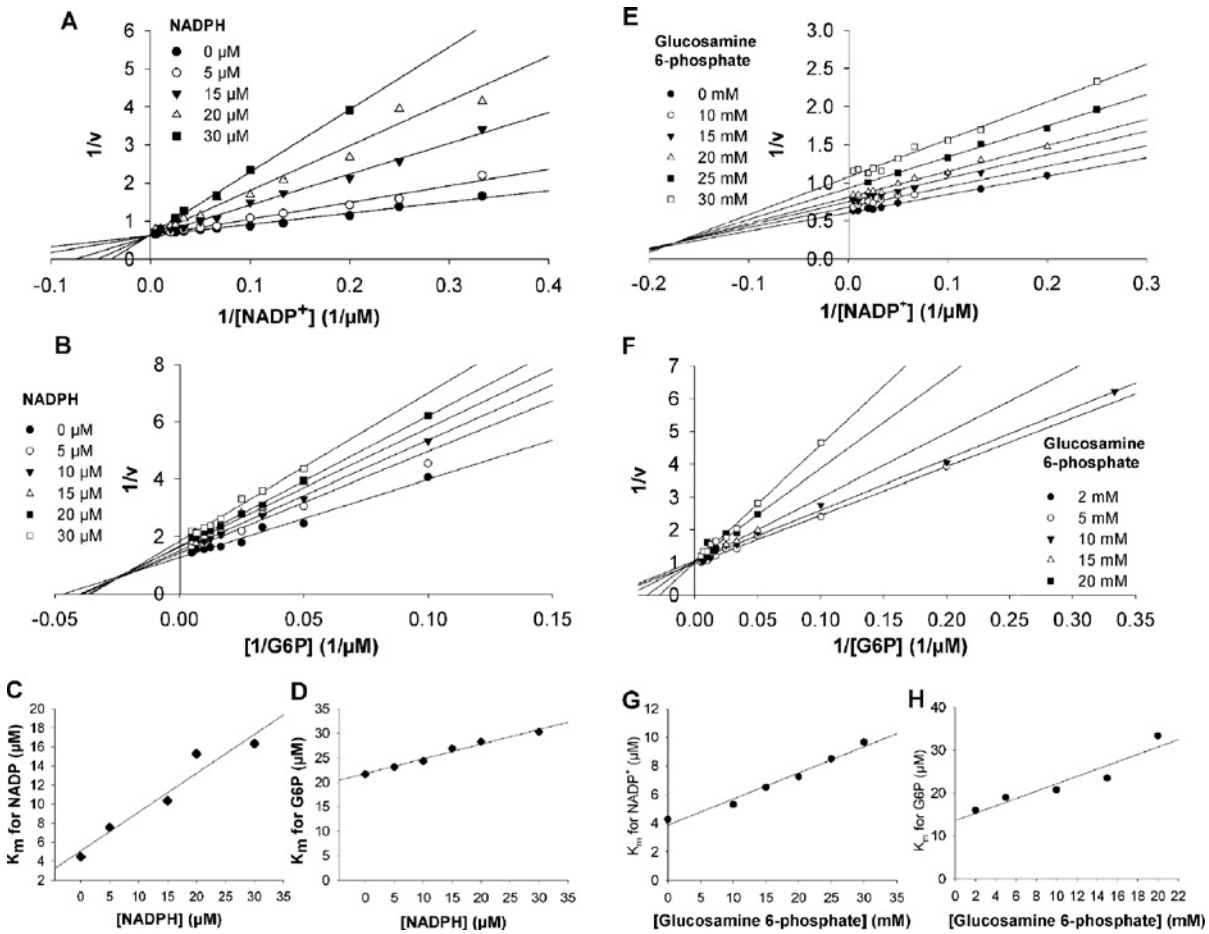

Figure 3 Inhibition studies of PfGluPho by the product inhibitor NADPH (A–D) and the dead-end inhibitor glucosamine 6-phosphate (E–H) with varied $NADP^+$ and varied G6P concentrations

Lineweaver–Burk plots were obtained at 60 μM G6P and different $NADP^+$ concentrations (**A** and **E**) or at 10 μM $NADP^+$ and different G6P concentrations (**B** and **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** and **G**) and G6P (**D** and **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.

inhibitor towards both G6P and $NADP^+$ (Figure S5 at http://www.BiochemJ.org/bj/436/bj4360641add.htm). $G6PD_{PfGluPho}$ is inhibited with an IC_{50} of 130 ± 11 μM, whereas 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 transferase [9]. This motivated us to test whether recombinant PfGluPho is S-glutathionylated using MALDI–TOF-MS 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 cysteine residues 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 4A and 4C). In contrast, incubation with GSH as well as DTT increases PfGluPho's G6PD activity by 25 %, whereas the 6PGL activity is not affected by reduction. Incubation of the S-glutathionylated PfGluPho with DTT showed that the inhibition of both activities could be partially reversed (~80 %) by DTT, showing that PfGluPho is inhibited by a reversible mechanism (Figures 4B and 4D). In contrast, hG6PD was not found to be regulated by GSSG, whereas h6PGL showed a similar inhibition by S-glutathionylation when compared with PfGluPho (Figures 4B and 4C).

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 PPP from *P. falciparum* with unique structural and functional characteristics. The present study demonstrates that PfGluPho shows remarkable differences to the corresponding human enzymes regarding the primary structure, oligomerization behaviour, substrate affinity and regulation, as well as the kinetic mechanism.

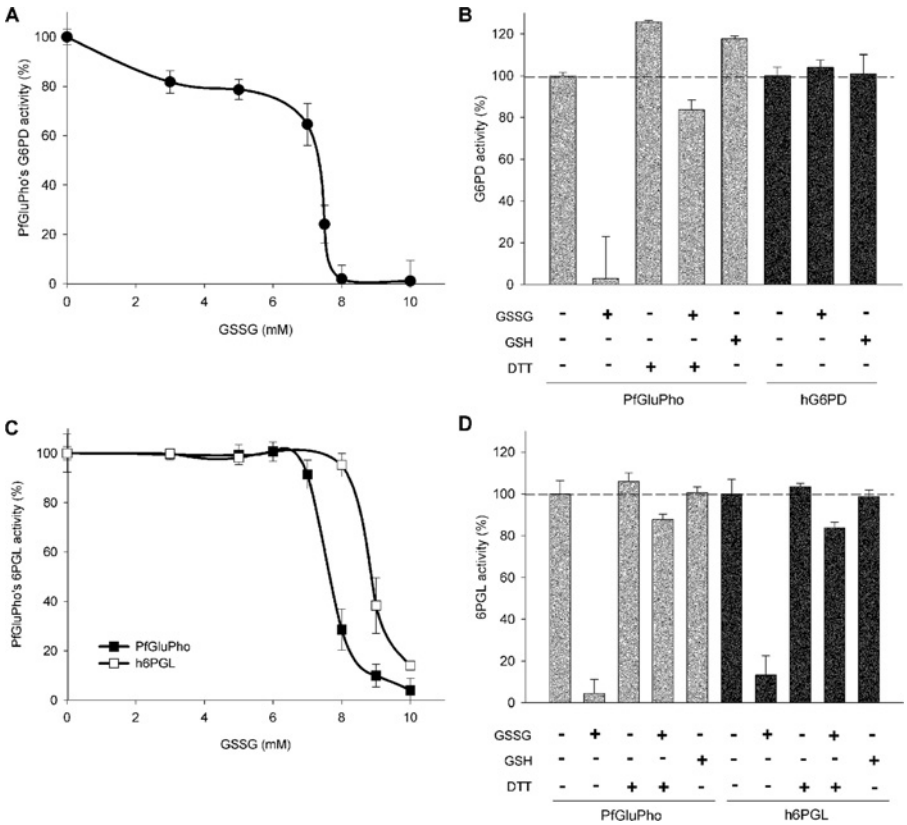

Figure 4 S-Glutathionylation of PfGluPho and hG6PD

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 °C. Activity is given as percentage of initial activity. (**B**) Activity of PfGluPho's G6PD (grey bars) and hG6PD (black bars) after incubation with glutathione. (**D**) Activity of PfGluPho's 6PGL (grey bars) and h6PGL (black bars) after incubation with glutathione. Each value is a mean ± S.D. from at least three independent determinations each including five measurements.

Previous attempts to clone the full-length PfGluPho failed, most probably 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 of protein per litre of *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 characterization, inhibitor tests and crystallization screening, and might be contaminated with G6PD from human RBCs. Cloning, overexpression and purification of full-length PfGluPho was challenging, but finally we were able to produce 2–3 mg of pure PfGluPho per litre of *E. coli* culture.

According to our studies, recombinant PfGluPho is a tetramer of 443 kDa (Supplementary 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], whereas hG6PD exists in transition between dimer and tetramer depending on ionic strength, pH and substrate concentration, with the dimer probably 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 studies and the results of the present study. 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 RBCs, 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 units/mg compared with 4.5 units/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. [27] who clearly documented a rapid equilibrium random-order mechanism for recombinant hG6PD. 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

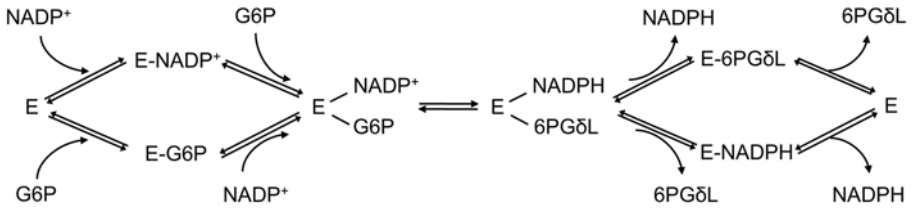

Figure 5 PfGluPho's G6PD reaction follows a rapid equilibrium random Bi Bi mechanism

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.

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. [27] previously.

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, whereas the values of Φ_0 ($1/k_{cat}$), Φ_{G6P} and $\Phi_{NADP^+\,G6P}$ 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 $\mu M \cdot s$, while Φ_{NADP^+} with 2deoxyG6P as a substrate was 0.9 $\mu M \cdot s$. 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. Owing to instability of the substrate, the enzymatic properties of 6PGL from various organisms including humans 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 hydrolysed and forms a 'dead-end' of the PPP [26]. However, according to the present study, both PfGluPho and h6PGL accept the γ-lactone as a substrate. Compared with 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-catalysed hydrolysis of 6PGδL is required to maintain a low $NADP^+$/NADPH ratio by avoiding that the uncatalysed 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]). MS 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 (Figure 4), indicating that the intracellular GSH/GSSG ratio regulates PfGluPho activity. hG6PD activity did not respond to glutathione, whereas h6PGL was affected by high glutathione concentrations (Figures 4B and 4D). A comparable glutathione-mediated inhibition has been shown for h6PGL previously [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 approximately 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.

The present 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.

AUTHOR CONTRIBUTION

Esther Jortzik, Boniface Mailu, Janina Preuss, Lars Bode, Stefan Rahlfs and Katja Becker conceived and designed the experiments. Esther Jortzik, Boniface Mailu, Janina Preuss and Marina Fischer carried out the experiments. Esther Jortzik, Stefan Rahlfs and Katja Becker analysed the data. Esther Jortzik and Katja Becker wrote the manuscript.

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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. et al. (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. et al. (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, Jr, E. F. (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. et al. (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 Signaling **10**, 1941–1988
32 Fratelli, M., Demol, H., Puype, M., Casagrande, S., Eberini, I., Salmona, M., Bonetto, V., Mengozzi, M., Duffieux, F., Miclet, E. et al. (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

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SUPPLEMENTARY ONLINE DATA

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

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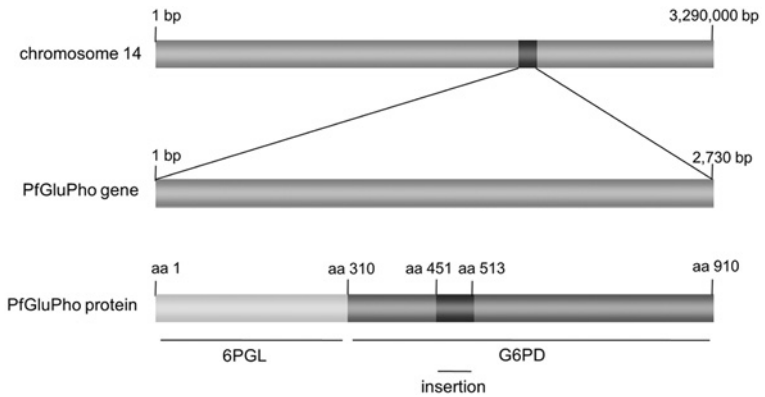

Figure S1 Schematic presentation of the PfGluPho gene and protein

aa, amino acid.

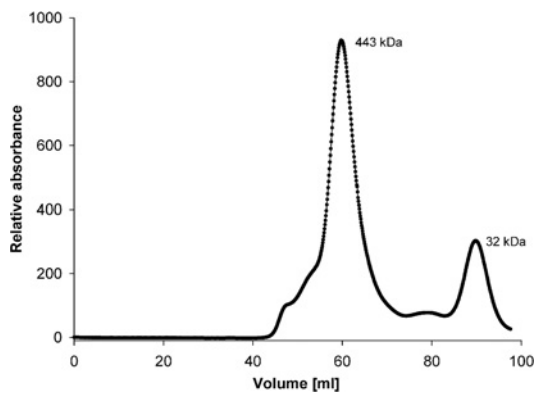

Figure S2 Oligomerization of PfGluPho

Under native conditions, PfGluPho elutes from the gel-filtration column as a tetramer with an apparent molecular mass of 443 kDa.

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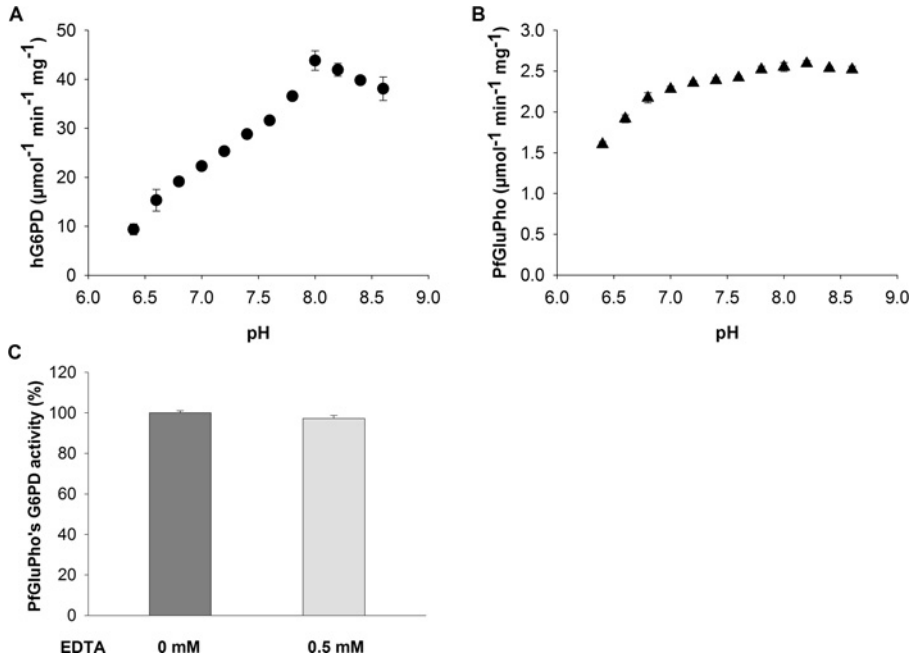

Figure S3 pH profile of hG6PD (A) and the G6PD activity of PfGluPho (B), as well as the effect of EDTA on the G6PD activity of PfGluPho (C)

(**A**) and (**B**) The G6PD activity was determined in the presence of 200 μM $NADP^+$ and 200 μM G6P in 0.1 mM Tris/HCl, 10 mM $MgCl_2$ and 0.5 mM EDTA at 25 °C. The pH was varied in steps of 0.2. (**C**) PfGluPho's G6PD activity was measured in the presence of 200 μM $NADP^+$ and 200 μM G6P in 0.1 mM Tris/HCl and 10 mM $MgCl_2$ (pH 8.0) at 25 °C either with 0 mM (dark grey bars) or 0.5 mM (light grey bars) EDTA. Each value is a mean $\pm$ S.D. from at least three independent determinations each including five measurements.

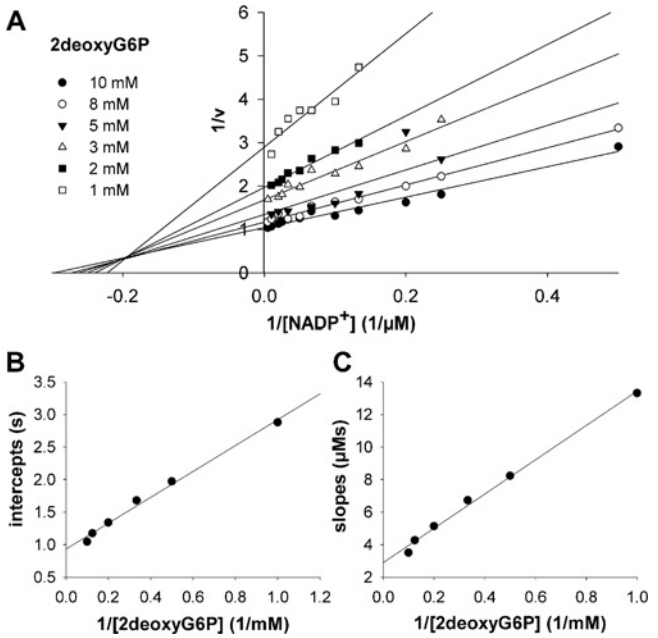

Figure S4 PfGluPho's double reciprocal plots for the reaction with NADP$^+$ and the alternative substrate 2-deoxy-glucose 6-phosphate (2deoxyG6P) as substrates

(**A**) Primary plots of 1/v against 1/[NADP$^+$] at various concentrations of 2deoxyG6P. (**B**) Secondary plots of intercepts of primary plots against 1/[2deoxyG6P]. (**C**) Secondary plots of slopes of primary plots against 1/[2deoxyG6P].

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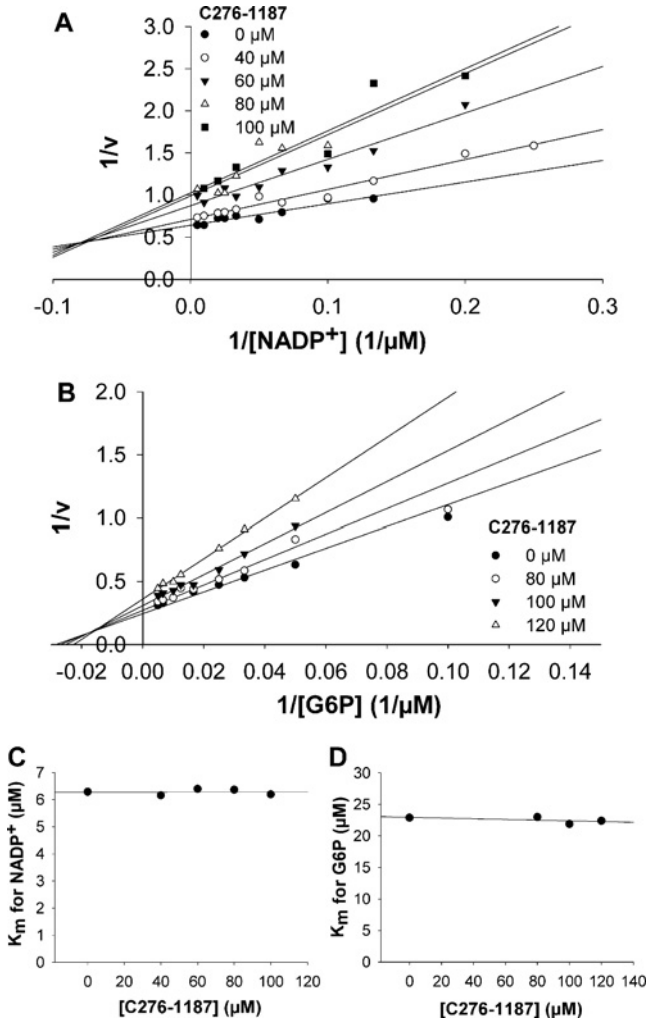

Figure S5 Inhibition of PfGluPho by compound C276-1187

Lineweaver–Burk plots were obtained at 20 μM NADP$^+$ and different G6P concentrations (**A**) or at 60 μM G6P and different NADP$^+$ concentrations (**B**) in the presence of different concentrations of C276-1187. The apparent K_m values for NADP$^+$ (**C**) and G6P (**D**) are plotted against the concentration of the compound C276-1187.