Glutathione synthetase from *Plasmodium falciparum*

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GSH is the major low-molecular-mass thiol in most organisms. The tripeptide maintains a reduced intracellular environment and protects cellular components from damaging oxidation. GSH is synthesized by the action of two ATP-dependent enzymic steps, in which γ -glutamylcysteine synthetase (γ -GCS) catalyses the ligation of glutamate and cysteine and subsequently glutathione synthetase (GS) adds glycine to the dipeptide. Recently it was shown that the synthesis of γ -glutamylcysteine is crucial for the survival of the erythrocytic stages of the malaria parasite *Plasmodium falciparum* by using the specific γ -GCS inhibitor buthionine sulfoximine. In order to investigate further the synthetic pathway of the tripeptide in the parasite, GS was cloned and expressed recombinantly. The deduced amino acid sequence of *P. falciparum* GS shares only a moderate degree of identity with other known GSs, but the residues responsible for substrate and co-factor binding are almost all conserved, with

the exception of the ones involved in γ -glutamylcysteine binding. The protein is active as a dimer, with a subunit molecular mass of 77 kDa, and the addition of reducing reagents such as dithiothreitol is essential in maintaining enzymic activity, indicating that thiol groups are important for stability and enzymic activity. The K_m^{app} values for γ -glutamyl- α -aminobutyrate, ATP and glycine were determined to be 107.1 μ M, 59.1 μ M and 5.04 mM, respectively, and the V_{max} of $5.24 \pm 0.7 \mu\text{mol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$ was in the same range as that of the mammalian enzymes. However, the negative co-operativity observed for γ -glutamylcysteine binding to the rat enzyme was not found for the parasite protein. This may be due to the alteration of several amino acids in the γ -glutamylcysteine-binding site.

Key words: glutathione, glutathione synthesis, malaria, oxidative stress.

INTRODUCTION

The tripeptide GSH is the major low-molecular-mass thiol in numerous organisms. It is considered as a thiol redox buffer responsible for maintaining the intracellular reducing environment, e.g. the thiol/disulphide ratio usually is between 10 and 100 [1]. In addition it is involved in the detoxification of reactive oxygen species, such as hydrogen peroxide, and thus in preventing oxidative damage to proteins, membranes and nucleic acids [2,3]. During these reactions GSH is oxidized to glutathione disulphide and its reduced state is maintained efficiently by the NADPH-dependent glutathione reductase. A number of reactions, such as the detoxification of xenobiotics by glutathione S-transferases, lead to the loss of GSH, which needs to be replenished by synthesis. The synthesis of GSH requires two separate enzymic reactions. The first and rate-limiting step of GSH synthesis is the ligation of glutamate and cysteine, catalysed by γ -glutamylcysteine synthetase (γ -GCS), which is followed by the reaction of glutathione synthetase (GS), responsible for the addition of glycine and the formation of GSH. It has been shown that the reduction of GS activity in mammals leads to either mild or debilitating diseases [4,5]. Total loss of GS activity was not reported, suggesting that the enzyme is essential for the survival of mammals [4]. In contrast, in *Saccharomyces cerevisiae* the presence of GS appears not to be essential for survival. Grant and co-workers [6] showed that the deletion of the GS gene does not impair the ability of the yeast cells to withstand oxidant stress, although the growth of the mutants is poor compared with

the wild type. It was presumed that γ -glutamylcysteine (γ -GC) acts as a substitute for GSH in yeast, whereas in mammalian cells this substitution appears not to be possible without causing disease.

The protozoan parasite *Plasmodium falciparum* causes malaria tropica, which leads to millions of deaths in developing countries despite the availability of a variety of drugs. However, the emergence of drug resistance makes it impossible to control this devastating disease, and there is an urgent need to identify and validate new drug targets in the parasite metabolism. It has been shown in many studies that *Plasmodium*-infected red blood cells are under enhanced oxidative stress [7–10] and it is assumed that interference with the parasite's antioxidative systems might be an approach to interrupt parasite development in the human host. Recently it was shown that GSH is essential for the survival of *P. falciparum* and that the regulatory mechanism involved in maintaining adequate GSH levels in the infected red blood cell is mainly the synthetic pathway [11–13]. The essential role of γ -GCS was shown not only for *Plasmodium* but also for another protozoan parasite, *Trypanosoma brucei*, using the specific inhibitor of this enzyme, buthionine sulfoximine [14].

Here we report on the isolation of the gene encoding GS from *P. falciparum* and the recombinant expression and characterization of the protein. Knowledge of the primary structure of this protein is essential for investigating the structure, mechanism of action and physiological function of the enzyme in *P. falciparum* and will allow comparative studies with the human host enzyme. In addition these investigations will give insights into the

Abbreviations used: γ -GC, γ -glutamylcysteine; γ -GCS, γ -glutamylcysteine synthetase; γ -GluAbu, γ -glutamyl- α -aminobutyrate; GS, glutathione synthetase; PfGS, *Plasmodium falciparum* GS; DTT, dithiothreitol; Bis-Tris propane, 1,3-bis[tris(hydroxymethyl)methylamino]propane.

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The nucleotide sequence data for *Plasmodium falciparum* glutathione synthetase have been submitted to the GenBank Nucleotide Sequence Database under the accession no. CAC59841.

regulatory mechanisms responsible for maintaining adequate GSH levels in *Plasmodium*-infected red blood cells.

EXPERIMENTAL

Materials

[α - 32 P]dATP (3000 Ci/mmol) was purchased from Amersham Buchler, and γ -glutamyl- α -aminobutyrate (γ -GluAbu) was a product from Bachem. All other reagents were obtained from Sigma, unless indicated. The expression plasmid pJC40 was a kind gift from Dr Joachim Clos, Bernhard Nocht Institute, Hamburg, Germany [15].

Parasites and nucleic acids

P. falciparum 3D7 were maintained *in vitro* according to Jensen and Trager as described previously [16]. Parasite proteins were obtained after saponin lysis of infected red blood cells [17]. Genomic DNA of *P. falciparum* was isolated according to [18].

Recombinant expression of *P. falciparum* GS (PfGS)

The coding region of PfGS was amplified with *Pfu* polymerase (Stratagene) from genomic *P. falciparum* DNA using the sense oligonucleotide 5'-GCGCCATATGGAAAGAAAGGTAGATG-3', containing an *NdeI* restriction site, and the antisense oligonucleotide 5'-GCGCCTCGAGTCAATGTTTCAGTTAA-AAAAAAG-3', containing an *XhoI* restriction site, using the following PCR programme: 6 min at 95 °C, followed by 30 cycles of 1 min at 95 °C, 1 min at 48 °C and 2 min 30 s at 68 °C, followed by 2 min at 68 °C. The resulting PCR fragment was cloned into TOPO Blunt (Invitrogen) before it was digested with *NdeI* and *XhoI* and subcloned into pJC40 that had been cut previously with both endonucleases. The sequence of the GS insert was verified by automated nucleotide sequencing using an ABI 377 automatic sequencer (Applied Biosystems). Subsequently, competent *Escherichia coli* BL21 (DE3) cells were transformed with pJC40 containing the GS coding region. A single bacterial colony was picked, grown overnight at 37 °C and diluted 1:50 in fresh Luria-Bertani medium containing 50 μ g/ml ampicillin. The bacteria were grown at 37 °C until the D_{600} value reached 0.5, after which expression of the recombinant protein was induced with 1 mM isopropyl β -D-thiogalactoside and the temperature was reduced to 25 °C. After overnight culturing, the bacteria were harvested and the pellet was resuspended in 50 mM NaH_2PO_4 , pH 8.0, 300 mM NaCl, 10 mM imidazole and 1 mM dithiothreitol (DTT; buffer A) and subsequently sonified with a Branson sonifier. This was followed by a 1 h centrifugation at 100 000 *g* (Centricon T-1065; Kontron). The 100 000 *g* supernatant was loaded on to a Ni^{2+} -nitrilotriacetate column (2 ml; Qiagen) and subsequently washed with buffer A containing 20 mM imidazole to remove non-specifically bound contaminating proteins before PfGS was eluted with a linear gradient (10 column vol.) of buffer A and buffer C (50 mM NaH_2PO_4 , pH 8.0, containing 300 mM NaCl, 300 mM imidazole and 1 mM DTT). Subsequently, the fractions containing PfGS activity were pooled and concentrated using a Centricon cartridge (Amicon) with an exclusion size of 50 kDa before the pool was applied to an FPLC Sephadex S-200 gel-sizing column (2.6 cm $\times$ 60 cm) equilibrated previously with 0.1 M Bicine/Bis-Tris propane/Mes/1 mM DTT, pH 7.1 {where Bis-Tris propane is 1,3-bis[tris(hydroxymethyl)methylamino]propane}. The purity of the protein fractions was assessed by SDS/PAGE. Protein concentration was determined using the Bradford method [19] with BSA as a standard. The Sephadex S-200 column was calibrated with a molecular-mass marker kit (Sigma)

with the range 29–2000 kDa (carbonic anhydrase, 29 kDa; BSA, 66 kDa; alcohol dehydrogenase, 150 kDa; thyroglobulin, 669 kDa; Dextran Blue, 2000 kDa) to determine the molecular size of the active enzyme.

Enzymic assays and kinetic analyses

All steady-state kinetic analyses were performed with purified recombinant GS. The enzyme activity was determined at 30 °C using a spectrophotometric assay which couples ADP production to NADH oxidation [20]. In the standard assay, γ -GluAbu was used instead of γ -GC to avoid the complication of thiol oxidation. For specific activity measurements, buffer (100 mM Bicine/Bis-Tris propane/Mes, pH 7.5/10 mM MgCl_2 /75 mM KCl) was mixed with 0.2 mM NADH, 5 units of pyruvate kinase (type III rabbit muscle), 10 units of lactic acid dehydrogenase (type II rabbit muscle), 5 mM sodium phosphoenolpyruvate and the GS substrates to a final reaction volume of 1.0 ml. The concentrations of ATP, γ -GluAbu and glycine were 5, 2 and 40 mM, respectively. The assay was initiated by addition of the enzyme (3–5 μ g) and the change in absorbance at 340 nm, was followed (Uvikon 933; Kontron). It was noted that upon addition of γ -GluAbu the pH of the assay mixture fell dramatically. Therefore great care was taken to ensure that the pH of this particular substrate was monitored before being used in the enzyme assay. For the determination of K_m values, the respective dilutions of ATP, γ -GluAbu and glycine were prepared in 100 mM Bicine/Bis-Tris propane/Mes, pH 7.1, and the pH of each measurement was confirmed before the reaction. Two of the substrates were made saturating while the third was used at various concentrations. The pH optimum was determined using 50 mM Bicine/Bis-Tris propane/Mes buffer between pH 5.5 and 10.0.

RESULTS

PfGS gene and deduced amino acid sequence

A nucleotide sequence from *P. falciparum* chromosome 5 with similarity to GS of other organisms was identified on the Sanger Centre website at <http://www.sanger.ac.uk/Projects/P-falciparum/>.

Using sequence-specific oligonucleotides in a PCR, a 1968-nucleotide fragment was amplified from both cDNA and genomic DNA encompassing the entire coding region of the putative PfGS. According to these results the GS of *P. falciparum* does not contain any introns. The deduced amino acid sequence encodes a protein of 655 amino acids with a moderate degree of overall identity to the amino acid sequences of human (18.2%), *Arabidopsis thaliana* (19.8%) and yeast (18.5%) GS proteins (Figure 1). The PfGS polypeptide is about 150–200 amino acids longer than the other three sequences shown in Figure 1, due to insertions typical for a *Plasmodium* protein. The predominant amino acids within the insertions are asparagine, glutamine and glutamic acid. Despite the low degree of identity between the *Plasmodium* and the other GS sequences, there are several regions in the C-terminal part of the protein which appear to be highly conserved and thus may have an essential function for protein structure and catalysis. Almost all substitutions in the ATP-, Mg^{2+} - and sulphate-binding sites are conservative. However, in the GSH-binding site of PfGS, Ser-151 (human GS) is substituted by an Ala, and in the γ -GC-binding site there are several non-conservative substitutions in the PfGS compared with the mammalian protein. For instance, Leu-254 in human GS is replaced by a rather bulky tyrosine residue in PfGS, and Leu-286 of human GS is exchanged for a phenylalanine in the *Plasmodium* sequence.

P. falc.	MERKVDIFYKVIEWE	VLNYFTCPNGKNEYL	SYERIKSLIKDMIAF	LNTSEYIIFTNSYKN	EYNIDFLYNPKLFSF	TLLPHRLDKKLLLELC	90
H. sap.	MATNWGSLQ	QNAAFLEQTLSSTIK	DKQQLEELARQAVDR	ALAEGVLLRTSQEPT	SSEVVSY----	APF TLFPSLVPSALLEQA	65
A. thal.	MESQKPIFDLEKL	DEEFVQKLVYDALVW	SSLHGLVVGDKSYQK	SGNVPGVG-LMHAPI	ALLPTAFPEAYWKQA		72
S. cerev.	MAHYPP	SKDQLNELIQEVNQW	AITNGLSMYPPKFEE	NPSNASVS-----PV	TIYPTPIPRKCFDEA		61
P. falc.	KYSTLLYSELFDMNV	CDLSYLLSIFENIKG	-----HDFVKKMLE	VCKKYVGDNSDSDN	SDNSNNSNSSNNSN	FRNIKDDIRCVIGRS	175
H. sap.	YAVQMDENLLVDAVS	QNAAFLEQTLSSTIK	-----QDDFTARLFD	IHKQVLK-----	-----	-EGIAQTVELGLNRS	126
A. thal.	CNVTPLFNELIDRV	LDGKFLQDLSLRTK	-----VDVFTSRLLD	IHSKMLE-----	-----	-RNKKEDIRLGLHRF	133
S. cerev.	VQIQPVFENELIARIT	QDMAQPD SYLHKTE	ALALSDSEFTGKLWS	LYLATLKS-----	-----	-QYKQKQNFRLGIFRS	129
P. falc.	DYMTNRNNDIIDDKE	KIDDENINDKDIKQI	EYNTISVAFGNLSSI	LFNGHKYILKQIYKE	YFPYIGNEEEQKEVN	DILDKKFDNNFVQGI	265
H. sap.	DYMFQRSADGS----	-----PALKQI	EINTISASFGGLASR	TPAVHRHVLVSLSKT	K-----	-EAGKILSNPSKGL	188
A. thal.	DYMLDEETN-----	-----SLIQI	EMNTISCSFPGLSRL	VSQHLQSLRSYQDQ	IG-----	IDSERVPINTSTIQF	194
S. cerev.	DYLIDKKKG-T----	-----EQIKQV	EFNTVSVSFAGLSEK	VDRLHSYLNRRANKYD	PKGFI-----	YNDQNMVISDSGYLL	195
P. falc.	ITCFKKCHDIYISEY	KPLLGSCHKVIMMSIL	HEDDFN ⁺ SDKYKTTN	ELNKNINQKALTIN	EIKLLFESKKLFLNY	KDETLLIDSLKRIKKN	355
H. sap.	ALGIKAWELYGSP-	-----NALVLLIA	QEKERNIFDQRAIEN	ELLARN-----	-----IHVIRRTFEDI	SEKGLDQDR-----	252
A. thal.	ADALAKAWLEYSNP-	-----RAVVMVIV	QPEERNMYDQHLSS	ILREKHN-----	-----IVVIRKTLAEV	EKEGVSQDE-----	259
S. cerev.	SKALAKAVESYKSQ	SSST-TSDPIVAFIV	QRNERNVFDQKVLEL	NLLEKFG-----	-----TKSVRLTFDDV	NDKLFIDDKTGKL--	270
P. falc.	EYNYPNEIFKPGKLL	IDLNDIEDILNVDCI	NINDILKNINKYERN	IFEISVLYFRALYTP	NHFNENI-WKIREMF	EFSDAIKIPSLPYQL	444
H. sap.	-----	-----	-----RLFVD	GOEIAVVYFRDGYMP	RQYSLQN-WEARLLL	ERSHAAKCPDIATQL	301
A. thal.	-----	-----	-----TLIVG	GQAVAVVYFRSGYTP	NDHPSESEWNARLLI	EESSAVKCPSIATHL	309
S. cerev.	-----	-----	-----FIRDT	EOEIAVVYVYRTGYTT	TDYTSKDWEARLFL	EKSFAIKAPDLLTQL	320
P. falc.	VGSKKIQMLLLDNDI	LKKYISLNLNKEKKS	DEQITKDMTLLKKT	ALQVDPSONISAHII	QDAINNENYLLKPKQ	REGGRNHLHGNOVKQ	534
H. sap.	AGTKKVQQLSRPGM	LEMLLPGQP-----	-EAVARLRATFAGLY	SLDVGEE---GDQAI	AEALAAPSFRVLKPKQ	REGGGNNLYGEEMVQ	381
A. thal.	TGSKKIQQLAKPGV	LERFLDNK-----	-EDIAKLKRCFAGLW	SLDDSE-----IV	KQAIKPKGLFVMPKQ	REGGGNNIYGDDVRE	384
S. cerev.	SGSKKIQQLLTDEGV	LGYISDA-----	-EKSSLLKTFVKIY	PLDDTKL---GREGK	RLALSEPSKYVLKPKQ	REGGGNNVYKENIPN	399
P. falc.	KLKLFYDHNKKQTLS	HYVLMQRLFPSTFTA	IHCRTQEMKNEICTT	NISNEQQKKKPSHLI	QFSPEKSISEFSLFH	NFIYKKNKILNEQK	624
H. sap.	ALKQLKDEER--A-	SYILMEKIEPEPFEN	CLLRPGS-----	-----	PARVVQCISELGIFF	VYVRQKTLVMNKHV	445
A. thal.	NLLRLQKEGEGNA-	AYILMQRIFFKVSNM	FLVREG-----	-----	VYHKHQAISELGVYG	AYLRSKDEVIVNEQS	449
S. cerev.	FLKGIEERHWD---	AYILMELIEPELNEN	NIILRDN-----	-----	KSYNEPIISELGIYG	CVLFNDEQVLSNEFS	462
P. falc.	GYLVRTKNYTENEGG	AICGISSLDSFFLTEH	655				
H. sap.	GHLRLTKAIEHADGG	VAAGVAVLDNPYPV	474				
A. thal.	GYLMTKIASSEDEGG	VAAGFGVLDIYILI	478				
S. cerev.	GSLLRSKFNTSNEGG	VAAGFGCLDSIILI	491				

Figure 1 Alignment of the *PfGS* amino acid sequence with the *Homo sapiens*, *A. thaliana* and *S. cerevisiae* sequences

For maximal alignment, several gaps were introduced (—). The shaded box indicates the conserved glycine-rich loop. Asterisks, arrows and crosses indicate residues responsible for ATP, γ -GC and GSH binding, respectively, in human GS that are conserved between *H. sapiens* and *P. falciparum*. *A. thal.*, *A. thaliana* (accession no. CAA74136); *H. sap.*, *H. sapiens* (accession no. AAA99146); *S. cerev.*, *S. cerevisiae* (accession no. XP_009471); *P. falc.*, *P. falciparum* (accession no. CAC59841).

The *PfGS* sequence possesses seven potential glycosylation sites at positions 150, 153, 156, 216, 498, 580 and 632. The first four are potential O-glycosylation sites (O-linked *N*-acetylglucosamine addition) and the last three are potential N-linked glycosylation sites. It needs to be further investigated whether these glycosylation sites are actually used in the erythrocytic stages of *Plasmodium*. Further, there is an abundance of cysteine residues (eleven) present in the *PfGS* when compared with the mammalian protein, which only possesses three cysteines. One of these cysteine residues (Cys-422) appears to be involved in the catalytic activity of the human enzyme [21], although structural analysis of mammalian GS revealed that this particular residue is not in contact with any of the substrates during the enzymic reaction [22]. The equivalent residue in *PfGS* is a serine. The abundance of cysteine residues in *PfGS* might have a functional implication for its catalytic activity, and the fact that the recombinant enzyme requires stabilization with DTT and EDTA supports this suggestion.

Recombinant expression and steady-state kinetic analyses of *PfGS*

As shown in Figure 2, *PfGS* was purified by Ni²⁺-nitrilotriacetate chromatography and subsequent gel filtration to apparent homogeneity. The use of the gel-sizing column revealed that the protein forms dimers with identical subunits of 77 kDa. The recombinant protein is unstable (loss of 50% of its activity within 24 h) and the addition of 1 mM DTT and 1 mM EDTA was required to stabilize it, suggesting that cysteine residues are either involved in the catalytic reaction or have structural functions that affect the stability of the protein. Despite the addition of DTT/EDTA, the protein lost its activity upon freezing, and after 4 days at 4 °C the activity was reduced by 25%. Storage with 10–25% glycerol and precipitation with ammonium sulphate did not improve the protein's stability. The optimum pH for the enzymic assay performed was determined to be 7.1. The steady-state kinetic parameters of *PfGS* were determined at this pH and are summarized in Table 1. The

Figure 2 SDS/PAGE of recombinantly expressed and purified *P/GS*

Left-hand lane, molecular-mass standards (Life Technologies); right-hand lane, 5 μg of recombinant *P/GS* was separated by SDS/PAGE (7.5% gel) and visualized subsequently with Coomassie Brilliant Blue. The apparent molecular mass of the His-tagged monomeric protein was 80 kDa.

Table 1 Comparison of molecular masses and kinetic parameters of *P/GS* with GCs from *H. sapiens* and *Rattus norvegicus*

Molecular masses were determined for three different enzyme preparations. The kinetic parameters were determined in triplicate on three different enzyme preparations. Means $\pm$ S.D. are given.

Species ...	<i>P. falciparum</i>	<i>H. sapiens</i> * [†]	<i>R. norvegicus</i> †
Molecular mass (dimer; kDa)	154 $\pm$ 8	105	105
V_{max} ($\mu\text{mol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$)	5.24 $\pm$ 0.7	6.01 $\pm$ 1.4	11.3
$k_{\text{cat}}^{\text{app}}$ (s^{-1})	6.71 $\pm$ 0.93	5.25	13.0
$K_{\text{m}}^{\text{app}} \gamma\text{-GluAbu}$ (μM)	107.1 $\pm$ 23.1	650 $\pm$ 220	42
$K_{\text{m}}^{\text{app}} \text{ATP}$ (μM)	59.1 $\pm$ 10.0	220 $\pm$ 30	37
$K_{\text{m}}^{\text{app}} \text{glycine}$ (mM)	5.04 $\pm$ 0.82	1.34 $\pm$ 0.34	0.913

* Data from [27].
† Data from [28].

specific activity of the recombinant protein was determined to be 5.24 units $\cdot$ mg⁻¹. The apparent K_{m} values for ATP, glycine and γ -GluAbu were determined to be 59.1 μM , 5.04 mM and 107.1 μM using Michaelis–Menten and double-reciprocal Lineweaver–Burk plots (Figure 3).

DISCUSSION

The tripeptide GSH is the most important low-molecular-mass thiol in an aerobic cell. It prevents oxidation of proteins and other cellular components and maintains the intracellular thiol redox status. GSH is maintained in its reduced state by glutathione reductase and despite the high efficiency of this enzymic reaction GSH is lost from the cell by, for example, detoxification of xenobiotics and subsequent excretion from the cell. In order to maintain GSH levels adequately it is synthesized by a distinct two-step reaction involving two ATP-dependent synthetases: γ -

Figure 3 Michaelis–Menten and Lineweaver–Burk double-reciprocal plots for the substrates of *P/GS*

The enzyme assay was carried out by varying one substrate concentration in the presence of saturating concentrations of the other two substrates (see the Experimental section). One unit of enzyme activity is defined as that which causes 1 μmol of ATP to be consumed per min. Top panel: the apparent K_{m} for γ -GluAbu was determined to be 107 μM . Middle panel: the apparent K_{m} for ATP was determined to be 59 μM . Bottom panel: the apparent K_{m} for glycine was determined to be 5.04 mM. U, units.

GCS and GS. We have cloned GS from *P. falciparum* and the gene encodes a polypeptide of 655 amino acids. The sequence is thus much larger than the GS sequences known in other organisms, which is attributable to several insertions in the *Plasmodium* sequence. Many *Plasmodium* proteins have insertions, but it is still not known what the function of these inserts is or whether they have a function at all. Deletion of inserts from *P. falciparum* glutathione reductase had effects on the stability and co-factor binding of the recombinant protein [23]. It is therefore likely that some of these insertions are actually responsible for structural features of the parasite's proteins. Another interpretation has been that the abundance of asparagine, glutamine and glutamic acid has to do with circumvention of the host-cell immune system [24].

Although the overall identity between the *Plasmodium* GS amino acid sequence and those of other organisms is rather low, there are regions which are highly conserved in all proteins. The structure of human GS has been solved to a resolution of 2.1 Å in a complex with ADP, GSH, a SO_4^{2-} ion and two Mg^{2+} ions [22]. This complex allowed presumptions as to which amino acids are involved in substrate and product binding during catalysis. Most of the residues implicated in catalysis are conserved in *PfGS*. The only non-conservative exchange that may have an impact on GSH binding is at position 151 in human GS, equivalent to position 213 in *PfGS*, where a serine residue is changed into an alanine residue. The main-chain amide of Ser-151 in human GS is thought to be involved in assisting to stabilize the charge on the tetrahedral adduct of the reaction. The other features, which may have an impact on the overall structure of the *Plasmodium* protein, are the inserted sequences, which might form loops between the actual binding sites of the protein. Another residue, found to be essential for enzymic activity in human GS, is Cys-422, replaced by serine in the equivalent position in *PfGS*. This residue has no implication for substrate or product binding, and it was suggested that it is involved in maintaining some important structural feature of the protein [22]. A highly conserved region in the eukaryotic as well as *E. coli* GS sequences is Lys-160 and the region between Gly-164 and Gly-167, which corresponds to Lys-517 to Lys-525 in the *P. falciparum* sequence (see Figure 1). The *E. coli* sequence is very well characterized and it is known that Lys-160 is involved in binding of the adenine ring and α -phosphate of ATP. The three glycine residues are thought to form a flexible loop which protects the γ -glutamylcysteinylphosphate intermediate in the substrate-binding site from the reaction with bulk water [25]. Using a site-directed mutagenesis approach it was shown that the residues Lys-367/Pro-368 and Gly-374 in *A. thaliana* GS are essential for activity of the enzyme and structural analyses verified this function in human GS [22,26]. Since this region of *PfGS* is highly conserved, it is very likely that the equivalent residues will also have an essential function in the *Plasmodium* protein.

PfGS was recombinantly expressed in *E. coli* BL 21 (DE3) and the purified protein had a specific activity of 5.24 units $\cdot$ mg⁻¹, which is comparable with that of the mammalian enzyme [27,28]. The protein is active as a homodimer with a subunit size of 77 kDa. The apparent K_m value determined for ATP (59 μM) was found to be similar to the K_m^{app} value of the rat enzyme, whereas the K_m^{app} for glycine (5.0 mM) was four to five times higher when compared with mammalian GS [27,28]. The K_m^{app} for γ -GluAbu (107 μM) was six times lower when compared with the human enzyme but 2.5 times higher than the respective K_m of the rat enzyme. The negative co-operative effect of γ -GluAbu observed for the rat enzyme was not found for the *Plasmodium* enzyme. Interestingly, the amino acid residues involved in γ -GC

binding are quite distinct in the parasite enzyme. In particular, the exchange of Leu-254 into Tyr-397 and Leu-284 into Phe-429 is surprising because two bulky residues (one of them even polar) are introduced into a position of the protein equivalent to a hydrophobic pocket in the human enzyme [22]. Mutations of both leucine residues in the human enzyme result in impaired substrate binding [4]. The two amino acid replacements in the plasmodial γ -GluAbu-binding site are possibly responsible for the abolition of co-operativity.

In future studies we will characterize the catalytic mechanism of *PfGS* further and also address the question of whether the protein is essential for parasite survival in the red blood cell.

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