Glutathione synthetase from *Plasmodium falciparum*

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INTRODUCTION

The tripeptide GSH is the major low-molecular-mass thiol in most organisms. It is considered as a thiol redox buffer responsible for maintaining the intracellular reducing 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} values for γ-glutamyl-x-aminobutyrate, ATP and glycine were determined to be 107.1 μM, 59.1 μM and 5.04 mM, respectively, and the *V*_{max} of 5.24 ± 0.7 μmol·min⁻¹·mg⁻¹ was in the same range as that of the mammalian enzymes. However, the negative cooperativity 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.

Abbreviations used: γ-GC, γ-glutamylcysteine; γ-GCS, γ-glutamylcysteine synthetase; γ-GluAbu, γ-glutamyl-x-aminobutyrate; GS, glutathione synthetase; PG5, *Plasmodium falciparum* GS; DTT, dithiothreitol; Bis-Tris propane, 1,3-bis[(tris(hydroxymethyl)methylamino)propane. 1 To whom correspondence should be addressed (e-mail s.muller@dundee.ac.uk).

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**

\([x-^32P]dATP\) (3000 Ci/mmol) was purchased from Amersham Buchler, and \(\gamma\)-glutamyl-\(\alpha\)-amino butyrate (\(\gamma\)-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 Jensen [18].

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

The coding region of P/GS was amplified with *Pfu* polymerase (Stratagene) from genomic *P. falciparum* DNA using the sense oligonucleotide 5'-GCCGCGCATGAGGAAAGAGGTGATAG-G-3', containing an *NdeI* restriction site, and the antisense oligonucleotide 5'-GCCGCTCGAGCTATGCTCAGTAA-AAAAGG-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 \(\mu\)g/ml ampicillin. The bacteria were grown at 37°C until the *D*~max~ value reached 0.5, after which expression of the recombinant protein was induced with 1 mM isopropyl-\(\beta\)-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 NaHPO\(_4\), pH 8.0, 300 mM NaCl, 10 mM imidazole and 1 mM diithiothreitol (DTT; buffer A) and subsequently sonicated with a Branson sonifier. This was followed by a 1 h centrifugation at 100000 g (Centricon T-1065; Kontro). The 100000 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 P/GS was eluted with a linear gradient (10 column vol.) of buffer A and buffer C (50 mM NaHPO\(_4\), pH 8.0, containing 300 mM NaCl, 300 mM imidazole and 1 mM DTT). Subsequently, the fractions containing P/GS 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-sieving 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 29–200 kDa (carbonic anhydrase, 29 kDa; BSA, 66 kDa; alcohol dehydrogenase, 150 kDa; thryoglobulin, 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, \(\gamma\)-GluAbu was used instead of \(\gamma\)-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 II 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, \(\gamma\)-GluAbu and glycine were 5, 2 and 40 mM, respectively. The assay was initiated by addition of the enzyme (3–5 \(\mu\)g) and the change in absorbance at 340 nm, was followed (Uvikon 933; Kontron). It was noted that upon addition of \(\gamma\)-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, \(\gamma\)-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**

**PGS 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 P/GS. 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 P/GS 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 P/GS, Ser-151 (human GS) is substituted with a rather bulky tyrosine residue in P/GS, and Leu-286 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 P/GS 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 P/GS, Ser-151 (human GS) is substituted with a rather bulky tyrosine residue in P/GS, and Leu-286 of human GS is exchanged for a phenylalanine in the *Plasmodium* sequence.
The \( P/GS \) 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 \( P. falciparum \). Further, there is an abundance of cysteine residues (eleven) present in the \( P/GS \) 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 \( P/GS \) is a serine. The abundance of cysteine residues in \( P/GS \) 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 \( P/GS \)

As shown in Figure 2, \( P/GS \) was purified by Ni\(^{2+}\)-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 and 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 \( P/GS \) were determined at this pH and are summarized in Table 1. The
**Figure 2** SDS/PAGE of recombinantly expressed and purified PfGS

Left-hand lane, molecular-mass standards (Life Technologies); right-hand lane, 5 μg of recombinant PfGS 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 PfGS with GCs from *H. sapiens* and *Rattus norvegicus*

<table>
<thead>
<tr>
<th>Species</th>
<th><em>P. falciparum</em></th>
<th><em>H. sapiens</em></th>
<th><em>R. norvegicus</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Molecular mass (dimer; kDa)</td>
<td>154 ± 8</td>
<td>105</td>
<td>105</td>
</tr>
<tr>
<td>$V_{\text{max}}$ (μmol·min$^{-1}$·mg$^{-1}$)</td>
<td>5.24 ± 0.7</td>
<td>6.01 ± 1.4</td>
<td>11.3</td>
</tr>
<tr>
<td>$K_{\text{cat}}$ (s$^{-1}$)</td>
<td>6.71 ± 0.93</td>
<td>5.25</td>
<td>13.0</td>
</tr>
<tr>
<td>$K_{\text{m}}^{\gamma}$-GluAbu (μM)</td>
<td>107.1 ± 23.1</td>
<td>650 ± 220</td>
<td>42</td>
</tr>
<tr>
<td>$K_{\text{m}}^{\text{ATP}}$ (μM)</td>
<td>59.1 ± 10.0</td>
<td>220 ± 30</td>
<td>37</td>
</tr>
<tr>
<td>$K_{\text{m}}^{\text{glycine}}$ (mM)</td>
<td>5.04 ± 0.82</td>
<td>1.34 ± 0.34</td>
<td>0.913</td>
</tr>
</tbody>
</table>

* Data from [27].
† Data from [28].

Specific activity of the recombinant protein was determined to be 5.24 units·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 PfGS

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 has effects on the stability and co-factor binding of the recombinant protein [23]. It is therefore likely that some of these inserts 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 *Pf*GS. 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 *Pf*GS, 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 enzymatic activity in human GS, is Cys-422, replaced by serine in the equivalent position in *Pf*GS. 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 z-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 *Pf*GS is highly conserved, it is very likely that the equivalent residues will also have an essential function in the *Plasmodium* protein.

*Pf*GS was recombinantly expressed in *E. coli* BL21 (DE3) and the purified protein had a specific activity of 5.24 units/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 $K_{app}$ value of the rat enzyme, whereas the $K_{app}$ for glycine (5.0 mM) was four to five times higher when compared with mammalian GS [27,28]. The $K_{app}$ 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 *Pf*GS further and also address the question of whether the protein is essential for parasite survival in the red blood cell.

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**REFERENCES**


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