GLUTATHIONE-*S*-TRANSFERASES FROM CHLOROQUINE-RESISTANT AND -SENSITIVE STRAINS OF *PLASMODIUM FALCIPARUM*: WHAT ARE THEIR DIFFERENCES?

P Rojpibulstit[1], S Kangsadalampai[1], T Ratanavalachai[1], J Denduangboripant[2] and P Chavalitshewinkoon-Petmitr[3]

[1]Division of Biochemistry, Faculty of Medicine, Thammasat University (Rangsit Campus), Pathum Thani; [2]Department of Biology, Faculty of Science, Chulalongkorn University, Bangkok; [3]Department of Protozoology, Faculty of Tropical Medicine, Mahidol University, Bangkok, Thailand

Abstract. Glutathione-*S*-transferases (GSTs) from chloroquine-resistant (CQR, K1) and -sensitive (CQS, T9/94) strains of *Plasmodium falciparum* were studied. The enzymes from both strains exhibited the optimal pH for enzyme catalysis, at pH 7.5, and were stable at temperatures below 60ºC. They also showed the highest specific activities toward CDNB and moderate activities to ethacrynic acid (40% of the activity to CDNB) but little or no activity for other substrates. K_m and V_{max} values for CDNB and GSH, calculated by Lineweaver-Burk plot from both CQR- and CQS-GSTs, were not statistically different ($p<0.05$). However, the GSTs activity from CQR appeared to be significantly higher than that from CQS. Therefore, we proposed that GSTs from both malarial strains are identical in their functional domain but different in level of gene expression. Furthermore, protein sequence alignment between *P. falciparum* GST and GSTs from other organisms suggested that the malarial enzyme is closely similar to other GSTs in *Sigma, Alpha, Mu* and *Pi* subclasses, probably most to the *Alpha* group. Characterization of the purified malarial GST in detail would reveal more precise classification and better understanding of its role in malarial detoxification.

INTRODUCTION

Malaria is one of the deadly tropical diseases in humans which threatens 200 to 300 million cases worldwide. Up to 2 million deaths have been reported each year (WHO, 1992). It is caused by intra-erythrocytic protozoan parasites in the genus *Plasmodium,* and *P. falciparum* is the most virulent and lethal species to humans. Although the incidence of infected cases and the mortality rate appeared to be constant in the late 1990s, the problem of antimalarial drug resistance resulting from drug overuse remains global (Malaria Foundation International). Chloroquine-resistant *P. falciparum* has been reported in Southeast Asia, South America and recently throughout Africa. Chloroquine plays a role as an antimalarial drug by interfering with the polymerization of toxic heme that releases during proteolysis of hemo-

globin (Slater, 1993). Of interest, in resistant parasites, the accumulation of chloroquine inside the food vacuole was reported to be diminished. Therefore, concentration of the chloroquine used in resistant malaria should be sufficient to function properly. Previous independent studies suggested that development of chloroquine-resistance is related to a mutation in the *pfmdr1* gene, which is homologous to a multidrug-resistant gene of mammalian tumor cells (Foote *et al*, 1989; 1990; Cowman and Karcz, 1991). One extensive investigation revealed that malarial parasites became resistant to chloroquine by expelling the drug rapidly in an unaltered form, thereby reducing levels of drug accumulated in the malarial vesicles (Krogstad *et al*, 1987). This efflux involved an ATP-driven P-glycoprotein pump, which is similar to that in mammalian multidrug-resistant tumor cell lines (Morrow and Cowan, 1988; Endicott and Ling, 1989). However, it has been reported that the mechanism of rapid chloroquine efflux has been found in both chloroquine-resistant and -sensitive *P. falciparum* (Bray *et al*, 1992, 1994). Thus, other mechanisms involving drug

Correspondence: P Rojpibulstit, Division of Biochemistry, Faculty of Medicine, Thammasat University, Pathumthani 12121, Thailand.
Tel. 66 (0) 2926-9710; Fax: 66 (0) 2926-9711
E-mail: panadda_rojpibulstit@hotmail.com

resistance have been proposed, including an increase in detoxification enzymes, of which glutathione-*S*-transferase (GST) has been studied in great detail.

GSTs (E.C. 2.5.1.18) are a family of enzymes that catalyze the formation of covalent bonding between glutathione and xenobiotic compounds, including drugs, herbicides, and insecticides (Mannerwick, 1985; Hayes and Wolf, 1988; Hayes and Pulford, 1995), as well as endogenous substrates, such as toxic products from tissue damage (Jakoby, 1978; Awasthi *et al*, 1983; Pickett and Lu, 1989; Rushmore and Pickett, 1993; Armstrong, 1994). GSTs have also been studied in a number of infectious microorganisms and recognized as one of the promising enzymes for potential drug targets (Vuilleumire, 1997; Vuilleumier and Pagni, 2002).

In malarial parasites, markedly increased GST activities in chloroquine-resistant *Plasmodium* species have been reported (Dubois *et al*, 1995; Platel *et al*, 1999; Srivastava *et al,* 1999). It was noted that elevation of the enzyme activities in the chloroquine- resistant strain is proportional to the degree of drug pressure (Srivastava *et al,* 1999). Recently, Harwaldt *et al* (2002) reported no difference in GST activities between other chloroquine-sensitive and resistant-strains grown without drug pressure. This finding reinforces the possibility that the rise in enzyme activities would depend not only on strain but also on presence of drug.

However, it is still not clear that elevated GST activity in chloroquine-resistant malaria is the result of structural or transcriptional modification of the enzyme. Therefore, it was the aim of this study to determine the K_m, V_{max} and other characteristics of the GSTs from the chloroquine-resistant strain and to compare them with those from the sensitive strain. It was anticipated that the result of this study may provide better understanding of the way GST activity is enhanced in chloroquine-resistant *P. falciparum*.

MATERIALS AND METHODS

Malarial parasites (chloroquine-sensitive strain; T9/94 and -resistant strain; K1) were synchronized and cultured for 3 days without changing the medium supplemented with 15% human plasma (Chavalitshewinkoon and Wilairat, 1991). The infected erythrocytes were washed with cold sodium phosphate isotonic buffer, pH 7.5, and lysed with 0.15% (v/v) saponin at 37ºC, for 10 minutes. After centrifugation for 10 minutes at 2,000g, the parasite pellet was washed twice with the same isotonic buffer and kept at -80ºC until used. The purity of the parasite preparations was verified by determining RBC acetylcholinesterase activity using the method of Ellman *et al* (1961).

Unless otherwise stated, all procedures in the cell extraction scheme were performed at 4ºC. One volume of parasite pellet (approximately 10^{11} parasites) was resuspended in two volumes of cold extraction buffer [0.1 M $NaPO_4$ buffer, pH 6.5, 1 mM EDTA, 0.01% NP-40 and 1 mM phenylmethylsulfonylfluoride (PMSF)]. The parasite suspension was then homogenized by Teflon pestle tissue homogenizer (Thomas Scientific, USA). The homogenate was centrifuged at 100,446*g* for 45 minutes at 4ºC in a refrigerated supra-speed centrifuge (Sorvall, RC-28S, Dupont, USA) and the supernatant was removed for determination of GST activity using the modified method of Habig *et al* (1974), as described below. The amount of total protein in the crude extract was quantitated using bicinchonic acid reagent, according to the method of Smith *et al* (1985), with bovine serum albumin as a standard protein.

Enzyme activity was assayed with GSH and 1-chloro-2,4-dinitrobenzene (CDNB) as substrates, using the modified method of Habig *et al* (1974). In brief, the enzyme activity was measured by determining an increase in color production at 340 nm of S-(2,4-dinitrophenyl) glutathione, a conjugated form of GSH and CDNB. The reaction condition was carried out at 37ºC; 0.1 M $NaPO_4$ buffer, pH 7.5 with 1 mM EDTA, 7.5 mM GSH, 1 mM CDNB (dissolved in ethanol). Negative control was performed by omitting the cell lysate. Glutathione-*S*-transferase from bovine liver (Sigma Chemical, St Louis, USA) was used as positive control. The reaction was initiated by addition of the enzyme. An increased absorbance at 340 nm was followed for 1 minute in a Shimadzu UV1201 equipped with a thermoelectrically temperature-controlled cell-holder

(Shimadzu TCC-240A). One unit of enzyme activity was defined as the amount of GST required to produce 1 micromole of conjugated product per minute, using an extinction coefficient of 9.6 cm^{-1} mM^{-1}. Specific activity was expressed as units per mg of total protein. Measurement was repeated 10 times for each strain.

The determination of K_m and V_{max} of the enzyme were performed by varying the concentration of GSH (0.5, 1.0, 2.5, 5.0, 7.5 and 10 mM) in the reaction with fixed 1.0 mM CDNB and, on the other hand, the concentration of CDNB was varied (0.2, 0.4, 0.5, 0.8, 1.0 and 1.5 mM) with fixed 7.5 mM GSH. The kinetic parameters, K_m and V_{max}, were determined by Lineweaver-Burk plot based on non-linear regression analysis using GraphPad Prism version 3.02 (free trial version from http://www.graphpad.com, GraphPad Software, CA, USA).

To elucidate the substrate specificity of the malarial GST, enzyme activities were determined for a number of substrates: 1,2-dichloro-4-nitrobenzene (DCNB), *p*-nitrobenzyl chloride, *p*-nitrophenethyl bromide, trans-4-phenyl-3-buten-2-one and ethacrynic acid, by the method of Habig *et al* (1974).

To find out to which subclass *Plasmodium* GST belongs in the GST family, its amino acid sequence (AAK 00582) was used as a query to search similar sequences from the protein sequence database with BLASTP (Altschul *et al,* 1990) service, National Center for Biotechnology Information (http://www.ncbi.nih.gov). GST sequences found with significant alignment score were then aligned with that of *Plasmodium* GST using the ClustalX version 1.83 program (Thompson *et al,* 1997). Neighbor-joining (NJ) trees, both unrooted- and midpoint-rooted trees, were produced from the aligned sequence matrix with the program PAUP* version 4.0b10 for Macintosh (Swofford, 2002), using BioNJ method, and ties (if encountered) were broken randomly. Bootstrap statistic supporting values from 1,000 replicate analyses for each tree branch were also calculated with PAUP*.

RESULTS

The GST activities from chloroquine-resistant (CQR) and chloroquine-sensitive (CQS) *P. falciparum* were assessed at various temperatures and pH. The results, in Fig 1, suggested that GST from either CQR or CQS strains is stable at temperatures below 60ºC and prefers to catalyze a conjugation reaction *in vitro* (and/or *in vivo*) at 37ºC, pH 7.5. At this optimal condition, the specific activities of CQR and CQS enzymes were 1.92 ± 0.13 and 0.95 ± 0.05 U/mg total protein, respectively (significant difference, $p < 0.05$).

Table 1
Specific activities of *Plasmodium falciparum* GST with various substrates.

Substrate[a]	Specific activity (µmole/min/mg protein)	
	CQR	CQS
1-chloro-2,4-dinitrobenzene (CDNB)	1.92 ±0.12 (100%)[b]	0.95 ±0.05 (100%)[b]
1,2-dichloro-4-nitrobenzene (DCNB)	0.03±0.01 (1.35%)[b]	ND[c]
p-nitrobenzyl chloride	0.26±0.01 (13.55%)[b]	ND[c]
p-nitrophenethyl bromide	ND[c]	ND[c]
Trans-4-phenyl-3-buten-2-one	ND[c]	ND[c]
Ethacrynic acid	0.78±0.08 (40.96%)[b]	0.39±0.02 (40.67%)[b]

[a]Except the reaction with CDNB, which used 7.5 mM GSH, most experiments used 5 mM GSH.
[b]Number in parenthesis indicates relative activity compared with CDNB.
[c]ND : Not detectable is activity too low to be measured by this method.
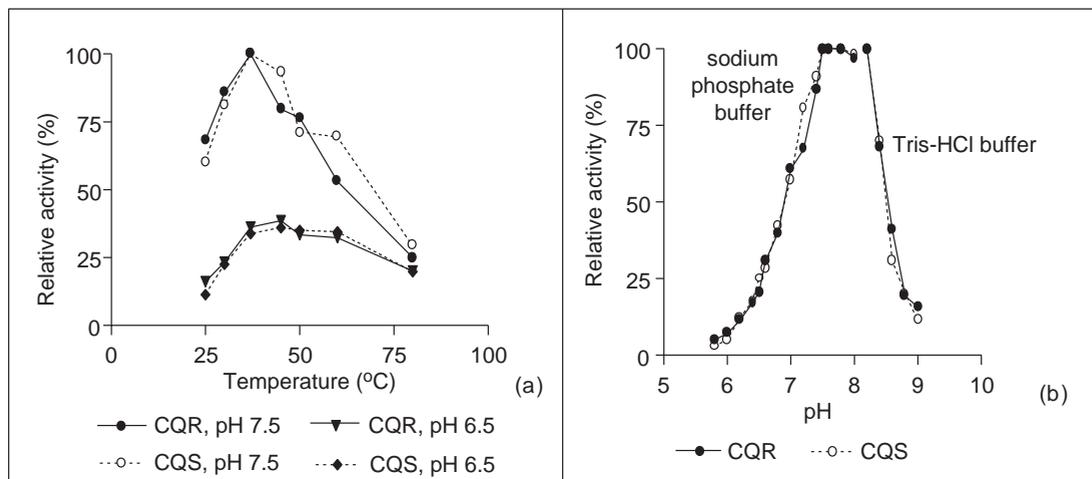
Fig 1–Temperature and pH profiles of chloroquine-resistant and -sensitive *P. falciparum* GSTs.

    a= temperature profile at pH 6.5 and 7.5. The maximum activity (at 37ºC) was taken as 100%. The relative activity was presented as a mean of at least three separate assays.

    b= pH profile. The buffers used were 0.1 M sodium phosphate buffer with 1 mM EDTA pH 5.8-8.0 and Tris–HCl buffer pH 8.2-9.0. The maximum activity (sodium phosphate buffer pH 7.5) was taken as 100%.

Apart from the GST activity, enzyme kinetics were studied to differentiate the CQR GST from the CQS enzyme. The initial velocities (V_0) of the enzymes was plotted against GSH and CDNB concentrations (Fig 2). K_m and V_{max} of the GSTs from both strains (calculated from double reciprocal plot in Fig 2) are shown in Fig 3. In addition, malarial GSTs exhibit the highest activity to CDNB and moderate activity to ethacrynic acid (40% of activity to CDNB) but little or no activity for other substrates (Table 1). These data agree with that of the recombinant PfGST. Malarial GST, therefore, could be classified into one of *Alpha, Mu* or *Pi* subclasses. This suggestion is very well supported with the BLASTP searching result that only GST sequences of *Sigma, Alpha, Mu* and *Pi* subclasses, not *Delta, Theta, Zeta* or *Omega* was found significantly similar to *P. falciparum* GST (with E value from 9e-19 to 3e-05, and protein identities from 31% to 24%) (Fig 4).

DISCUSSION

In our study, glutathione-*S*-transferases (GSTs) from chloroquine-resistant (CQR, K1) and –sensitive (CQS, T9/94) strains of *Plasmodium falciparum* have been investigated. GST activities obtained in this study (1.92 U/mg protein at pH 7.5 for chloroquine-resistant strain) were 100 times higher than previously reported (0.017 U/mg at pH 6.5 for the same strain) by Harwaldt *et al* (2002). This is likely to result from elevated pH, which enhances a spontaneous reaction between GSH and CDNB (Harwaldt *et al*, 2002). Although this argument could be taken into account, the GST activity in our study even at the same pH 6.5 (0.94 U/mg protein), is still 50 times higher than that reported by Harwaldt *et al* (2002). One possible explanation is that the isolated parasite suspensions in our study were not, or very weakly, contaminated by RBC, as undetectable RBC acetylcholinesterase was observed (data not shown). Thus, any substances from the contaminated RBC that could interfere with the measurement of enzyme activity, particularly contaminated RBC protein, were infinitesimal, resulting in the high GST activity.

Of particular note is the finding that the kinetic parameters of CQR and CQS enzymes were not only similar, but also not different from recombinant *Pf*GST (Harwaldt *et al*, 2002). This finding, as well as temperature and pH profiles, led us to conclude that the GSTs from both strains
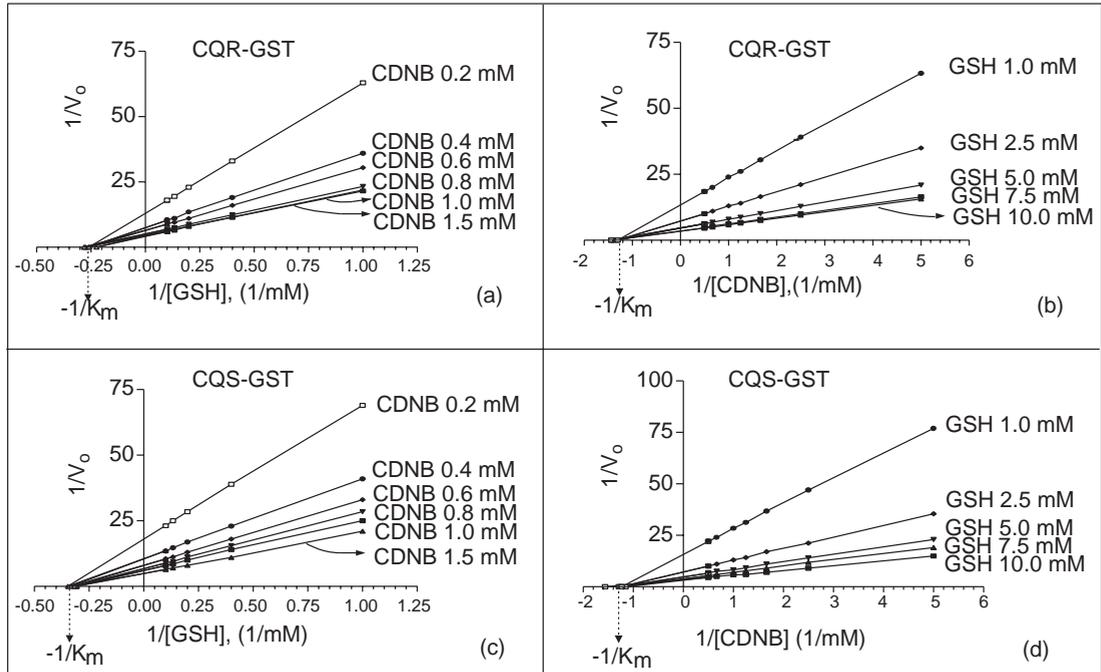
Fig 2–Double-reciprocal plots of *P. falciparum* GSTs at various concentration of GSH and CDNB.
a = CQR-GST activity at various GSH concentration ; b = CQR-GST activity at various CDNB concentration; c = CQS-GST activity at various GSH concentration; d = CQS-GST activity at various CDNB concentration.
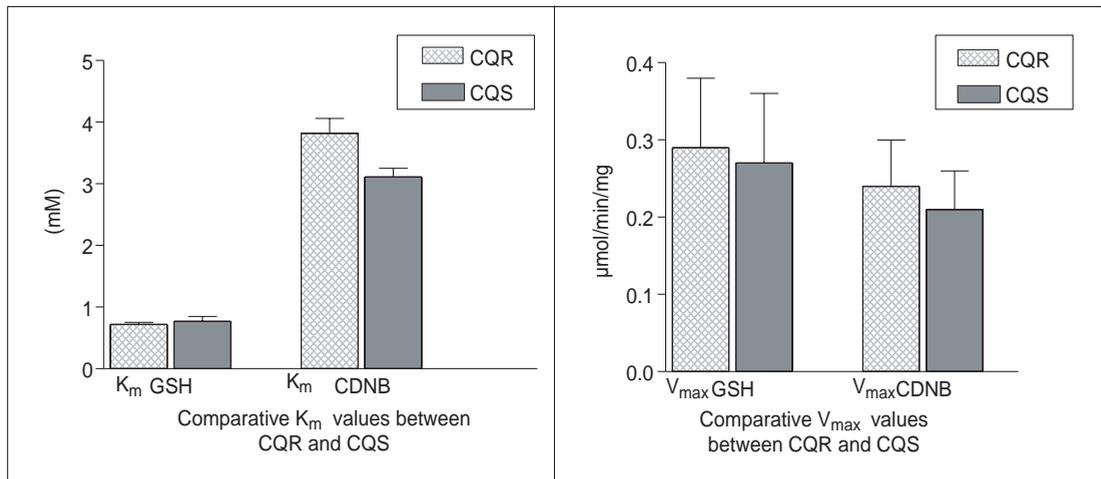
Fig 3–K_m (left) and V_{max} (right) of the GSTs from chloroquine-resistant and -sensitive *P. falciparum*.

could be functional-domain-identical, and the increase in GST activity in resistant *P. falciparum* could be the result of modified gene expression. This conclusion comes from the fact that, if there are any structural changes in the CQR-GST, different K_m should be present. Further study of the

CQR-GST mRNA by northern blot analysis would reveal more insights into the enzyme expression step.

Alternatively, based on the unrooted neighbor-joining tree representing sequence relationships of *P. falciparum* GST and other known GSTs
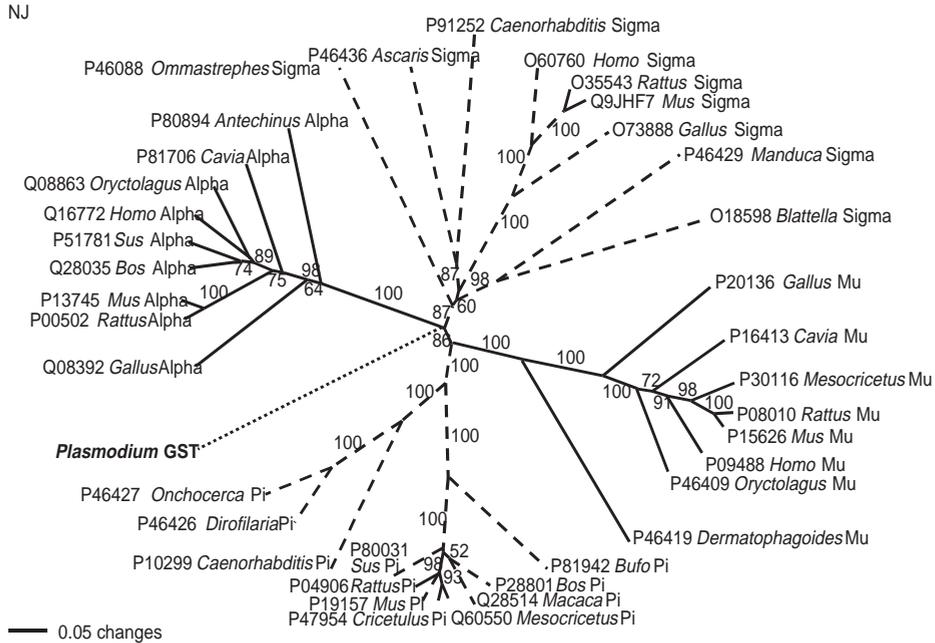
Fig 4–Unrooted neighbor-joining tree of the amino acid sequence matrix of 38 GSTs (238 amino acid characters) including *P. falciparum* GST (AAK 00582). Bootstrap supporting values were indicated along each internal branch. Numbers in front of taxon names are the protein-database accession numbers.
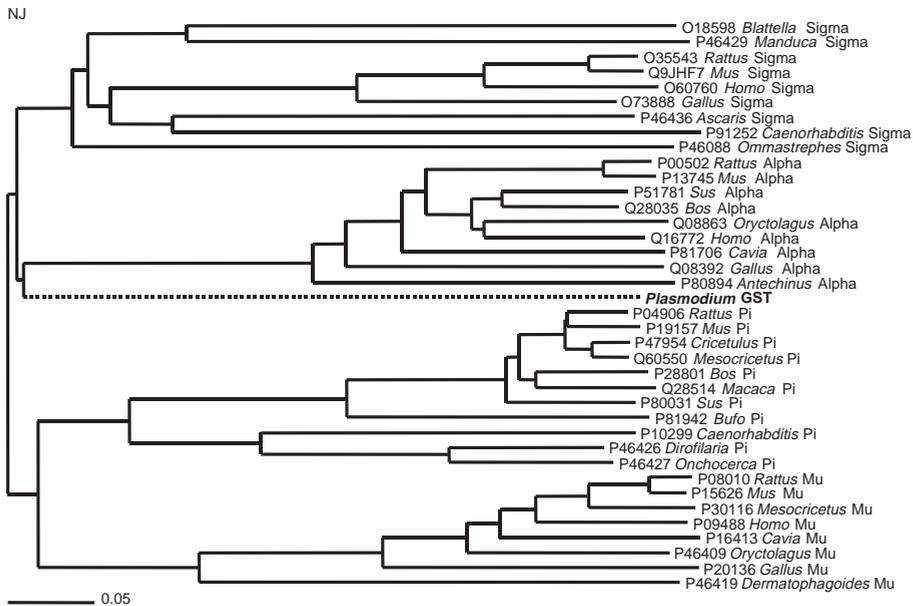
Fig 5–Genetic relationship of *P. falciparum* GST (AAK 00582) and other known GSTs revealed by midpoint-rooted NJ tree of the same matrix, as in Fig 4. The rooting point was suggested by PAUP* to be the internal branch between all four GST subclasses.

retrieved from BLASTP (Fig 4), the malarial enzyme was not placed specifically inside any particular GST subclass. Its position was nevertheless closer to the *Alpha* and *Sigma* group than the other two. Using midpoint-rooting technique to root the NJ tree (Fig 5), it is suggested that the malarial GST should belong to the *Alpha* group of the GST superfamily. These data, however,

contrast with allocation of the enzyme into the *Mu*-subclass as suggested by Harwaldt *et al* (2002). Hence, classification of the malarial GSTs is still a subject of controversy. Further characterization of purified native or recombinant enzyme by determination of isoelectric point, crystal structure, and other substrates and inhibitors, would reveal more precise classification of malarial GST.

In conclusion, although the role of GSTs in drug detoxification processes in *P. falciparum* is still not clear, the information in the present study and some previous reports (Dubois *et al*, 1995; Platel *et al*, 1999; Srivasatava *et al*, 1999; Harwaldt *et al,* 2002; Liebau *et al*, 2002) strongly support the concept that the chloroquine-resistant mechanism in *P. falciparum* involves an increase in glutathione-*S*-transferase enzyme. Understanding the role of *P. falciparum* GST in chloroquine resistance is important, especially for a drug target approach to eradicating malarial parasites. As recombinant GSTs have recently been constructed (Harwaldt *et al,* 2002; Liebau *et al*, 2002), site-directed mutagenesis could be used to create mutant enzymes. Characterization of these variant GSTs would reveal some important information on the protein structure and function relationships of malarial GSTs.

ACKNOWLEDGEMENTS

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