Investigations into aspects of central metabolism in the human malaria parasite *Plasmodium falciparum*

A thesis submitted to the University of Manchester for the degree of

Doctor of Philosophy (PhD)

in the Faculty of Life Sciences

2011

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Abstract

The University of Manchester
Martin Read
Doctor of Philosophy
Investigations into aspects of central metabolism in the human malaria parasite *Plasmodium falciparum*. 2011.

This thesis combines four published research papers and a book chapter investigating aspects of central metabolism in the human malaria parasite *Plasmodium falciparum*. The publications are preceded by a statement which explores features of the research not fully described in the published texts, incorporates a review of the development over time and present state of relevant scientific knowledge and discusses the place of the individual papers and book chapter within malaria research. An assessment of the impact of each publication on its field of study is also included. A general discussion of the combination of papers as representative of the progress of research into the metabolism of malaria parasites concludes the statement section.

The first publication is a chapter from a book, which describes detailed methods for the *in vitro* cultivation of *P. falciparum*. Such methodology, both robust and reliable, is a prerequisite for any investigation of parasite metabolism. The following publications are all primary research papers.

The second publication describes the isolation and characterisation of the gene encoding the glycolytic pathway enzyme enolase from *P. falciparum*. The inferred amino acid sequence included peptide insertions found only in the enolases of higher plants and other photosynthetic organisms. This raised implications concerning the deep evolutionary history of the malaria parasite and related species.

The third is concerned with the elucidation of the molecular basis of resistance to the antimalarial drug sulfadoxine. Resistance was found to result from point mutations within the dihydropteroate synthetase domain of the bifunctional protein hydroxymethylpterin pyrophosphokinase-dihydropteroate synthetase, an enzyme of the parasite folate pathway. Additionally, it was discovered that the presence of exogenous folate has an antagonistic effect on sulfadoxine in some parasites of a defined genotype. This highlighted the importance of folate salvage in parasite metabolism.

Fourth is a paper representing the discovery of a novel metabolism in both *P. falciparum* and the related apicomplexan parasite *Toxoplasma gondii*. The use of parasite genes in rescuing an *Escherichia coli* tyrosine auxotroph resulted in a proof of function of the products of these genes as pterin-4a-carbinolaminedehydratases. Pterin recycling, hitherto undetected in apicomplexans, was therefore added to the known metabolic processes of these organisms.

The final paper describes an investigation into the subcellular distribution of the folate pathway enzyme serine hydroxymethyltransferase (SHMT) within *P. falciparum* erythrocytic stage parasites. The use of confocal laser scanning microscopy and immunofluorescent techniques showed that SHMTc, the sole enzymatically active parasite SHMT protein, was found in the cytoplasm but also showed a stage-specific localisation to both the mitochondrion and apicoplast organelles. The otherwise enigmatic, enzymatically inert, SHMTm paralogue revealed a possible function, when in complex, in allowing targeted localisation of SHMTc to the mitochondrion. The spatial distribution of SHMTm also suggested a possible role in the morphogenesis of elongating apicoplasts during schizogony.

University of Manchester

PhD by published work Candidate Declaration

Martin Read
Faculty of Life Sciences
Investigations into aspects of central metabolism in the human malaria parasite *Plasmodium falciparum*

i. Co-author contributions:

Chapter 2: **Read, M. and Hyde, J.E.** (1993) Simple *in vitro* cultivation of the malaria parasite *Plasmodium falciparum* (erythrocytic stages) suitable for large-scale preparations, in *Protocols in Molecular Parasitology*, J. E. Hyde, Editor. Humana Press, Totowa, New Jersey. 43-55.

All practical methods described were used, adapted or developed by Martin Read. Martin Read composed the body of the chapter; John E. Hyde composed the introduction.

Chapter 3: **Read, M., Hicks, K.E., Sims, P.F.G. and Hyde, J.E.** (1994) Molecular characterization of the enolase gene from the human malaria parasite *Plasmodium falciparum* - evidence for ancestry within a photosynthetic lineage*. Eur. J. Biochem.* **220**, 513-520.

All the experimental work was undertaken by Martin Read. The CHEF gel chromosomal separation was carried out in collaboration with Karen E. Hicks. John E. Hyde and Paul F.G. Sims supervised the study.

Chapter 4: **Wang, P., Read, M., Sims, P.F.G. and Hyde, J.E.** (1997) Sulfadoxine resistance in the human malaria parasite *Plasmodium falciparum* is determined by mutations in dihydropteroate synthetase and an additional factor associated with folate utilization*. Mol. Microbiol.* **23**, 979-986.

The experimental work was intentionally split in order to eliminate any possibility of the subconscious influence of knowledge of previously characterised isolate or progeny genotypes on the assays. All preparation of parasites for assay was undertaken by Martin Read and the actual drug assay, on parasites given ciphered names, was undertaken by Ping Wang. Martin Read also developed the method for 96-well plate parasite culture and was instrumental in the adoption of a vacuum-manifold and paper disc method for harvesting labelled parasites for scintillation counting. John E. Hyde and Paul F.G. Sims supervised the study.

PhD by published work Candidate Declaration (continued)

Co-author contributions:

Chapter 5: **Wang Q, Hauser V, Read M, Wang P, Hanson AD, Sims P.F.G. and Hyde, J.E.** (2006) Functional identification of orthologous genes encoding pterin recycling activity in *Plasmodium falciparum* and *Toxoplasma gondii. Mol. Biochem. Parasitol*. **146**, 109-112.

This paper was initially a 3rd year undergraduate research project undertaken by Vicia Hauser. Martin Read was the bench supervisor for the project, and therefore was responsible for detailed experimental design, instruction of the student in all the methods used, trouble-shooting problems and general supervision. Some procedures were repeated to produce figures and data more suitable for publication by Qi Wang, and Ping Wang undertook some confirmatory DNA sequencing of constructs. Andrew Hanson collaborated with the group in an advisory capacity and John E. Hyde and Paul F.G. Sims supervised the study.

Chapter 6: **Read, M., Müller, I. B., Mitchell, S. L., Sims, P. F. G., Hyde, J. E.** (2010). Dynamic subcellular localization of isoforms of the folate pathway enzyme serine hydroxymethyltransferase (SHMT) through the erythrocytic cycle of Plasmodium falciparum. *Malaria Journal*, **9** (351), 20.

All the experimental work was undertaken by Martin Read, excepting the parasite transformation which was undertaken by Ingrid B. Müller. Two micrographs used in the paper were from preparations produced by Martin Read and Sarah L. Mitchell when the latter was being instructed in the immunofluorescence method. John E. Hyde and Paul F.G. Sims supervised the study.

ii. All of the work presented herein was carried out whilst the author was a member of staff of The University of Manchester Institute of Science and Technology or The University of Manchester.

iii. I hereby declare that no portion of the work presented in this thesis has been submitted in support of an application for any other degree or qualification of this or any other university or of any professional or learned body.

I confirm that this is a true statement and that, subject to any comments above, the submission is my own original work.

Signed: ..... Date: .....

Copyright statement

Statement

(Part i)

Candidate's degrees, other qualifications and research experience

Degrees

University of Hull, 1982.

Degree Subject: Special Zoology. Awarded **BSc** Honours, Second Class (Division II). Ancillary subjects: Physiology, Geology.

Manchester University Institute of Science and Technology (UMIST), 1995.

MSc by Research and Thesis (equivalent to an MPhil). Degree awarded for a thesis entitled "Tubulin in the Erythrocytic Stages of *Plasmodium falciparum.*"

Other qualifications

Institute of Biology (now the Society of Biology) Chartered Biologist (CBiol) 1990.

Research experience

September 1986 – September 1989: employed as a Research Technician (initially Grade 3 then Grade 5), Department of Biochemistry and Applied Molecular Biology, UMIST. September 1989 to May 2000: Research Assistant, Department of Biochemistry and Applied Molecular Biology, UMIST (subsequently known as the Department of Biomolecular Sciences, UMIST). May 2000 to 2004: Research Associate, Department of Biomolecular Sciences UMIST. Following the merger of the universities (2004 to the present): Research Associate, Faculty of Life Sciences, The University of Manchester.

Statement

(Part ii)

List of publications submitted

i. Chapter 2: **Read, M. and Hyde, J.E.** (1993) Simple *in vitro* cultivation of the malaria parasite *Plasmodium falciparum* (erythrocytic stages) suitable for large-scale preparations, in *Protocols in Molecular Parasitology*, J. E. Hyde, Editor. Humana Press, Totowa, New Jersey. 43-55.

ii. Chapter 3: **Read, M., Hicks, K.E., Sims, P.F.G. and Hyde, J.E.** (1994) Molecular characterization of the enolase gene from the human malaria parasite *Plasmodium falciparum* - evidence for ancestry within a photosynthetic lineage. *Eur. J. Biochem.* **220**, 513-520.

iii. Chapter 4: **Wang, P., Read, M., Sims, P.F.G. and Hyde, J.E.** (1997) Sulfadoxine resistance in the human malaria parasite *Plasmodium falciparum* is determined by mutations in dihydropteroate synthetase and an additional factor associated with folate utilization. *Mol. Microbiol.* **23**, 979-986.

iv. Chapter 5: **Wang, Q., Hauser, V., Read, M., Wang, P., Hanson, A.D., Sims, P.F.G. and Hyde, J.E.** (2006) Functional identification of orthologous genes encoding pterin recycling activity in *Plasmodium falciparum* and *Toxoplasma gondii. Mol. Biochem. Parasitol*. **146**, 109-112.

v. Chapter 6: **Read, M., Müller, I.B., Mitchell, S.L., Sims, P.F.G., Hyde, J.E.** (2010). Dynamic subcellular localization of isoforms of the folate pathway enzyme serine hydroxymethyltransferase (SHMT) through the erythrocytic cycle of Plasmodium falciparum. *Malaria Journal*, **9** (351), 20.

Chapter 1

Statement

(Part iii)

Comprising:

Introduction.

An overview of the work presented in each publication and its place within malaria research.

General discussion.

1.1 Introduction

Malaria remains a heavy burden on the populations of some of the poorest regions of the world due to the mortality and morbidity it imposes; it is also a serious impediment to economic growth. Malaria is one of the most severe of infectious diseases, resulting in ca. 250 million cases and ca. 1 million deaths each year (World Health Organization, 2009). The malaria parasite *Plasmodium falciparum* is by far the most important species of its genus in causing human disease (Miller *et al*., 2002). In addition to its impact as a disease-causing agent this parasite, and its relatives, have many scientifically interesting facets to their biology including metabolic pathways which show distinctions from their equivalents found in the 'classic' metabolisms derived from higher plants, higher animals and yeast. The divergent aspects of parasite metabolism have been of great importance in the development of drug therapy and prophylaxis as metabolic differences between the human host and the parasite can be exploited to enable the application of drug therapies that have limited side-effects on the host. The development of future antimalarials will also be largely constrained by the comparative metabolisms of the parasite and its human host.

There have been, arguably, two major advances which have allowed or greatly facilitated detailed investigation of parasite metabolic pathways, and parasite biology in general: the first was the development of *in vitro* cultivation of erythrocytic stage *P. falciparum* parasites by Trager and Jensen in 1976, and the second the sequencing of the complete genome of the parasite, which was published in 2004 (Trager and Jensen, 1976; Hall and Gardner, 2004). The articles presented within this thesis cover almost two decades of work, none of which would have been possible in the absence of methods for the *in vitro* cultivation of *P. falciparum*. Some of the papers were completed before the publication of the parasite genome sequence and others after.

Chapter 1 contains commentaries on the publications, their methodology, place in the relevant fields of study and related scientific developments subsequent to their publication. This is supplementary to the introductory material and discussions presented in the publications themselves. The papers are presented in chronological order rather than grouped strictly by subject.

However, there is a methodological flow and other connecting elements between the papers in the order presented.

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The second publication describes the isolation and characterisation of the gene encoding the glycolytic pathway enzyme enolase from *P. falciparum*. The inferred amino acid sequence included peptide insertions found only in the enolases of higher plants and other photosynthetic organisms. This raised implications concerning the deep evolutionary history of the malaria parasite and related species.

The third is concerned with the elucidation of the molecular basis of resistance to the antimalarial drug sulfadoxine. Resistance was found to result from point mutations within the dihydropteroate synthetase domain of the bifunctional protein hydroxymethylpterin pyrophosphokinase-dihydropteroate synthetase, an enzyme of the parasite folate pathway. Additionally, it was discovered that the presence of exogenous folate has an antagonistic effect on sulfadoxine in some parasites of a defined genotype. This highlighted the importance of folate salvage in parasite metabolism.

Fourth is a paper representing the discovery of a novel metabolism in both *P. falciparum* and the related apicomplexan parasite *Toxoplasma gondii*. The use of parasite genes in rescuing an *Escherichia coli* tyrosine auxotroph resulted in a proof of function of the products of these genes as pterin-4a-carbinolaminedehydratases. Pterin recycling, hitherto undetected in apicomplexans, was therefore added to the known metabolic processes of these organisms.

The final paper describes an investigation into the subcellular distribution of the folate pathway enzyme serine hydroxymethyltransferase (SHMT) within *P. falciparum* erythrocytic stage parasites. The use of confocal laser scanning microscopy and immunofluorescent techniques showed that PfSHMTc, the sole enzymatically active parasite SHMT protein, was found in the cytoplasm but also showed a stage-specific localisation to both the mitochondrion and

apicoplast organelles. The otherwise enigmatic, enzymatically inert, PfSHMTm paralogue revealed a possible function, when in complex, in allowing targeted localisation of PfSHMTc to the mitochondrion. The spatial distribution of PfSHMTm also suggested a possible role in the morphogenesis of elongating apicoplasts during schizogony.

1.2 Cultivation of *P. falciparum* erythrocytic stage parasites **(Relating to the publication presented as chapter 2)**

1.2.1 The development of *P. falciparum* culture methods

The investigation of any aspect of parasite metabolism requires that parasites be cultivated. Cultivation by infecting a surrogate host, for example *Aotus* monkeys, with human malaria parasites imposes serious constraints including the relatively low parasitaemias achievable, and therefore low yields of parasites, high cost and the need for specialised animal facilities (Collins *et al.*, 1973). The use of model systems using other malaria species infective to rodent hosts, but not of humans, also have obvious disadvantages as inter-specific metabolic variation may be considerable. The development of *in vitro* culture of *P. falciparum* was, therefore, a necessary prerequisite for the detailed elucidation of the metabolic pathways of this parasite. Parasites can be grown to yield nucleic acids, proteins or metabolites. They can also be challenged by drugs and their resistance to the drugs quantified. Additionally they can be observed directly, and the use of electron or light microscopy can reveal, often with the use of specific reporter molecules, aspects of parasite metabolism *in situ.*

In 1976 Trager and Jensen published the first method for the *in vitro* culture of *P. falciparum* (Trager and Jensen, 1976). This is generally known as the “candle-jar method.” Parasites were cultured in Petri dishes within an airtight container in which the oxygen concentration was lowered and the carbon dioxide increased by burning a candle until it was extinguished. The lower O_2 and higher CO_2 concentrations produced by burning the candle mimicked the partial pressures in which malaria parasites exist within the human host. Following this breakthrough the disadvantages of the method: a relatively high tendency for bacterial and fungal contamination, easy spillage from shallow Petri dishes and limited space inside airtight chambers were overcome in an *ad hoc* way in many malaria laboratories around the world. The introduction of tissue culture flasks, developed primarily for mammalian cell culture, and specialised low O_2 and high CO_2 gas-mix cylinders for use with sealed flasks or incubators supplied with piped CO_2 employed with flasks incorporating gas-permeable membranes, greatly improved the reliability of

parasite culture. Trager produced a “continuous flow” method for parasite culture in 1979, but the method’s lack of robustness resulted in only limited subsequent use (Trager, 1979).

1.2.2 The book chapter on culture methods

The book chapter, “Simple *in vitro* cultivation of the malaria parasite *Plasmodium falciparum* (erythrocytic stages) suitable for large-scale preparations,” (Read *et al*., 1993a) included as chapter 2 of this thesis reflects the parasite cultivation methods used and developed in the Molecular Parasitology Group of UMIST, and later the University of Manchester, headed by Professor John Hyde. These culture methods were based on those developed at Edinburgh in the laboratory of Prof. John Scaife (Zolg *et al.* 1982). The chapter addressed basic methods in the culture of *P. falciparum* in detail; one of the remits of the book the chapter was a part of was to be inclusive of those details of methodology usually unreported in most research papers due to constraints of space. Safety matters were discussed and a ‘trouble shooting’ element was included. The chapter also incorporated practical instructions on growing large preparations of parasites suitable for the bulk extraction and purification of nucleic acids and proteins. The present author adapted or developed most of the methods described in the chapter, especially those aspects particularly related to growing large numbers of parasites.

In the book chapter particular problems were addressed relating to the use of human blood plasma, specifically plasma which had not been heat-treated, as a culture medium additive. In the absence of ready access to human serum, human plasma can be employed to culture parasites; indeed untreated plasma can support higher parasitaemias than can either heat-treated plasma or serum (Read and Hyde, 1988). Heat-treatment was often used by blood transfusion services as an alternative to producing serum in order to prevent the problematic clotting that can be caused by the presence of active clotting factors. Methods which minimise the tendency for untreated plasma to clog sterile filters, and which prevent the gelling of complete media, were described.

1.2.3 Subsequent developments in the culture of *P. falciparum*

Since the publication of the book a number of developments in parasite culture have occurred. From an early stage in the history of *in vitro* parasite

culture medium, additives other than human serum and plasma have been employed to greater or lesser effect (Asahi and Kanazawa, 1994). Studies on the components of complete media necessary to sustain parasite growth have highlighted the essential role that the fatty acid content of serum, plasma or their derivatives plays (Mitamura *et al.,* 2000). In particular lysophosphatidylcholine containing C-18 unsaturated fatty acids was found to support the growth of parasites, in the presence of bovine albumin (Asahi *et al*., 2004).

The identification of the chemical components of medium necessary for parasite growth, especially pantothenic acid (Saliba *et al*., 1998), plus difficulties in obtaining human blood products, combined with a greater batch consistency and ease of ordering, has made bovine serum fractions, such as Albumax I, Albumax II (Gibco-Invitrogen) and bovine serum fraction V (Sigma), the medium additives of choice for most laboratories. Though the ease of use of bovine serum fractions has outweighed other factors, personal observation has shown that the parasitaemias obtainable using bovine products are significantly lower than those achievable using human blood derived additives; a phenomenon also recently noted by Preechapornkul and co-workers (Preechapornkul *et al.*, 2010). They compared parasite growth rates in medium containing 0.5% Albumax II and medium containing 10% human serum and found that the former gave growth rates (mean ± standard deviation) of 0.69±0.28, whilst the latter gave growth rates of 0.91± 0.40 (growth rates calculated according to the method described in Trager and Jensen, 1976).

The use of a bovine serum derived fraction, in this case Albumax I (Gibco-Invitrogen), as a medium additive, proved to be essential in developing a reliable method for measuring anti-folate drug resistance in parasites. This will be further discussed in section 1.4.3 and chapter 4.

Relatively recently, hollow-fibre bioreactors have begun to be employed in malaria culture. The hollow fibres in such a bioreactor are semi-permeable and separate the parasitised blood cells from the perfusing medium, they provide a large surface area for gas and nutrient exchange and therefore allow a much higher haematocrit to be employed than do other methods. Limitations on the achievable parasitaemias of this approach have been reported and it is perhaps too early to predict whether this, or similar methodologies, will replace

the use of conventional tissue culture flasks for the large-scale culture of parasites (Li *et al*., 2003; Preechapornkul *et al.*, 2010).

1.2.4 Publication impact

"Simple *in vitro* cultivation of the malaria parasite *Plasmodium falciparum* (erythrocytic stages) suitable for large-scale preparations" has been cited 10 times (ISI Web of Science) or 19 times (Google Scholar) in publications. The methods described in the chapter allowed the production of nucleic acids and proteins for many projects undertaken in the Hyde group in Manchester and was instrumental in facilitating the development of a number published drug assays, such as that described in chapter 4.

1.3 Glycolysis in malaria parasites (Relating to the publication presented as chapter 3)

1.3.1 Overview

One of the few salient facts known about the metabolism of malaria parasites before the revolution in knowledge generated by the molecular techniques developed from the mid 1980s onward was the important role played by glycolysis. The uninfected erythrocyte has modest energetic needs and glucose consumption is relatively low (Jensen *et al*., 1983). However, the glucose consumption rate of a parasite infected erythrocyte has been estimated to increase up to 100-fold (Roth et al., 1982). Given the apparent prominence of glycolysis in parasite biology a project to characterise genes from this pathway was undertaken. Two *P. falciparum* genes were fully characterised, 3-phosphoglycerate kinase and enolase (2-phospho-D-glycerate hydrolase), and two further genes, glyceraldehyde-3-phosphate dehydrogenase and triose-phosphate isomerise, were isolated and partially characterised (Hicks *et al*., 1991; Read *et al*., 1994). One of the papers resulting from this study, "Molecular characterisation of the enolase gene from the human malaria parasite *Plasmodium falciparum; e*vidence for ancestry within a photosynthetic lineage," is included as chapter 3 of this thesis.

1.3.2 The glycolytic pathway in *P. falciparum*

The erythrocytic stage parasite is bathed in a glucose-rich environment within the bloodstream of its host and has little requirement for any other nutrient as an energy source. Approximately 60 to 70% of the glucose taken up by *P. falciparum* is oxidised to lactic acid, which is then excreted (Jensen *et al*., 1983). This relatively low percentage, compared with >90% in uninfected erythrocytes, reflects the proportion of glucose which is incorporated into the nucleic acids, lipids and glycosylated proteins required by the actively growing and reproducing parasite (Olszewski and Llinás, 2011). Asexual parasites are highly dependent on glycolysis and the removal of extracellular glucose results in the almost immediate acidification of parasite cytosol, and inhibitors of the *P. falciparum* hexose transporter cause parasite death (Saliba *et al*., 2004). This dependence has suggested that glycolysis could be a potential target pathway for future antimalarials (Woodrow and Krishna, 2005, Granchi *et al*., 2010).

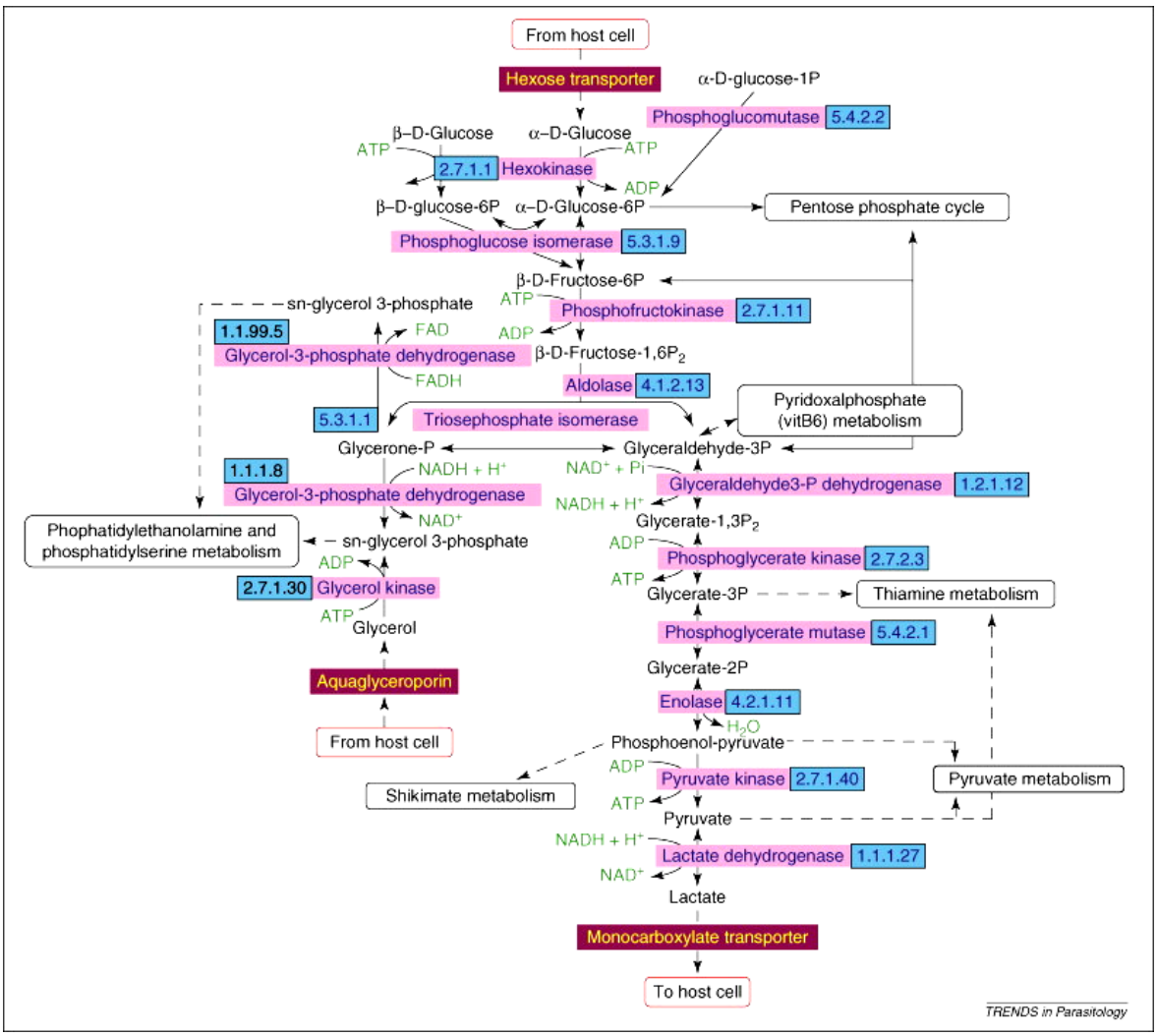

Fig. 1 The glycolytic pathway of *P. falciparum* and associated metabolism. Reproduced from Ginsburg, 2008.

Of the genes encoding the enzymes of the glycolytic pathway in *P. falciparum*, seven of the ten were characterised by 'classic' molecular technology between 1988 and 2000: hexokinase, glucose-6-phosphate isomerase, aldolase, triose phosphate isomerase, glyceraldehyde 3-phosphate dehydrogenase, phosphoglycerate kinase and enolase. The gene encoding the fermentation-step enzyme, converting pyruvate to lactate, lactate dehydrogenase was characterised in 1993. Subsequently the use of bioinformatics methods, in conjunction with the complete genome of *P. falciparum,* has largely filled the gaps in the pathway (Woodrow and Krishna, 2005). However, the limitations of automated *in silico* reconstructions of metabolic pathways in *Plasmodium* have been highlighted (Ginsburg, 2008).

Any metabolic step inferred from bioinformatics data must be functionally proven before it can be accepted as canonical.

The glycolytic pathway enzymes are, unsurprisingly, expressed in relatively high abundance and many were isolated and identified from parasite extracts using proteomics methods, two dimensional electrophoresis and mass spectrometry, at a relatively early stage in the application of these to malaria research (Nirmalan *et al*., 2004).

1.3.3 The enolase paper - methodology

The gene encoding *P. falciparum* enolase, the enzyme catalysing the conversion of 2-phosphoglycerate to phosphoenolpyruvate, was isolated and characterised by classic molecular biology methods. Advances, particularly in the availability of the complete genome of *P. falciparum* and in deoxyribonucleic acid (DNA) sequencing, would today allow the laboratory work, which took approximately two years to accomplish, to be carried out in a matter of days or weeks at most. An outline of the methodology used to isolate and characterise the enolase gene is given within the paper.

In the absence of a fully sequenced genome, isolation of the gene was dependent on the generation of DNA probes complementary to one or more regions of the gene. This was achieved through the use of oligomer primer pairs designed for sections of highly conserved sequence within the gene, and optimised for parasite codon usage (*P. falciparum* DNA has a very high AT content). The primers were then used in polymerase chain reactions (PCR) to generate the probes. Isolation was then entirely dependent on the presence of the target gene within DNA libraries. In the case of the enolase gene only part of it was to be found, and solely in one of several libraries screened, approximately 25% of the 5'-terminal region of the encoded protein was missing. This part of the gene was isolated by a combination of inverse polymerase chain reaction (PCR) on a circularised genomic DNA restriction fragment, and 5' rapid amplification of cDNA ends (RACE). Sequencing of the gene was by manual methods largely using cycle sequencing, which was a relatively new development, in place of Klenow fragment (*Escherichia coli* derived DNA polymerase I) sequencing (Sanger *et al*., 1977). DNA sequencing was laborious and very much a craft at the time, with a high degree of

experimental skill required at every step from pouring the ultra-thin polyacrylamide gels to interpreting the banding on the exposed X-ray film. DNA sequencing is now much faster and largely automated, using fluorescent rather than radioactive labelling, capillaries replacing full size electrophoresis gels and automated rather than visual interpretation.

The chromosomal location of any sequence of interest is now readily to be found in the database of the *P. falciparum* genome, PlasmoDB (http://plasmodb.org/plasmo); however, before this facility was available chromosomal location had to be determined experimentally. Linear agarose electrophoresis will not resolve large DNA molecules such as chromosomes, therefore pulsed-field gel electrophoresis (PFGE), using a contour-clamped-homogeneous-electric-field (CHEF) apparatus (Bio-Rad) was employed. This apparatus uses phased switching between four pairs of electrodes surrounding the agarose gel to separate chromosomes (Van der Ploeg *et al*, 1985; Sambrook and Russel, 2001). The preparation of the parasites and the phasing of the electrode switching were both problematic, having to be developed empirically for the particular parasite isolates used. The switching parameters also had the distressing tendency to drift between experiments; conditions used successfully for one experiment might not work for another, apparently identical experiment, conducted a week later.

1.3.3 The enolase paper – results

The *P. falciparum* enolase gene mapped to chromosome 10, and encoded a predicted protein of 48.7kD. The encoded protein showed a 60-70% identity to other eukaryote enolases and all amino acid residues implicated in catalysis and cofactor/substrate binding were conserved. The degree of identity of the *P. falciparum* protein to the enolases of other organisms was the highest of all the parasite glycolytic enzymes to their homologues. The high degree of similarity of the parasite enolase to that of humans would seem to preclude the enzyme as a useful potential antimalarial drug target; a conclusion stated in the paper itself.

If the overall architecture of the gene and its encoded protein was unremarkable, one notable feature was apparent. Amino acid insertions or deletions found elsewhere only in the enolases of higher plants, were found in

that of *P. falciparum.* Six small insertions/deletions were evident, and even more striking was a single pentapeptide insertion, EWGWS, which corresponded to the motif EWGWC found in such diverse higher plants as *Arabidopsis*, maize and tomato. Previous work on the extrachromosomal genome of the apicoplast organelle had revealed rRNA genes and RNA polymerase subunit genes with striking similarities to those of chloroplasts. An evolutionary connection between malaria parasites and photosynthetic organisms had therefore been indicated (reviewed in Palmer, 1992). The amino acid insertions in the enolase gene, however, were the first demonstration of this connection at the level of a protein-encoding nuclear gene. In the paper it was noted that the level of overall similarity of the *P. falciparum* protein was no greater to those proteins containing the pentapeptide motif than to those lacking the insertion. The counterintuitive lack of a greater similarity amongst genes possessing the insertion was accompanied by the surprising fact that the enolase of *Chlamydomonas reinhardtii*, a photosynthetic unicellular alga, lacked the pentapeptide insertion. Taken together, these features suggested that the phylogeny of the enolase gene was probably complex, perhaps including elements of horizontal transfer affecting only parts of genes.

1.3.4 Subsequent progress in knowledge of parasite glycolysis, enolase and horizontal gene transfer

Work on the glycolytic pathway of *P. falciparum* and related organisms has continued. This has included the filling in of gaps in the array of characterised pathway genes, as was discussed earlier. One glycolytic enzyme, lactate dehydrogenase (LDH), was used as the target molecule of an antibody-based diagnostic tool (optiMAL – a dipstick) for the detection of malarial infection (Woodrow and Krishna, 2005).

Perhaps the most important new insight into parasite glycolysis and carbon metabolism in general has been afforded by the application of mass spectrometry-based metabolomics methods. The central paradox of carbon metabolism in malaria parasites was the presence of most tricarboxylic acid (TCA) cycle genes but little apparent need for this metabolism for the parasite's energetic requirements. It has been revealed that TCA metabolism in the parasite is not cyclic, but rather branched, the major carbon sources being the

amino acids glutamate and glutamine. As a result of this branched nature, a number of reactions proceed in reverse of the standard direction. The branches comprise two largely independent pathways: an oxidative branch from 2-oxoglutarate to succinyl-CoA, succinate, fumarate and malate, and a reductive branch from 2-oxoglutarate to isocitrate, citrate, oxaloacetate and malate. The succinyl-CoA required for heme biosynthesis is produced by the oxidative branch. However, the reductive branch produces two-carbon units during the citrate cleavage step in contrast to consuming two-carbon units in the form of acetyl-CoA. This work has also shown that, unlike glycolysis in most organisms, glycolysis in malaria parasites is essentially uncoupled from TCA metabolism (Olszewski *et al*. 2010; Olszewski and Llinás, 2011; Ginsburg, 2010).

The enolase gene of *P. falciparum* was heterologously expressed in *E. coli* in 2004, allowing the enzymatic characterisation of the recombinant protein to be undertaken. The enzyme formed an active homodimer, and had a substrate affinity similar to that of mammalian enolases (Pal-Bhowmick *et al*., 2004). This work confirms that the gene isolated and characterised in Read *et al.*, 1994 encoded the functional enolase of *P. falciparum.* The same laboratory discovered that the enolase protein was located, in addition to the cytoplasm, in the nucleus and also on the surface of the merozoite. Evidence that *P. falciparum* enolase was immunogenic in populations living in malaria endemic areas of India was also noted (Pal-Bhowmick *et al*., 2007).

Moonlighting, non-glycolytic, functions have been suggested for plasmodial enolase, partly as a result of the cellular locations described above. The strongest case made thus far is for a probable involvement by the enolase protein in the functioning of the parasite food vacuole to which it also localises. A yeast strain exhibiting vacuolar disruption due to enolase deficiency was used in a complementation experiment. Transformation of this deficient yeast with *P. falciparum* enolase restored vacuolar morphology and function (Das *et al*., 2011).

In the related apicomplexan parasite *Toxoplasma gondii* localisation of enolase to the nucleus has also been indicated. *T. gondii* has a complement of two isoforms of the enzyme, and their nuclear location has been demonstrated

to be related to parasite replication, perhaps indicating a regulatory role for the protein (Ferguson *et al*., 2002). Of particular interest is that the *T. gondii* isoforms were heterologously expressed and the pentapeptide insertion, plus a dipeptide insertion also found in *P. falciparum* and plant enolases, was deleted. The insertions were shown to have a synergistic effect on the Km value for glycerate-2P, with the greatest effect provided by the pentapeptide insertion. The deletion of these peptides produced a decrease in the affinity of the enzyme for its substrate. A similar effect was shown for *P. falciparum* enolase; the deletion of the pentapeptide insert resulted in an approximately100-fold decrease in Kcat/Km and caused the dissociation of enzyme dimers (Vora *et al*., 2009). These results strongly suggest that the amino acid insertions found in the enolases of land plants and apicomplexan parasites contribute materially to their enzymatic activities. The functional difference bestowed by the amino acid insertions casts doubt on the conclusion stated in Read *et al.* 1994 dismissing the enolase of *P. falciparum* as a potential drug target; this assertion may have been unduly pessimistic.

The presence of the pentapeptide insertion in enolases of evolutionary distant organisms such as apicomplexans and higher plants provoked enquiry from molecular taxonomists. Initially, the hypothesis that the amino acid insertion into the enolase of apicomplexans could have entered the nucleus of apicomplexan parasites by transfer from the photosynthetic endosymbiont which also donated the apicoplast was advanced (Dzierszinski et al., 1999). However, the insert was also found to exist in alveolates outside the Apicomplexa, charophyte green algae and *Chlorarachnion* but not in chlorophytes and red algae. As the red algae are considered the most likely donor of the apicoplast their role in the transfer of the enolase insertions would seem unlikely (Keeling and Palmer, 2001). Keeling and Palmer put forward a proposition that the ancestral alveolate ingested a charophyte alga and the enolase gene from this food source, instead of replacing entirely the predator's own enolase, recombined with it, resulting in a mosaic gene (Keeling and Palmer, 2001). Further work indicated that insertions were also to be found in the enolase enzymes of some ciliates, but not in dinoflagellates which are considered to be a sister group to the alveolates. The rodent malaria parasite *Plasmodium yoelii* was surprisingly found to possess two enolase genes, one of

which did not contain the pentapeptide. At present the distribution of enolases containing insertions and those which are insertion-free throughout various taxa suggests a complex evolutionary history, probably including horizontal transfer of whole genes, parts of genes and deletion events (Harper and Keeling, 2004).

1.3.5 Publication impact

"Molecular characterisation of the enolase gene from the human malaria parasite *Plasmodium falciparum; e*vidence for ancestry within a photosynthetic lineage" has been cited 42 times (ISI Web of Knowledge) or 35 times (Google Scholar) in relevant literature. The paper helped to precipitate a considerable body of subsequent scientific enquiry, not least in the field of molecular taxonomy.

1.4 Sulfadoxine resistance and the folate pathway

(Relating to the publication presented as chapter 4)

1.4.1 The folate pathway as a target for antimalarial drugs

Folate is an essential cofactor for the malaria parasite; parasites can make use of exogenous folate, salvaged from the host plasma, but appear to have a sufficiently great reliance on *de novo* folate synthesis that effective disruption of this metabolic pathway leads to parasite death. A drug-induced blockade of the folate pathway results in decreased synthesis of pyrimidines and a resultant arrest in DNA replication; methionine synthesis and the conversion of glycine to serine are also decreased. All these metabolic processes involve the transfer of one-carbon units (Cowman, 2001; Hyde, 2005).

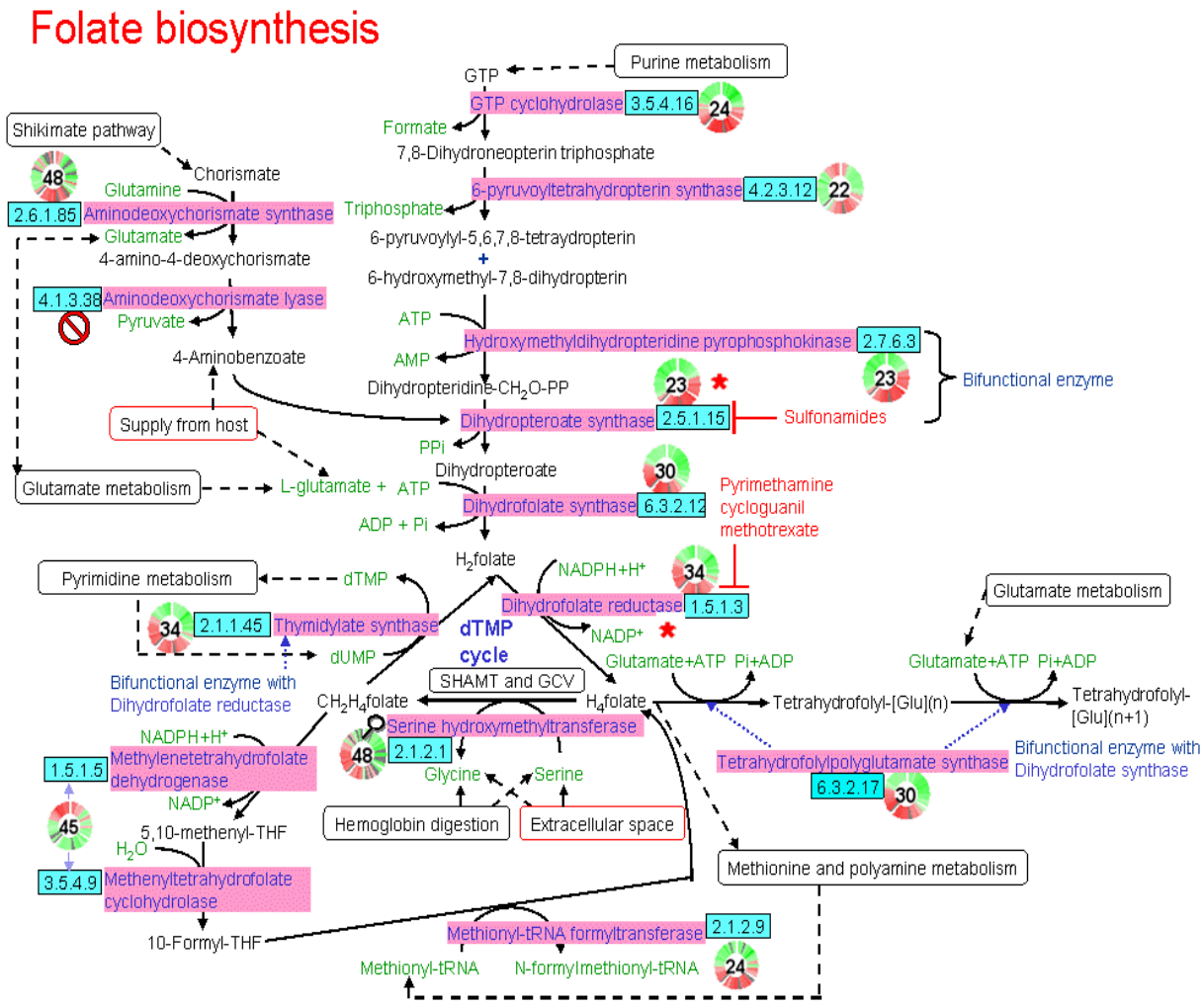

Fig. 2 The folate pathway of *P. falciparum* and associated metabolism. Reproduced from Ginsburg, Hagai, "Malaria Parasite Metabolic Pathways" http://sites.huji.ac.il/malaria/

The folate pathway has two well established targets for drug therapy: the enzymatic activity of the dihydrofolate reductase (DHFR) domain of the bifunctional dihydrofolate reductase-thymidylate synthetase (DHFR-TS) protein and the activity of the dihydropteroate synthetase (DHPS) domain of the bifunctional hydroxymethylpterin pyrophosphokinase-dihydropteroate synthetase (HPPK-DHPS) protein. Pyrimethamine and proguanil (which is metabolised *in vivo* to the active form cycloguanil) are drugs inhibiting DHFR activity, whilst a number of sulfa drugs, including sulfadoxine (a sulfonamide) and dapsone (a sulfone), inhibit DHPS activity. DHPS is found only in the parasite, being absent from humans and other metazoans. DHFR, however, is present in both host and parasite. The efficacy of pyrimethamine and proguanil is therefore dependant on a several hundred-fold lower binding for the drugs to the human DHFR enzyme.

Synergistic combinations of either pyrimethamine or proguanil with sulfa drugs, such as the highly successful sulfadoxine-pyrimethamine combination (Fansidar™), first used in the late 1960s, have proved useful as cheap alternatives to combat chloroquine-resistant parasites. With the introduction of artemisinin-based combination therapy as the most widely used first-line antimalarial, the importance of antifolates as a 'fall-back' measure to treat chloroquine-resistant infections has declined. However, antifolates retain an important role in 'intermittent preventative treatment' in areas of high malaria transmission (Warsame *et al*., 2010). Additionally, sulfadoxine-pyrimethamine remains in widespread use when artemesinin combinations are considered too expensive, or are unavailable (Müller and Hyde, 2010).

1.4.2 Dihydropteroate synthetase and sulfadoxine resistance

The gene (*hppk-dhps*) encoding the bifunctional enzyme hydroxymethylpterin pyrophosphokinase-dihydropteroate synthetase (HPPK-DHPS) of *P. falciparum* was isolated and characterised in 1994 by Darren Brooks and co-workers, including the present author, and contemporaneously by Triglia and Cowman (Brooks *et al*., 1994; Triglia and Cowman, 1994). The enzymatic function of DHPS is to couple *para*-aminobenzoic acid (PABA) to 7,8-

dihydropterin to produce dihydropteroate, the step immediately before dihydrofolate synthesis.

It had been previously shown that the molecular basis of resistance to the folate analogs pyrimethamine and cycloguanil is directly linked to a small number of specific amino acid alterations within the DHFR domain of the bifunctional DHFR-TS enzyme (Cowman *et al*., 1988; Snewin *et al*., 1989; Peterson *et al.,* 1990; Hyde, 1990). The studies published in 1994 indicated a possibly similar role in sulfadoxine resistance of mutations in the DHPS domain of HPPK-DHPS. However, the extent and nature of the contribution of these mutations to sulfadoxine resistance was unknown. A study designed to examine the contribution of mutations in DHPS to sulfadoxine resistance was undertaken and the resultant paper, "Sulfadoxine resistance in the human malaria parasite *Plasmodium falciparum* is determined by mutations in dihydropteroate synthetase and an additional factor associated with folate utilization," is included as chapter 4 of this thesis.

1.4.3 The sulfadoxine resistance paper

In the investigation of the potential role of DHPS mutations in sulfadoxine resistance a vital tool was available, the products of a sexual cross between the isolates HB3 and Dd2 undertaken by Wellems and co-workers (Wellems et al., 1990). The parental line HB3 proved to be very sensitive, and Dd2 very resistant to sulfadoxine, as was predicted by earlier work (Brooks *et al*., 1994). It was anticipated that any parental variation in sulfadoxine resistance and previously characterised parental variation in pyrimethamine resistance would be inherited unlinked by the cross progeny, as the two target genes are located on different chromosomes.

At the inception of the work leading to the paper it was unclear as to the extent that malaria parasites could make use of exogenous folate, though the effect of folate levels in growth medium on sulfadoxine assays had been previously noted (Chulay et al., 1984; Watkins et al., 1985). In initial experiments it proved difficult to obtain reproducible IC_{50} values for sulfadoxine for the same isolate or progeny over several weeks. The most reasonable explanation for this variation was that variable folate levels in the human blood plasma used as a medium additive and in the donated blood cells themselves

were affecting the abilities of some parasites to grow in the presence of sulfadoxine. This strong indication that parasite growth was being affected by the presence of exogenous folate made it imperative to control for folate and PABA levels during the growth of parasites. Custom medium was obtained depleted of folate and PABA, as had been used by other groups for previous investigations of sulfadoxine resistance; however, additional means of controlling folate concentrations in the parasite growth environment were adopted: human plasma was substituted by a bovine serum fraction (Albumax I) and a single donor of blood was utilised throughout. These methods and a regime of adapting parasites to low-folate medium before growth in folate-free medium gave consistently reproducible results.

The improved assay indicated that sulfadoxine resistant and sensitive progeny differed in IC_{50} values by approximately three orders of magnitude. The values obtained were very close to those for one or other of the parent isolates. Moreover the IC_{50} values correlated exactly with the distribution of wild-type and mutant *dhps* genotypes.

Having shown that mutations in the *dhps* gene were responsible for modulating sulfadoxine resistance in the absence of folate, the effect on sulfadoxine resisitance of adding measured concentrations of folate into assay cultures was investigated. Of the parent isolates the IC_{50} value of HB3 was affected very little by the presence of exogenous folate, whereas the susceptibility of Dd2 to sulfadoxine was markedly reduced by relatively low concentrations of folate in the culture medium. Of the progeny, some showed similar levels of antagonism of sulfadoxine by folate as was found in Dd2, the "folate effect," whilst others were unresponsive to folate. These folate phenotypes were unrelated to the presence or absence of mutations in *dhps.* Further experimentation indicated that the ability for folate to antagonise the effect of sulfadoxine was related to the inheritance pattern of a restriction fragment polymorphism marker that mapped to the same region of chromosome 4 as the *dhfr-ts* gene. The folate effect was found to be directly related to *dhfr* genotype; those progeny having the Dd2 genotype showing the folate effect, those having the HB3 genotype being unresponsive to the presence of exogenous folate. This suggested that the effect was dependent on the *dhfr* genotype, or on that of a gene closely linked to *dhfr* on chromosome 4.

1.4.4 The development of research into sulfadoxine resistance and the folate effect.

Following the elucidation of the mechanism of sulfadoxine resistance, through specific amino acid alterations in the DHPS enzyme of the parasite, many field studies have been carried out to assess the levels of, and changes to, drug resistance in endemic areas. These studies have often combined the assessment of sulfadoxine resistance with that of pyrimethamine, as the two are administered together. Many thousands of samples from across the world have been typed to date for the highly resistant *dhfr* triple mutant (N51I, C59R, S108N) genotype and the *dhps* double mutant (A437G and K540E) genotype. Most typing studies have used PCR or restriction enzyme-based tests (Wang *et al*., 1997b; Pearce *et al.* 2009; Naidoo and Roper, 2010; Müller and Hyde, 2010). Recently, a bioinformatic meta-analysis of data generated by field studies in Africa has highlighted an increasing prevalence of resistant genotypes. The conclusion of this analysis was that the loss of efficacy of the sufadoxine-pyrimethamine drug combination required further monitoring and that a centralised resistance data network, as proposed by the Worldwide Antimalarial Resistance Network, would be invaluable in allowing timely interventions to combat drug resistance (Sridaran *et al*., 2010).

A study utilising the transfection of parasites allowed the roles of specific mutations of *dhps* in sulfadoxine resistance to be further defined. A series of mutant *dhps* alleles derived from *P. falciparum* variants found in field isolates conferred varying levels of sulfadoxine resistance to transformed parasites. High levels of sulfadoxine resistance were found to be the result of an accumulation of mutations, with A437G being the most important substitution. This study furnished formal proof that the mechanism of resistance to sulfadoxine in *P.falciparum* involved mutations to the *dhps* gene (Triglia *et al*., 1998).

In the paper presented as chapter 4 it was proposed, with reservations, that the “folate effect” might be linked to the resistant *dhfr* genotype (Wang, *et al*., 1997a). Parasites were assayed in the simultaneous presence of both pyrimethamine and sulfadoxine in a study published in 1999; this showed that the presence of pyrimethamine reduced the folate effect to a significant degree

when compared to parasites challenged solely by sulfadoxine (Wang, *et al.*, 1999). Subsequent work, following the publication of the complete *P. falciparum* genome, indicated that the "folate effect" phenotype correlated solely with the coding sequence of the *dhfr* domain. In turn this suggested that a property of the mutation S108N confers the ability of exogenous folate to antagonise the action of sulfadoxine (Wang et al., 2004). The role in sulfadoxine resistance of factors other than amino acid substitutions in DHPS, including exogenous folate levels, was underlined in a field isolate study from Kenya (Mberu *et al*., 2002).

The role of *Plasmodium falciparum* multidrug resistance protein 1 (PfMRP1) in sulfadoxine-pyrimethamine resistance has recently been revealed. It is proposed that PfMRP1 affects intracellular folate homeostasis in parasites; the intracellular folate concentration having important effects on the activities of antifolate drugs. The 1466K allele of the gene encoding PfMRP1 is implicated in increased resistance to sulfadoxine-pyrimethamine, a resistance not linked to the previously characterised mutations in parasite DHFR and DHPS (Dahlström *et al*., 2009).

1.4.5 Publication impact

"Sulfadoxine resistance in the human malaria parasite *Plasmodium falciparum* is determined by mutations in dihydropteroate synthetase and an additional factor associated with folate utilization" has been cited 160 times (ISI Web of Knowledge) or 168 times (Google Scholar) in relevant literature. The paper played a vital role in elucidating the molecular mechanisms of resistance to an important antimalarial, therefore many field study papers, and others, have referenced it.

1.5 Pterin recycling in *Plasmodium falciparum* and *Toxoplasma gondii* (Relating to the publication presented as chapter 5)

1.5.1 *Toxoplasma gondii*

Pterin recycling in *P. falciparum* was explored in parallel with that of the related organism *Toxoplasma gondii.*

T. gondii is another obligate intracellular parasite belonging to the same phylum (Apicomplexa) as *Plasmodium;* it is the causative agent of the disease toxoplasmosis. The definitive hosts of this parasite are felid carnivores and it can probably infect, as an intermediate host, all warm-blooded animals (mammals and birds) including humans. It is prevalent in most areas of the world and is of veterinary and medical importance because it may cause abortion or congenital disease in its intermediate hosts. Infection, if acquired postnatally, is usually well controlled by the host's immune system. Infection usually consists of an acute phase when rapidly multiplying tachyzoite stage parasites infect a range of tissues; this is followed by a latent phase during which slowly replicating bradyzoite stage parasites form tissue cysts in muscles and the brain. In immunocompromised humans, such as transplant patients on immunosuppressive drug regimes or *acquired immune deficiency syndrome* (AIDS) patients, toxoplasmosis can be fatal (Tenter *et al*., 2000).

1.5.2 Pterin recycling

Before the study presented as chapter 5 was undertaken virtually nothing was known concerning the pterin metabolism of apicomplexan parasites. Pterin-4a-carbinolaminedehydratase (PCD) is an enzyme catalysing the first of two reactions which recycle tetrahydrobiopterin (BH_4), the cofactor of aromatic amino acid hydroxylases (AAHs). The BH_4 cofactor participates in the hydroxylase reactions that convert tryptophan or phenylalanine to tyrosine; BH_4 is vital to the function of nitric oxide synthase, and, in metazoan animals, it is involved in the synthesis of neurotransmitters such as serotonin and dopamine. PCD dehydrates the pterin-4a-carbinolamine formed in the hydroxylase reaction, giving a quinonoid, (q)-dihydropterin, this is then reduced to a

tetrahydrobiopterin by dihydropterin reductase (DHPR, also termed q-dihydropterin reductase) (Thony *et al*., 2000; Cameron *et al*., 2008).

PCDs and the AAH enzymes they support are best known from metazoan animals but have been found in higher plants and some bacteria, such as *Pseudomonas aeruginosa (*Naponelli *et al*., 2008). A number of parasitic protozoa, including the Kinetoplastida, require exogenous pterin, acquiring it through the action of a specific transporter (Cunningham and Beverley, 2001).

1.5.3 The pterin recycling paper

The paper "Functional identification of orthologous genes encoding pterin recycling activity in *Plasmodium falciparum* and *Toxoplasma gondii*" is included as chapter 5 of this thesis.

The experiments leading to the abovementioned paper grew out of contacts with Andrew Hanson of the University of Florida, who, though working largely on higher plants, had overlapping interests in the folate pathway with ourselves. Through him access was available to the plasmid pJZ9-4 and the *E. coli* tyrosine auxotroph strain JP2255, which had been used by Roy Jensen (University of Florida) to characterise the PCD orthologue of *Pseudomonas aeruginosa* (Zhao *et al*., 1994; Song *et al.*, 1999).

This work followed the completion of sequencing the genome of *P. falciparum* and also the availability of genomic data for *T. gondii* (http://ToxoDB.org), (Kissinger *et al*., 2003). Although the genes encoding PCD were not originally annotated within the databases, finding the relevant PCD gene sequences was considerably easier than formerly, and could be accomplished entirely *in silico* by searching the databases of predicted translation products for the histidine motifs separated by 16 residues ($HHX_{16}H$) characteristic of PCD amino acid sequences.

Once candidate genes had been located oligomeric DNA primers perfectly matched to the target sequences were synthesised and used to generate the genes by PCR from cDNA libraries of the two organisms. Both genes had identically positioned introns making a cDNA origin for the amplification a necessity. The amplified genes were cloned into a suitable

plasmid and transformed into the *E. coli* tyrosine auxotroph. The phenylalanine hydroxylase gene of *P. aeruginosa,* that was necessary and thus allowed the completion of the exogenous pterin recycling system which would allow the conversion of phenylalanine to tyrosine, was also introduced to the auxotroph. The two apicomplexan PCD genes were expressed in the bacteria and were capable of rescuing the *E. coli* mutant.

This relatively simple rescue experiment proved the identity and functionality of the PCD proteins encoded by the genes derived from the *P. falciparum* and *T. gondii* genomes and introduced a novel aspect of metabolism into the sum of knowledge of apicomplexan biology.

1.5.4 Pterin recycling in the Apicomplexa, recent advances

The crystal structure of *T. gondii* PCD was determined in 2007. The researchers found that the enzyme could process (*R*)- and (*S*)-forms of pterin-4a-carbinolamine. There was a high degree of sequence and structural conservation of the parasite enzyme when compared with mammalian orthologues, suggesting that PCD has little promise as a potential drug target (Cameron *et al.*, 2008).

A paper by Andrew Hanson and colleagues looked at PCDs from a wide range of organisms. Plants were found to have two isoforms, one with PCD catalytic activity found in the mitochondria and another without this activity in the plastid. A number of organisms with functional PCDs were identified including angiosperms and yeast, where no AAH enzymes were evident to utilise the BH_4 cofactor produced by pterin recycling. Preliminary results suggested a role for the PCDs of these organisms in molybdopterin cofactor metabolism. It was also suggested that PCD could support hitherto unrecognised pterin-dependant enzymes (Naponelli *et al*., 2008).

Two AAH encoding genes were discovered in *T. gondii,* one of which was only expressed during the formation of bradyzoites. The recombinant AAH proteins produced from these genes proved to be bifunctional enzymes which catalysed the conversion of phenylalanine to tyrosine and tyrosine to L-3,4-dihydroxyphenylalanine (L-DOPA); this activity is unlike those of previously characterised metazoan enzymes which can convert only a single amino acid substrate. L-DOPA, a psychoactive chemical, is the precursor of dopamine,

and L-DOPA of parasite origin may be involved in the generation of those behavioural changes which have been noted in animals infected with *T. gondii* (Gaskell *et al*., 2009). A role for *T. gondii* PCD in recycling pterin and thus supplying BH_4 to support AAH activity has therefore been indicated. However, no *P. falciparum* AAH genes have been identified to date and the role, or roles, of the PCD enzyme in the wider metabolism of this organism remains unclear.

1.5.5 PTPS and the missing link in the plasmodial folate pathway

Interest in the pterin metabolism of *P. falciparum* led, indirectly, to the filling of a gap in the characterisation of the enzymes in the folate pathway of the organism. As related in the pterin paper (chapter 5) a gene encoding an enzyme other than PCD, but also associated with pterin metabolism in other organisms, was found in the *P. falciparum* genome. This was a putative orthologue of 6-pyruvoyl-tetrahydropterin synthase (PTPS).

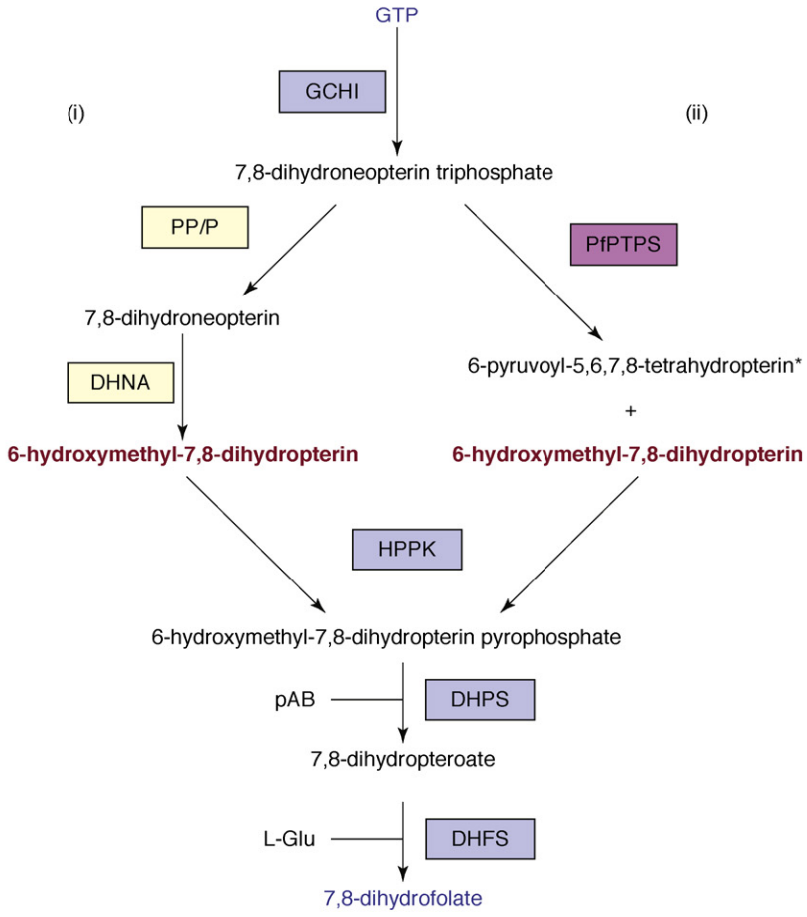

Fig. 3 The classic pathway of folate biosynthesis (i) and the role of PTPS in *P. falciparum* (ii). Reproduced from Hyde *et al*., 2008.

All but one of the genes encoding *P. falciparum* folate pathway enzymes had been isolated and characterised prior to 2008, the majority in the Hyde laboratory in Manchester. The remaining gene in the classical folate pathway yet to be characterised was that for dihydroneopterin aldolase (DHNA) which catalyses the third step in the pathway. The application of sophisticated bioinformatics techniques failed to identify a candidate gene and biochemical analysis of parasite extract also failed to find evidence of DHNA activity. The absence of any indication of DHNA in the parasite led to a consideration of possible routes around this metabolic lacuna. A gene encoding a PTPS orthologue, which had been identified in the parasite genome from earlier interest in pterin metabolism, suggested itself as a possible alternative to DHNA. It was discovered that parasite PTPS produced two products, the predominant of which was 6-hydroxymethyl-7,8-dihydropterin, the substrate for the fourth step in folate biosynthesis (the other product was 6-pyrovoyl-5,6,7,8-tetrahydropterin). This metabolic step therefore provided a bypass for the missing DHNA activity (Dittrich *et al*., 2008).

It has since become apparent that this alternative route for folate biosynthesis is not confined to *Plasmodium* but is found in other apicomplexans and various other taxa including heterokonts and some bacteria (Hyde *et al*., 2008). Conventionally, in animals, fungi and certain bacteria, PTPS is the second enzyme in the pathway of BH_4 synthesis. In these organisms PTPS converts DHNTP to 6-pyruvoyltetrahydropterin, which is then reduced by sepiapterin reductase to BH_4. The animal form of PTPS has been termed a PTPS-II type. Many bacteria have a PTPS paralogue, PTPS-I type, which has no role in BH_4 metabolism, but catalyses a step in the biosynthesis of the modified tRNA base queuosine. Some bacteria, such as *Cyanobacteria,* have both PTPS-I and PTPS-II type enzymes. The plasmodial PTPS, which functionally replaces DHNA, is classed as a PTPS-III type (Pribat *et al*., 2009).

1.5.6 Publication impact

"Functional identification of orthologous genes encoding pterin recycling activity in *Plasmodium falciparum* and *Toxoplasma gondii*" has been cited 4 times (ISI Web of Science and Google Scholar) in publications. This study introduced a

novel addition to known apicomplexan metabolism and facilitated finding the sole hitherto-uncharacterised gene of the folate pathway of malaria parasites.

1.6 Subcellular localisation of the folate pathway enzyme serine hydroxymethyltransferase (SHMT)

(Relating to the publication presented as chapter 6)

1.6.1 Background

The work on the subcellular localisation of the folate pathway enzyme serine hydroxymethyltransferase (SHMT) was the result of the intersection of a number of interests: the first was the folate pathway and how it functions in malaria parasites, the second was parasite cell biology and the last an interest in immunofluorescence microscopy as a tool for elucidating parasite internal structure.

The latter interest dates back to a study of microtubular structures and tubulin post-translational modification in erythrocytic stage parasites published in 1993 (Read *et al*., 1993b). This work showed, using light microscopy and immunofluorescence methods, the morphological changes in microtubular structures within the parasite through multiple mitotic events and revealed post-mitotic structures in the late schizont. The most surprising aspect of parasite cell biology from this work was evidence that the multiple mitotic events in erythrocytic schizogony were asynchronous. Mitotic asynchrony in *P. falciparum* has recently been confirmed by Arnot and co-workers who followed individual live parasites, which were fluorescently labelled, through erythrocytic schizogony (Arnot *et al*., 2010).

The subject of chapter 6, the enzyme serine hydroxymethyltransferase (SHMT), is part of the thymidylate cycle (see Fig. 2 – labelled ‘dTMP cycle’) within the folate pathway, which essential for DNA synthesis. SHMT reversibly catalyses the conversion of serine to glycine, whereby the hydroxymethyl group of the former is transferred to 5,6,7,8-tetrahydrofolate (THF) yielding 5,10-methylenetetrahydrofolate (5,10-methylene-THF), which is then used by thymidylate synthase (TS) as the one-carbon donor to convert deoxyuridine monophosphate (dUMP) to deoxythymidine monophosphate (dTMP).

There has been a great deal of research done on the biochemistry of the folate pathway in malaria parasites, partly due to the pathway being a target for important antimalarial drugs, but no work previous to that presented here had

been undertaken on the subcellular location of folate pathway enzymes or their metabolites. In higher plants subcellular compartmentalisation is a marked feature of the folate pathway (see Fig. 4).

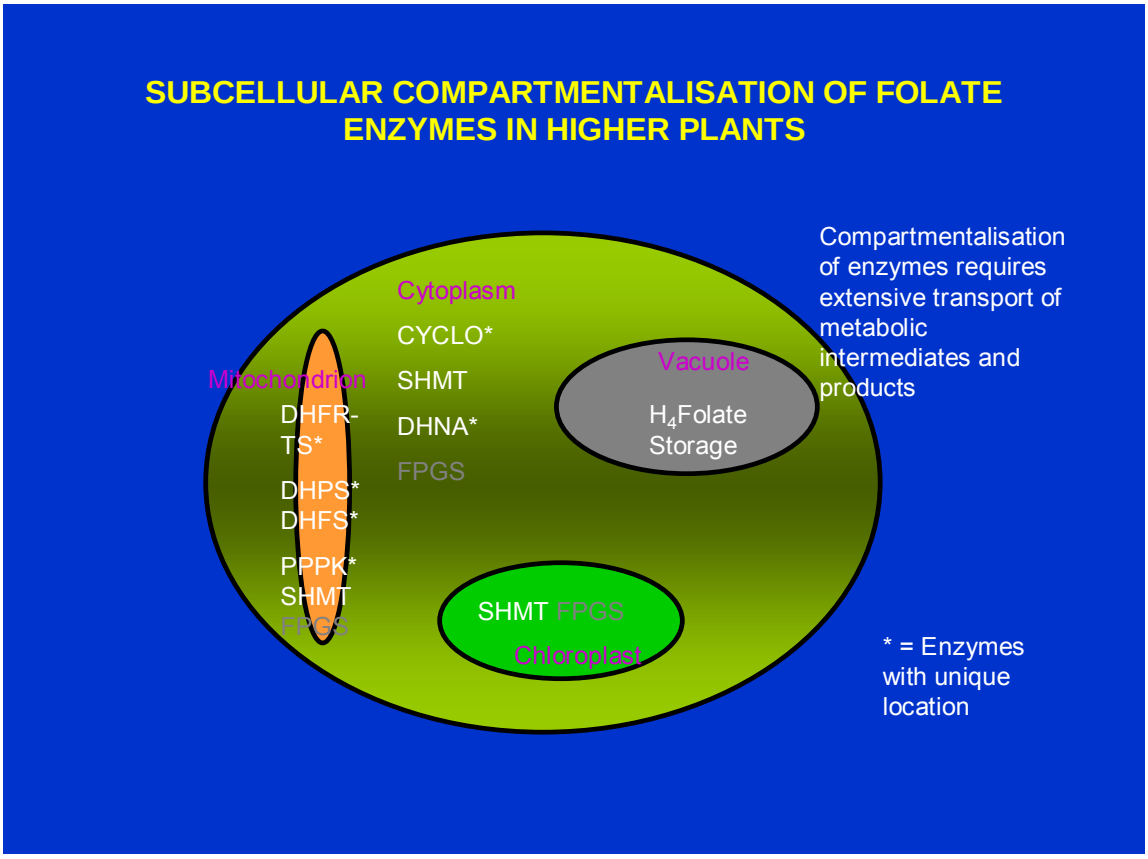

Fig. 4 Subcellular compartmentalisation of folate pathway enzymes in higher plants. SHMT isoforms are found in the cytoplasm and within two organelles: the chloroplast and the mitochondrion (CYCLO indicates GTP cyclohydrolase, other enzyme abbreviations are conventional – FPGS, folate polyglutamate synthase essential for folate homeostasis).

In plants many folate pathway enzymes are found in unique subcellular locations, for example DHPS is found only within the mitochondrion, whilst DHNA is entirely cytoplasmic. Isoforms of SHMT, in contrast, are found in the cytoplasm and in two organelles: the mitochondrion and in the chloroplast (Chen *et al*., 1997). A surprisingly close evolutionary relationship has been shown to exist between plants and the Apicomplexa, as was discussed earlier, thus the possibility of plasmodial folate pathway enzymes being found within organelles was considered a potentially rewarding subject for investigation.

The subcellular locations of a number of folate pathway enzymes: SHMT, DHFR, dihydrofolate synthase (DHFS) and guanosine triphosphate cyclohydrolase (GTPCH) were investigated in parallel, however, only the results for SHMT were in a state to be publishable when funding for the research failed to be renewed and further experimental work became impossible.

In *P. falciparum* there is apparently only one gene encoding a functional SHMT, referred to as PfSHMTc in the paper. This precludes the differentiation of isoforms of the enzyme into specific subcellular compartments as is found in plants. A second open reading frame occurs in *P. falciparum* that encodes a protein with an 18% identity to PfSHMTc and incorporates a putative mitochondrion-specific tag. This protein is described in the paper as PfSHMTm. It displays an almost complete lack of conservation of the amino acids that constitute the active site residues of all other SHMT isoforms. The subcellular distribution of both proteins was investigated.

The results of the research undertaken into the cellular distribution of SHMT were published in the paper "Dynamic subcellular localization of isoforms of the folate pathway enzyme serine hydroxymethyltransferase (SHMT) through the erythrocytic cycle of Plasmodium falciparum," which is included as chapter 6 of this thesis.

1.6.2 SHMT paper – problematic aspects of methodology

The major experimental problem to overcome in this study was that of gaining images of sufficient brightness when using immunofluorescence methods to visualise non-structural proteins in relatively low abundance. A number of proteomics studies have demonstrated the low abundance of folate pathway enzymes (Nirmalan *et al*., 2007; O'Cualain *et al*., 2010). The slightly unorthodox method adopted of exposing chemically fixed parasitised blood to both primary and secondary antibodies entirely in suspension, rather than attached to microscope slides, proved the most effective way of ensuring maximum exposure of target proteins to the relevant antibodies. Variations of methods involving the fixation of parasitized blood to the surface of microscope slides were tried, but gave uniformly weak fluorescence.

It was evident that extraction, using the non-ionic detergent Triton x100, of the parasites was advantageous to ensure penetration of antibodies to

subcellular compartments. Indeed a control preparation not employing Triton indicated that detergent extraction was a requirement for localised fluorescence using the apicoplast-specific antibody anti-acyl carrier protein (anti-ACP). This strongly suggested that extraction was an absolute necessity for the entry of antibodies to the apicoplast. However, detergent extraction introduced certain practical problems. Red blood cells from different donors, and indeed blood cells from a single donor with increasing time after donation, vary in robustness when challenged with Triton. A preparation of parasitised blood which lyses readily in Triton gives a different absolute exposure of the parasites to the applied antibodies, both primary and secondary, than a preparation of more robust erythrocytes. This produced a tendency for a variation in brightness of fluorescence, including that of the YOYO-1 stained DNA, between preparations which was difficult to predict.

Attempts were made to produce organelle preparations by differential lysis. It was hoped that the organelles, especially the apicoplast and mitochondrion, could be separated from the cytosolic components of the parasite by lysing the parasite but not the organelles. Organellar extracts, representing an organellar proteome, could then be probed with antibodies on a western blot. It was found that one round of freeze-thawing of parasites in water, of parasites that had been previously released from their erythrocytes by saponin lysis, lysed virtually all apicoplasts. However, mitochondria were considerably more robust and could withstand three to five rounds of freeze-thaw. Though not very useful for our purposes the method is potentially useful for those solely interested in the mitochondrion, as this organelle is difficult to investigate in isolation from the apicoplast to which it is closely applied, or even conjoined, through much of the erythrocytic cycle (Kobayashi *et al*., 2007).

1.6.3 SHMT paper – results

The first indication of the probable presence of folate pathway enzymes within an organelle was the occurrence of a discrete, elongated area of more intense fluorescence bordering the food vacuole (indicated by haemozoin) in some parasites at the late trophozoite to early schizont stages when using anti-DHFS. This was observed before any attempt was made to visualise the apicoplast or mitochondrion using probes specific to these organelles.

Further experimentation using organelle-specific markers showed that in addition to SHMT, which will be further discussed, DHFR, DHFS and GTPCH were all detectable within the apicoplast by immunofluorescence (M. Read, unpublished results).

To a degree these results ran counter to previous work which suggested that proteins found within organelles require targeted transit peptides in order to gain entry to the organelle (Waller *et al*., 2000; Foth *et al*., 2003). However, because of the difficulties in obtaining the separation of pure organellar preparations from *P. falciparum,* the complete organellar proteomes are unknown. It is probable that many proteins, particularly housekeeping enzymes, are found in multiple subcellular compartments, their movements possibly mediated by protein/protein interactions, by multiple distinct receptors and import machineries recognising different parts of a protein or vesicular transport mechanisms. If a protein were to be found in more than one organelle, and especially if a flux of protein to and from multiple organelles was occurring, then logic would dictate that transport mechanisms other than targeting using unique organelle-specific transit peptides attached to each protein would have to be employed. Because of the lack of a detectable apicoplast transit peptide motif in either PfSHMTc or PfSHMTm and their apparent presence within apicoplasts a great deal of effort was expended on the various controls described in the paper; this effort was made to minimise the possibility that the organellar location of these proteins, as visualised by fluorescence, was artifactual in origin.

The central result from the study was that PfSHMTc and PfSHMTm were to be found in the cytoplasm and in both the mitochondrion and apicoplast and that the organellar location of the enzymes varied in a dynamic manner through the erythrocytic cycle. These results are discussed fully in the paper.

An aspect of the paper which was novel in parasite research was the use of the Imaris image analysis software which allowed a quantification of the relative fluorescence co-localisation between anti-SHMT fluorescence and the fluorescence of organelle-specific probes (Costes *et al*., 2004). This enabled an estimate of the proportion of each SHMT protein to be found partitioned between an organelle and the cytoplasm. PfSHMTc showed a maximum

apicoplast location of 22.3% and mitochondrial location of 5.8%, for PfSHMTm this was even higher at 36.3% and 12.9% respectively. Therefore, mostly during the active mitotic division phase of schizogony, a considerable proportion of both proteins could be found within organelles. As was remarked on earlier, unpublished results from work carried out in parallel with SHMT localisation indicated that other folate enzymes were to be found within the apicoplast. Thus, sequestration of folate pathway enzymes, including the pyrimethamine target DHFR, within organelles could have implications on the effectiveness of anti-folate drugs. Target enzyme located within a membrane-bound organelle may not have as much, or indeed any, exposure to drugs in comparison to the same enzyme found in the cytoplasm.

The discovery of complementary amino acid sequence motifs on both PfSHMTc and PfSHMTm suggestive of the ability to form a stable but readily reversible heterotetramer is intellectually satisfying. It suggests a manner in which PfSHMTc might gain targeted entry into the mitochondrion when in complex with PfSHMTm, which contains an experimentally verified mitochondrion-specific transit peptide. It also gives the otherwise enigmatic, catalytically inactive, PfSHMTm protein a cellular function. The concentration of PfSHMTm in the tips of elongating apicoplasts, described in the paper, is also implies another possible role for this protein – an involvement in the morphogenesis of apicoplasts during schizogony.

Though the generation of hetrotetramers of PfSHMTc and PfSHMTm gives a possible mode of entry of enzymatically active SHMT into the mitochondrion it is unclear how both proteins gain entry to the apicoplast. However, the mitochondrion and apicoplast in *P. falciparum* are closely apposed or may even be connected by a junction through most of the erythrocytic cycle (Kobayashi *et al*., 2007; Sato, 2011). Pino and co-workers have shown that dual targeting of proteins to both mitochondrion and apicoplast occurs in *T. gondii* (Pino *et al*., 2007), and they state: “This study shows that dual protein targeting to the mitochondrion and apicoplast may be a widespread phenomenon in apicomplexan parasites, and that this complex targeting activity is made possible by more than one molecular mechanism.” It is apparent that more than one possible mechanism exists to allow the entry of both SHMT proteins, and indeed other folate enzymes, into the apicoplast.

1.6.4 Publication impact

As a relatively recent paper, published in December 2010, in a field generally requiring a long optimisation period before useful data can be produced no publications have yet cited the SHMT subcellular localisation paper. However, the paper, published in an online journal in order to allow the many micrographs to be optimally viewable, reached “Highly Accessed” status within a few days of it becoming available.

1.7 General Discussion

The book chapter and papers which form the core of this thesis have a number of connections and interlocking themes. The chapter on *in vitro* cultivation of *P. falciparum* is concerned with the basic techniques on which all of the investigation of parasite biology described in the following papers is predicated. Without a robust and reliable culture method the metabolism of any unicellular organism is a closed book. The primary papers are all analyses of aspects of central metabolism within the parasite. They are presented in chronological order, though other orderings or groupings were possible. Presenting them in this manner has allowed the advances in practical techniques, equipment and other experimental resources to be shown as a readily comprehensible progression.

Arguably the key resource to become available over the period covered by the research presented here is the complete *P. falciparum* genome sequence. It is difficult to overstate the revolution that this development has made to research into the biology of the malaria parasite. The increased facility in isolating genes of interest, and in assigning their chromosomal location, afforded by the annotated genome is best illustrated by a comparison of the methods used in the enolase paper and those of the PCD paper. Other developments, especially in PCR techniques, DNA sequencing and in heterologous expression systems have made the isolation and characterisation of genes and the proteins they encode very much faster and less labour intensive. In the absence of recent advances in light microscopy, particularly the development of laser scanning confocal microscopy, co-localisation quantification methods and the increasingly wide range of commercial fluorochrome-conjugated secondary antibodies, the work presented in the SHMT paper would have been impossible.

Other advances in techniques and approaches have been made over the period covered by the publications herein, some of which have been alluded to already. Proteomics, the study of protein structure and function often at the level of a biological system or of the whole organism, and metabolomics, a similar approach directed at the small molecules such as metabolic substrates and cofactors found in biological systems, have already yielded important

advances in knowledge of parasite metabolism. Bioinformatics, the *in silico* investigation and modelling of biological structures and processes, has produced many useful pointers to aspects of metabolism that have yet to be investigated directly. However, the metabolic pathways or steps indicated by these methods require experimental verification before they can be accepted as entirely accurate. The recently developed field of systems biology, incorporating a holistic approach to biological systems in opposition to the usual reductionist scientific method, has yet to produce tangible advances in parasite knowledge and has, to date, failed in general to achieve concrete results commensurate with its early promise.

The particular papers contained in the thesis were selected, in part, because they represent various points along the continuum of research required for the characterisation of a metabolic pathway in an organism. The work on PCD shows the discovery of a novel metabolic process in malaria parasites, one with little or no prior indication. The isolation and DNA sequencing of the enolase gene was carried out on a metabolic pathway which had been partially characterised and this study filled in a missing enzyme-encoding gene from a metabolism well known in other organisms. Both the sulfadoxine resistance and SHMT papers describe aspects of what can be learned about specific enzymes, and their place in the biology of the parasite, once relevant genetic data are available. The sufadoxine resistance paper made use of previously characterised parasite genotypes and a sexual cross to investigate the mechanism of resistance to an important antimalarial drug. The SHMT paper was reliant on gene sequences to heterologously generate recombinant proteins, or protein fragments, to use in the generation of antibodies. Knowledge of a metabolic pathway in the absence of information on its location within a cell is incomplete. The subcellular location of enzymes can provide important information on the relation of a particular pathway to the general metabolism and biology of an organism, point to possible moonlighting functions of enzymes, and, in pathogens, indicate the possibility of sequestration of drug target enzymes within membrane-bound organelles. Such sequestration of enzymes may present an impediment to therapy as the enzymes may be partially or wholly shielded from drug action.

All of the papers exhibit an aspect of scientific research worth a degree of comment, that of serendipity. The paradigm of scientific serendipity is the well known story of the discovery of penicillin by Alexander Fleming. He noted that the growth of *Penicillium notatum* mould, an accidental contaminant, on an agar plate of *Staphylococcus* destroyed colonies of the bacterium in their vicinity. This fortuitous event presaged the development of antibiotics which in turn revolutionised the treatment of infectious diseases of bacterial origin (Hare, 1970). The work on enolase was intended to characterise the gene encoding this enzyme as a prelude to studying its biochemical properties, however, it also gave an intriguing glimpse into the deep evolutionary history of the Apicomplexa and hinted at the lateral transfer of parts of genes between unrelated species. Earlier studies had implicated point mutations within the *dhps* domain of the *hppk-dhps* gene as the determinant of resistance to sulfadoxine. This was confirmed by the work presented here, but an unexpected role for the salvage of exogenous folate in antagonising the effect of the drug, in parasites of a particular genotype, was also discovered. The detection of pterin cycling activity within *P. falciparum* drew attention to a gene, *ptps*, which proved to encode the missing link in the enzymes of the malarial folate pathway. Finally, exploring the subcellular location of SHMT gave unexpected insights as to possible roles for the otherwise enigmatic, enzymatically inert, PfSHMTm protein, both in allowing entry of the enzymatically active PfSHMTc protein into the mitochondrion and also, as indicated by distinct concentrations of PfSHMTm within the distal regions of elongating plastids, a potential function in the morphogenesis of apicoplasts during schizogony.

Since the mid 1980s there has been considerable progress in understanding the metabolic processes occurring in malaria parasites. The present state of knowledge is probably most easily comprehended by perusal of the very useful “Malaria Parasite Metabolic Pathways” website maintained by Hagai Ginsburg (http://sites.huji.ac.il/malaria/). Although all the enzymes on this site that are flagged as definitely existing in the parasite have been at least identified at the level of the encoding gene within the genome, the existence of other enzymes is merely inferred, either from the detection of metabolic end products, or from the identification or characterisation of other genes of the relevant pathway. The various metabolic pathways of the parasite have been

unevenly investigated. Arguably those most fully explored are the glycolytic, folate, shikimate and, recently, TCA metabolisms. The shikimate pathway, though incompletely characterised in *P. falciparum*, is especially interesting as it does not occur in animals and therefore presents a potential target for drug development (McConkey, 1999). Shikimate metabolism also has links into both glycolysis and the folate pathway.

As to the aims and achievements of the work presented here, those of the individual studies are outlined within each publication, and have been further addressed earlier in this chapter. Unlike the conventional PhD, which of necessity is constrained to cover a relatively narrow field of research conducted over three or four years, the format of the PhD by publication has allowed a series of interconnected studies undertaken over two decades to be presented as a synthetic whole. This has permitted elements of scientific history, including the development of technical innovation and evolution of scientific approaches, to be presented to an unusual extent. Furthermore, a standard thesis shows the body of knowledge on a subject only up to the point when the practical work was completed, this thesis includes many descriptions of work undertaken subsequent to a study being published, allowing a more complete description of the development of a particular research field over time. If this thesis has been successful in highlighting the developmental nature of research into malaria metabolism over the last quarter of a century it will have achieved the aims its author intended.

Though there remain very large gaps in our knowledge of malaria parasite metabolism and parasite biology in general, a great deal of very painstaking and successful research has been carried out in recent decades. The parasitological community has now a vastly increased understanding of how malaria parasites function. The publications presented here have played a small but, hopefully, not entirely insignificant part in this process. So as not to finish on a hubristic note, however modest, it is sobering to note that malaria as a disease is imposing much the same levels of mortality and morbidity on some of the poorest populations in the world as it did when the earliest of the works contained in this thesis was published almost twenty years ago.

Chapter 2

Simple *in vitro* cultivation of the malaria parasite *Plasmodium falciparum* (erythrocytic stages) suitable for large-scale preparations

Published in 1993 as the fourth chapter in the book:

Protocols in Molecular Parasitology, J. E. Hyde, Editor. Humana Press, Totowa, New Jersey. Pages 43-55.

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CHAPTER 4

Simple In Vitro Cultivation of the Malaria Parasite *Plasmodium falciparum* (Erythrocytic Stages) Suitable for Large-Scale Preparations

Martin Read and John E. Hyde

1. Introduction

Malaria represents the world's greatest public health problem in terms of number of people affected, and the levels of morbidity and mortality. The protozoan malaria parasites (*Plasmodium* spp.) are transmitted by infected female mosquitoes when feeding on blood. Parasites soon enter liver cells, and after several days of multiplication, are released into the bloodstream where further cycles of asexual reproduction occur, giving rise to the clinical symptoms of malaria. Some erythrocytic parasites will differentiate into presexual forms (gametocytes; *see* Chapter 6), which when taken up by mosquitoes in further blood meals, mature into gametes and undergo a sexual cycle. With the eventual release of infective sporozoites into the mosquito salivary glands, the life cycle of the parasite is completed.

Research into the most pathogenic of the human malaria parasites, *Plasmodium falciparum*, has expanded dramatically in the last fifteen years, not only because of the advent of recombinant DNA technology, but also because of the demonstration in 1976 that the

From *Methods in Molecular Biology, Vol 21 Protocols in Molecular Parasitology*
Edited by John E Hyde

organism could be cultured in vitro essentially indefinitely *(1)*. This rendered *P. falciparum* much more accessible as an experimental organism, and since that time, improvements to and simplifications of the original "candle-jar" method have been made (e.g., *2–4)*. In a small number of laboratories, continuous culture of *P. falciparum* has been successfully automated to cope with the more repetitive aspects of the procedure, particularly when large amounts of parasite material are required *(5,6)*, but this entails setting up specialized and complex equipment that can be prone to irritating and costly problems.

The great majority of laboratories culture the asexual blood stages of malaria parasites that provide DNA, RNA, and protein suitable for most types of experimentation. There are many variables involved in culturing, and many factors that contribute to optimal parasite growth. The method for cultivation of asexual stages described here is relatively simple, requires the minimum of components, and is not particularly labor intensive. It has been successfully used in our laboratory for over seven years to provide the large numbers of parasites required for DNA, and especially RNA studies.

2. Materials

2.1. Equipment

1. A class 2 sterile flow cabinet, preferably with a gas supply so that a burner (fitted, if possible, with a foot or hand switch) for flaming bottle necks can be used inside it. All manipulations of parasite cultures and media must be undertaken in conditions of scrupulous sterility *(see* Note 1).
2. A supply of sterile plastic tissue culture flasks (e.g., Falcon or Nunclon); 50 and 250 mL are the most useful sizes.
3. Singly-wrapped sterile disposable graduated plastic pipets (2 and 10 mL).
4. Pasteur pipets (long-form) plugged with cotton wool. These can be wrapped in aluminum foil, in groups of six, for autoclaving.
5. An automatic pipetor for use with serological pipets (invaluable, because a great deal of repetitive pipeting is necessary).
6. Autoclavable screw-capped glass storage bottles (e.g., Duran); 250-mL, 500-mL, and 1-L bottles are the most suitable. These bottles must be silane treated *(see* Note 2) before use with blood or nonheat-treated plasma as untreated glass tends to promote clotting.
7. Sterile 10- and 50-mL plastic centrifuge tubes.

8. Equipment for both small-scale (up to 500 mL) and large-scale (500 mL to 4 L) filter-sterilization is required for preparation of medium. For small-scale filtration a 25-mm diameter Swinnex type filter holder (Millipore) is adequate when used with a 20-mL syringe. For large-scale filtration, a 90-mm diameter disk filter in an autoclavable steel tripod type holder is necessary. A 40-mm diameter prefilter holder is also useful. A Millipore peristaltic pump (cat. no. XX80 200 00, using 3/16 in. internal diameter silicone tubing) is the recommended means of passing medium through the large-scale filters. The prefilter is a 40-mm AP15 depth filter, and the 25- and 90-mm filters are 0.22 μm pore-size membranes (Millipore).
9. A cylinder of a 5% CO_2, 5% O_2, 90% N_2 mixture is necessary for gassing the culture flasks, because the parasites prefer a high CO_2, low O_2 environment.
10. A 37°C incubator reserved solely for use with *P. falciparum* cultures.
11. A microscope capable of good resolution with the use of a 100x oil immersion objective lens, together with prewashed microscope slides. Slides with frosted ends are recommended for ease of indexing as they can be written on with a pencil.

2.2. Reagents

1. Human blood is the primary reagent in the culture of *P. falciparum*; type O^+ is used because it is compatible with serum or plasma from any blood group. Whole blood is the most widely used form, however, we have found that various forms of plasma-reduced blood (e.g., SAG-M blood or red cell concentrate) can be used without detriment *(7)*. Blood and blood products can often be obtained gratis from blood transfusion services. Blood is stored in silanized bottles at 4°C and is usable up to 1 mo from the donation date *(see* Note 3).
2. Components of human serum are essential additives to the culture medium, and human serum is conventionally added to 10% of the final volume. However, supplies of such serum can be problematic as many blood bank laboratories no longer produce it, preferring the convenience of working directly with plasma. This need not cause difficulties because nonheat-treated plasma, when used as a medium additive, produces results as good as, or better than serum *(7,8)*. The tendency for untreated plasma to form clots can be overcome by the use of plastic or silanized glass storage vessels *(see* Note 4).

Plasma is combined from at least 2 donors (preferably 4–6) before use to minimize variations of quality between individuals. It is aliquoted into 10- and 50-mL plastic centrifuge tubes (suitable for small and large scale cul-

tures, respectively) and stored at –20°C. These frozen aliquots have a useful life of approx 6 mo.

3. The culture medium used is RPMI 1640 containing 25 m*M* HEPES and 0.3 g/L L-glutamine (e.g., GIBCO-BRL, Gaithersburg, MD, cat. nos. 079-3018A, 1 L powder; 041–2400H, 100 mL liquid). To this is added $NaHCO_3$, to 2 g/L, glucose to 4 g/L final conc. (the medium is supplied containing 2 g/L glucose) and gentamycin to 50 mg/L. Liquid medium in the form of 100-mL bottles is convenient for use in small-scale cultures. Medium in powder form, to be made up to 1 L final volume, is more economical for large-scale preparative cultures (since they require up to 4 L from setting up to harvesting). Medium is stored at 4°C, both before and after the addition of 10% plasma or serum. After the plasma or serum is added, the medium has a life of about 1 mo.

For a stock solution, glucose is dissolved to 40% (w/v). This can be autoclaved, but at not more than 10 psi for 15 min to avoid caramelization, after which it is stored at 4°C. Gentamycin is made up to 50 mg/mL, filter sterilized, aliquoted into 1 mL amounts, and stored at –20°C.

4. Giemsa stain (improved R66, "Gurr," Merck), diluted 1:10 in Sorensen buffer (often sold as "Giemsa stain buffer solution concentrate"), is used to stain parasites in thin blood smears for microscopy *(see* Note 5).
5. Phosphate buffered saline (PBS) is used for washing red blood cells before use. For 1 L: 8.0 g NaCl, 0.2 g KCl, 1.14 g anhydrous Na_2HPO_4, 0.2 g KH_2PO_4. Adjust pH to 7.4 and autoclave before use. Store at 4°C.
6. Ringer's saline is used in the liquid N_2 preservation of parasites. For 1 L: 9 g NaCl, 0.42 g KCl, 0.25 g $CaCl_2$. Autoclave before use and store at 4°C.
7. Dimethyl sulfoxide (DMSO), cell culture grade, in aseptically filled ampoules (Sigma).

3. Methods

3.1. Preparation of Medium

1. If human serum is being used, go to step 2. When plasma is added to the medium it has a tendency to clog filters during sterilization. This can be minimized by use of the following simple procedure. The insoluble protein often evident as a cloudiness in plasma cannot be separated out by bench centrifugation under normal circumstances. However, when thawing frozen aliquots of plasma at 37°C, at the point where the last frozen part has just melted, the insoluble protein forms a flocculent white mass. In this state the protein can be pelleted by spinning in a bench centri-

fuge for 5 min at 2500 rpm (ca. 800*g*). Removal of this protein fraction does not compromise the ability of the medium to support parasite growth.

2. Small amounts of medium are most easily prepared using 100-mL bottles of sterile proprietary medium. Plasma or serum is passed through a 0.22-μm Swinnex filter directly into the medium using a 10- or 20-mL syringe. The other additives are from presterilized aliquots *(see* quantities in Section 2.2.).
3. Larger amounts of medium (up to 4 L) are prepared from RPMI powder. This is dissolved in a flask (well rinsed in deionized water) in deionized, distilled water *(see* Note 6), and the additives described in Section 2.2. (except plasma or serum) mixed in. The pH is adjusted to 7.45 (at room temperature) with 3*M* NaOH. The plasma or serum is now added and mixed in thoroughly with a magnetic stirrer. The complete medium is filtered using a peristaltic pump through an AP15 prefilter (40 mm diameter) then a 0.22-μm filter (90 mm diameter), or Sterivex cartridge *(see* Note 7) into a sterile storage bottle *(see* Note 8). If serum has been used, the AP15 prefilter can normally be omitted.

3.2. Washing Blood

Blood is washed immediately before use in culturing to remove leukocytes and the preservatives added on donation. Blood is placed in a centrifuge tube and an equal volume of PBS is added and mixed by inversion. For small scale preparations 10-mL sterile centrifuge tubes are adequate; for larger preparations (>20 mL) 50-mL tubes are more appropriate. Spin in a bench centrifuge at 2500 rpm (ca. 800*g*) for 5 min. The white blood cells form a pale layer (the buffy coat) on the surface of the red blood cells. This, together with the clear supernatant, can be taken off using a serological pipet. The procedure is repeated three times for use in standard maintenance cultures, or five times when cultures are destined for eventual DNA or RNA extraction (to ensure minimal contamination by host nucleic acids, *see* Note 9). After washing, resuspend the remaining packed red blood cells in an equal volume of complete medium (50% hematocrit, *see* next section).

3.3. Setting up a Culture

Two terms may usefully be defined: *hematocrit* refers to the percentage volume of cells to liquid in blood; *parasitemia* is the term used to express the percentage of blood cells containing parasites.

1. From microscopical observation *(see* Section 3.6.) the parasitemia of an existing culture can be ascertained. The volumes of parasitized and fresh blood needed to set up a new culture at a different (lower) parasitemia at any hematocrit can be calculated as follows:

Vol of freshly washed blood (at 50% hemat.) needed for new culture=

$$\frac{(\text{New hemat.} \times \text{Final vol of culture required})}{50\%} \quad (1)$$

Vol of inoculum of parasitized blood=

$$\frac{(\text{New parasitemia} \times \text{Vol of fresh blood [from above]})}{\text{Original parasitemia}} \quad (2)$$

Note that the parasitized blood in the layer on the floor of a culture flask is deemed to be at 50% hematocrit when the overlying medium has been pipeted off. If the volume of the inoculum calculated from Eq. (2) is 10% or less than the volume of fresh blood from Eq. (1), then it can be ignored. If not, then reduce the volume of fresh blood used by the volume of the inoculum.

2. In 50-mL tissue culture flasks, 1 mL of blood (50% hematocrit) is added to 10 mL of complete medium (i.e., approx 5% final hematocrit). In the larger (250-mL) flasks, 10 mL of blood is added to 50 mL of medium (i.e., approx 10% final hematocrit, *see* Note 10). Both blood and medium must be at 37°C before the inoculum of parasitized blood is added *(see* Note 11). The inoculum can be added using a micropipet. Micropipets reserved solely for tissue culture work should be used if possible, while tips containing an inert filter plug (e.g., Aerogard, Alpha Laboratories, Eastleigh, Hants, UK) prevent cross-contamination *(see* Note 12).
3. After the flasks have been inoculated they must be gassed. Pass the gas via a tube from the cylinder through a plugged disposable sterile 10-mL pipet, into the neck of the flask. Small flasks are gassed for 15 s, the larger flasks for 30 s *(see* Note 13). The flasks are then incubated at 37°C after closing the lids tightly.
4. Cultures are typically initiated at a parasitemia of 0.5% which will produce (in 50-mL flasks) a parasitemia of 15–25% over the course of 5 d *(see* Note 14). The health of the parasites suffers if they are in a culture of high parasitemia for more than a few days, so that parasitemias in excess of 30% are not normally achievable.

3.4. Changing Medium

1. Medium is normally changed daily. This can cause problems at weekends; however, this can be avoided as the need for medium change is less at low parasitemias. A culture set up on a Friday at 0.5% parasitemia, 5% hematocrit

(50-mL flask) will not require a change of medium until the following Monday. 250-mL flasks set up at 0.5% parasitemia, 10% hematocrit will require a medium change on Sunday (but for an alternative strategy, *see* Section 3.5.).

2. The parasitized blood forms a layer on the floor of the flask, so that spent medium can be drawn off from above by pipet. Flasks should be removed very carefully from the incubator to the microbiological cabinet to minimize disturbance of the settled cells. Otherwise, a significant amount of parasitized blood will be drawn off with the spent medium. The latter can be removed from small flasks using a Pasteur pipet and an automatic pipetor. Make sure that a fresh pipet is used for each flask to prevent the spread of any possible contamination from one flask to another *(see* Note 12). This method can also be used (substituting 10-mL pipets) for small numbers of large 250-mL flasks. For large numbers of 250-mL flasks, however, this approach is somewhat inefficient and tiring. A more effective method is to use a Pasteur pipet connected by an autoclavable silicone tube to the side arm of a Buchner flask (to trap the medium) which is in turn attached via an upper outlet through a bung to a peristaltic pump.
3. Fresh medium at 37°C is then added, using a pipet for small flasks or pouring directly from the storage bottle for large flasks (bottle necks should be flamed frequently).
4. Resuspend the red blood cells by gently swirling the flasks. Finally, gas the flasks before placing them in a 37°C incubator once again *(see* Notes 13,15,and 16).

3.5. Modified Method for Large-Scale Parasite Preparations Suitable for DNA/RNA Extraction

1. Set up 16–20 large flasks on Friday at 2% hematocrit (2 mL blood + 50 mL medium per flask), 0.1% parasitemia. These can be left over the weekend without a medium change *(see* Note 17).
2. On the following Monday, parasitemias of 3–6% should have been reached. Medium is changed, then 8 mL of freshly washed blood is added to each flask to bring the hematocrit to 10%.
3. The medium is changed daily until Thursday of the same week when the flasks can be harvested for DNA/RNA *(see* Chapter 11 for protocols). At harvest, parasitemias of 4–8 % can be expected, giving roughly 5×10^{10} parasites in the total preparation.

3.6. Thin Blood Smears and Microscopy of Parasites

1. Before making a blood smear, the glass slide to be used should be annotated with a pencil to show the date, parasite strain, and any other relevant information

2. Concentrated red blood cells from the floor of a culture flask are taken up in a Pasteur pipet with a roughly equal volume of the overlying medium (approx 10 µL total). This is placed as a drop close to the margin of a microscope slide.
3. Using the short edge of another slide, smear out the drop over the surface; this must be done with a swift and smooth action (some practice is needed to produce good results). If carried out correctly, the smear will consist of a monolayer of well spaced red blood cells. Take at least two slides from each flask.
4. Each slide is flamed very briefly, then fixed in 100% methanol by irrigation from a wash bottle. The slides are allowed to air dry, meanwhile, re-gas the flasks and return them to the 37°C incubator.
5. When dry, stain the slides for a minimum of 20 min in Giemsa (which stains nuclear material red/purple) diluted 1:10 in Sorensen buffer.
6. Wash the slides under running water for no more than 20 s. After air-drying, they are ready for viewing.
7. Lens immersion oil is placed directly on the blood smear (no coverslip is necessary). Slides treated in this manner can be stored indefinitely.
8. The parasitemia is calculated by counting 500–1000 red blood cells and noting the number containing parasites (double or triple infections are counted as one). A graticule (to divide the field of view into conveniently sized squares) is indispensable for accurately counting cell numbers, as is a hand-held counter *(see* Note 18).

3.7. Liquid N_2 Preservation of Parasites

1. To preserve parasite strains in liquid N_2, cultures are grown (in small flasks) to a parasitemia of 15–20%. A reasonably high proportion of these parasites should be at the early ring stage, as these best survive liquid N_2 preservation.
2. The parasitized blood from such a culture is brought to a hematocrit of 50% by centrifugation and resuspension (as described in Section 3.2.) in complete medium.
3. 0.5 mL of this suspension is then placed in a sterile 3-mL cryotube and 0.5 mL of 20% DMSO in Ringer's saline is added. This is immediately snap-frozen by immersion in liquid N_2.

3.8. Retrieval of Parasites from Liquid N_2

1. Thaw cryotubes in a water bath at 37°C for 2 min.
2. Transfer the thawed contents to a microfuge tube and centrifuge (12,000–14,000 rpm; ca. 10,000*g*, for 1 min), then remove the supernatant.
3. Resuspend the pellet in 1 mL of 10% sorbitol in PBS (this is added very slowly with continuous mixing, *see* Note 19).

4. A further centrifugation follows, the supernatant is removed once more and the pellet is resuspended in 1 mL 5% sorbitol in PBS (again with mixing). This is centrifuged again and the supernatant removed.
5. Wash the cells in 1 mL complete medium, then place in a culture flask with 10 mL complete medium plus 0.5 mL newly washed blood.

4. Notes

4.1. General

1. The source of any bacterial or fungal contamination can be investigated by streaking out blood, medium, and other reagents on suitable agar plates. The possibility of an improperly functioning sterile cabinet must also be considered. Contamination by mycoplasmas is more problematic as they are less easily detected and are difficult to treat (although commercial antimycoplasma preparations are available). In all cases of contamination, after investigating the source of the infection, the use of uncontaminated parasites retrieved from liquid N_2 or from other known noninfected sources is recommended.
2. Glass storage bottles are silane-coated as follows: Bottles are washed with conc. hydrochloric or chromic acid and rinsed thoroughly with distilled water. Dried bottles are partially filled with dimethyldichlorosilane solution (Merck) and the entire internal surface brought into contact with the liquid. After pouring off, the bottles are baked at 180°C overnight, then washed extensively with deionized, distilled water.
3. The majority of blood donors in temperate countries are unlikely to carry anti-*Plasmodium* antibodies. However, the possibility of blood used in culture containing such antibodies should be kept in mind.
4. Heat-treated plasma (i.e., plasma held at 56°C for 1 h, routinely carried out in blood transfusion centers) may also be used. This reagent, however, although not prone to clotting, has two disadvantages: It produces less vigorous cultures, yielding lower parasitemias, and it does not filter-sterilize well, tending to clog filters *(7)*. We therefore specifically request that our plasma be left untreated.
5. The improved R66 version of Giemsa is claimed to have a longer shelf life than other formulations, but other Giemsa stains will produce acceptable slides
6. Water quality is a vital factor. All medium and solutions that come into contact with parasitized blood must be made with deionized, distilled water, because parasites are very sensitive to dissolved impurities.
7. A Sterivex 0.22-μm disposable cartridge (Millipore) can be used in place of a 90-mm filter, but will not filter more than 1 L of medium containing plasma before blocking. A 45-μm (40 mm diameter) filter can be

added to the prefilter, to extend the filtering capacity of the Sterivex cartridge. However, when a 90-mm filter (steel tripod) is used, the 45-μm filters tend to clog long before the main filter and are inconvenient to replace during filtration.

8. Storage bottles for medium, blood, and solutions should not be washed using a detergent, since parasites are affected by traces that remain after rinsing. Excess or outdated blood or medium is poured from storage bottles into a hypochlorite solution for disinfection; the bottles are immediately filled with water (to prevent proteinaceous material "baking" onto the glass) and autoclaved. The bottles can now be washed using a bottle brush in tap water followed by extensive rinses in deionized distilled water. The bottles are then autoclaved once more before reuse
9. A single mammalian white blood cell contains roughly as much DNA as 500 malaria parasites. The extra washes are to ensure that essentially no human DNA is carried over into subsequent experiments.
10. The medium:blood ratio in the larger flasks is lower and somewhat suboptimal, but using 10 mL of blood, gas exchange with the cells becomes too inefficient if the ratio is made as high as in the smaller flasks. Although final parasitemias attainable in the large flasks will be lower than in the smaller flasks, the great saving in labor involved in processing the smaller number of large flasks required to reach a given overall number of parasitized cells easily offsets this disadvantage.
11. To save time, the medium on being taken from 4°C can be rapidly warmed in a microwave oven; however, care is needed to avoid overheating, and calibrations are usefully performed beforehand on bottles with equivalent volumes of water.
12. Cross-contamination of strains has been known to occur in a number of laboratories. To avoid this, clearly and indelibly marked flasks and tubes should be used if strains are being grown in parallel, and great care should be taken never to use the same pipet or pipet tip when processing flasks containing different parasites. If more than one strain is being brought out of liquid N_2 storage into flask cultures, it is safer to carry out the operation consecutively with each strain, rather than simultaneously with all of them.
13 When gassing a culture flask, the flow rate should be moderate, but strong enough to ruffle the surface of the liquid.
14. Several well characterized strains, as well as a large number of other strains (*P. falciparum* and rodent parasites), are available from the WHO Registry of Standard Strains of Malaria Parasites, c/o D. Walliker, ICAPB, Genetics Building, University of Edinburgh, West Mains Road,

Edinburgh EH9 3JN, Scotland, UK. For the introduction into culture of new strains derived from clinical cases, *see* ref. *9*.

15. For the maintenance of parasite isolates in continuous culture (in small flasks), a cycle of subculturing on Mondays and Fridays is recommended.
16. When introducing a new batch of complete medium or blood it is prudent to initially retain a parallel culture maintained in a previous batch. This will guard against any unsuitability or contamination in the new reagent.
17. When setting up a large-scale culture (e.g., for DNA extraction), set up one or two small flasks at the same time in order to maintain the isolate for continuing culture (if desired).
18. The methods described here include devices designed to avoid difficulties arising in the culture of *P. falciparum*. However, problems do occur, and it is important that the operator can recognize the early stages of distress in the parasites. To achieve this, it is recommended that a culture grown to a fairly high parasitemia is left without a medium change after the 5th day and slides taken from it over the following few days. These will show the deterioration in the condition of the parasites, the early stages of which are recognizable by the "condensed" appearance of the parasites, which are also more intensely stained with Giemsa. Loss of health is usually caused by adverse factors in blood or plasma, and changing one or both of these is the first remedy to try. Note that blood more than 4 wk after donation can lose its ability to support parasite growth quite rapidly.
19. The method for achieving continuous mixing when adding sorbitol solutions in the retrieval of parasites from liquid N_2 requires some dexterity. With one hand, the sorbitol solution is slowly added using a micropipet, while simultaneously, using the other hand, the blood suspension is repeatedly pumped (with a gentle action) in and out of a Pasteur pipet.
20. Parasites in culture can be metabolically labeled with radioactive compounds such as ^{35}S-methionine or ^{3}H-glucosamine after switching into the appropriate medium. For details, *see* refs. *10–12*.

4.2. Safety

21. Infections with laboratory cultured *P. falciparum* blood-stage forms are extremely rare under conditions of routine maintenance. The use of syringes with needles to inoculate cultures should obviously be avoided. In the unlikely event of a glass Pasteur pipet carrying malaria-infected blood penetrating the hand of the operator, the incident should immediately be reported according to local accident regulations. As a precau-

tion, the drug-resistance status of each strain cultured should be ascertained (if possible) so that appropriate antimalarial drugs can be prescribed if infection is suspected.

22. Blood and plasma/serum should only be obtained from reliable sources, where screening for the presence of dangerous pathogens (e.g., HIV and hepatitis viruses) is routinely undertaken.
23. Spent medium is treated by addition of a hypochlorite disinfectant (e.g , Chloros) to a conc. of 10% free chlorine before disposal.

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Chapter 3

Molecular characterization of the enolase gene from the human malaria parasite *Plasmodium falciparum* - evidence for ancestry within a photosynthetic lineage

Published in 1994 in:

The European Journal of Biochemistry, volume 220,

pages 513-520

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Eur. J. Biochem. 220, 513–520 (1994)

Molecular characterisation of the enolase gene from the human malaria parasite *Plasmodium falciparum*

Evidence for ancestry within a photosynthetic lineage

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(Received October 18/December 7, 1993) – EJB 93 1567/2

We have isolated and characterised the gene encoding the glycolytic enzyme enolase (2-phospho-D-glycerate hydrolase) from the human malaria parasite *Plasmodium falciparum*. This was achieved using a combination of cDNA sequencing and inverse-PCR techniques. The gene maps to chromosome 10 of the parasite. We have also mapped two further glycolytic enzyme genes, glyceraldehyde-3-phosphate dehydrogenase and triose-phosphate isomerase, to chromosome 14. The enolase gene encodes a protein of 446 amino acids (48.7 kDa), and all amino acid residues implicated in substrate/cofactor binding and catalysis are conserved in the malarial enolase molecule. The predicted protein sequence displays approximately 60–70% identity to enolase molecules of other eukaryotes, the closest relationship with its homologues seen amongst the seven fully described glycolytic pathway enzymes of *P. falciparum*. Of particular significance in this well conserved molecule is a characteristic 5-amino-acid insertion sequence that is identical in position and virtually identical in primary structure to that which is otherwise found uniquely in plant enolase proteins. This pentapeptide, together with other features of the plasmodial sequence, points to a common ancestry with photosynthetic organisms at the level of a protein-encoding nuclear gene, thus extending earlier analyses of nuclear small-subunit ribosomal RNA genes, and of an extrachromosomal circular 35-kb DNA element found in *P. falciparum*, which have also indicated such a relationship.

The glycolytic pathway has a number of features which suggest that it is particularly important to the blood-stage forms of the human malaria parasite *Plasmodium falciparum*, and thus make it an attractive candidate for experimental investigation. The level of glycolysis of parasite-infected cells is about 100-times greater than that observed in uninfected cells [1, 2] and can result in clinical hypoglycaemia as the infection progresses. ATP generation during this process is predominantly achieved by an almost quantitative conversion of glucose to lactate, providing the major source of energy in blood-stage malaria parasites. This increased activity has been shown to be associated with the appearance of parasite-encoded glycolytic enzymes [3]. Biochemical studies have suggested that several of these enzymes have physical and catalytic properties that are quite distinctive compared to their homologues in the human host [3–6], including a relative insensitivity to the fall in intracellular pH as large amounts of lactic acid are produced [7]. Such differences may therefore reflect the need of the parasite for an enhanced rate of glucose catabolism. For these reasons, we and others have initiated studies of the genes encoding the glycolytic enzymes of *P. falciparum*, which will provide information necessary for evaluating this pathway as a potential chemotherapeutic target. This will be aided by the wealth of structural data, derived from other sources, that is available for all eleven of the glycolytic enzymes.

Six genes in the glycolytic pathway of *P. falciparum* have been cloned and fully sequenced; they encode fructose bisphosphate aldolase [6, 8], glucose-6-phosphate isomerase (GPI) [9], phosphoglycerate kinase (PGK) [10], hexokinase [11], lactate dehydrogenase (LDH) [12] and triose-phosphate isomerase (TPI) [13]. A seventh gene, encoding glyceraldehyde-3-phosphate dehydrogenase (GAPDH) has been partially sequenced [14]. In this study, we characterise the gene encoding enolase (2-phospho-D-glycerate hydrolase). This

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Abbreviations. CHEF, contour-clamped homogeneous electric field; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GPI, glucose-6-phosphate isomerase; LDH, lactate dehydrogenase; PCR, polymerase chain reaction; PFGE, pulsed-field gel electrophoresis; PFK, phosphofructokinase; PGK, phosphoglycerate kinase; PGM, phosphoglycerate mutase; RACE, rapid amplification of cDNA ends; TPI, triose-phosphate isomerase.

Enzymes. Hexokinase (EC 2.7.1.1); glucose-6-phosphate isomerase (EC 5.3.1.9); phosphofructokinase (EC 2.7.1.11); aldolase (EC 4.1.2.13); triose-phosphate isomerase (EC 5.3.1.1); glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12); phosphoglycerate kinase (EC 2.7.2.3); phosphoglycerate mutase (EC 5.4.2.1); enolase (EC 4.2.1.11); pyruvate kinase (EC 2.7.1.40); lactate dehydrogenase (EC 1.1.1.27).

Note. The novel nucleotide sequence data reported here have been submitted to the EMBL/GenBank sequence data bank and are available under accession number U00152.

metalloenzyme catalyses the conversion of 2-phosphoglycerate to phospho*enol*pyruvate, which is the ninth reaction of the eleven-step pathway from glucose to lactic acid, and the only dehydration reaction in this series. Biochemical assay indicates a very high level of activity of this enzyme in the blood stages of malaria parasites [3]. Our comparison of this well conserved molecule with enolase sequences from a range of organisms reveals further evidence from the nuclear genome that the *falciparum* malaria parasite may have evolved from a photosynthetic ancestor, a surprising possibility that has also been suggested by studies of nuclear ribosomal RNA genes [15–18], as well as analysis of a 35-kb extrachromosomal DNA molecule found in the parasite [19–21].

MATERIALS AND METHODS

Parasite material

The K1 and 3D7 strains of *P. falciparum* were maintained in erythrocytic culture [22], RNA and DNA extracted and processed as previously described [10, 23].

Polymerase chain reaction (PCR)

Oligonucleotide primers were constructed to four regions of the gene that, from known enolase sequences, were predicted to be highly conserved. The 420-bp PCR product selected as a probe of our parasite gene libraries (see Results) derived from the following pair of primers: (1) 5′-CA(T/C)GC(A/T)GG(A/T)AATAAATT(A/G)GC(A/C/T)ATGCA-(A/G)GA based on the conserved amino acid motif HAGN-KLAMQE (residues 159–168 in yeast enolase numbering); (2) 5′-CCA(A/G)TC(A/G)TCTTGATC(A/G)AA(T/A/G)GG-(A/G)TC(T/C)TC based on the motif EDPFDQDDW (complementary strand, residues 295–303 in yeast).

The primers were designed on the basis of *P. falciparum* codon usage, with limited redundancies built in to increase the likelihood of perfect base-pair matching at the 3′ end [24, 25]. PCR were set up as previously described [25] in 100 µl containing 0.6 µg genomic DNA and 300 ng of each primer, utilising the hot start procedure [26] to minimise production of non-specific products. For inverse PCR, a series of ligations was set up, containing from 0.01 ng/µl to 10 ng/µl genomic DNA previously cut with the desired restriction enzyme. PCR using appropriate internal primers was performed on the circularised fragments and the product isolated from low-melting-point-agarose gels and purified on a Promega 'Magic' PCR column. To obtain fragments corresponding to the extreme N-terminus of the protein, 5′-RACE (rapid amplification of cDNA ends) [27] was performed, using a commercially available kit, on total *P. falciparum* RNA according to the manufacturer's (Gibco-BRL) instructions. A series of three nested primers was paired with the universal anchor primer provided in the kit.

DNA sequencing and analysis

DNA sequence was derived from double-stranded material by cycle sequencing or single-stranded M13mp9 subclones of λ library clones, using standard universal and reverse primers, together with a range of internal primers in both directions, designed as new sequence was acquired. Cycle sequencing (USB deltaTaq kit) was carried out on either unpurified asymmetric PCR products or double-stranded PCR products excised from low-melting-point gels and purified using Promega 'Magic' PCR columns. M13 sequencing was performed using Sequenase enzyme (USB). The PILEUP and GAP programs of the University of Wisconsin Genetics Computer Group package were used to align protein sequences and calculate percentage amino acid identities.

Pulsed-field gel electrophoresis (PFGE)

Parasites from cultures of K1 and 3D7 strains were processed for pulsed-field gel electrophoresis, the chromosomes electrophoresed on a contour-clamped-homogeneous-electric-field (CHEF) apparatus, and Southern blots prepared and probed exactly as previously described [28], except that pulse times were 6 min for 68 h, followed by 12 min for 42 h.

RESULTS

Isolation of the *P. falciparum* enolase gene

All four primer pairs designed from conserved regions of known enolase molecules gave PCR products of the size expected from parasite genomic DNA, assuming no introns occurred between them. A 420-bp product obtained using the primer pair shown in Materials and Methods was selected for further analysis. Sequencing of this product revealed an open reading frame with significant similarity to known enolase proteins in the corresponding region. The PCR fragment was then used as a probe to isolate clones from our λ NM1149 libraries of the K1 isolate of *P. falciparum*. No clones were found in either our *Hin*dIII or *Eco*RI genomic libraries; subsequent Southern blotting experiments indicated that the relevant fragments would be too big to be accommodated by the vector. However, a number of positive clones were isolated from our cDNA library. All of these yielded an identical *Eco*RI insert of 1.3 kb. This was found to encode all but the N-terminal 25% of the expected protein. To obtain the remaining coding sequence, two strategies were adopted. Inverse PCR experiments were carried out on a circularised 730-bp *Mae*II fragment shown to contain the upstream portion of the gene by Southern blotting experiments, and the PCR products analysed by cycle sequencing. In addition, the 5′-RACE technique was used with a series of nested primers to obtain a PCR product derived from the 5′ end of the mature mRNA of the K1 enolase gene. This product was cloned into a vector carrying a single T overhang to complement the A overhang produced at the 3′ ends of PCR products [29], using a commercial kit (Invitrogen, pCR-II vector). Several clones, positively identified by probing with an internal oligonucleotide, were then sequenced to confirm the remaining N-terminal portion of the coding region.

Sequence analysis of the *P. falciparum* enolase gene

The complete sequence of the enolase gene of *P. falciparum* is shown in Fig. 1. The gene encodes a protein of 446 residues (48.7 kDa), very close to the length of enolase molecules from other eukaryotes. The initiation codon was assigned by analogy to known enolase sequences (of which there are now over 20). This assignment is strongly supported by the nucleotide context of the ATG start codon, AAAATGG, where the purine at −3 and the G at +4 are characteristic of a functional initiation signal [30], the lack of alternative start codons anywhere in the vicinity, and an

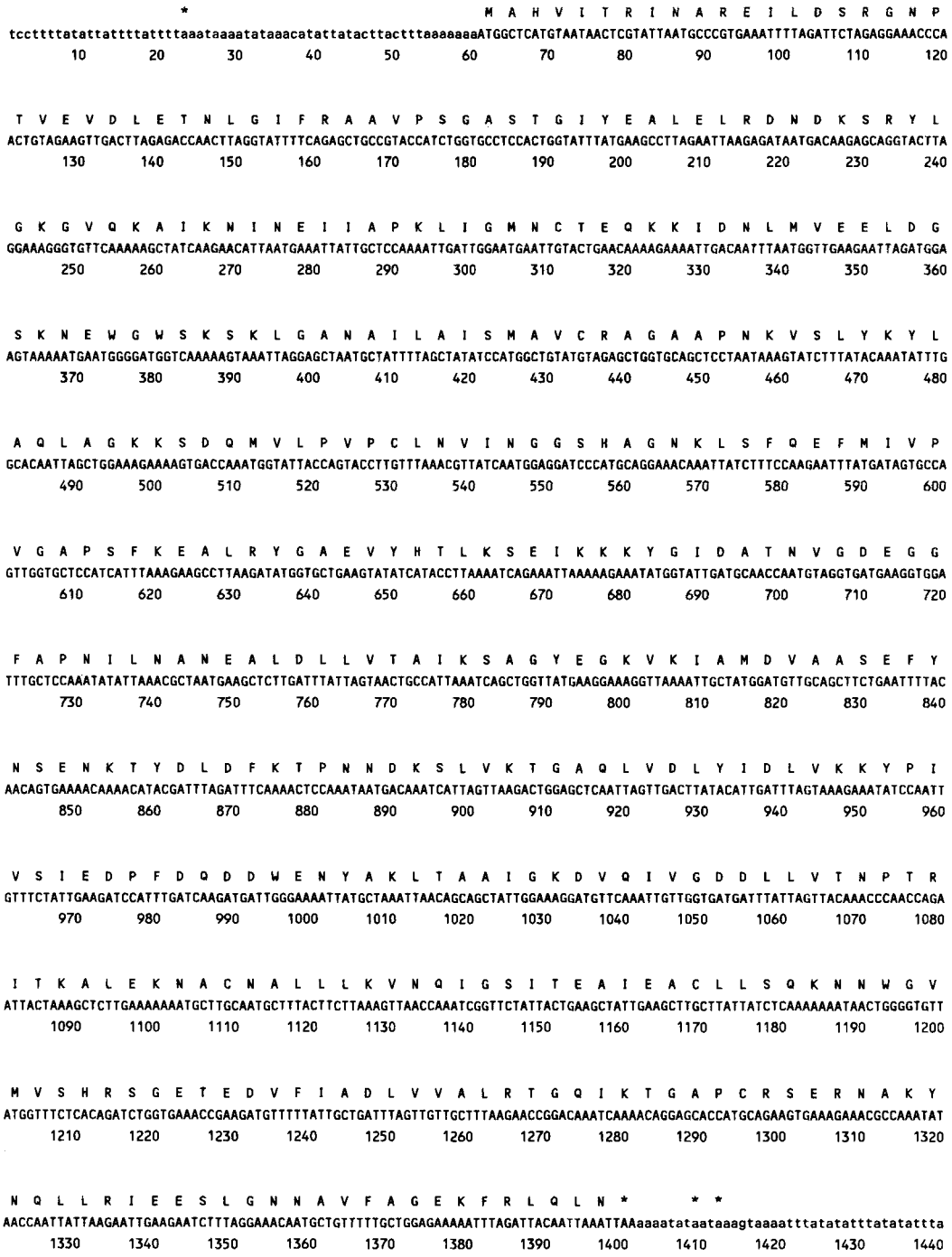

Fig. 1. Complete nucleotide sequence of the K1 enolase gene and its derived amino acid sequence. Lower-case letters indicate non-coding regions; (*) in-frame stop codons.

in-frame stop codon 39-bp upstream of this ATG, lying in a region of very high (92%) A+T content (Fig. 1).

The alignment of the predicted enolase protein of *P. falciparum* with enolases from a representative range of other organisms is shown in Fig. 2, and the levels of amino acid identity amongst a wider spectrum of eukaryotic enolase molecules are presented in Table 1. The lowest level of identity relative to the malarial sequence is seen with the enolases of the unicellular fungi *Candida* and yeast (61−63%), while the α, β and γ forms of the enzyme from the human host show 68−70% identity. Overall, the figures in Table 1 represent the highest degree of identity to homologous molecules from other organisms yet seen amongst the seven fully described glycolytic pathway enzymes of *P. falciparum* (Table 2). The degree of identity to the enolases of the higher plants tomato, maize and *Arabidopsis*, at 68−69%, is very similar to the comparisons with vertebrates. However, a detailed analysis of their primary sequences leads to the fascinating observation that the *P. falciparum* molecule shares features with the plant enolases that are not seen in other molecules from eukaryotic or prokaryotic sources. The most striking of these is a pentapeptide insertion sequence, EWGWC, located in a highly conserved region of the plant molecules [31, 32]. While all non-plant species lack this mo-

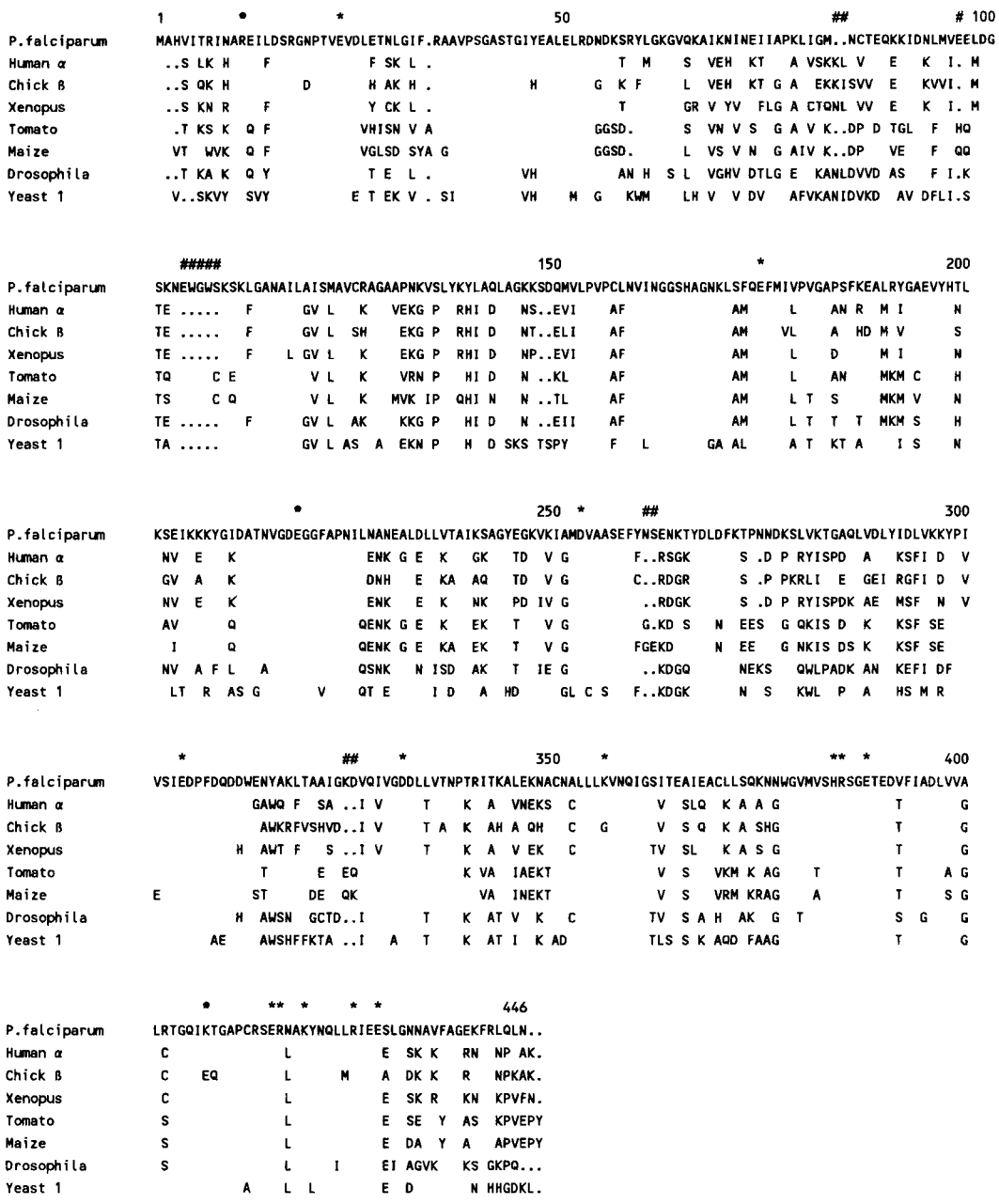

Fig. 2. Alignment of the predicted amino acid sequence of *P. falciparum* enolase with those of human (the parasite host), yeast (whose crystal structure is well characterised) and a number of other eukaryotic molecules. Swissprot protein database accession numbers are as follows: human α, P06733; chick β, P07322; *Xenopus*, P08734; tomato, P26300; maize, P26301; *Drosophila*, P15007; yeast 1, P00924. (*) Conserved residues implicated in substrate binding and catalysis; (#) insertions or deletions which the *P. falciparum* sequence has in common with plant enolases, but not those of other eukaryotes.

tif, *P. falciparum* enolase contains an almost identical insertion, EWGWS (residues 104–108), in the equivalent position (Fig. 3). In addition, the plant enolases display six other smaller (1 or 2 residue) insertions or deletions relative to those from eukaryotic sources outside of the plant kingdom. All but two of these are found in *P. falciparum* enolase, and are indicated in Fig. 2. Conversely, *P. falciparum* enolase shares only one insertion (residue 58) and one deletion (between residues 33 and 34) with molecules from non-plant sources that are absent from the plant enolases.

Despite significant divergence from other characterised enolase molecules, the *P. falciparum* sequence retains all of the residues considered to be important in binding both substrate and cofactor (Fig. 2), as identified by X-ray crystallography of the yeast molecule [33–35]. These include Asp253 (246 in yeast), Glu304 (295) and Asp331 (320), whose carboxylate groups coordinate the divalent metal ion ligand required for altering the enzyme conformation, thus allowing substrate binding; Lys356 (345) and Arg385 (374), which interact with the phosphate group of the substrate phosphoglycerate; Lys407 (396) and His384 (373), which interact with the carboxylate group of the substrate; and Glu175 (168) and Glu218 (211), which are involved in abstracting a proton in the dehydration step. In addition, further residues forming important ion-pair interactions are conserved. Secondary-structure analysis of the yeast and *P. falciparum* se-

Table 1. Amino acid identities amongst eukaryotic enolase sequences.

	Amino acid identities amongst enolase sequences of									
	Arabidopsis	tomato	maize	yeast	*Candida*	*Drosophila*	*Xenopus*	chick	duck	human α
	%									
P. falciparum	69.2	69.2	68.2	62.7	61.2	64.0	68.7	65.7	70.0	68.4
Arabidopsis		90.1	86.0	55.1	56.4	63.7	64.8	61.6	68.5	66.1
Tomato			87.2	55.5	56.6	65.4	66.9	62.5	69.9	70.1
Maize				51.2	56.8	61.9	63.7	56.7	64.7	64.7
Yeast 1					77.3	64.6	61.7	60.5	62.1	62.6
Candida						66.5	63.0	61.2	64.7	63.5
Drosophila							74.9	70.3	73.1	72.4
Xenopus								78.5	89.8	87.8
Chicken β									81.3	81.8
Duck α										92.1

```
                  99              115
P.falciparum      DGSKNEWGWSKSKLGAN
Arabidopsis       DGTQNEWGWCKQKLGAN
Tomato            DGTQNEWGWCKEKLGAN
Maize             DGTSNEWGWCKQKLGAN
Chlamydomonas     DGTDN.....KGKLGAN
Yeast 1/2         DGTAN.....KSKLGAN
Candida           DGTPN.....KSKLGAN
Drosophila        DGTEN.....KSKFGAN
Xenopus           DGTEN.....KSKFGAN
Chicken ß         DGTEN.....KSKFGAN
Duck α            DGSEN.....KSKFGAN
Human α/ß/γ       DGTEN.....KSKFGAN
Halobacterium     DGTDD.....FSGIGAN
E.coli            DGTEN.....KSKFGAN
Zymomonas         DGTPN.....KGKFGAN
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Fig. 3. The pentapeptide insertion of *P. falciparum* enolase aligned with the corresponding region in other enolase molecules from both eukaryotic and prokaryotic sources. Swissprot accession numbers additional to those given for Fig. 2 are, *Arabidopsis*, P25696; *Chlamydomonas*, P31683 (partial sequence); yeast 2, P00925; *Candida*, P30575; duck α, P19140; human β, P13929; human γ, P09104; *Halobacterium*, P29201; *E. coli*, P08324 (partial sequence). The *Zymomonas* sequence was translated from the EMBL/GenBank entry M99380.

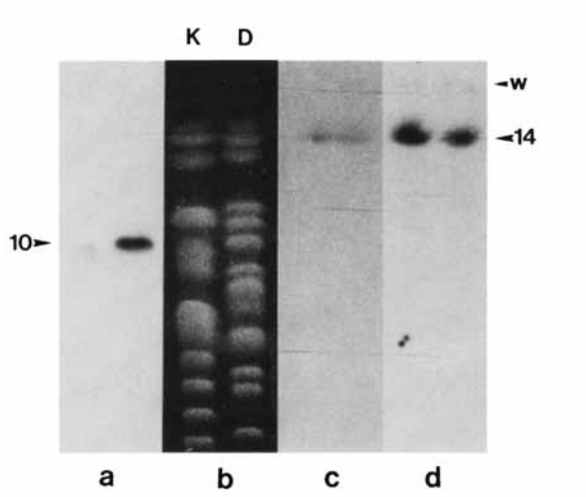

Fig. 4. Chromosomal mapping of the *P. falciparum* enolase gene. Parasite chromosomes from cultures of K1 (track K) and 3D7 (track D) strains were separated by electrophoresis and specific genes located by Southern blot analysis. (b) The ethidium bromide stained gel. Filters from three such identical pairs of tracks were each probed with a PCR fragment derived from the coding region of enolase (a), GAPDH (c) and TPI (d). The numbers indicate the chromosomal assignments of the three genes; w marks the position of the wells.

quences by the Chou-Fasman [36] and the Garnier-Osguthorpe-Robson [37] algorithms yielded results that were similar for each molecule in the extent to which the various stretches of secondary structure were predicted; neither method by itself predicted all of the components of the 8-fold $\beta+\alpha$ barrel [33] for either of the molecules, but the combined results obtained with the two methods predicted virtually all of the elements of this feature for both the yeast and malarial molecules. Taken together, the complete conservation of key residues in the *P. falciparum* molecule, and the strong similarity in predicted secondary structure, suggest that the mechanism of enolase catalysis in the parasite closely resembles that of other eukaryotes, including its human host.

Copy number, chromosomal location and expression of the enolase gene

To assess the copy number of the enolase gene, K1 genomic DNA was either singly digested with eight different restriction enzymes or double-cut with three different pairs of enzymes, and probed with various fragments derived from the coding region. In all eleven cases, the observed pattern corresponded to that expected for a single gene (data not shown). We note that all other glycolytic pathway genes (and indeed most other housekeeping genes) in *P. falciparum* that have been assessed for copy number have also been found to be single copies. Hybridisation of the enolase gene to Southern blots of chromosomal DNA separated on PFGE gels gave a single signal with each of the two strains tested (K1 and the reference strain 3D7; Fig. 4), also consistent with a single-copy gene. With both the K1 and 3D7 strains, the signal corresponded to chromosome 10, which is clearly resolved in the case of 3D7, and partially resolved from chromosome 8 in K1 (chromosome 9 in the latter is truncated and runs faster than chromosome 8 [28]). We also mapped two other glycolytic pathway genes that we have cloned from K1, namely those encoding TPI and GAPDH, both of which were found on chromosome 14 (Fig. 4). Thus, as summarised in Table 2, glycolytic pathway genes are located on at least 5 of the 14 chromosomes of *P. falciparum*. However, while the first enzyme in the pathway (hexokinase) and the character-

Table 2. Characteristics of *P. falciparum* glycolytic enzymes and their genes. Enzymes are listed in their functional order in the glycolytic pathway.

Enzyme	Amino acid identity to human homologue(s)	Chromosomal location of gene	Reference
	%		
Hexokinase	26	8	11
GPI	34	14	9
PFK	unknown	unknown	
Aldolase	50–54	14	6, 8, 10
TPI	43	14	13, this work
GAPDH	(77)[a]	14	14, this work
PGK	59–60	9	10
PGM	unknown	unknown	
Enolase	68–70	10	this work
Pyruvate kinase	unknown	unknown	
LDH	27–30	13	12

[a] Based on a partial sequence of 53 residues, corresponding to a highly conserved region of the molecule representing approximately 16% of the total coding sequence.

ised enzymes in the later part (PGK, enolase and LDH) are all encoded on different chromosomes, the remainder (GPI, aldolase, TPI and GAPDH) are found on the largest chromosome, number 14 (Table 2). All of the latter enzymes occur sequentially in the first half of the glycolytic pathway, together with phosphofructokinase (PFK), whose gene is as yet uncharacterised. Depending upon where PFK maps, it may therefore be the case that at least a substantial and contiguous part of the glycolytic pathway is encoded by a cluster of genes on a single chromosome. This will only be revealed by eventual fine mapping of this large (approximately 3.2 Mb) chromosome.

In most organisms enolase is an abundant product. The level of enolase activity in *P. falciparum*-infected erythrocytes is certainly considerable, about 15-times higher than that in uninfected cells, and, along with hexokinase, higher than that for any other parasite glycolytic enzyme [3]. Correspondingly high levels of protein and mRNA transcript might therefore be expected, and preliminary Northern blots of total RNA from mixed asexual parasites suggested that enolase mRNA (approximately 1.8 kb) was indeed abundant (data not shown). In yeast and *Candida*, where enolase is strongly expressed, the codon bias of the corresponding genes is marked [38, 39], with only those isoacceptor tRNA molecules that are the most abundant being recruited for protein synthesis. We thus examined the codon bias of the *P. falciparum* enolase gene for evidence of a similar phenomenon but found that it showed overall significantly less bias than both the *Candida* and yeast genes. It is likely that the organism has little scope to further bias its codon usage in highly expressed genes, presumably due to the intrinsically skewed base composition of all *P. falciparum* genes.

DISCUSSION

The most striking result to emerge from our characterisation of the *P. falciparum* enolase gene is the presence of the pentapeptide insertion EWGWS, and its near identity to the EWGWC motif found in the enolase molecules of the higher plants. This motif is not present in the enolase sequences from a wide range of other non-plant organisms, both eukaryotic and prokaryotic (Fig. 3), and must be regarded as highly significant because tryptophan residues are particularly rare in *P. falciparum* proteins [40]. Moreover, a search of the OWL non-redundant composite protein sequence database (21.9 million residues) revealed that the EWGWC motif is unique to plant enolases. The presence of this highly characteristic motif thus represents an important pointer to the evolutionary history of this parasite. From comparison of the yeast protein crystal structure [33], this insert is seen to fall in a region lacking regular secondary structure between the J and K α helices in the smaller, non-catalytic N-terminal domain, where presumably it has little effect on tertiary folding or catalytic activity. It therefore seems unlikely that it reflects a major functional difference between plant and non-plant enolases.

One source of evidence that *P. falciparum* may have evolutionary links with the plant kingdom has arisen from DNA sequence analyses of an extrachromosomal circular DNA molecule of 35 kb, which indicated that the latter may be the remnant of the plastid genome of a photosynthetic ancestor [19–21, 41]. Several features of this 35-kb circle, and in particular the sequence and architecture of its rRNA genes [20] and RNA polymerase subunit genes [21], most closely resemble those found in chloroplasts. Moreover, hybridisation studies indicate that all other members of the phylum Apicomplexa studied to date contain a homologue of the *P. falciparum* molecule [42]. Such studies cannot yield direct evidence about the lineage of the *Plasmodium* nuclear genome. However, molecular phylogeny studies of genomic sequences, mainly those encoding small subunit ribosomal RNA molecules [15–18] (reviewed in [43]), as well as morphological comparisons [15, 16, 43], suggest that the closest relatives of the Apicomplexa are the dinoflagellates. This is a diverse grouping of largely marine protists that includes both photosynthetic algal-like species and non-photosynthetic parasitic members that probably evolved from photosynthetic precursors.

Unfortunately, no enolase sequences from other protozoan organisms or dinoflagellates have been reported. If the Apicomplexa shared a common ancestor with, or were descended from the dinoflagellates, then it would be expected that some or all of the latter would encode the pentapeptide insertion in their enolase genes. However, the data of Fig. 3 show that this sequence motif cannot merely be a common feature of all protists on the one hand, or all photosynthetic organisms on the other, since it is absent from the enolase of the unicellular green alga *Chlamydomonas reinhardtii*. This organism is a member of the Chlorophyta, the division of the algae from which higher plants probably originated [43], and it is thus most curious that *P. falciparum*, which is undoubtedly overall much more distantly related to the higher plants than is *Chlamydomonas* [43], should share a distinctive sequence motif with them that the latter lacks. The sequencing of more enolase genes from protists and lower plants is required to shed further light on this apparent paradox. One intriguing but very speculative possibility to explain the paradox is that this highly host-specific human pathogen has evolved from a parasite of higher plants that acquired that part of the enolase gene encoding the pentapeptide insert by some type of horizontal transmission mechanism, long after the divergence of the green algae and multicellular plants. Note however that the data in Table 1 strongly argue against

the acquisition of a complete enolase gene from a higher plant.

Whatever its precise origin in evolutionary history, this nuclear remnant pointing to a relationship with a photosynthetic lineage may still be observable only because the glycolytic pathway is one of the most ancient and best preserved of all metabolic pathways, with a very low mutation rate within its genes [44]. The high degree of conservation amongst enolase sequences is evident from Table 1, and it may be particularly relevant that enolase is the most conserved of the seven complete glycolytic enzyme sequences reported to date from *P. falciparum* (Table 2). Further analysis of other previously characterised genes from this parasite may reveal additional relationships with similar evolutionary implications.

All the fully characterised genes in the parasite glycolytic pathway have been found to be present as a single copy. The very rapid turnover of glucose by the parasite in erythrocytes is therefore not assisted by gene amplification, nor apparently by codon bias, as described in Results. Given the apparent abundance of the transcripts (see above and [11]) and the gene products of the enolase, hexokinase and pyruvate kinase genes [3], it is likely that their promoters are particularly strong. This may eventually be usefully exploited in a *P. falciparum* transformation system once these promoters can be identified and characterised. The conservation of known active-site and other functional residues suggests that the structural properties and mechanism of catalysis of parasite enolase is likely to resemble those of the host quite closely, unlike the case with LDH, for example, where some active-site residues are different [12]. In general, the greater the difference between isofunctional parasite and host molecules, the greater the likelihood that inhibitors able to discriminate between them and selectively target only the parasite metabolism could be found. As seen in Table 2, there is considerable variation in the degree of primary-structure conservation of these enzymes relative to those of the host. Our analysis suggests that amongst the malarial glycolytic enzymes, enolase would not be a favourable target for chemotherapeutic intervention. Moreover, in other systems, enolase has never been found to have regulatory properties [33]. Its major interest at this stage therefore perhaps lies in its distinctive sequence and the further clue that it gives us as to the unexpected origins of the most pathogenic of the human malaria parasites.

We are most grateful to the Medical Research Council, UK, for financial support. This work benefited from the use of the SEQNET facility, Daresbury, England.

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Chapter 4

Sulfadoxine resistance in the human malaria parasite *Plasmodium falciparum* is determined by mutations in dihydropteroate synthetase and an additional factor associated with folate utilization

Published in 1997 in:

Molecular Microbiology, volume 23, pages 979-986

Wang, P., Read, M., Sims, P.F.G. and Hyde, J.E.

Molecular Microbiology (1997) 23(5), 979–986

Sulfadoxine resistance in the human malaria parasite *Plasmodium falciparum* is determined by mutations in dihydropteroate synthetase and an additional factor associated with folate utilization

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Summary

Sulfadoxine/pyrimethamine (Fansidar) is widely used in Africa for treating chloroquine-resistant falciparum malaria. To clarify how parasite resistance to this combination arises, various lines of *Plasmodium falciparum* were used to investigate the role of naturally occurring mutations in the target enzyme, dihydropteroate synthetase (DHPS), in the parasite response to sulfadoxine inhibition. An improved drug assay was employed to identify a clear correlation between sulfadoxine-resistance levels and the number of DHPS mutations. Moreover, tight linkage was observed between DHPS mutations and high-level resistance in the 16 progeny of a genetic cross between sulfadoxine-sensitive (HB3) and sulfadoxine-resistant (Dd2) parents. However, we also demonstrate a profound influence of exogenous folate on IC_{50} values, which, under physiological conditions, may have a major role in determining resistance levels. Importantly, this phenotype does not segregate with *dhps* genotypes in the cross, but shows complete linkage to the two alleles of the dihydrofolate reductase (*dhfr*) gene inherited from the parental lines. However, in unrelated lines, this folate effect correlates less well with DHFR sequence, indicating that the gene responsible may be closely linked to *dhfr*, rather than *dhfr* itself. These results have major implications for the acquisition of Fansidar resistance by malaria parasites.

Introduction

Malaria parasites cause about two million deaths annually and serious morbidity in a much greater number of victims. The spread of parasites resistant to the major therapeutic agent, chloroquine, has led an increasing number of African countries to adopt the antifolate combination of pyrimethamine (PYR) and sulfadoxine (SDX) as the treatment of choice for *Plasmodium falciparum* malaria (Bloland *et al.*, 1993). Inevitably, clinical resistance to this combination (PSD; Fansidar) is increasingly arising in these regions. A detailed understanding of the underlying mechanisms of such resistance would be a major contribution to better management of this problem, and could extend the effective life of PSD and related antifolate combinations.

PYR and SDX are inhibitors of the folate biosynthetic pathway, targeting, respectively, dihydrofolate reductase (part of a bifunctional protein with thymidylate synthetase, DHFR-TS; Bzik *et al.*, 1987) and dihydropteroate synthetase (bifunctionally combined with hydroxymethylpterin pyrophosphokinase, PPPK-DHPS; Brooks *et al.*, 1994; Triglia and Cowman, 1994). DHPS couples *p*-aminobenzoic acid (PABA) to a 7,8-dihydropterin, giving dihydropteroate in the step preceding dihydrofolate synthesis. DHFR activity is essential for maintenance of a constant supply of tetrahydrofolate cofactors for key 1-C transfer reactions, in which only the fully reduced forms are functional. In the many field isolates of *P. falciparum* studied to date (e.g. Peterson *et al.*, 1991; Basco *et al.*, 1995), PYR resistance is always associated with a mutation in DHFR to give Asn at residue 108, but higher levels of resistance result if this mutation is accompanied by Cys-59 to Arg and/or Asn-51 to Ile (reviewed in Hyde, 1990). Similarly, previous sequencing studies by ourselves (Brooks *et al.*, 1994) and others (Triglia and Cowman, 1994), in which the *pppk–dhps* gene was initially characterized, identified altered sequences near conserved areas of the DHPS domain that may underlie an element of resistance to sulfadoxine.

While the primary importance of mutations in DHFR to PYR resistance is clear, the relative contribution of mutations in DHPS to SDX resistance is at present less well defined. Older data (Watkins *et al.*, 1985; Milhous *et al.*, 1985; Dieckmann and Jung, 1986; Krungkrai *et al.*, 1989) have indicated that mechanisms other than an altered target enzyme may also be involved. However, these earlier studies were limited by a lack of knowledge about

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DHPS sequence variation, suboptimal assays for SDX resistance, and comparisons between unrelated parasite strains. In this work, we have quantitatively assessed the role of mutations in DHPS, as well as another potential factor in SDX resistance, namely the influence of exogenous folate, an important vitamin in the human host. To this end, as well as investigating a number of parasite lines whose *dhps* genotypes we have identified as different, we have exploited one of the two available genetic crosses between clones of *P. falciparum*. Progeny from the HB3–Dd2 cross (Wellems *et al.*, 1990) provide a unique opportunity to explore details of SDX resistance, since, as shown here, the parental lines are, respectively, highly SDX-sensitive and highly SDX-resistant. Moreover, we find that the 16 best-characterized progeny (Walker-Jonah *et al.*, 1992) include representatives of both parental *dhps* genotypes, as well as both parental *dhfr* types (unlinked, as *dhps* and *dhfr* reside on different chromosomes; Brooks *et al.*, 1994). Thus we have a powerful means of quantifying aspects of SDX resistance against a controlled genetic background, and thus ultimately of clarifying the importance of both SDX- and PYR resistance in the clinical loss of parasite sensitivity to PSD.

Results

dhps *genotypes of the parents and progeny of the HB3–Dd2 cross*

Using polymerase chain reaction (PCR) diagnostic tests developed to assay mutations at critical positions in the *dhps* gene (Wang *et al.*, 1995), and confirmatory DNA sequencing, we found that the parental clone HB3 contained the residues Ser-436, Ala-437, Ala-581 and Ala-613, like the It.D12, It.G2.F6, FCR3 and M24 lines described previously (Brooks *et al.*, 1994). The Dd2 parent, however, carried the residues Phe-436, Gly 437, Ala-581 and Ser-613, consistent with previous sequencing of the W2 line (Brooks *et al.*, 1994) from which it ultimately derives (Wellems *et al.*, 1990) (Table 1). By comparison with the data in Brooks *et al.* (1994), it was thus anticipated that HB3 would be SDX sensitive, while Dd2 would be SDX resistant (as verified below), and that the genetic cross of these parasites was potentially informative. Sequence analysis of the HB3–Dd2 cross progeny showed that 4 of the 16 had inherited the Dd2-type *dhps* mutations (progeny nos 1, 2, 3 and 7), while the other 12 had inherited the HB3 sequence at this locus (Table 2, column 2), confirming the usefulness of the cross.

Table 1. SDX resistance levels and amino acid residue variations in DHPS for different lines of *P. falciparum*.

		Residue					
Line	Origin	436	437	540[a]	581	613	IC_{50} (ng ml^{-1})[b]
HB3[c]	Honduras	Ser	Ala	Lys	Ala	Ala	4.0 ± 1.9
M24	Kenya	Ser	Ala	Lys	Ala	Ala	3.5 ± 0.7
FCR3	Gambia	Ser	Ala	Lys	Ala	Ala	7.0 ± 2.8
3D7	Africa	Ser	**Gly**	Lys	Ala	Ala	73 ± 53
K1	Thailand	Ser	**Gly**	Lys	**Gly**	Ala	223 ± 36
Dd2[c]	Indochina	**Phe**	**Gly**	Lys	Ala	**Ser**	3970 ± 1194

a. This position is included as recent studies have indicated that this, too, can be a site of mutation in certain lines (T. Triglia, J. Menting, C. Wilson and A. F. Cowman, submitted; C.-S. Lee, P. Wang and J. E. Hyde, in preparation).
b. Figures quoted are means of 4–7 determinations with standard deviations, except for M24 and FCR3, which were assayed twice; 4 ng ml^{-1} = 12.9 nM; 3970 ng ml^{-1} = 12.8 μM.
c. Parents of the genetic cross used in this study.

Table 2. Characteristics of parents and progeny of the HB3-Dd2 cross.

Line	DHPS status[a]	$IC_{50\ SDX}$ in the absence of folate/PABA (ng ml^{-1})[b]	Folate effect[c]	DHFR status[d]
Parent				
Dd2	D	3970 ± 1194	Yes	D
HB3	H	4 ± 2	No	H
Progeny				
1	D	420 ± 136	No	H
2	D	1685 ± 167	Yes	D
3	D	3461 ± 455	No	H
4	H	6 ± 2	No	H
5	H	7 ± 1	No	H
6	H	4 ± 1	No	H
7	D	3010 ± 206	Inter	D
8	H	19 ± 9	Yes	D
9	H	11 ± 6	No	H
10	H	8 ± 1	Yes	D
11	H	3 ± 1	No	H
12	H	3 ± 1	Yes	D
13	H	8 ± 1	No	H
14	H	6 ± 1	No	H
15	H	5 ± 2	Yes	D
16	H	8 ± 1	No	H

a. D = Dd2-type, i.e. carries the three DHPS mutations S436F, A437G, and A613S; H = HB3-type, i.e. wild-type DHPS.
b. Mean of 3–6 determinations and standard deviation.
c. Yes = added folate increases IC_{50} to such a high level that measurement is impossible at the highest [SDX] used (25 μg ml^{-1}). Inter = added folate has a pronounced effect, but IC_{50} is just measurable at *c.* 20 μg ml^{-1}). No = measured IC_{50} remains close to that in column 3, even in the presence of 100 ng ml^{-1} folate.
d. D = Dd2-type, i.e. carries the three DHFR mutations S108N, N51I, and C59R. H = HB3-type, i.e. carries the one DHFR mutation S108N.

Sulfadoxine assays of various P. falciparum *lines of known* dhps *genotype in the absence of folate and PABA*

In vitro tests for parasite SDX resistance require culture medium depleted of folate and PABA, which antagonize SDX (Chulay *et al.*, 1984; Watkins *et al.*, 1985; Milhous

et al., 1985). However, despite the use of such medium for many years, published data for IC_{50} (50% inhibitory concentration) values show massive discrepancies, varying from 10 nM (3.1 ng ml^{-1}; Milhous *et al.*, 1985) to 150 nM (46.5 ng ml^{-1}; Watkins *et al.*, 1987), 1 μM (0.31 μg ml^{-1}; Chulay *et al.*, 1984) to as high as 3 mM (930 μg ml^{-1}; Schapira *et al.*, 1986) for SDX-sensitive strains, often with relatively small differences between types classified as resistant or sensitive. By systematically monitoring the variables involved, we refined this assay to give consistently reproducible data, with large differences between the most sensitive and most resistant strains. This involved substitution of quality-controlled lipid-rich albumin for culture plasma or serum, regularizing the supply of erythrocyte host cells from a single known donor, and culturing the parasites firstly in a low-folate medium, followed by a complete growth cycle of 48 h in medium devoid of folate and PABA immediately before entry into the drug test. This procedure represents a significant improvement over earlier protocols, and allowed us to monitor accurately differences in SDX responses among different lines, and between parental levels and those of the individual progeny of the HB3–Dd2 cross.

The modified SDX test was first applied to a number of *P. falciparum* lines with different *dhps* genotypes, and IC_{50} values in the absence of folate and PABA were measured (Table 1). The lines chosen varied in the mutant positions identified by earlier studies (Brooks *et al.*, 1994; Triglia and Cowman, 1994) and in the experiments just described. Lines isogenic with HB3 in *dhps* showed the lowest IC_{50} values. Moreover, we consistently observed a significant and reproducible difference between the sensitivities of HB3 and 3D7, which differ only at residue 437 of DHPS. The lower IC_{50} figure for HB3 indicates that Ala-437 should be regarded as wild type and Gly as mutant. On this basis, K1 DHPS has two mutations, at 437 and 581, and showed a level of resistance *c.* 50-fold greater than that of the wild type, while Dd2 DHPS has three mutations, at 436, 437 and 613, a combination that gave the highest level of resistance of the lines tested, about 20-fold higher again than that of K1 (Table 1). To date, no parasites have been observed that carry mutations in all four of these positions.

These data suggest that mutations in the *dhps* gene are of biological and chemotherapeutic relevance. Given that Dd2 and HB3 represented, respectively, the most resistant and the most sensitive parasite types that we assayed (Table 1), we next turned to the genetic cross of these cloned lines to examine in greater depth the role of the mutations at positions 436, 437 and 613. The rationale here was to examine to what extent levels of SDX resistance showed linkage to *dhps* genotypes in the progeny of the cross, and thus to consolidate the likelihood of a causal relationship, as indicated by the above data.

In vitro *SDX susceptibility of progeny of the HB3–Dd2 cross in the absence of folate and PABA*

The SDX inhibition profiles obtained for the parents of the genetic cross were very reproducible and the IC_{50} values were measured as 4.0 ± 1.9 ng ml^{-1} (12.9 ± 6.1 nM) for HB3 and 3970 ± 1194 ng ml^{-1} (12.8 ± 3.9 μM) for Dd2 (Table 1). This difference of three orders of magnitude in IC_{50} values is considerably greater than most previous estimates for parasites reported as SDX sensitive or resistant, again reflecting the improved nature of the assay. When we measured inhibition levels for each of the 16 progeny under these conditions, the values obtained closely resembled those of one or other of the parents, and correlated completely with the wild-type and mutant *dhps* genotypes (Table 2, columns 2 and 3). (For reasons that are unclear, progeny 1 consistently displayed an IC_{50} value that, while clearly indicative of resistance, was only about 100-fold higher than the wild-type value, rather than being about 1000-fold higher, as for the other mutant progeny, 2, 3 and 7.) Overall, the data in Table 2 provide strong confirmatory evidence that the *dhps* mutations directly affect the ability of the drug to inhibit parasite growth. Consistent with this, recent measurements of K_i values on recombinant versions of the PPPK–DHPS protein carrying the various mutations show that they have a marked effect on the affinity of the drug to the purified enzyme (T. Triglia, J. Menting, C. Wilson and A. F. Cowman, submitted, and see below).

Antagonism of SDX action by exogenous folic acid on parasites of the HB3–Dd2 cross

We noted that where the same lines had been assayed both here and in our earlier work (Brooks *et al.*, 1994), there were significant differences between the absolute values of the IC_{50} measurements. Older data had indicated the critical influence of folate concentration on the outcome of SDX assays (Chulay *et al.*, 1984; Watkins *et al.*, 1985; Milhous *et al.*, 1985). It was therefore likely that these discrepancies arose from the more rigorous manner in which folate levels were controlled in the present work. The HB3–Dd2 cross family, the improved SDX assay and knowledge of *dhps* genotypes gave us the first opportunity to examine this effect of folate systematically and evaluate its impact on SDX resistance, relative to modifications in the DHPS enzyme. This is an important consideration, given the ubiquity of folate cofactors in human cells and plasma. We therefore repeated the above IC_{50} measurements while monitoring the effect of adding increasing amounts of exogenous folic acid (0–100 ng ml^{-1}) to the samples.

First, the parental lines HB3 and Dd2 were tested and their responses to added folate were found to be

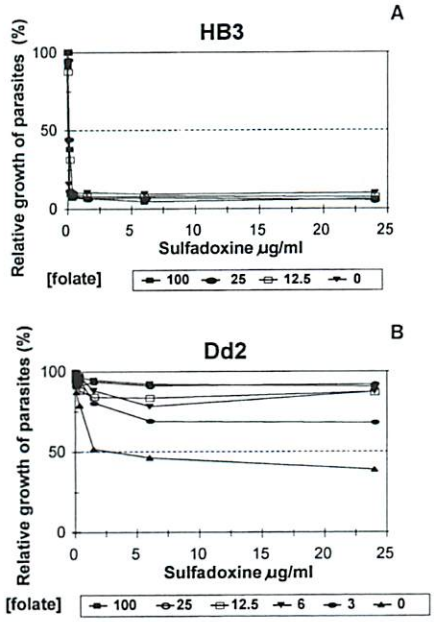

Fig. 1. Comparison of the susceptibilities to SDX of the parental lines used in the genetic cross, HB3 (A) and Dd2 (B), and the influence of increasing concentrations of exogenous folic acid (in ng ml^{-1}) on inhibition by SDX. The absence (A) or presence (B) of the folate effect was confirmed for each parent ≥6 times.

remarkably different (Fig. 1). Thus, the SDX susceptibility of HB3 was altered only to a very minor degree, even in the presence of 100 ng ml^{-1} folate, while that of Dd2 was markedly reduced in only 3 ng ml^{-1} folate. Moreover, at concentrations above 6 ng ml^{-1}, this antagonism was so pronounced that very little reduction of growth in Dd2 occurred over the concentration of SDX used, even up to 100 μg ml^{-1}, making calculation of IC_{50} values impossible.

Second, we carried out identical folate titrations for each of the 16 progeny of the cross to monitor this phenomenon, which we term the folate effect. Certain of the progeny responded in a fashion similar to that of the Dd2 parent, while others were essentially unresponsive to folate, as for the HB3 parent. Crucially, however, these phenotypes did not correlate with the presence or absence of mutations in the *dhps* gene. Thus, for example, progeny nos 8, 10 and 12, which are wild type for *dhps*, displayed the folate effect to the same degree as Dd2, while progeny nos 1 and 3, which are mutant in *dhps*, were non-responders (Fig. 2; Table 2, columns 2 and 4). We conclude from these data that the status of at least one other gene strongly influences the susceptibility of a particular parasite line to SDX inhibition under conditions in which exogenous folate is provided.

The association of dhfr *genotype with SDX susceptibility of the cross family in the presence of folate*

The above results demonstrate that different parasites can vary considerably in the degree to which added folic acid antagonizes their sensitivity to SDX inhibition. As *P. falciparum* has been shown to be capable of utilizing exogenous folate (Krunkrai *et al.*, 1989), a likely explanation for

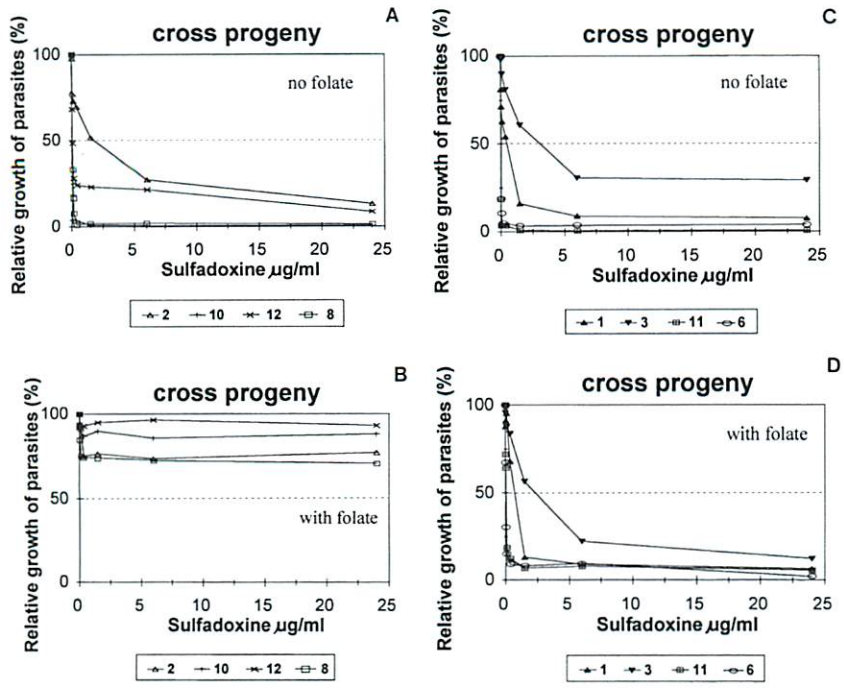

Fig. 2. Representative responses to folic acid addition (100 ng ml^{-1}) by progeny of the HB3–Dd2 cross. For clarity, not all progeny are included. Panels A (no folate) and B (plus folate) show four of the progeny that display the strong folate effect characteristic of the parent Dd2. Those in panels C (no folate) and D (plus folate), like the parent HB3, are hardly affected, in terms of their SDX susceptibility, by added folate. All 16 progeny reproducibly displayed one or the other of these phenotypes (summarized in Table 2). Errors in the measurements were similar in magnitude to those given in Tables 1 and 2 for assays carried out in the absence of folate.

these data is that different parasite strains vary markedly in this ability, as previously speculated (Milhous *et al.*, 1985). Interestingly, we noticed that amongst the parents and progeny of the HB3–Dd2 cross, there was a good correlation between the apparent ability of a given parasite to utilize exogenous folic acid in overcoming SDX inhibition and the inheritance pattern of an anonymous restriction fragment length polymorphism (RFLP) marker (pC4.H31) that mapped to the same region (*c.* 360 kb) of chromosome 4 as the *dhfr-ts* gene (Walker-Jonah *et al.*, 1992). We therefore explored this relationship further by sequencing the *dhfr* domains of all 16 of the cross progeny. We found that the correlation between *dhfr* genotype and the presence or absence of the folate effect was absolute; all progeny that carried the Dd2-type *dhfr* gene (mutated in codons 51, 59 and 108) showed a strong folate effect, while those with the HB3-type *dhfr* gene (mutated only in codon 108) were non-responders (Table 2, columns 4 and 5). These results strongly suggested that the folate effect is dependent either upon the nature of the *dhfr* gene itself, or of a gene that is closely linked to the latter on chromosome 4.

The effect of added folate to other lines of P. falciparum *differing in* dhfr

Given the possible clinical relevance of the above observations, it was important to establish whether parasite lines other than Dd2 showed a similar folate effect, and, if so, what the mutational status of their *dhfr* gene was. Therefore, further experiments were conducted in which a number of lines were challenged with increasing SDX in the presence or absence of 100 ng ml^{-1} folate (Table 3). Only one line (FCR3), other than HB3, was classified as a non-responder. Five others resembled Dd2 in displaying a clear folate effect. However, here there was no obvious correlation between the presence or absence of a folate effect and the mutational status of the DHFR amino acid sequence, indicating that the latter is not necessarily directly responsible for the folate effect.

Table 3. Incidence of the folate effect amongst different lines of *P. falciparum*.

Line	Origin	DHFR Residue[a] 51	59	108	Folate effect[b]
3D7	Africa	Asn	Cys	Ser	Yes
Tak9/96	Thailand	Asn	Cys	Ser	Yes
M24	Kenya	Asn	Cys	Ser	Yes
FCR3	Gambia	Asn	Cys	Thr/Asn	No
HB3	Honduras	Asn	Cys	Asn	No
FCB	Africa	Asn	Cys	Thr/Asn	Yes
K1	Thailand	Asn	Arg	Asn	Yes
Dd2	Indochina	Ile	Arg	Asn	Yes

a. Key residues determining levels of pyrimethamine resistance. For each line, the status of these residues was verified by sequencing of the *dhfr* gene after the SDX/folate assays. FCR3 and FCB showed mixed sequence.
b. As defined for Table 2, using 100 ng ml^{-1} folate.

Discussion

After chloroquine, PSD currently represents the only effective antimalarial formulation affordable in Africa on a mass scale (Foster, 1991). One explanation for the developing resistance to PSD in this region is that observed moderate levels of resistance to PYR alone are mediated by parasites carrying just the single Asn-108 DHFR mutation, but that these are sufficiently sensitive to SDX that PSD can still clear them. The transition to PSD resistance might then occur in two ways. First, further mutation in DHFR might confer sufficient PYR resistance such that even the synergistic PYR/SDX combination is no longer effective, or, second, the emergence of sufficient SDX resistance might yield the same result – possibly both events are necessary. It is thus essential to analyse parasite resistance to both components of PSD in order to clarify the association between altered parasites and clinical failure. This should improve prediction of PSD resistance and help to rationalize treatment strategies and the possible use of other cheap antifolate combinations that would avoid the development costs of new drugs. In this work, we have attempted to elucidate the basis of parasite resistance to the SDX component of PSD.

It has long been known that the presence of compounds related to SDX influences *in vitro* susceptibility to the drug (Chulay *et al.*, 1984; Watkins *et al.*, 1985; Milhous *et al.*, 1985), and the large variations in reported $IC_{50\ SDX}$ values reflect the difficulties in completely controlling such levels and achieving reproducibility. Our preliminary studies confirmed that even minute levels (ng ml^{-1}) of folic acid, in particular, could have a major effect on the outcome of assays. We therefore took stringent steps to eliminate, as far as possible, these compounds from the assay and to ensure that all other components were carefully controlled. This consistently yielded more reliable measurements which, together with the recent discovery of variability in the target enzyme of SDX (Brooks *et al.*, 1994; Triglia and Cowman, 1994), permitted a more meaningful analysis of the molecular basis of SDX resistance than was hitherto possible.

The data thus obtained (Tables 1 and 2) demonstrate that the various mutations identified in the *dhps* gene have a significant and cumulative effect on SDX susceptibility in live parasites. In particular, the observed tight linkage of SDX-resistance levels to *dhps* genotype in the progeny of the HB3–Dd2 cross (Table 2) provides compelling evidence for the importance of these mutations. The effect of changing amino acid 437 is clearly seen in the

IC_{50} values for the various lines in Table 1, comparing 3D7 with HB3, M24 and FCR3. These data suggest that position 437 might be analogous to position 108 in DHFR-TS, where mutation gives rise to intermediate PYR resistance that is much increased by combination with further mutations elsewhere in the molecule. While the level of SDX resistance is also greatly enhanced (1–2 orders of magnitude) when the 437 mutation is combined either with that at 581, as in the DHPS of K1, or with those at 436 and 613, as in Dd2, the two situations are not entirely parallel. Thus, all PYR-resistant strains isolated from natural infections carry the mutant Asn-108 residue in DHFR, whereas in the case of SDX-resistant strains, there are parasites such as SLD6 in which residue 437 of DHPS is wild type but one or more of the other positions are mutant (Brooks *et al.*, 1994; C.-S. Lee, P. Wang and J. E. Hyde, in preparation).

Conclusions similar to those above also arise from a complementary study in which K_i values for SDX binding to different forms of the isolated recombinant PPPK–DHPS enzyme have been measured (T. Triglia, J. Menting, C. Wilson and A. F. Cowman, submitted). In those experiments, a difference of approx. three orders of magnitude was seen between wild-type enzyme and that carrying the three mutations found in Dd2-type parasites. It appears, therefore, that the resistance levels of live parasites that we observe in zero folate can be satisfactorily accounted for by alterations in the affinity of SDX for its target enzyme. However, our quantification of the extent to which SDX susceptibility is modulated by exogenous folate strongly suggests that under physiological conditions this phenomenon may be the determining factor in the efficacy of SDX against certain parasite lines, regardless of their *dhps* status. Thus for parasites like Dd2, a folate concentration as low as *c.* 5 ng ml^{-1} renders SDX ineffective over concentrations of drug attainable in plasma after chemotherapeutic administration (*c.* 40–80 μg ml^{-1}; Weidekamm *et al.*, 1982), while for parasites of the HB3 type, folate concentrations at levels much higher than those found in plasma have only a marginal effect on SDX susceptibility.

The lack of correlation between the *dhps* mutation status in a given parasite line and the effect of added folate on the $IC_{50\,SDX}$ indicates that mutation(s) elsewhere must be responsible for the observed response. The highest correlation (15/16 progeny) of the folate effect to RFLP markers was with an anonymous fragment lying in the same quarter of chromosome 4 as the *dhfr-ts* gene. Sequencing of the corresponding *dhfr* domains revealed that all six progeny displaying the folate effect had inherited their *dhfr* gene from the Dd2 parent, while the other 10 all carried the HB3-type gene. However, when six further unrelated lines were examined, no clear correlation of folate effect with DHFR sequence was observed. The formal possibilities that would reconcile these two sets of data are either that the gene underlying the folate effect is closely linked to *dhfr* but is not *dhfr* itself, or that a property of the *dhfr* gene product other than its primary sequence (such as its level of expression) is involved. Transfection of the *dhfr* gene from Dd2 into a folate non-responder line like HB3 or FCR3 may help to resolve this question, an approach that is now feasible (Wu *et al.*, 1996).

What might be the role of the gene product involved in the folate effect? It appears that parasites of the Dd2 type are much better able to utilize exogenous folate than the HB3 type, and thus perhaps possess a more efficient uptake system, or a way of reducing sufficient of the folic acid directly to the DHF, and hence THF form, or an ability to induce breakdown of the folate molecule and re-use the components more efficiently. That the parasite can utilize folic acid at all may be considered unusual. Dietary folic acid is rapidly reduced and methylated in the gut to 5-methyltetrahydrofolate (Baker *et al.*, 1994), the predominant form found in both plasma (5–30 nM) and erythrocytes, where it is 30–100-fold more concentrated. However, in the latter, the folate exists almost exclusively as polyglutamated forms (Chanarin, 1980). It has been suggested that *Plasmodium* cannot use this rich store of reduced folate in the cells it invades (Ferone, 1977), because of a lack of a hydrolase (Krungkrai *et al.*, 1989) to remove the polyglutamate moieties, but this has not been directly measured. Our data are consistent with the erythrocyte being a negligible source of folate, because if the parasites were able to utilize this source to any degree, we would not expect the very low levels of added exogenous folate to give the large differential effects that we observe. However, a parasite type like Dd2 might have acquired the ability to efficiently take up monoglutamate forms from the extracellular milieu which, using its own pteroylpolyglutamate synthetase, it could then build up to the favoured pentaglutamate form (Krungkrai *et al.*, 1989).

With regard to the folate moiety itself, *Plasmodium berghei* was reported to lack any folate reductase activity (Ferone and Hitchings, 1966), and apparently neither wild-type nor mutant DHFRs from *P. falciparum* can reduce folic acid under standard conditions (W. Sirawaraporn, personal communication). A more likely fate may be cleavage of the folate molecule at the 9–10 bond to yield the pterin aldehyde and *p*-aminobenzoylglutamic acid. We note with respect to this last possibility that the DHFR from an antifolate-resistant strain of *E. coli* is capable of breaking down folate in this way (Poe, 1973). Moreover, human erythrocytes themselves have a latent activity that will cleave this bond under acidic conditions (Braganca *et al.*, 1957). Another possibility then is that Dd2-type parasites have an enhanced capability for activating a catabolic step of this nature.

Whatever the exact molecular basis of the folate effect, our results have important implications for the response of parasites to PSD. Parasite resistance to drugs spreads in the wild by frequent recombination events amongst different strains during the sexual cycle in infected mosquitoes. Acquisition of a mutant *dhps* gene will confer a level of resistance to SDX that broadly reflects the number of point mutations it is carrying. Acquisition of a mutant *dhfr* gene, as well as bestowing PYR resistance, may also reduce susceptibility to SDX by the co-acquisition of a gene closely linked to *dhfr*, which somehow enables the parasite to utilize exogenous folate to a much higher degree than in other strains. Most of the laboratory lines that we tested in addition to Dd2 also displayed the folate-effect phenomenon. Moreover, in Gambian children, folate supplements have been shown to compromise the effectiveness of Fansidar (van Hensbroek *et al.*, 1995). However, it remains to be established how widespread this phenotype is amongst parasites in the field, what the variation in its magnitude is, and thus how important it is on a global scale, relative to the incidence of *dhps* mutations, in reducing the effectiveness of the SDX component of PSD in malaria chemotherapy. Our results may also have implications for the treatment of infections by other parasitic organisms, such as *Toxoplasma gondii* and *Pneumocystis carinii*, where antifolate combinations containing sulfa drugs are also used.

Experimental procedures

Parasite lines

The parental *P. falciparum* HB3 and Dd2 lines, as well as the 16 well-characterized progeny of their cross, have been described previously (Wellems *et al.*, 1990; Walker-Jonah *et al.*, 1992), as have the lines 3D7, Tak9/96, FCR3, K1 and M24 (summarized in Brooks *et al.*, 1994). FCB is an uncloned African isolate. In the present work, progeny from the HB3–Dd2 cross are identified by a number from 1 to 16 for simplicity. Relative to their original nomenclature, these are: 1 (3B-B1); 2 (QC-13); 3 (B1-SD); 4 (QC-01); 5 (B4-R3); 6 (SC-05); 7 (TC-08); 8 (GC-03); 9 (3B-A6); 10 (1B-B5); 11 (3B-D5); 12 (SC-01); 13 (QC-34); 14 (QC-23); 15 (TC-05); 16 (GC-06).

For routine culture, parasites were grown in standard RPMI 1640 medium, 10% human plasma and supplements as described (Read and Hyde, 1993). In preparation for testing their SDX susceptibility, they were transferred into medium containing 0.5% Albumax I (Gibco-BRL; added from 10% stock in sterile water) instead of plasma or serum, custom RPMI medium supplied free of folic acid and PABA (Gibco-BRL), 1 $\mu g\,ml^{-1}$ hypoxanthine, with 100 $ng\,ml^{-1}$ of folic acid added back as a supplement. After they were sufficiently adapted to this medium (as judged by a healthy morphological appearance and a normal growth rate that enabled parasitaemias of $\geq 5\%$ to be easily achieved), the parasites were transferred to the same medium without folic acid 2 d before entering the SDX test described below.

In vitro *sulfadoxine assay*

To obtain reproducible and credible data for SDX inhibition levels, it was necessary to develop a modified assay, more extensive details of which will be presented elsewhere (P. Wang *et al.*, in preparation). Briefly, parasites were set up in the above medium at an initial 0.5% parasitaemia, 225 µl volume, in 96-well microtitre plates (Costar). SDX (stock solution 12–25 $mg\,ml^{-1}$ in tissue-culture grade DMSO) was added to samples over a concentration range of 0–24 $\mu g\,ml^{-1}$ (0–77 µM) or 0–100 $\mu g\,ml^{-1}$ (0–323 µM), as appropriate, with the volume of added drug solution being kept at 1 µl. After 48 h of incubation with the drug, 0.5 µCi of [^{3}H]-hypoxanthine (Amersham; 10–30 $Ci\,mmol^{-1}$) in 25 µl of test medium was added to each well to monitor growth (Chulay *et al.*, 1983) and samples were processed after a further 16 h. This entailed harvesting of the well contents onto 24 mm Whatman GF/C filters and removal of excess label by washing with 30 ml of distilled water. Filters were counted in 4 ml of Ecoscint scintillant (National Diagnostics) and data were analysed using locally developed curve-fitting software. IC_{50} values were defined as the concentration of SDX at which parasite growth was reduced to half that of the untreated controls.

To study the folate antagonism of SDX resistance, folic acid (pteroylmonoglutamic acid) was dissolved to 100 $\mu g\,ml^{-1}$ (227 µM) in 1 M $NaHCO_3$, sterilized by filtration, and added to cultures at the desired concentration. To control for the danger of cross-contamination inherent in culturing several parasite lines simultaneously, genotypes of all cultures were re-checked by PCR after completion of data collection for the SDX assays.

DNA sequence analysis

Polymerase chain reactions for rapid examination of mutant positions in the *dhps* gene were carried out as before (Wang *et al.*, 1995). For DNA sequencing, PCR products spanning the domains of interest were amplified from DNA extracted from individual parasite cultures and directly cycle-sequenced using an ABI PRISM dye terminator kit (Perkin Elmer) and an ABI 373 automated fluorescence sequencer.

Acknowledgements

The parasite lines resulting from the HB3–Dd2 cross were a generous gift of Dr Tom Wellems (NIH, Bethesda). We thank Dr Alan Cowman (WEHI, Melbourne) for stimulating discussions, and the Wellcome Trust and the Medical Research Council for financial support.

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Chapter 5

Functional identification of orthologous genes encoding pterin recycling activity in *Plasmodium falciparum* and *Toxoplasma gondii*

Published in 2006 in:

Molecular and Biochemical Parasitology, volume 146,

pages 109-112

Wang, Q., Hauser, V., Read, M., Wang, P., Hanson, A.D., Sims, P.F.G. and Hyde, J.E.

Available online at www.sciencedirect.com
SCIENCE DIRECT
Molecular & Biochemical Parasitology 146 (2006) 109–112
MOLECULAR & BIOCHEMICAL PARASITOLOGY

Short communication

Functional identification of orthologous genes encoding pterin recycling activity in *Plasmodium falciparum* and *Toxoplasma gondii*

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Received 22 September 2005; accepted 3 November 2005
Available online 28 November 2005

Keywords: Apicomplexa; Complementation; Malaria; Pterin-4a-carbinolamine dehydratase; Pterin metabolism; Toxoplasmosis

In higher eukaryotes, pteridine cofactors such as tetrahydrobiopterin (BH_4) are essential for a range of enzyme-catalysed reactions, including hydroxylation of aromatic amino acids and cleavage of ether lipids by specific monooxygenases, as well as production of nitric oxide by NO synthase [1]. Among the protozoa, it has long been known that trypanosomatids are pteridine auxotrophs that salvage the necessary molecules from the host organism [2]. Although pterins (i.e. naturally occurring pteridines with 2-amino and 4-oxy substitutions on the pteridine ring) are recognised as a growth factor for trypanosomatids, their precise functions in these organisms are not clear, but may include roles in dealing with oxidative stress and in parasite differentiation [3,4]. Interestingly, there is also evidence that *Leishmania* can synthesise folates from biopterin by a route that differs from the standard folate biosynthetic pathway found in other microorganisms [5]. These observations led us to consider a possible role for pterin metabolism in the apicomplexan parasites.

The conventional route to pterin synthesis, lacking in trypanosomatids, initially involves the conversion of GTP to 7,8-dihydroneopterin triphosphate by GTP cyclohydrolase I (GTPC; EC 3.5.4.16), an enzyme that is present in both *P. falciparum* [6] and *T. gondii* (Smith, Hyde and Sims, unpublished data). This product can then be utilised by many microorganisms (but not higher eukaryotes, other than plants) in a biosynthetic pathway leading to tetrahydrofolate and its derivatives, which are also key enzyme cofactors, or it can be acted upon by 6-pyruvoyltetrahydropterin synthase (PTPS; EC 4.2.3.12) and sepiapterin reductase (SR; EC 1.1.1.153) to yield BH_4.

Although much has been learnt about folate biosynthesis and salvage in *P. falciparum* [7] and to a lesser extent, in *T. gondii* [8], almost nothing is known about the pterin content of these organisms and any metabolic role such molecular species might play. The only annotated gene in the complete *P. falciparum* genome sequence hinting at the existence of pterin metabolism is PFF1360w, putatively encoding a PTPS orthologue, although there is no obvious orthologue of SR. However, we discovered an unannotated gene located between PF11_0095 and PF11_0096 whose predicted product bore histidine motifs separated by 16 residues characteristic of pterin-4a-carbinolamine dehydratase (PCD; EC 4.2.1.96) (Fig. 1). PCD is an enzyme that in many organisms is essential for the recycling of the BH_4 moiety, which is oxidised to the dihydro- level when acting as a cofactor for amino acid hydroxylations (e.g. Phe to Tyr, Tyr to L-dopa, Trp to 5-hydroxytryptamine) and other reactions. PCD executes a dehydration step, removing as water an –OH group introduced onto the 4a position of the pterin ring in the first step of BH_4 utilisation (Fig. 2). This gene may have been missed in the original annotation, as it is very short, with a coding length of 324 bp split by a 142 bp intron between codons 39 and 40. We also found an equivalent gene in the *T. gondii* database with a 315 bp ORF and a single intron (271 bp) in the same relative position (between codons 44 and 45) as in the *P. falciparum* gene (Fig. 1).

To test the function of a putative PCD gene biochemically is not straightforward as the carbinolamine substrate is not readily available. We therefore took advantage of the fact that phenylalanine hydroxylation systems are relatively rare in prokaryotes to set up a microbiological complementation test in an *Escherichia coli* tyrosine auxotroph [9]. Thus, *E. coli* lacks both phenylalanine hydroxylase and PCD genes, but does have a dihydropteridine reductase activity (DHPR; EC 1.6.99.7), which is necessary

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0166-6851/$ – see front matter
doi:10.1016/j.molbiopara.2005.11.002

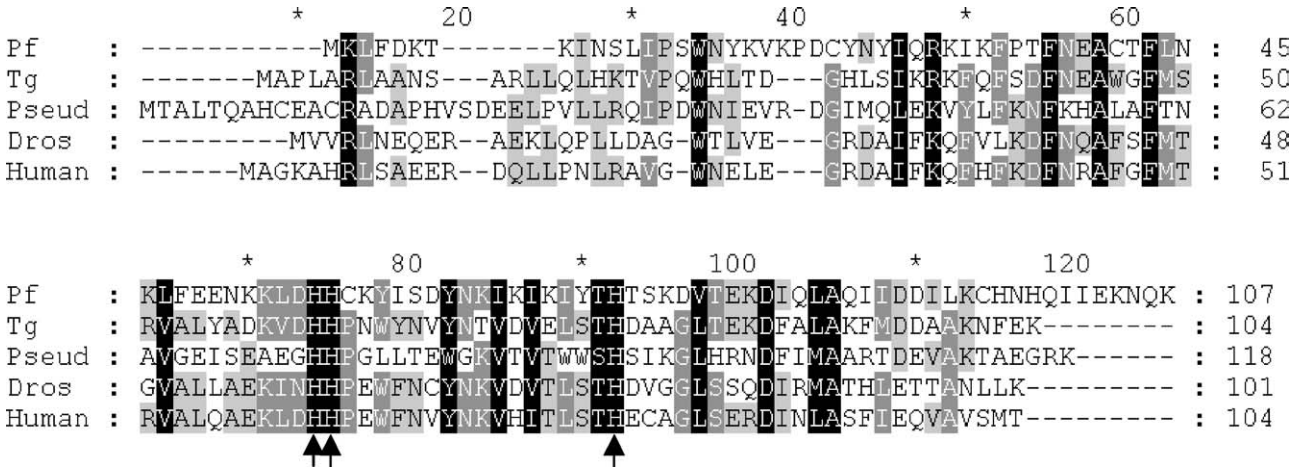

Fig. 1. Alignment of pterin-4a-carbinolamine dehydratase (PCD) sequences. Pf, *P. falciparum* (this work, accession no. DQ223776); Tg, *T. gondii* (this work, DQ223777); Pseud, *Pseudomonas aeruginosa* (P43335); Dros, *Drosophila melanogaster* (AAC25196); Human, *Homo sapiens* (P80095). Arrows indicate the three conserved His residues involved in binding the pterin ligand [13].

for completion of a pterin recycling pathway (Fig. 2). However, phenylalanine hydroxylase and PCD genes both occur in certain bacteria including the gamma-proteobacterium *Pseudomonas aeruginosa* [10]. Thus, when transformed into *E. coli*, the products of these two genes can combine with the endogenous DHPR to establish a pterin recycling assay with the necessary positive controls.

Our strategy was thus to assay for recycling by the successful synthesis of Tyr from Phe, dependent upon the introduced *P. aeruginosa* phenylalanine hydroxylase and PCD activities, and then test the putative *P. falciparum* and *T. gondii* PCD genes for function by substituting them for the *P. aeruginosa* PCD gene in the positive control. Without pterin recycling, the synthesis of Tyr is inadequate for viability. After PCR amplification of the *P. falciparum* and *T. gondii* genes from cDNA libraries, the products were cloned into pGEM-T or pGEM-T Easy (Promega) in such a way that they were positioned downstream of the *lacZ* promoter with a stop codon in-frame with the *lac* gene situated shortly before the PCD ATG start codon. This was to truncate the synthesis of the beta-galactosidase alpha-peptide product. We were thus depending upon translational reinitiation at the start codon of a correctly orientated PCD gene in order to observe activity. To complete the recycling system, the *P. aeruginosa* phenylalanine hydroxylase gene (*phhA*) was introduced into the *E. coli* host (JP2255) on a compatible pACYC177-based plasmid, pJSll [9].

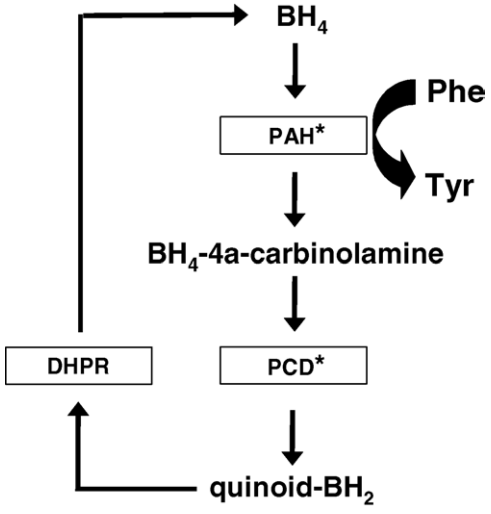

Fig. 2. Recycling of tetrahydrobiopterin (BH_4) via the pterin-4a-carbinolamine dehydratase (PCD) activity after acting as a cofactor in the hydroxylation of phenylalanine by phenylalanine hydroxylase (PAH). The redox cycle is completed by dihydropterin reductase (DHPR). Asterisks indicate enzymes absent in *E. coli* but present in *P. aeruginosa*.

For both *P. falciparum* and *T. gondii*, clones were tested for their ability to rescue the *E. coli* mutant to a degree comparable to that observed with the *P. aeruginosa* positive control, as judged by growth rate on the minimal medium agar plates containing phenylalanine (Fig. 3A and B). All positive clones were found to contain the putative PCD gene orientated in the sense direction with respect to the *lacZ* promoter. To further confirm that the activity was not spurious but was a direct result of the genes we had inserted, the *T. gondii* ORF was reversed in the plasmid while the *P. falciparum* gene was cut with *Bst*BI and the two-base overhang filled in with Klenow polymerase, causing the PCD reading frame to be disrupted about half-way into the coding sequence. Neither of these constructs was able to rescue the *E. coli* auxotroph (Fig. 3C and D). We thus conclude that the genes we have identified in *P. falciparum* and *T. gondii* indeed encode PCD activity.

Our observations point to a new area of metabolism that merits investigation in these apicomplexan organisms. It is not yet clear what role pterins might play in these parasites, although observations made in trypanosomatids are suggestive [4]. As found in *E. coli* and *P. aeruginosa*, although both *P. falciparum* and *T. gondii* appear to have a gene encoding PTPS, albeit functionally untested, there seems to be no equivalent of an SR-encoding gene whose product could convert 6-pyruvoyltetrahydropterin to BH_4, at least in the case of *P. falciparum*. It has been suggested in the case of the bacterial systems that one or more pterin cofactors other than BH_4, such as

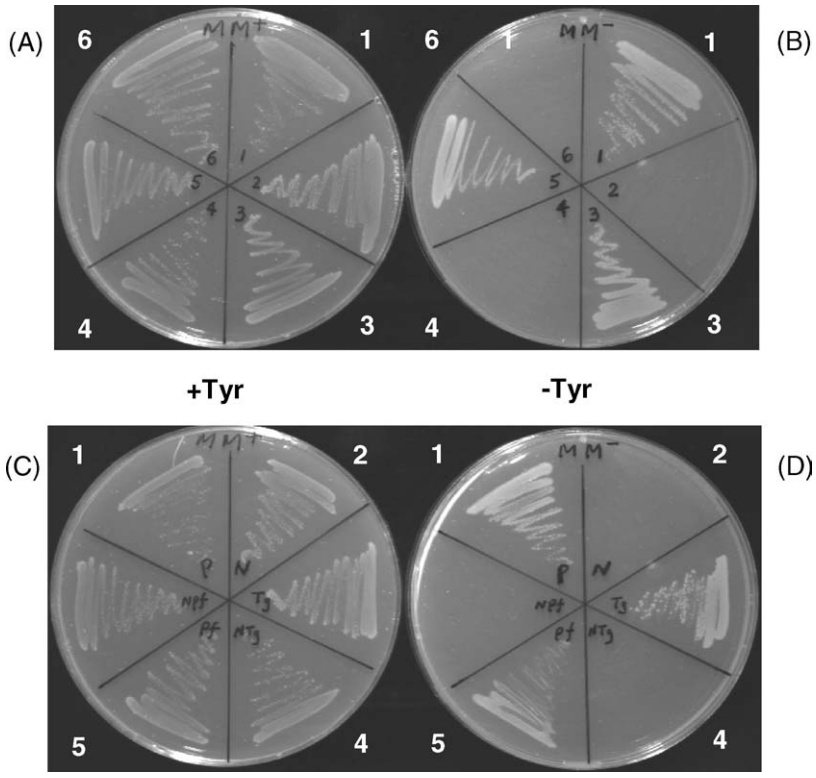

Fig. 3. Complementation tests in the *E. coli* tyrosine auxotroph JP2255 [9] of the putative *P. falciparum* and *T. gondii pcd* genes. Panels A and B: sector 1, positive control carrying *Pseudomonas phhB* plus *Pseudomonas phhA* genes; 2, negative control carrying *Pseudomonas phhB* only; 3, *T. gondii* cDNA clone tg2 plus *Pseudomonas phhA*; 4, *T. gondii* cDNA clone tg2 alone; 5, *P. falciparum* cDNA clone pf3 plus *Pseudomonas phhA*; 6, *P. falciparum* cDNA clone pf3 alone. Panels C and D: sectors 1 and 2, controls as above; 3, *T. gondii* cDNA clone tg2 plus *Pseudomonas phhA*; 4, *T. gondii* clone tg2 with inverted insert plus *Pseudomonas phhA* gene; 5, *P. falciparum* cDNA clone pf3 plus *Pseudomonas phhA* gene; 6, *P. falciparum* clone pf3 with insert frameshifted ca. half-way along the ORF, plus *Pseudomonas phhA* gene. Note that the *Pseudomonas phhB* gene (introduced on plasmid pJZ9-4 [9]) encodes PCD. Growth was for 48 h at 37 °C on minimal medium plates supplemented with 10 $\mu g\,ml^{-1}$ streptomycin, 50 $\mu g\,ml^{-1}$ phenylalanine, with (A and C) or without (B and D) 50 $\mu g\,ml^{-1}$ tyrosine.

tetrahydroneopterin, might be involved in the recycling reaction described above [9]. The feasibility of this is reinforced by the fact that chemical studies of the rat PCD enzyme have demonstrated a lack of sensitivity to the nature of the 6-substituent, and to the stereochemistry of this or the 4a-hydroxyl group [11], indicative of a flexible binding pocket. Interestingly, it has also been shown in humans that the PCD protein plays a second role as a transcriptional regulator (known as DCoH) in the nucleus [12], although this may be a function only found in higher organisms. PCD genes appear to be quite widely distributed in bacteria, but the known enzymes that use a pterin cofactor, such as the hydroxylases described above, are much less common. Thus there probably remain to be discovered in bacteria, and possibly in apicomplexans, other enzymes that produce a carbinolamine intermediate requiring recycling via PCD. The identification and functional demonstration of a pterin recycling activity reported here is a first step in exploring the likely importance of pterin metabolism in *P. falciparum* and *T. gondii*.

Acknowledgements

We thank Roy A. Jensen (University of Florida) for *E. coli* strain JP2255 and plasmid pJZ9-4, and the Wellcome Trust, UK (Grant No. 056845) for financial support.

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Chapter 6

Dynamic subcellular localization of isoforms of the folate pathway enzyme serine hydroxymethyltransferase (SHMT) through the erythrocytic cycle of Plasmodium falciparum

Published online in 2010 in:

Malaria Journal, volume 9 (351)

Read, M., Müller, I. B., Mitchell, S. L., Sims, P. F. G., Hyde, J. E

Due to the many small colour micrographs this paper is more advantageously viewed in an electronic format.

It may be accessed online at:

http://www.malariajournal.com/content/pdf/1475-2875-9-351.pdf

alternatively, a disc containing the article is available, attached to the back cover of the thesis.

Erratum: Figure 4 legend. The description of (D) should read:
(D) Mitotic schizont developmentally a little later than (C) showing an apicoplast in the early stages of ramification. The area of intense PfSHMTc fluorescence follows the 'Y' shape of the apicoplast closely.

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Dynamic subcellular localization of isoforms of the folate pathway enzyme serine hydroxymethyltransferase (SHMT) through the erythrocytic cycle of *Plasmodium falciparum*

Martin Read[1], Ingrid B Müller[2], Sarah L Mitchell[1,3], Paul FG Sims[1], John E Hyde[1*]

Abstract

Background: The folate pathway enzyme serine hydroxymethyltransferase (SHMT) converts serine to glycine and 5,10-methylenetetrahydrofolate and is essential for the acquisition of one-carbon units for subsequent transfer reactions. 5,10-methylenetetrahydrofolate is used by thymidylate synthase to convert dUMP to dTMP for DNA synthesis. In *Plasmodium falciparum* an enzymatically functional SHMT (PfSHMTc) and a related, apparently inactive isoform (PfSHMTm) are found, encoded by different genes. Here, patterns of localization of the two isoforms during the parasite erythrocytic cycle are investigated.

Methods: Polyclonal antibodies were raised to PfSHMTc and PfSHMTm, and, together with specific markers for the mitochondrion and apicoplast, were employed in quantitative confocal fluorescence microscopy of blood-stage parasites.

Results: As well as the expected cytoplasmic occupancy of PfSHMTc during all stages, localization into the mitochondrion and apicoplast occurred in a stage-specific manner. Although early trophozoites lacked visible organellar PfSHMTc, a significant percentage of parasites showed such fluorescence during the mid-to-late trophozoite and schizont stages. In the case of the mitochondrion, the majority of parasites in these stages at any given time showed no marked PfSHMTc fluorescence, suggesting that its occupancy of this organelle is of limited duration. PfSHMTm showed a distinctly more pronounced mitochondrial location through most of the erythrocytic cycle and GFP-tagging of its N-terminal region confirmed the predicted presence of a mitochondrial signal sequence. Within the apicoplast, a majority of mitotic schizonts showed a marked concentration of PfSHMTc, whose localization in this organelle was less restricted than for the mitochondrion and persisted from the late trophozoite to the post-mitotic stages. PfSHMTm showed a broadly similar distribution across the cycle, but with a distinctive punctate accumulation towards the ends of elongating apicoplasts. In very late post-mitotic schizonts, both PfSHMTc and PfSHMTm were concentrated in the central region of the parasite that becomes the residual body on erythrocyte lysis and merozoite release.

Conclusions: Both PfSHMTc and PfSHMTm show dynamic, stage-dependent localization among the different compartments of the parasite and sequence analysis suggests they may also reversibly associate with each other, a factor that may be critical to folate cofactor function, given the apparent lack of enzymic activity of PfSHMTm.

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Background

Malaria parasites are a major cause of mortality and morbidity, resulting in over a million deaths each year and 350 to 500 million clinically significant malaria infections [1]. Folate metabolism is the target of a number of anti-malarial drugs, which, though compromised by the occurrence and spread of resistance within parasite populations, remain important in treatment and prophylaxis [2,3]. For almost all organisms, the folate pathway is essential in maintaining a constant supply of cofactors that act as donors or acceptors of one-carbon (C_1) units in a variety of biosyntheses. In malaria parasites, the most prominent of these is the synthesis of pyrimidines required for DNA replication [4]. Unlike mammals, *Plasmodium falciparum* cannot salvage thymidine and thus relies completely on the folate-dependent production of dTMP. The folate pathway can be conveniently divided into two main sections: the first five enzyme activities effect the *de novo* biosynthesis of the basic folate moiety, 7,8-dihydrofolate (DHF), with further enzymes interconverting the fully reduced form 5,6,7,8-tetrahydrofolate (THF) to the various derivatives utilized in C_1 transfer reactions. Plants and most microorganisms, including many protozoa, are able to synthesize folates *de novo*. In contrast, higher organisms must obtain folate from the diet or commensal microorganisms. It has been shown that *P. falciparum* has the ability to exploit both *de* novo synthesis and folate salvage routes for its metabolic needs [5-7].

The later part of the folate pathway directly relevant to DNA replication is termed the thymidylate cycle. In this, dihydrofolate reductase (DHFR; EC 1.5.1.3) catalyses the reduction of DHF to THF. Serine hydroxymethyltransferase (SHMT; EC 2.1.2.1), the subject of this study, reversibly catalyses the conversion of serine to glycine, whereby the hydroxymethyl group of the former is transferred to THF yielding 5,10-methylenetetrahydrofolate (5,10-methylene-THF), which is then used by thymidylate synthase (TS; EC 2.1.1.45) as the C_1 donor to convert dUMP to dTMP. Concomitantly, the folate cofactor is oxidized to the dihydro-form, making a functional cycle that is capable of reducing this back to THF essential for continued DNA synthesis. A further activity, folylpolyglutamate synthase (FPGS; EC 6.3.2.17), part of a bifunctional protein also carrying dihydrofolate synthase (DHFS; EC 6.3.2.12) [8-10] adds a variable length polyglutamate tail to reduced folate cofactors, a phenomenon involved in subcellular storage and the retention of folates within the cell [11-13].

Despite much research detailing the biochemistry of the folate pathway and the genetic basis of resistance to antifolate drugs, there has been very little investigation of the subcellular location of folate pathway enzymes or their metabolites in malaria parasites. In other eukaryotes there is substantial evidence for the compartmentalization of folate metabolism within the cell. In particular, fully reduced substituted folates (such as 5-methyl-THF) appear not to exchange between mitochondrial and cytoplasmic compartments, suggesting that limited transport of intermediates between subcellular compartments may be an important factor in enzyme localization [14].

Consistent with this, the majority of methyl derivative forms are associated with the vacuole and cytosol, whereas formyl derivatives make up the greatest proportion of folates located within organelles, at least in plants [15]. In these organisms, the first two enzymes of biosynthesis, GTP cyclohydrolase I (GTPCH; EC 3.5.4.16) and dihydroneopterin aldolase (DHNA; EC 4.1.2.25), are found exclusively in the cytoplasm, whereas most of the remaining enzymes are located exclusively in the lumen of the mitochondrion. SHMT and FPGS are exceptional as they are found in both the cytoplasm and mitochondrion as well as the plant chloroplast. The two forms of SHMT, SHMTc and SHMTm, occur as distinct proteins encoded by different genes [16], but the chloroplast enzyme appears not yet to have been characterized as a separate isoform. Distinct SHMT isoforms are also found in the cytoplasm and mitochondria of yeast and the trypanosomatid *Leishmania major* [17], and in mammals, the distribution of both SHMTc and SHMTm differs between tissues and at different stages of development [18,19]. Glycine formed from SHMT acting on serine can feed into the glycine cleavage complex (GCV) of the mitochondrion, which provides an additional source of C_1 units by transferring the α-carbon of glycine onto THF [20]. *Plasmodium falciparum* SHMT, described here as PfSHMTc, is encoded by a single copy gene at locus PFL1720w [8] and its enzymic properties are well characterized [21-23]. A second open reading frame has also been identified (PF14_0534) that encodes a product with an 18% identity to PfSHMTc and incorporates a putative mitochondrion-specific tag [24]; this protein is described here as PfSHMTm. However, its sequence displays an almost complete lack of conservation of those amino acids that constitute the active site residues of all other SHMT isoforms, both cytoplasmic and mitochondrial, consistent with a failure to detect SHMT activity in a recombinant form of the protein [23]. The metabolic or other function of this related gene product thus remains to be identified, and particularly whether it could act in conjunction with a GCV in plasmodial mitochondria. Components of a potential GCV have been identified bioinformatically in *P. falciparum* [24], although experimental evidence for their mitochondrial location has thus far only been established for the H-protein [25].

The multiplicity of environments that the parasite must accommodate in its complex life cycle suggests that adaptability in folate metabolism and its enzymes is highly probable, and that variation in enzyme localization over the life cycle might occur. Additionally the parasite exhibits a number of unusual developmental features that could result in differences in folate metabolism from other eukaryotes. The schizogonic nature of asexual reproduction, with its repeated and apparently asynchronous cryptomitoses, results in an atypical cell cycle [26,27]. Peculiarities in the timing and duration of events associated with DNA replication may result in temporal variation in the demand for pyrimidine synthesis [28]. Here, patterns of localization of PfSHMTc during the erythrocytic cycle of *P. falciparum* are investigated because of its key role in dTMP synthesis, the strongly modulated level of transcriptional control of its gene and its relatively higher levels of expression compared to other enzymes in the folate pathway [29-31]. In parallel, the localization of the enigmatic PfSHMTm protein is investigated, which shows similarities in its behaviour, but with distinct and important differences from the PfSHMTc isoform.

Methods

Cloning and heterologous expression of the *pfshmt* genes

The full-length cytoplasmic *pfshmt* gene (PFL1720w) was amplified from cDNA previously cloned into pMALc2x (New England Biolabs). The intronless full-length *shmt* homologue PF14_0534 was amplified from K1 isolate genomic DNA, and both products were cloned into the pET-46 Ek/LIC vector (Novagen). The cytoplasmic *pfshmt* clone was expressed in the BL21 (DE3) pLysS expression host whilst the PF14_0534 ORF was expressed in Rosetta 2 (DE3) pLysS (Novagen). Cultures of both clones were harvested using the Bugbuster kit (Novagen) and the insoluble phases subjected to SDS-PAGE and subsequently blotted onto nitrocellulose. Fractions were loaded on the PAGE gel to give equal protein quantities in the bands of interest between the two expressed protein products. Western blots were probed with anti-PfSHMTc IgY or anti-PfSHMTm IgY, or anti-polyhistidine IgG primary antibodies (see below) followed by the appropriate AP conjugate secondary antibodies (Promega), and developed using standard methods [32].

Parasite culture and transfection

Parasites (either K1 or 3D7) were grown in 25 cm^2 tissue culture flasks with 1 ml of blood (type O; 50% haematocrit) and 10 ml of medium as described [33]. Flasks were harvested at a parasitaemia of 8 - 15%. The use of synchronous cultures was investigated but yielded no significant advantage owing to the inherent asynchrony of the repeated mitoses in individual cells [26,34]. The developmental stage of a particular parasite within asynchronous cultures was ascertained through its size, haemozoin development, number of nuclei and overall morphology. For GFP-labelling studies, 3D7 parasites were transfected with appropriate plasmid constructs encoding SHMT-GFP fusion proteins essentially as described [35], using the primers pfSHMTm-kpn-s (gcgcggtaccATGCTGAAGGAGTTTGTTAAAAATG) and pfSHMTm100-avr-as (gagacctaggGCAACCCCAATATTTCTTTTGTAA) to clone the truncated *pfshmtm* gene described in Results into the pARL1a- vector [36].

Western blotting of parasite extracts

Parasite extracts were also prepared for western blotting by freeze-thawing, in which 1 ml of blood at 50% haematocrit and *ca*. 10% parasitaemia was saponin lysed and washed in PBS. The resulting parasite pellet was resuspended in 0.1 ml of deionized water and subjected to 5 rounds of freezing and thawing. Following centrifugation the supernatant was recovered and 20 μl (equivalent to *ca*. 10^8 parasites) used per lane on 12% acrylamide SDS-PAGE gels. Protein was transferred to nitrocellulose using a Biorad Mini Protean II blotter; blots were probed with primary antibody and secondary alkaline phosphatase-conjugated antibodies (Promega).

Antibodies

The PfSHMTc primary polyclonal antibody (IgY) was raised in chickens against the denatured product of a 70-codon DNA segment [369-GIRIG...QWAKN-438] located towards the 3' terminus of the *pfshmt* gene (PFL1720w) expressed in *E. coli* as a GST fusion. The PfSHMTm primary polyclonal antibody (IgY) was raised in chickens against the denatured full-length gene product expressed in *E. coli* as a His-tagged fusion. The same gene product was additionally used to raise antibodies (IgG) in rabbits. All three antibodies were commercially produced by Eurogentec. The donated apicoplast-specific antibody, anti-acyl carrier protein (anti-ACP; IgG) was raised in rabbits [37], as were the donated antibodies against the cytoplasmic enzymes chorismate synthase and cyclin-dependent protein kinase 5 [38]. The donated 3D7 parasite transfectant strain carrying pSSPF2/PfACP-DsRED [39] was used to confirm data obtained with the anti-PfACP antibody and control for possible interactions between this and other primary antibodies used simultaneously. The secondary antibodies, Alexafluor (488, 546 and 594 nm) goat anti-chicken IgY and anti-rabbit IgG, were obtained from Molecular Probes, as were the MitoTracker Orange CMTMRos mitochondrial probe and the DNA stain YOYO-1 (491/509 nm).

Immunofluorescence: parasite fixation, permeabilization and staining

A preparative method was developed to maximize the fluorescence intensity of the target proteins by ensuring a high degree of penetration of both primary and secondary antibodies and sufficient incubation with the primary antibody. This was necessary as soluble enzymes are frequently found in relatively low concentrations and are thus less readily visualized than structural, membrane-associated or exclusively organelle-bound proteins. Moreover, malarial folate pathway enzymes are known from both transcriptional and proteomic measurements to be expressed at low levels [29-31]. Preservation of the erythrocyte membrane proved to be largely impracticable due to lysis caused by a combination of detergent extraction and the mechanical stresses inherent in the mixing and centrifugation steps. Transmission light images were thus relatively poor and often obscured by erythrocyte ghosts; they are included merely to indicate the position of the haemozoin within the parasite pigment vacuole. However, the high degree of preservation of the parasites themselves using this method is evident in the undistorted images of internal fine structure shown. Significantly, the control apicoplast-specific antibody (anti-PfACP; see below) showed no fluorescence within the apicoplast in the absence of detergent permeabilization, demonstrating that any immunofluorescence investigation of the internal distribution of proteins within parasites should always ascertain the necessity of such a step.

Giemsa-stained thin blood smears were taken from all cultures used for immunofluorescence imaging upon harvesting to ensure that parasites showed normal undamaged morphology and healthy growth. Cultures selected for mitochondrial staining were incubated at 37°C for 45 min with MitoTracker Orange CMTMRos freshly dissolved in dimethylsulfoxide to give 100 nM final concentration in the medium. A 1 ml volume of parasitized erythrocytes was harvested by centrifugation (3,000 g, 5 min). Pelleted cells were resuspended and fixed in 5 ml freshly prepared 3.7% (w/v) paraformaldehyde in phosphate buffered saline (PBS) for a minimum of 2 h at 4ËšC. Following fixation, the parasitized blood was centrifuged (as above) and washed twice in blocking/wash solution (1 ml PBS, 0.5% (w/v) BSA, 2% (v/v) bovine serum; Sigma) in parallel-sided, screw-capped microfuge tubes with rotational mixing at room temperature for 5 min followed by centrifugation (8,000 g, 30 s). Cells were then incubated in wash buffer plus 0.25% (v/v) Triton X-100 for 5 min to increase permeability. After a further three washes in wash buffer (also used in all subsequent washes), primary antibodies (diluted 1:100 in wash buffer) were added and incubated overnight at 4°C. The samples were then washed four times and incubated with fluorescent secondary antibodies, diluted to the manufacturer's specification (usually 2.5 μl in 1,000 μl), for 2 - 4 h at room temperature. This was followed by three washes, then the DNA was labelled by the addition to the cells suspended in 1 ml of wash solution of 20 μl YOYO-1 (diluted 1:1,000 in wash buffer) and incubation at room temperature for 5 min. The cells were then centrifuged, washed for 1 min and then immediately centrifuged again (as above). The pelleted cells were resuspended in 100 - 250 μl Mowiol (Harco, UK) mountant and mounted on microscope slides under a coverslip [32].

Microscopy

Parasites labelled for immunofluorescence were viewed by laser scanning confocal microscopy using a Zeiss Axiovert 200 M microscope with argon (548-514 nm) and helium/neon lasers (543 nm, 632.8 nm) using a 100× oil immersion objective lens. Images were viewed and analysed using a combination of Zeiss LSM image software and Imaris 5.7.1 software (Bitplane Scientific Solutions). The latter allows the qualitative display of combined colours from co-localized probes to be quantitatively analysed by providing measurements of their overlap in three dimensions by analysing z-stack scans taken through the whole span of the organelle [40]. This enables a much more accurate assessment of coincidence of the labels than is possible with single-plane images. Co-localization is expressed as a percentage of the individual fluorochrome volume and material (the latter derived from volume and fluorescence intensity) that occupies the same 'voxels' (three-dimensional pixels) as the second fluorochrome. For a single cell, similar values between volume % and material % co-localized indicate similar concentrations inside and outside the organelle, whereas a higher organellar material % compared to volume % indicates concentration of the target protein relative to the cytoplasm. Three-dimensional projections were created from scans with a z-axis interval of 0.2 μm. This was the minimum increment possible before the scans became excessively long, resulting in unacceptable levels of photobleaching. Scans were sequential, with each colour wavelength scanned in rotation for each single plane image or within each plane in a z stack. For clarity, orange wavelength fluorescence (Alexafluor 546, DsRED and MitoTracker) is false-coloured green, green wavelength fluorescence (Alexafluor 488 and YOYO-1) is false-coloured blue, but far-red fluorescence (Alexafluor 594) is unchanged in all of the images displayed. Transfected parasites expressing GFP-fusion protein endogenously were imaged as previously described using Hoechst 33342 to visualize the nuclei [35] and MitoTracker Red CMXRos (0.625 nM) to visualize the mitochondrion. All images presented are

representative examples of each feature as seen in multiple samples.

Imaging controls

Antibody extracts from pre-immune yolk showed no bands when applied to western blots of total parasite proteins in parallel with the antigen-specific antibodies. Control slides labelled with secondary antibodies alone, or with combinations of secondary antibodies (used to control for artifactual interactions), showed no visible fluorescence when scanned using identical microscope settings and computer processing parameters as those used in producing the images shown. As the anti-PfSHMTc antibody was raised to a GST fusion polypeptide, the possibility of it recognising a plasmodial GST orthologue was excluded using a commercial polyclonal anti-GST IgG (GE Healthcare) on parasites as described above, which also showed no visible fluorescence. Haemozoin auto-fluoresces at a number of wavelengths, however its crystalline nature makes its fluorescence easily recognized and an appropriate choice of filters avoided interference with any of fluorochromes used.

To provide controls against the possibility that the preparative method used might artifactually produce organellar fluorescence in a non-specific manner, polyclonal antibodies against two unrelated enzymes, chorismate synthase and cyclin-dependent protein kinase 5, were also employed, which had been previously characterized as showing a simple cytoplasmic distribution in *P. falciparum* [38]. These control antibodies were employed with 3D7 parasites expressing the DsRED labelled apicoplast-specific protein PfACP [39]. Parasites were treated with control antibody in parallel procedures alongside parasites treated with anti-PfSHMTc and anti-PfSHMTm antibodies. No level of apicoplast-specific fluorescence was observed with either control antibody, which produced a generalized staining of the parasites with no evidence of fluorescence adopting the shape of apicoplasts (see Additional file 1 Negative control images for organellar staining). Furthermore, to exclude the possibility that artifactual interactions between the apicoplast-specific antibody anti-PfACP and the anti-PfSHMT antibodies were occurring, the latter were also used in conjunction with the above DsRED-transfected parasites, yielding identical patterns as those obtained using two primary antibodies simultaneously (see Results).

Results

Antibody specificity with respect to PfSHMTc and PfSHMTm

The *pfshmt* gene from *P. falciparum* (PFL1720w) [41] encodes a product that has been functionally characterized as a conventional cytoplasmic SHMT [21-23]. However, a predicted SHMT-like gene product (PfSHMTm, encoded on PF14_ 0534) was also identified that carries a putative mitochondrial signal sequence [24] with 18% amino acid identity and 44% similarity to PfSHMTc, but lacks almost all (16 of 21) of the known, very highly conserved residues [42] contributing to the active site in SHMT orthologues from other organisms, whether cytoplasmic or organellar (See Additional file 2 Sequence alignments of the PfSHMT isoforms). Despite the relatively low level of identity, it was essential to establish the specificity of the anti-PfSHMTc and anti-PfSHMTm antibodies that had been raised to be certain of the identity of the protein yielding positive signals. Both full-length open reading frames were therefore cloned in *Escherichia coli* expression systems and equal amounts of protein products processed for western blotting. The anti-PfSHMTc antibody recognized the heterologously expressed cognate protein (Figure 1B) and blots of total parasite lysates from two lines, K1 and 3D7, showed a single band also at the predicted size (49.8 kDa) for the full length PfSHMTc protein (Figure 1D). Importantly, there was no evidence for cross-reaction with the PfSHMTm product of PF14_0534 (Figure 1B), whereas control anti-His-tag antibodies recognized both recombinant products essentially equally (Figure 1A). This engendered confidence that subsequent immunofluorescence signals using the cognate antibody arose solely from PfSHMTc. In the case of PfSHMTm, this antibody was raised to the whole protein (unlike the anti-PfSHMTc antibody), some cross-reaction with PfSHMTc was not unexpected and was evident on blots against recombinant protein. However, this was approximately fourfold less intense than that seen in recognising the cognate PfSHMTm protein (Figure 1C). Against parasite extracts, the anti-PfSHMTm antibodies gave a predominant band with the same mobility as the recombinant protein (Figure 1E). It was noted on all blots that PfSHMTm ran slightly ahead of PfSHMTc, despite its somewhat higher predicted molecular weight (55.2 kDa). These differences in specificity led us to conclude that the differences seen below in immunofluorescence images of parasites probed with anti-PfSHMTc from those produced using anti-PfSHMTm are a reliable indicator of biologically significant variations in the distribution of the respective target proteins.

Cytoplasmic distribution of the PfSHMT isoforms

SHMT subcellular distribution in a number of organisms shows a partition between cytoplasmic SHMT and distinct isoforms of the enzyme located within organelles. As only PfSHMTc has thus far been confirmed as enzymatically active in *P. falciparum* [21-23], a single cellular location might be predicted. However, initial probing using its cognate antibody showed that PfSHMTc does not follow such a simple distribution

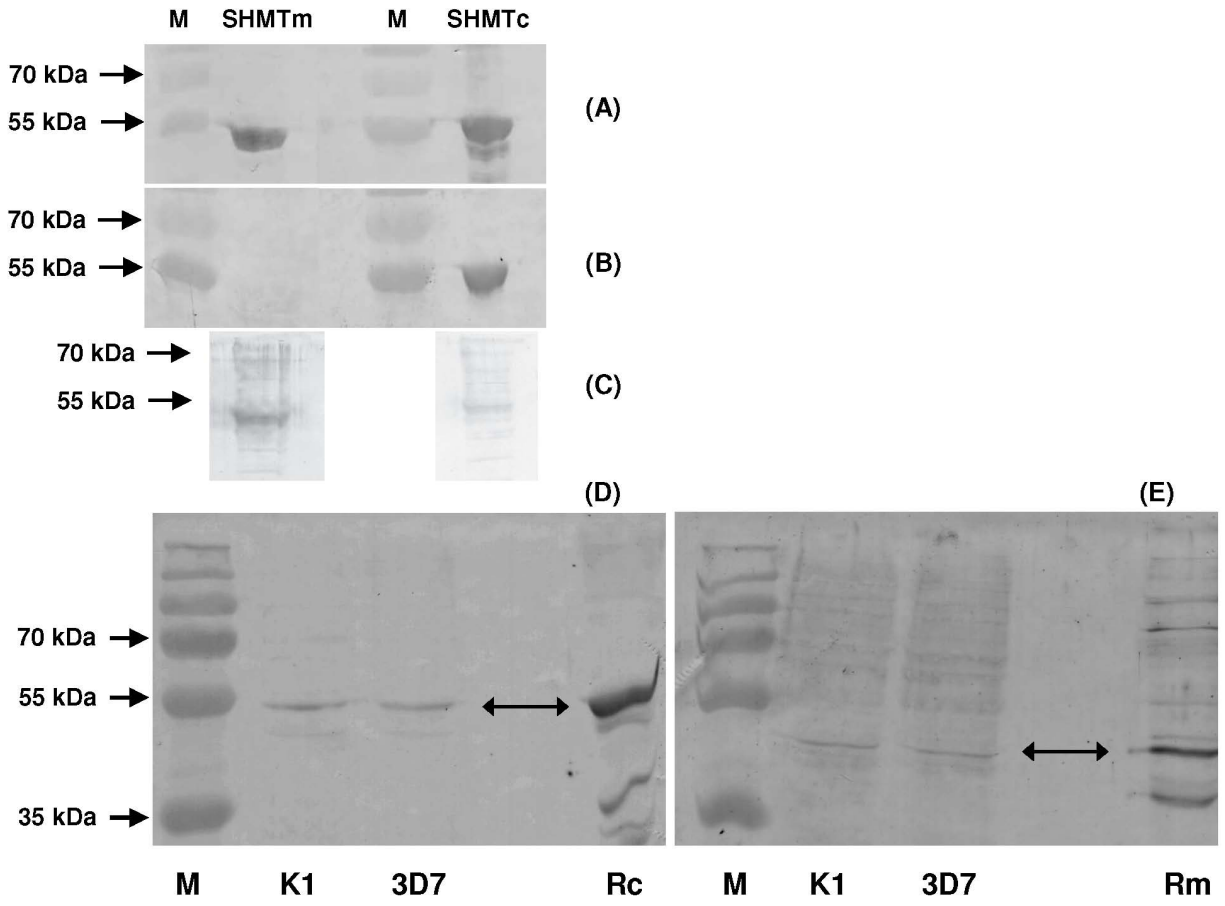


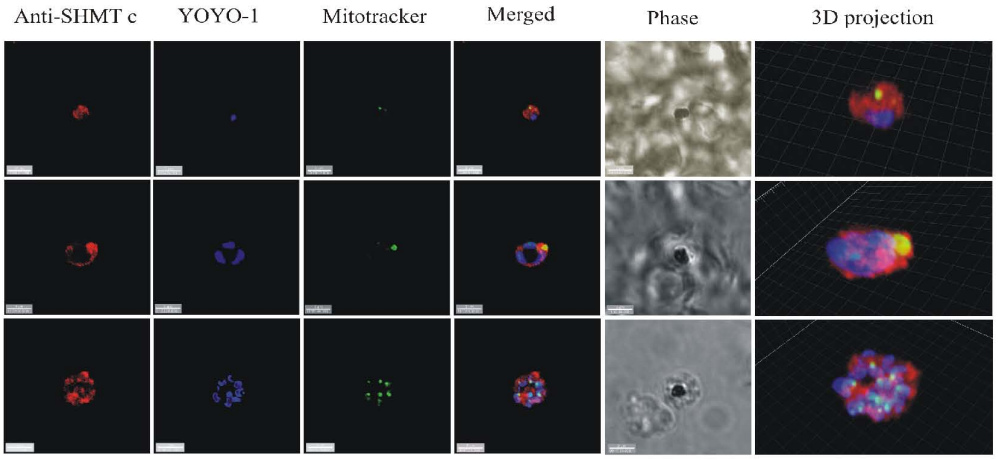
Figure 1 Specificity of the polyclonal anti-PfSHMT preparations. Full-length His-tagged recombinant protein (500 ng) expressed from the genes encoding PfSHMTc (PFL1720w) and PfSHMTm (Pf14_0534) probed on western blots with (A) anti-polyhistidine IgG, (B) the anti-PfSHMTc and (C) the anti-PfSHMTm preparations used for subsequent immunofluorescence studies. Panels D and E are western blots of total parasite extracts from K1 and 3D7 probed with anti-PfSHMTc (D) and anti-PfSHMTm (E). Rc, recombinant PfSHMTc; Rm, recombinant PfSHMTm; M, prestained molecular weight markers.

pattern during the erythrocytic cycle. All stages showed an expected generalized cytoplasmic staining and this, by visual examination and volumetric analysis by the Imaris software, is where the majority of the PfSHMTc molecules are located for most of the time. However, fluorescence brightness within the cytoplasm was not uniform and constriction of cytoplasm between organelles, especially nuclei, produced a patchy appearance (Figure 2). The PfSHMTm protein (Figure 3) showed an almost identical cytoplasmic distribution to that described for the PfSHMTc enzyme, as can also be seen in Figure 4, 5, 6, 7, 8 and 9, in which the anti-PfSHMTc and anti-PfSHMTm antibodies are used in various combinations with organellar labels. However, images obtained where both antibodies were used in combination did show some minor differentiation in cytoplasmic localization and relative concentration within individual parasites, exemplified by Figure 5C, D and 5F.

Mitochondrial localization of PfSHMTc

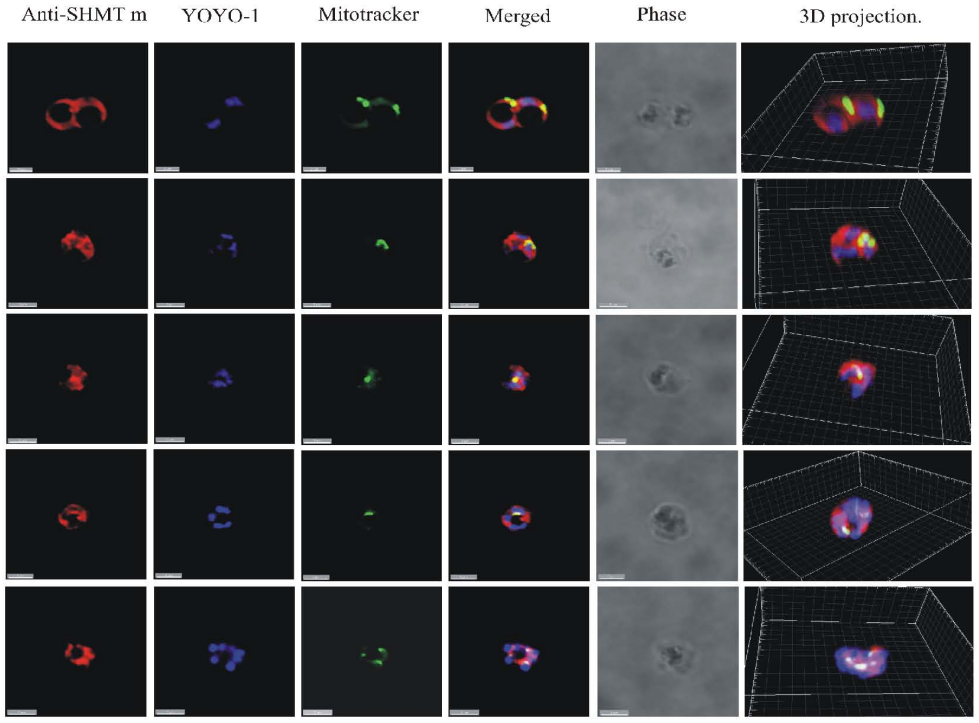
The mitochondrion and the apicoplast undergo a similar, though not simultaneous, morphological evolution during the development of erythrocytic stage parasites. The two organelles are found in close physical association and a junction between their respective membranes has been described [43,44]. The organelles increase in size, and in the case of the K1 isolate used here, were often observed to adopt a globular shape in the early schizont stage; thereafter they lengthen and ramify, eventually dividing to allow one of each organelle to associate with each individual developing merozoite [45]. These organelles thus have a requirement for folate pathway metabolites for the synthesis of DNA precursors needed for the replication of their genomes.

The mitochondria, visualized using MitoTracker, showed some evidence of associated PfSHMTc fluorescence throughout the erythrocytic cycle but



		Sc	MIT
(A)	V%	2.9	62.3
	M%	3.0	64.4
(B)	V%	6.3	12.5
	M%	5.8	12.3
(C)	V%	2.2	17.9
	M%	2.2	18.3

Figure 2 PfSHMTc immunofluorescence images showing localization in the mitochondrion. (A) Mid-trophozoite showing the association of a small mitochondrion with PfSHMTc fluorescence. (B) Early schizont showing association of an enlarged globular mitochondrion with a region of more intense PfSHMTc fluorescence. (C) Late schizont showing very little co-localization of mitochondria with areas of PfSHMTc fluorescence. Mitochondria are closely aligned to nuclei and show some co-localization with YOYO1 staining (scale bars 3 μm). The associated table shows the percentage volume (V%) and material (M%) co-localization data for PfSHMTc (Sc) and MitoTracker (MIT) fluorescence.



		Sm	MIT
(A)	V%	12.1	39.0
	M%	12.9	40.6
(B)	V%	10.5	83.5
	M%	10.8	84.3
(C)	V%	8.7	80.7
	M%	10.0	81.6
(D)	V%	4.5	86.5
	M%	5.0	88.4
(E)	V%	6.8	87.9
	M%	7.4	89.0

Figure 3 PfSHMTm immunofluorescence images showing localization in the mitochondrion. (A) Two late trophozoites. (B-D) Mitotic schizonts. (E) Post-mitotic schizont. The images show the persistence of co-localization of PfSHMTm fluorescence with the mitochondria throughout the developmental cycle (scale bars 3 μm). The associated table shows the percentage volume (V%) and material (M%) co-localization data for PfSHMTm (Sm) and MitoTracker (MIT) fluorescence.

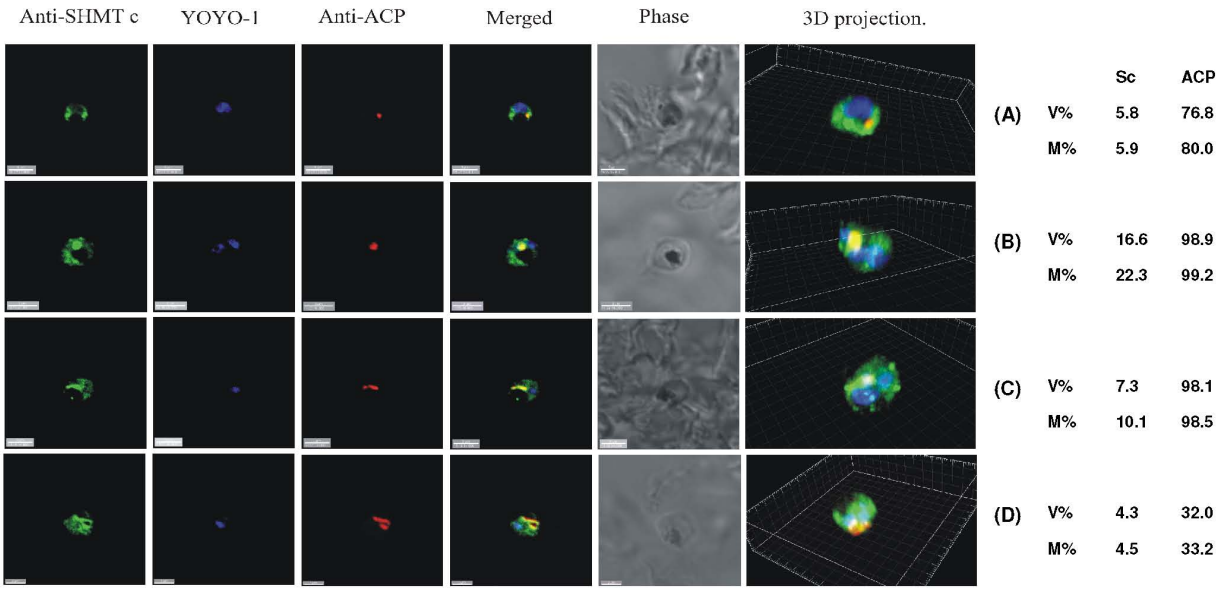


Figure 4 PfSHMTc immunofluorescence images showing localization in the apicoplast. (A) Mid-trophozoite showing the co-localization of plastid specific fluorescence with PfSHMTc fluorescence. (B) Early mitotic schizont showing very marked co-localization of plastid specific fluorescence (enlarged globular apicoplast) with PfSHMTc fluorescence. (C) Mitotic schizont showing very marked co-localization of plastid specific fluorescence with PfSHMTc fluorescence. The plastid here is in the early stages of elongation. Note also the small punctate concentration of PfSHMTc fluorescence on the periphery of the unstained region of the parasite corresponding to the pigment vacuole. (D) Mitotic schizont developmentally a little later than (C) showing a mitochondrion in the early stages of ramification. The area of intense PfSHMTc fluorescence follows the 'Y' shape of the mitochondrion closely (scale bars 3 μm, except (D) which is 2 μm). The associated table shows the percentage volume (V%) and material (M%) co-localization data for PfSHMTc (Sc) and acyl carrier protein (ACP) fluorescence.

predominantly during the stages associated with DNA replication. In many early and late parasites, the mitochondria were physically very small and consequently it could not be concluded with any certainty that PfSHMTc fluorescence was within the organelle lumen or merely in the adjacent cytoplasm. Indeed some early to mid-trophozoites showed no evidence of PfSHMTc fluorescence within their mitochondria. However, some mid-trophozoites showed a more convincing co-localization, e.g. Figure 2A, while the larger mitochondria found in very late trophozoites and early schizonts, such as shown in Figure 2B, clearly showed PfSHMTc fluorescence within the lumen, though at a similar concentration to that in the immediately surrounding cytoplasm. Figure 2C is an example of a post-mitotic schizont where very little co-localization remains.

Calculation of the levels of co-localization of PfSHMTc and MitoTracker reinforces this qualitative conclusion. The percentage of PfSHMTc material co-localizing varied between 2.2% and 5.8% (Figure 2) and the percentage volume of PfSHMTc co-localized showed only a similar, or slightly higher, value in comparison, confirming that the mitochondrion does not accumulate a noticeably higher concentration of PfSHMTc than that found in the cytoplasm. The three-dimensional projection within Figure 2C gives a particularly good view of a post-mitotic schizont showing the close spatial connection between the nuclei and mitochondria destined to occupy the same daughter merozoite. However, the mitochondria in this late stage parasite showed little evidence of PfSHMTc staining.

Mitochondrial localization of PfSHMTm

The use of anti-PfSHMTm revealed a different pattern of mitochondrial co-localization. The PfSHMTm protein, in contrast to PfSHMTc, was found strongly associated with the mitochondria throughout the erythrocytic cycle, from early trophozoites to late, post-mitotic, schizonts. The mitochondrion was always found within regions of relatively high intensity PfSHMTm fluorescence (Figure 3A and 3E) and in many instances the shape of the PfSHMTm fluorescence conformed to the shape of mitochondria (Figure 3B, C and 3D). Importantly, there were no instances of scanned images where mitochondria were found without associated PfSHMTm fluorescence or where such fluorescence was visibly lower than that of the adjacent cytoplasm. However, the quantitative analysis for Figure 3 gave very similar figures for percentage PfSHMTm material co-localized with the MitoTracker compared with

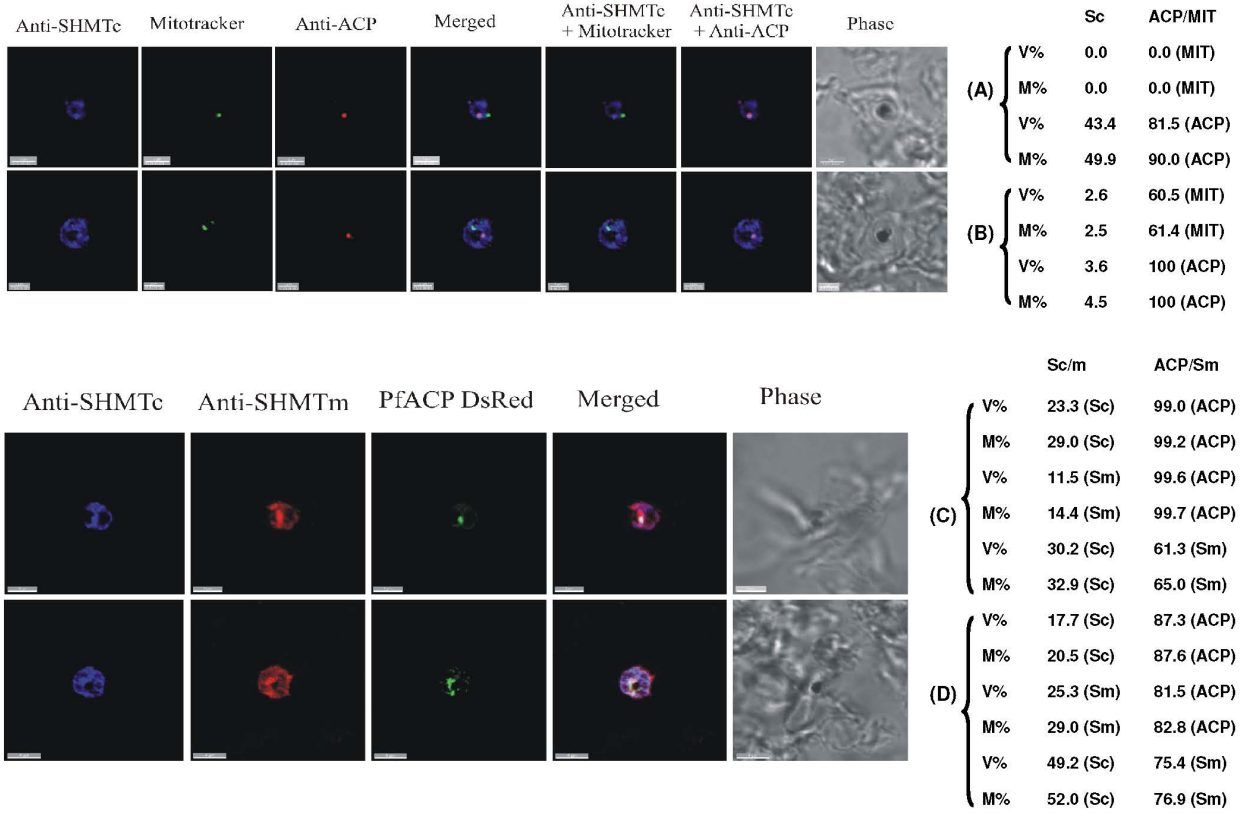


Figure 5 Triple-labelling experiments. (A) and (B) Combined mitochondrial and apicoplast images probed with anti-PfSHMTc. These do not show nuclear morphology, therefore the erythrocytic cycle stage cannot be precisely ascertained; however, the size of the organelles and overall size of the parasites in (A) and (B) suggest that both are mid trophozoites. In (A) the parasite is probed with anti-PfSHMTc, MitoTracker and anti-ACP (plastid). The plastid is coincident with an area of marked PfSHMTc fluorescence, whereas the mitochondrion shows no evidence of coincident PfSHMTc fluorescence. In (B) the parasite is probed with anti-PfSHMTc, MitoTracker and anti-ACP (plastid). The plastid is coincident with a discrete area of PfSHMTc fluorescence, whereas the mitochondrion is located in a pocket of lower PfSHMTc fluorescence. (C) Parasite is probably a late trophozoite and (D) a mitotic schizont. Both parasites were expressing DsRED-labelled ACP and were probed with both anti-PcSHMTc (IgY) and anti-PfSHMTm (IgG). The distribution of the two SHMT fluorescence signals are similar but not identical, and both co-localize with the apicoplast (scale bars (A) and (C), 3 μm, (B) 2 μm, (D) 4 μm). The associated table shows the percentage volume (V%) and material (M %) co-localization data for PfSHMTc (Sc), PfSHMTm (Sm), MitoTracker (MIT) and acyl carrier protein (ACP) fluorescence.

percentage volume co-localized, suggesting that there was no active accumulation of PfSHMTm within the mitochondria above the levels in the cytoplasm. The percentages of PfSHMTm material co-localized with MitoTracker varied between 5.0% and 12.9%, a higher range of values than measured for PfSHMTc (2.2 - 5.8%).

Apicoplast localization of PfSHMTc

In contrast to the relatively weak spatial association between subcellular PfSHMTc distribution and the mitochondrion, the apicoplast exhibited a distinctly more pronounced relationship. The apicoplast was visualized in two ways: using antibodies to acyl carrier protein (anti-ACP), which is apicoplast specific [37,45] and using a transfected 3D7 line constitutively expressing DsRED-tagged PfACP [39]. The parasite shown in Figure 4A was at the mid-trophozoite stage, and although the apicoplast was still relatively small, PfSHMTc fluorescence was clearly co-localized with anti-ACP, indicating that it was within the lumen of this organelle. The parasites in Figure 4B and 4C are early schizonts, at which stage the apicoplast is considerably larger in absolute volume, as well as relative to overall cell volume. In the K1 isolate used in these images, the apicoplast often assumes first an enlarged globular form, which then elongates before ramifying. All of these parasites showed a bright PfSHMTc fluorescence coincident, or largely coincident, with the anti-ACP fluorescence that defines the position of the apicoplast, with the surrounding general cytoplasmic PfSHMTc fluorescence being perceptibly less bright. The parasite shown in Figure 4B displays the earlier globular apicoplast morphology, the parasite in Figure 4C contains an apicoplast

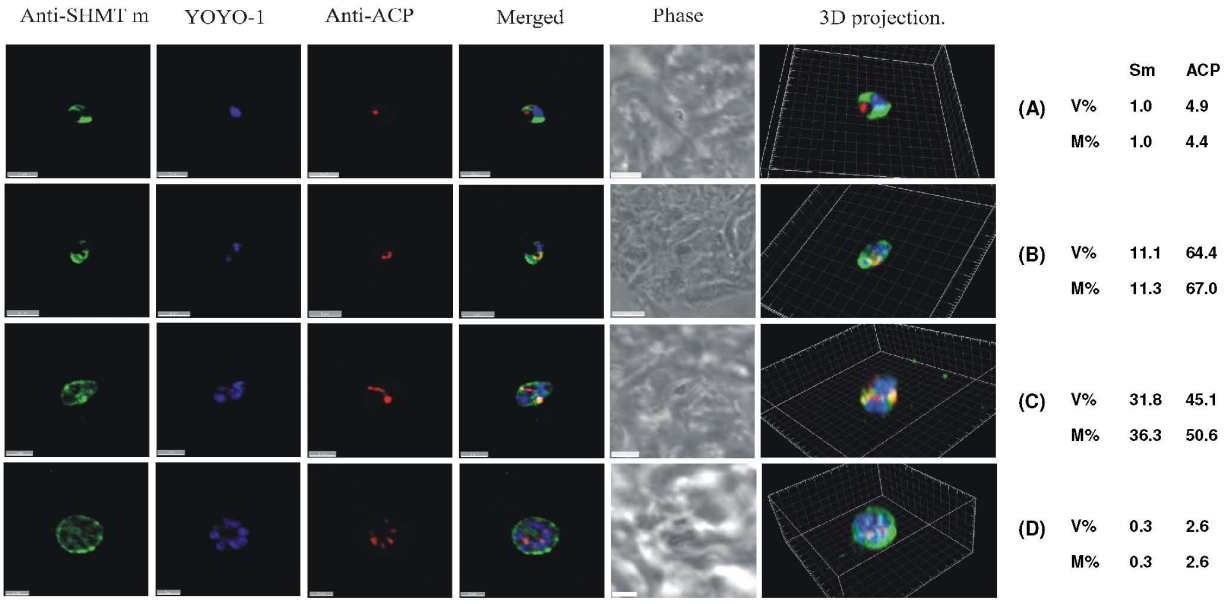


Figure 6 PfSHMTm immunofluorescence images showing localization in the apicoplast. (A) Early trophozoite showing no apicoplast PfSHMTm co-localization. (B) Early mitotic schizont with PfSHMTm fluorescence conforming closely to the 'C' shaped apicoplast. (C) Mitotic schizont with an elongating apicoplast, PfSHMTm fluorescence is concentrated within the distal portions of the organelle with little fluorescence in the medial section. (D) Post-mitotic schizont showing little spatial coincidence of PfSHMTm and the multiple apicoplasts (scale bars 3 μm, except (D). which is 2 μm). The associated table shows the percentage volume (V%) and material (M%) co-localization data for PfSHMTm (Sm) and acyl carrier protein (ACP) fluorescence.

that has started to elongate. The three-dimensional projection of the parasite in Figure 4C also allows a clear visualization of the small punctate concentrations of fluorescence that are suggestive of a vesicle-associated location of PfSHMTc, often seen in close proximity to the haemozoin containing pigment vacuole in trophozoite and early schizont stages. The parasite in Figure 4D shows an apicoplast in the ramifying stage of its development and the correspondence of the anti-PfSHMTc fluorescence to the 'Y' shaped apicoplast is striking. In the 3D7 transfectant expressing PfACP with a DsRED tag, the development of the apicoplast did not

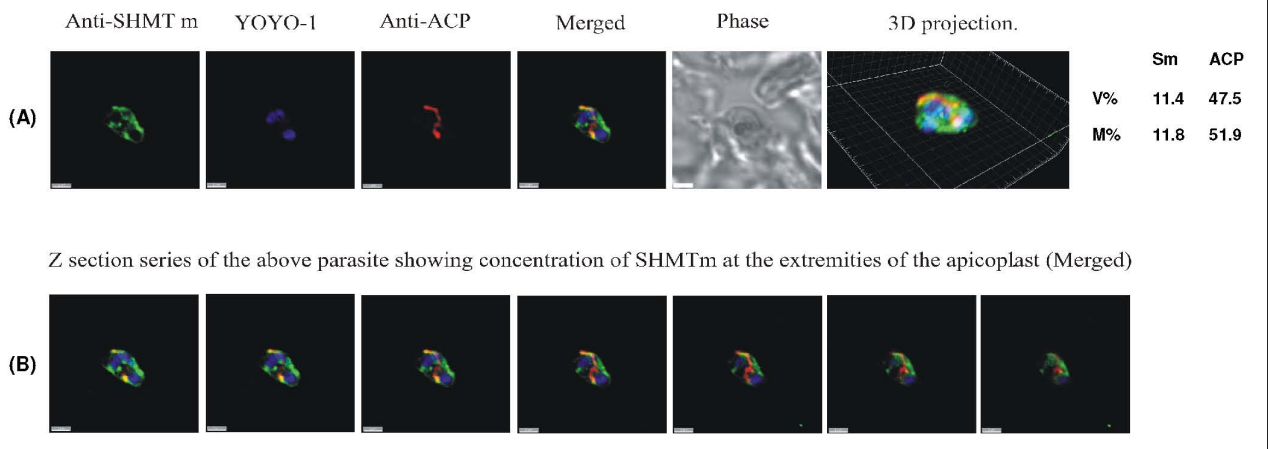


Figure 7 PfSHMTm apicoplast immunofluorescent images illustrating the concentration of fluorescence in the extremities of elongating apicoplasts. (A) Mitotic schizont with an elongating apicoplast. (B) A z-stack series with an interval of 0.2 μm through the same parasite showing the concentration of PfSHMTm fluorescence in the distal portions of the apicoplast and the relative lack of PfSHMTm fluorescence in its medial section (scale bars 2 μm).

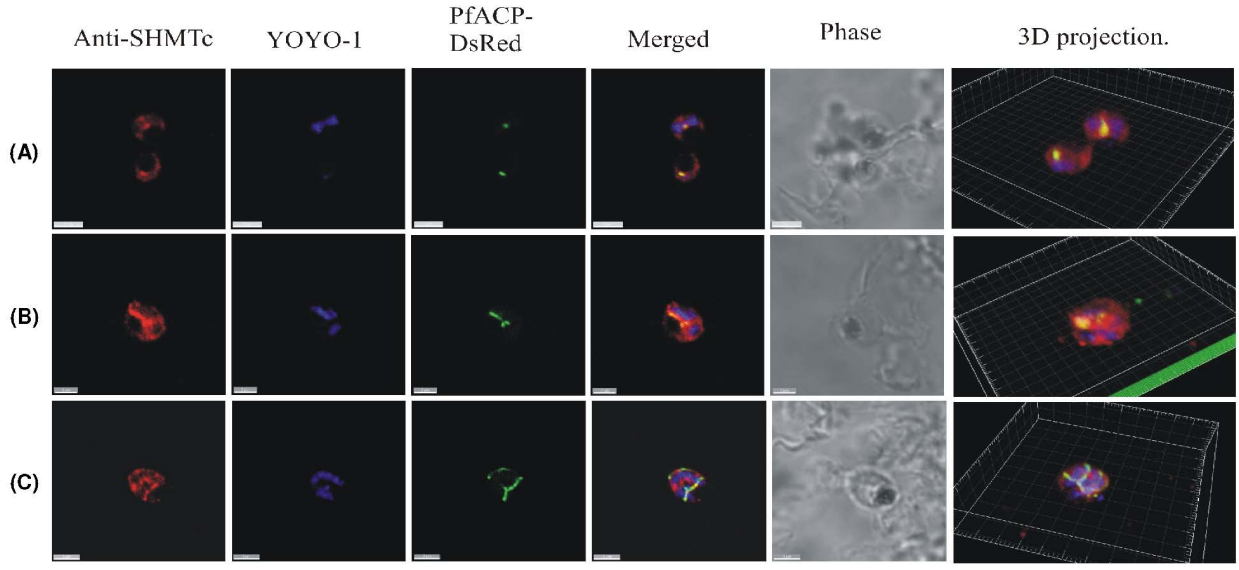


Figure 8 Positive control images using endogenously expressed DsRED-tagged ACP instead of anti-ACP antibodies. The use of only one primary antibody, anti-PfSHMTc, with expressed DsRED tagged Pf ACP, was aimed at eliminating any possibility of artifactual fluorescence arising from interactions between two primary antibodies used simultaneously. (A) Two parasites, upper parasite is undergoing its first division, lower parasite is a late trophozoite. (B) Mitotic schizont with elongating apicoplast. (C) Mitotic schizont with ramifying apicoplast. All parasites show co-localization of anti-PfSHMTc fluorescence with the apicoplast, closely following the shape of the organelle, identical results to those obtained using two primary antibodies (scale bars (A) and (C) 3 μm, (B) 2 μm).

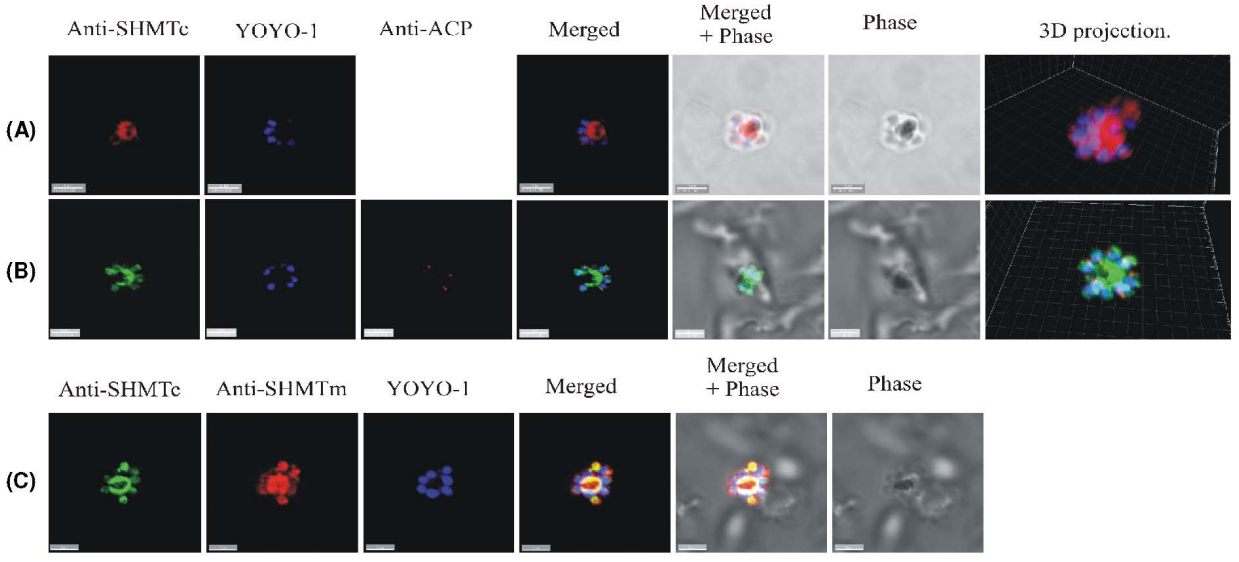


Figure 9 Late schizonts show a central concentration of PfSHMTc fluorescence. (A) Post-mitotic schizont showing a concentration of PfSHMTc fluorescence in the centre of the parasite, and overlapping the outer zone of haemozoin. PfSHMTc is largely excluded from the nuclei. (B) Post-mitotic schizont showing a concentration of PfSHMTc fluorescence in the centre of the parasite as well as at low intensity in the multiple small apicoplasts. Note the merozoite buds arranged in a radial pattern centred on the future residual body. (C) A post-mitotic parasite probed with both anti-PfSHMTc (IgY) and anti-PfSHMTm (IgG). Both SHMT proteins show a similar, but not identical distribution, as described for image series (A) and (B) above (scale bars 3 μm).

exhibit the globular stage often seen in K1 parasites, with narrow ramifying apicoplasts being far more evident (Figure 8B and 8C). However, the close coincidence of the apicoplast and PfSHMTc fluorescence was equally evident as when using K1 and two primary antibodies (see also below).

Quantitative analysis again supports the visual interpretation of the apicoplast data. The trophozoite shown in Figure 4A had a percentage material co-localization of PfSHMTc with anti-ACP of 5.9% and a percentage volume co-localization of 5.8%, indicating that the PfSHMTc fluorescence in this parasite was not appreciably higher within the apicoplast than without. The early schizont stage parasites in Figure 4B and 4C showed significantly higher percentages of PfSHMTc material co-localization of 10.1% and 22.3%, indicating that a considerable proportion of the PfSHMTc of these particular parasites was located within the comparatively small volume of the apicoplast. Moreover, the percentage material co-localized for PfSHMTc fluorescence in these two parasites was about one-third higher than the respective percentage volumes, reinforcing the visual impression that in these parasites PfSHMTc was at a higher concentration within the apicoplast than in the cytoplasm generally. A slightly later parasite (Figure 4D), showing a ramifying apicoplast, displayed a somewhat lower level of co-localization of PfSHMTc with anti-ACP of 4.5% at a concentration that is again no higher than that of the surrounding cytoplasm.

A direct comparison between mitochondrial and apicoplast PfSHMTc concentrations was made in triple staining experiments. In this case, the limitations of wavelengths available precluded using a dye to simultaneously stain the DNA so that the precise stage of the parasites viewed was not clearly discernible; however, the size of the organelles and overall size of the parasites suggest that those shown in Figure 5A and 5B are mid-trophozoites. In these experiments, PfSHMTc was stained using Alexafluor anti-chicken IgY 488 nm (false coloured blue), which proved to be especially prone to bleaching and therefore unsuited to the repeated exposure to laser light necessary in building a z-stack scan. Unlike the other images presented here, therefore, those showing both the mitochondrion and the apicoplast are from single plane scans where both organelles were in the same z-axis plane. The parasite in Figure 5A shows apicoplast-specific fluorescence located within a discrete region of bright PfSHMTc fluorescence, whereas in contrast, the mitochondrion appears to have no associated PfSHMTc fluorescence. The parasite in Figure 5B also shows the apicoplast fluorescence within a region of high PfSHMTc fluorescence whilst the mitochondrion occupies a pocket of lower intensity PfSHMTc fluorescence. Quantitative analysis confirmed the much more substantial association of PfSHMTc with the apicoplast than with the mitochondrion. As these figures refer to pixels in a single plane rather than voxels in a three-dimensional projection from a z-stack scan, extrapolation to volumetric values was unsafe in this particular case.

Apicoplast localization of PfSHMTm

Use of anti-PfSHMTm in conjunction with anti-ACP showed that the PfSHMTm protein was also found within the apicoplast. The temporal distribution of PfSHMTm within the apicoplast through the erythrocytic cycle was qualitatively similar to that of PfSHMTc. Thus, there was no discernible co-localization seen in the early trophozoite (Figure 6A), however, there was a marked presence of PfSHMTm fluorescence within the apicoplasts of both late trophozoites and mitotic schizonts (Figure 6B and 6C, Figure 7; see also Figure 10). The later, post-mitotic, schizonts showed a similar lowering of apicoplast-associated PfSHMTm fluorescence to that found using the PfSHMTc specific antibody (Figure 6D). However, the spatial distribution of the PfSHMTm fluorescence within the elongating apicoplasts of early schizonts was, in contrast, dissimilar to that shown by PfSHMTc. Whereas the latter exhibited fluorescence relatively uniformly across the apicoplasts (Figure 4C and 4D; Figure 8B and 8C), PfSHMTm was distinctly concentrated in their extremities, and was notably absent, or in very much lower concentration, within the medial sections of these organelles (Figure 6C). This phenomenon is further illustrated by the sequential z plane views (at 0.2 μm intervals) through the same parasite shown in Figure 7B, especially in the second panel

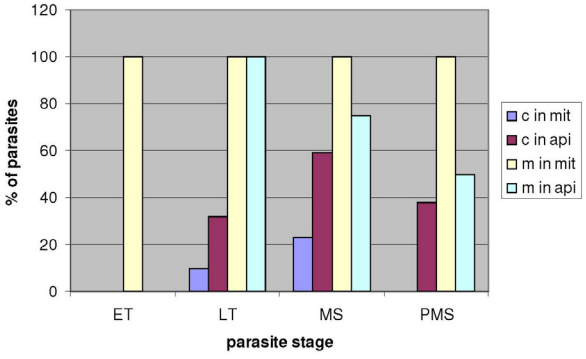


Figure 10 Organellar distribution of fluorescence through the erythrocytic cycle. Percentages of parasites (of the total number scanned for each stage) showing marked fluorescence for PfSHMTc (c) and PfSHMTm (m) in the mitochondrion (mit) and apicoplast (api). ET, early trophozoites; LT, late trophozoites; MS, mitotic schizonts; PMS, post-mitotic schizonts. For organellar localization of PfSHMTc, n = 82; for that of PfSHMTm, n = 76.

of this sequence, which clearly shows concentration of PfSHMTm fluorescence at the tips, and the fourth and fifth panels, where the lower degree of staining of the medial regions relative to the tips is apparent. The percentage co-localization of anti-PfSHMTm material with anti-ACP fluorescence was indicative of a low level of apicoplast PfSHMTm concentration in the trophozoite, e.g. 1.0% for Figure 6A, followed by much higher apicoplast PfSHMTm concentrations in the mitotically active schizont: e.g. 11.3% for Figure 6B, 11.8% for Figure 7A and 36.3% for Figure 6C. In the later, post-mitotic, schizonts, levels of co-localization fell back to lower values, the parasite shown in Figure 6D having a percentage of PfSHMTm material co-localizing with anti-ACP of only 0.3%.

Imaging using an endogenously expressed apicoplast marker

The simultaneous use of two primary antibodies, even when raised in different species, combined with their respective fluorochrome-conjugated secondary antibodies, raised the formal possibility that any observed co-localization was the result of fortuitous interactions between those antibodies. To eliminate this possibility, 3D7 transfected parasites expressing the apicoplast-specific protein ACP fused to the DsRED reporter were employed [39]. When these parasites were probed with the single anti-PfSHMTc antibody, the images obtained showed an identical incidence of co-localization of the PfSHMTc fluorescence with the apicoplast (Figure 8A-C) as was seen using the two antibody approach above, although the relatively low absolute brightness of the DsRED fluorescence made these images unsuited to quantitative evaluation. The conclusion from this result is that the images created using two primary antibodies are a true reflection of the sub-cellular distribution of the proteins investigated and that the same distribution is found in two independent lines of the parasite, K1 and 3D7.

The parasites expressing PfACP-DsRED were also simultaneously probed with antibodies to both PfSHMTc (IgY) and PfSHMTm (IgG), again employing single plane scans without a DNA-specific dye rather than z-stacks for this triple labelling experiment. The parasite in Figure 5C (estimated to be a mid to late trophozoite), and that in Figure 5D (an early schizont) both show overlapping, though not identical, PfSHMTc and PfSHMTm fluorescence distribution in the cytoplasm. Both parasites show PfSHMTc and PfSHMTm coincident with the apicoplast as indicated by white colouration in the relevant merged image. Quantitative image analysis reinforces the visual indication of co-localization of both PfSHMTc and PfSHMTm with each other, and with the apicoplast specific fluorescence. In particular the apicoplast specific fluorescence was almost entirely (between 82.8% and 99.7%,) co-localized with the signals from both isoforms of SHMT.

SHMT distribution in the post-mitotic schizont

In late, post-mitotic, schizonts, PfSHMTc fluorescence was characterized by a concentration in the central portion of the parasite. The peripheral regions of the parasite occupied by the nuclei and other constituents of the developing merozoites contained conspicuously lower levels of fluorescence, as shown in Figure 9A and 9B. The central area of late schizonts is the region that becomes the residual body upon completion of merozoite maturation and lysis of the erythrocyte, a prominent component of which is the pigment vacuole containing the crystalline haemozoin. In the very late schizont when the majority of the haemoglobin has been digested, the pigment vacuole occupies a large volume. Figure 9A and 9B show the central mass of dense haemozoin exhibiting no PfSHMTc staining but with marked PfSHMTc fluorescence in the region immediately surrounding it. To assess the relative frequency of this category of PfSHMTc distribution, 48 scans of post-mitotic schizonts were viewed, of which 15 showed a marked concentration of fluorescence in the centre of the schizont when compared to their periphery, an incidence of 31%. The use of anti-PfSHMTm antibody in conjunction with anti-PfSHMTc showed that PfSHMTm has a very similar concentration within the central region of the very late schizont (Figure 9C). Additional to this general distribution pattern, the post-mitotic schizont contains numerous small apicoplasts, each associated with a developing merozoite. Despite the diminutive size of these organelles, the persistence of PfSHMTc fluorescence within the 'daughter' apicoplasts was still discernible in some images, e.g. in Figure 9B, where the lower right plastid in the parasite clearly shows its presence. The three-dimensional projection shown in Figure 9B is interesting as it shows a relatively late stage of daughter merozoite biogenesis.

Nuclear localization

In most parasites viewed there was a distinctly lower PfSHMTc fluorescence within nuclei than was found in the cytoplasm. However, PfSHMTc fluorescence was very rarely entirely excluded from the nucleus (see especially Figure 4C and 4D; Figure 9B and 9C). The level of nuclear relative to cytoplasmic fluorescence was variable with higher levels of intranuclear PfSHMTc fluorescence seen in some late trophozoites and mitotic schizonts. Nuclear PfSHMTc fluorescence rarely approached the intensity of cytoplasmic fluorescence, however. In contrast, PfSHMTm showed very little evidence of nuclear localization throughout the erythrocytic cycle, with most

images showing an essentially complete exclusion of PfSHMTm fluorescence from nuclei (Figure 3D and 3E; Figure 6A, C and 6D).

Relative incidence of organellar SHMT fluorescence through the erythrocytic cycle

In view of the initially surprising results that PfSHMTc showed organellar co-localization patterns, a large number of z-axis scans of parasites were analysed in order to ascertain the relative incidence of organellar fluorescence for this isoform over the erythrocytic cycle. Parasites were assigned to one of four broadly defined developmental stages by examination of overall size, haemozoin development and nuclear morphology (Figure 10). These results emphasize the stage-specific dependence of organellar PfSHMTc fluorescence, which was undetectable in parasites up to and including the early trophozoite stages, visible from mid-trophozoites onward and peaking at the mitotic schizont stages. The corresponding analysis for PfSHMTm with respect to the mitochondrion is strikingly different in that 100% of parasites showed fluorescence in this organelle, regardless of the cell cycle stage. However, its incidence in the apicoplast was similar to that of PfSHMTc, in that it was not seen in the early trophozoite stage but peaked in the late trophozoite stage, although the percentage of parasites displaying this pattern was significantly higher than was the case for PfSHMTc.

GFP-tagging of SHMT via transfection

To support the immunofluorescence studies in a complementary manner, independent attempts were made in the two collaborating laboratories to produce transfected parasites expressing GFP-tagged, full length PfSHMTc and PfSHMTm endogenously, as well as shorter versions carrying a GFP-tag downstream of the first 100 amino acids of each protein (i.e. about one-quarter of their total length). Despite repeated transfections using several different protocols for these four constructs, viable parasites could only ever be recovered in the case of the truncated version of PfSHMTm + GFP. Fluorescence microscopy clearly located this hybrid protein in the mitochondrion (Figure 11), confirming the initial prediction based on sequence analysis that PfSHMTm carries a mitochondrial targeting signal at its N-terminus [24]. However, in contrast to the studies above using the anti-PfSHMTm antibody, no additional distribution in the cytoplasm or apicoplast was apparent, suggesting that localization to these areas was dependent upon properties of the full-length molecule.

Transcript analysis of PfSHMTc

In the original characterization of the gene encoding PfSHMTc, comparison of cDNA and genomic sequences, together with RACE analyses of the transcript start point in two independent laboratories [8,21], identified only a ~240 base 5' UTR on the mRNA which lacked any AUG motif upstream of the documented start codon. To confirm and extend this result, we carried out RT-PCR experiments using a range of internal primers based on genomic sequence extending up to 1 kb upstream of the start codon. However, no splice variants were detected (data not shown), nor could any putative splicing event using the normal GU and AG intron junction signals within this sequence create an alternative start codon. Thus there was no evidence that PfSHMTc might employ a conventional signal sequence that had previously been overlooked to gain access to the organellar compartments.

Discussion

SHMT is the principal agent by which one-carbon units are introduced onto folate carriers for subsequent essential transfer reactions, including the formation of thymidylate. Of the two SHMT isoforms expressed by *P. falciparum*, PfSHMTc is an enzymatically active member of the thymidylate cycle [21-23], whereas PfSHMTm is more enigmatic, as it lacks most of the conserved active site residues found in other SHMTs, whether cytoplasmic or organellar [24,42] and has been found to be inactive in studies of the recombinant protein [23]. With a combination of antibody probes and endogenous expression of tagged molecules, the cellular distribution of these two species across the parasite erythrocytic cycle has been investigated to gain possible insight into their biological function.

Although PfSHMTc lacks any obvious signal sequences and was expected to be confined to the cytoplasm, the most surprising result was that, although it is indeed in the cytoplasm that the majority population is found throughout erythrocytic development, PfSHMTc also localizes within parasite organelles, including the mitochondrion and particularly the apicoplast. Moreover, the distribution varies in a dynamic and developmental stage-dependent manner, consistent with a temporally mediated variation in the targeting of newly expressed enzyme protein between sub-cellular locations from a cytoplasmic pool. PfSHMTm, on the other hand, was identified as having an N-terminal mitochondrial targeting sequence [24], which was functionally confirmed here using a transfected parasite construct expressing GFP fused to its N-terminal domain. However, full-length PfSHMTm in its native state shows complex localization patterns that are similar to, but subtly different from those of PfSHMTc in the cytoplasm, mitochondrion and apicoplast.

Although the parasite preparations undergo a multistep protocol for immunofluorescence that necessarily

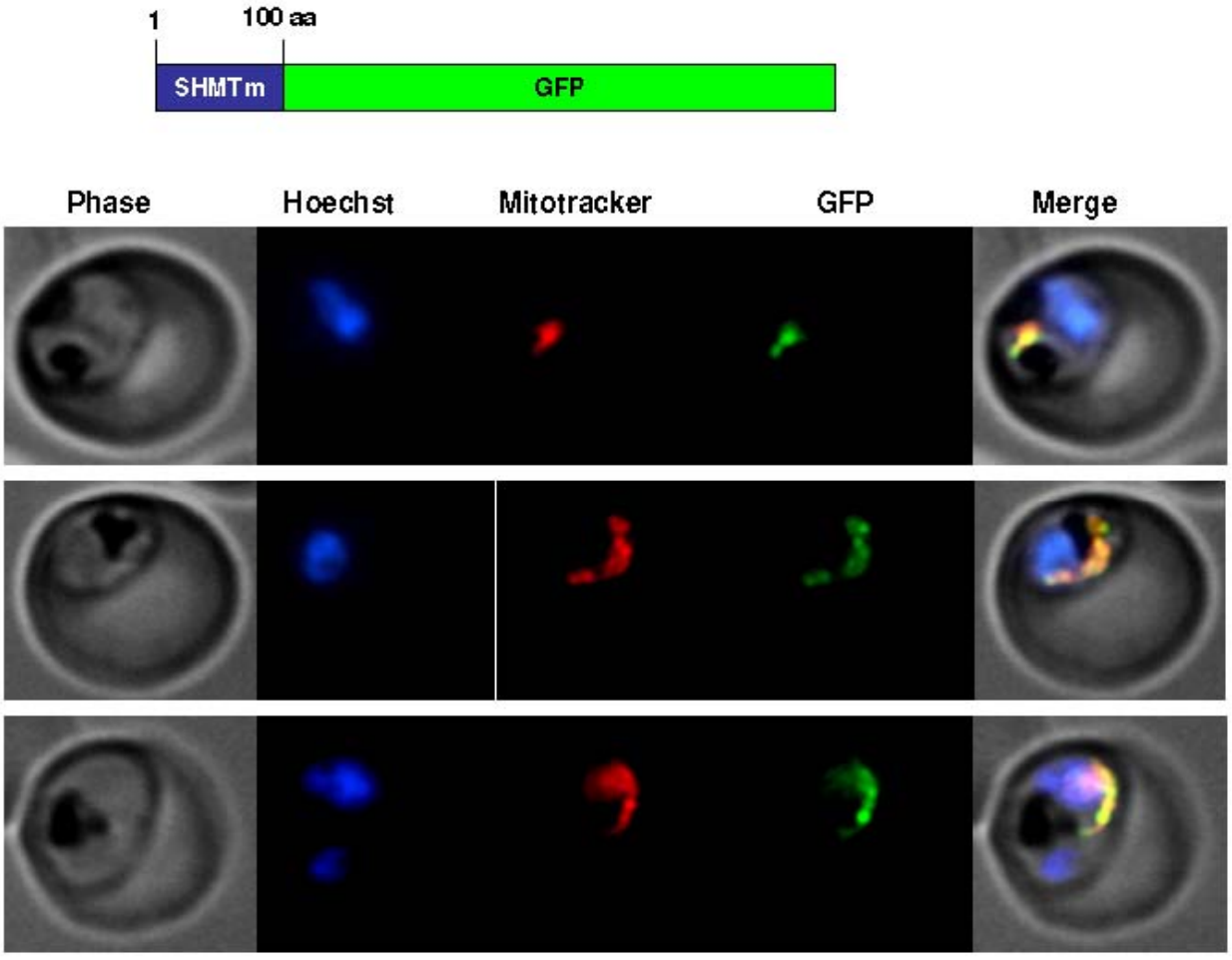


Figure 11 GFP-tagging of truncated PfSHMTm in transfected 3D7 parasites. Fluorescence images of parasites transfected to yield a GFP-fusion carrying the first 100 amino acids of PfSHMTm at the N-terminus. MitoTracker was also used to localize the mitochondrion, which showed complete coincidence with the GFP fluorescence (three examples shown).

includes a mild detergent permeabilization, there are strong reasons to believe that the results obtained are not artifactual. Thus (i) organellar fine structures of the parasites are preserved, (ii) both primary anti-PfSHMT antibodies show a high degree of specificity, (iii) antibodies against two known cytoplasmically located enzymes show no organellar co-localization, (iv) conversely, the anti-ACP primary antibody locates exclusively to the apicoplast as expected, with no evidence of staining elsewhere that might indicate a loss of organellar integrity, (v) the incidence of PfSHMTc in the organelles shows a strong stage-dependency, being absent in early trophozoites, peaking in late trophozoites and mitotic schizonts, then diminishing in later (post-mitotic) schizonts, (vi) a different stage dependency for PfSHMTm is evident, particularly in the case of the mitochondrion, where co-localization is seen in all parasites throughout the cycle, and (vii) localization patterns of PfSHMTc within the apicoplast are the same, regardless of whether two primary antibodies (anti-PfSHMTc and anti-PfACP) are employed as probes or anti-PfSHMTc alone plus the endogenous apicoplast fluorescence from transfected parasites in which PfACP is tagged with DsRED. The use of z-stack scanning and quantitation of overlapping fluorescence signals considerably increased confidence in assigning position compared to conventional 2 D analysis of images in a single plane, especially in the case of late trophozoites and early schizonts, where organelles were large enough to permit at least 3 and up to 7 or 8 planes within the organelle to be examined, as exemplified in Figure 7. Stages at which a specifically stained organelle was at its smallest unavoidably gave less clear-cut images that could be more easily compromised by adjacent cytoplasmic staining. Moreover the close apposition of the mitochondrion and apicoplast is also potentially problematic when both are very small or narrow,

although this was less of an issue with the larger organelles of K1, primarily used for this study, compared to 3D7. However, attempts to increase resolution further by scanning z-planes every 0.1 μm instead of 0.2 μm resulted in unacceptable levels of photobleaching before such lengthy scans could be completed.

The observed association of PfSHMTc with the mitochondrion and the apicoplast is not of equal degree. The mitochondrion shows relatively low levels of PfSHMTc fluorescence in the earlier and later stages of the cycle, with signal within the organellar lumen only obvious in the late trophozoite and early schizont stages, when the mitochondrion is expanding and then elongating, with significant synthesis of internal constituents [45]. However, even at maximum visibility, the fluorescence intensity, as confirmed by quantitative image analysis, does not exceed that found in the surrounding cytoplasm. In contrast to the mitochondrion, the apicoplast shows a significantly higher level of PfSHMTc association over a longer period of the erythrocytic cycle. PfSHMTc fluorescence within the apicoplast lumen is first detected in trophozoite stages before the organelle has expanded noticeably and persists into the small daughter plastids of very late schizonts. In both of these developmental stages, the concentration of PfSHMTc within the apicoplast does not exceed that in the cytoplasm. However, between these stages, in the early schizont, when the apicoplast is expanding maximally and subsequently elongating, PfSHMTc fluorescence inside this organelle becomes very marked, such that the concentration of PfSHMTc within it now exceeds that in the surrounding cytoplasm. The percentage of total PfSHMTc fluorescence co-localising with apicoplast-specific fluorescence was measured in some parasites at this stage at >20%, a considerable proportion of the cellular total.

The PfSHMTm protein, despite its N-terminal mitochondrial targeting sequence, shows a similar spatial and temporal distribution to that of PfSHMTc, albeit with some important variation. Thus, the level of localization of PfSHMTm within the mitochondrion is distinctly higher than that of PfSHMTc. Moreover, the occurrence of concentrations of PfSHMTm within the extremities of elongating apicoplasts and a corresponding paucity in the medial sections is a polarization not seen with Pf SHMTc and may suggest a more specific developmental role for PfSHMTm in this organelle. Given also the occurrence of Pf SHMTm in the cytoplasm, there are likely to be further signals downstream of the N-terminal domain yet to be characterized that contribute to the complex and dynamic distribution patterns, as discussed further below.

The data suggest a connection between these apparently rather short-lived associations and internal organellar metabolism. Both organelles must replicate their own genomes, prior to which local demand for folate pathway products would be high. Moreover, there is evidence for the existence of a glycine cleavage complex (GCV) in the mitochondrion [24], which is dependent on the provision of folate cofactor. Studies in other systems indicate that substituted tetrahydrofolates cannot freely exchange between mitochondrial and cytoplasmic compartments [14], suggesting that transport of folate enzymes into membrane-bound organelles may be essential. In the very late post-mitotic schizont, PfSHMTc is found concentrated in the centre of the parasite, in the region that forms the residual body on erythrocyte lysis and merozoite release. This association can be rationalized in that, after mitosis, demand for DNA precursors is low, however, the late schizont is very active in protein production for organellogenesis and other aspects of merozoite maturation. As amino acids are released from haemoglobin, the late concentration of PfSHMTc in and around this organelle may be connected with an increased demand for the reversible Ser/Gly interconversion function of the SHMT enzyme and/or methionine metabolism.

Although plant cells also exhibit a partitioning of SHMT across cytoplasm, mitochondrion and plastid, this association is a persistent feature [46], rather than the more transient phenomenon seen here in the malaria parasite. By contrast, the parasite must undergo rapid asexual reproduction at the blood stages, requiring parasite metabolism to be highly efficient in its production and use of folate pathway components. The locations of greatest demand for such products would thus vary through the processes of growth, repeated mitoses and cytodifferentiation in the erythrocytic cycle. It would, therefore, be advantageous to be able to translocate folate enzymes to varying subcellular locations as the demand for folates changed throughout development. The results of this study support a view of the cellular location of folate pathway enzymes being dynamic and responsive to the changing needs of the parasite over time.

Important questions that now need to be addressed are how PfSHMTm and, more puzzling, PfSHMTc, are targeted to the organelles investigated here and their precise function(s) therein. Dual targeting of proteins to the mitochondrion and plastid is frequently observed in other systems [47]. However, in organisms as diverse as plants, yeast and mammals, dedicated cytoplasmic and organellar isoforms of SHMT are employed to effect compartmentalized folate metabolism. Only PfSHMTm has a recognizable mitochondrial targeting sequence and neither isoform possesses the conventional bipartite topogenic signal associated with plastid targeting via the endoplasmic reticulum in *P. falciparum* and the closely

related *Toxoplasma gondii* [48,49]. Moreover, although a proportion of PfSHMTm is located in the mitochondrion throughout the erythrocytic cycle and also in the apicoplast in the middle to late stages, how can this isoform perform the necessary enzymic steps when its sequence (see Additional file 2 Sequence alignments of the PfSHMT isoforms) and the inactivity of the recombinant protein [23] strongly indicate that it cannot? It could be that the folate metabolism essential for the replication of their genomes is provided by the enzymatically competent PfSHMTc after gaining access to these organelles. The sequence and transcript analyses exclude the possibility of a hitherto unidentified upstream leader sequence that could splice onto the ORF as currently defined, but cryptic motifs further downstream in the encoded protein cannot be excluded, especially as numerous plastid proteins in other systems depend upon ill-defined, sometimes non-contiguous regions of the molecule [50]. However, another intriguing possibility is suggested by a closer analysis of the primary sequences of PfSHMTc and PfSHMTm, which reveals that each carries one or the other of two internal conserved sequence motifs (residues 138-146 in PfSHMTc and residues 262-277 in PfSHMTm), both of which are present in the SHMTs of higher organisms and are known to mediate intersubunit interactions (Figure 12). Thus, mammalian SHMTs are stable homotetramers, whereas bacterial SHMTs, which lack these motifs entirely, are homodimers [42]. The plasmodial proteins seem to represent a complementary pairing system thus far unique among SHMT types and this leads us to the hypothesis that while PfSHMTm is in itself apparently catalytically inactive, a dimer thereof might be able to form a stable but readily reversible heterotetramer with dimeric PfSHMTc, generating a complex in which the requisite SHMT activity is provided (from PfSHMTc) together with (an) organellar targeting sequence(s) (from PfSHMTm) that could be modulated as necessary. Such an arrangement would have parallels with the S-adenosyl-L-methionine decarboxylase (AdoMetDC) system of trypanosomes, where a catalytically inactive paralogue of AdoMetDC forms a heterodimer with AdoMetDC itself and thereby regulates the activity of the latter allosterically [51]. This scenario could explain why the localizations of the two PfSHMT isoforms show considerable overlap, and why our GFP construct attached to the first 100 amino acids of PfSHMTm, and thus lacking both of the above motifs, migrates solely to the mitochondrion. Moreover, many nuclear-encoded proteins destined for plastids and mitochondria in other organisms are translated on cytoplasmic ribosomes and imported (often long) after their synthesis [47], thus providing a credible precedent for encounters between PfSHMTc and PfSHMTm. However, it is certainly unclear at this point precisely how such a complex could be successfully translocated across the requisite membranes, although analyses of organellar protein transport found in other systems serve to emphasize the

		Motif 1	Motif 2
Human cyt	(tetramer)	157-KKKISATSI-165	270-RKGVKSVDPKTGKEILY-286
Human mit	(tetramer)	VKRISATSI	RKGVKAVDPKTGREIPY
Pf-c		138-KKKVSITSD-146	250-NK--KR-NP--G--IDQ-259
Pv-c		KKKVSITSD	NK--KR-NP--G--IEQ
Pk-c		KKKVSITSD	NK--KR-NP--G--IDQ
Py-c		KKKVSVTSD	NK--KR-NP--G--IEQ
Pb-c		KKKVSITSD	NK--KR-NY--G--IEQ
Pf-m		142-NIENNVSND-150	262-KKGYKCVN-TDGHIIEY-277
Pv-m		AEEGEANNP	KRGYKQVD-KEGKLIHY
Pk-m		SQEGEANSP	KRGYKQVD-TKGKLIHY
Py-m		KEESRMDKN	RKGFKNIS-TQGKILCY
Pb-m		KEESRMDKN	RKGFKNIS-TQGKILYY
E. coli	(dimer)	135----FSGK-L-139	242-K-G--------GSEELY-249

Figure 12 Potential interactions between PfSHMTc and PfSHMTm. The two protein sequence motifs identified in mammalian SHMTs (both cytoplasmic and mitochondrial isoforms; top two lines) essential for stable tetramer formation; bacterial SHMTs lack these motifs entirely and form stable dimers (bottom line; *E. coli* as example) [42]. SHMTc from *P. falciparum* and its orthologues from other *Plasmodium* species (*P. vivax*, *P. knowlesi*, *P. yoelii* and *P. berghei*, respectively) possess a highly conserved equivalent of Motif 1 only (blue text), whereas the SHMTm forms possess only Motif 2 (red text), suggesting that heterotetramers could be formed by a combination of $(PfSHMTc)_2$ and $(PfSHMTm)_2$.

considerable diversity of mechanisms associated with this phenomenon [50,52].

Conclusions

The two isoforms of SHMT in *P. falciparum*, PfSHMTc and PfSHMTm, exhibit complex distribution patterns across the cytoplasm and organelles of the parasite that are similar but differ in their levels of occupancy and cell-cycle stage dependency. PfSHMTm is confirmed as having an N-terminal mitochondrial targeting sequence whereas PfSHMTc lacks any obvious targeting signals. Interactions between the two isoforms suggested by sequence analysis may be involved in the dynamic patterns of localization observed and may be important in overcoming the apparent lack of catalytic competence of the PfSHMTm isoform. Further studies are required to establish whether such an association can occur and play a role in ensuring the provision of essential folate cofactors for replication of the nuclear, mitochondrial and apicoplast genomes.

Additional material

Additional file 1: Negative control images for organellar staining. The figure shows immunofluorescence images obtained using antibodies against known cytoplasmic enzymes.

Additional file 2: Sequence alignments of the PfSHMT isoforms. The figure shows alignment of the PfSHMTc and PfSHMTm sequences with cytoplasmic and mitochondrial SHMTs from other organisms.

Acknowledgements

We thank Chi Tang and Apolinar Maya-Mendoza (University of Manchester) for assistance with the microscopy, Geoffrey McFadden (University of Melbourne) for providing the anti-ACP antibody, Teresa Fitzpatrick (University of Zürich) and Barbara Kappes (University of Heidelberg) for the anti-CS and anti-PK5 antibodies, Shigeharu Sato (NIMR, London) for the 3D7 parasite transfectant strain carrying pSSPF2/PfACP-DsRED and Ping Wang (University of Manchester) for contributions to the transfection experiments. We are also grateful to the Wellcome Trust, UK (grant no. 073896) and BBSRC, UK (studentship for S.L.M), for financial support.

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Authors' contributions

MR developed the immunofluorescence protocols, carried out most of the experimental work in Manchester and drafted the manuscript. IBM constructed GFP transfection plasmids, produced parasite transfectants and carried out the imaging thereof. SLM contributed to the establishment of the immunofluorescence procedures. PFGS and JEH conceived the study, wrote parts of the manuscript and prepared the final version. All authors were involved in data interpretation and analysis and have approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

Received: 27 August 2010 Accepted: 3 December 2010
Published: 3 December 2010

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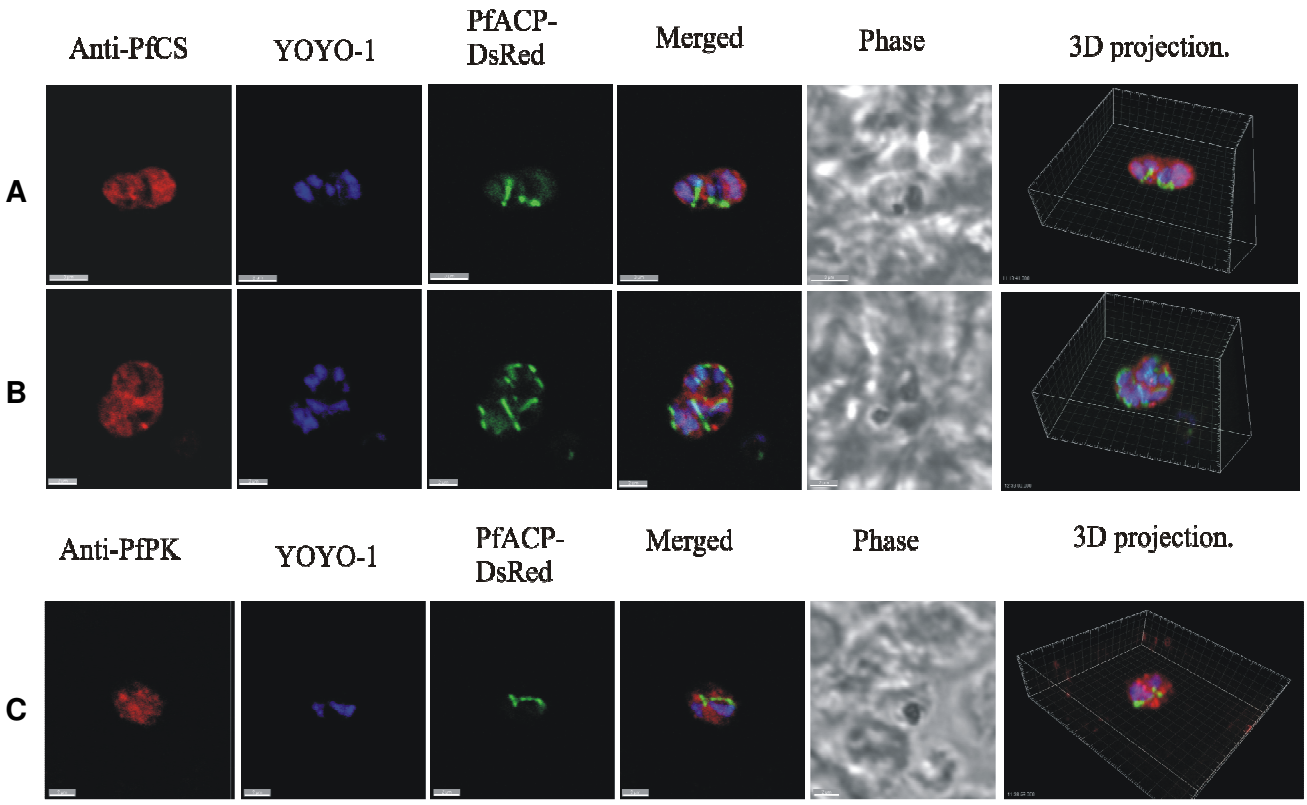
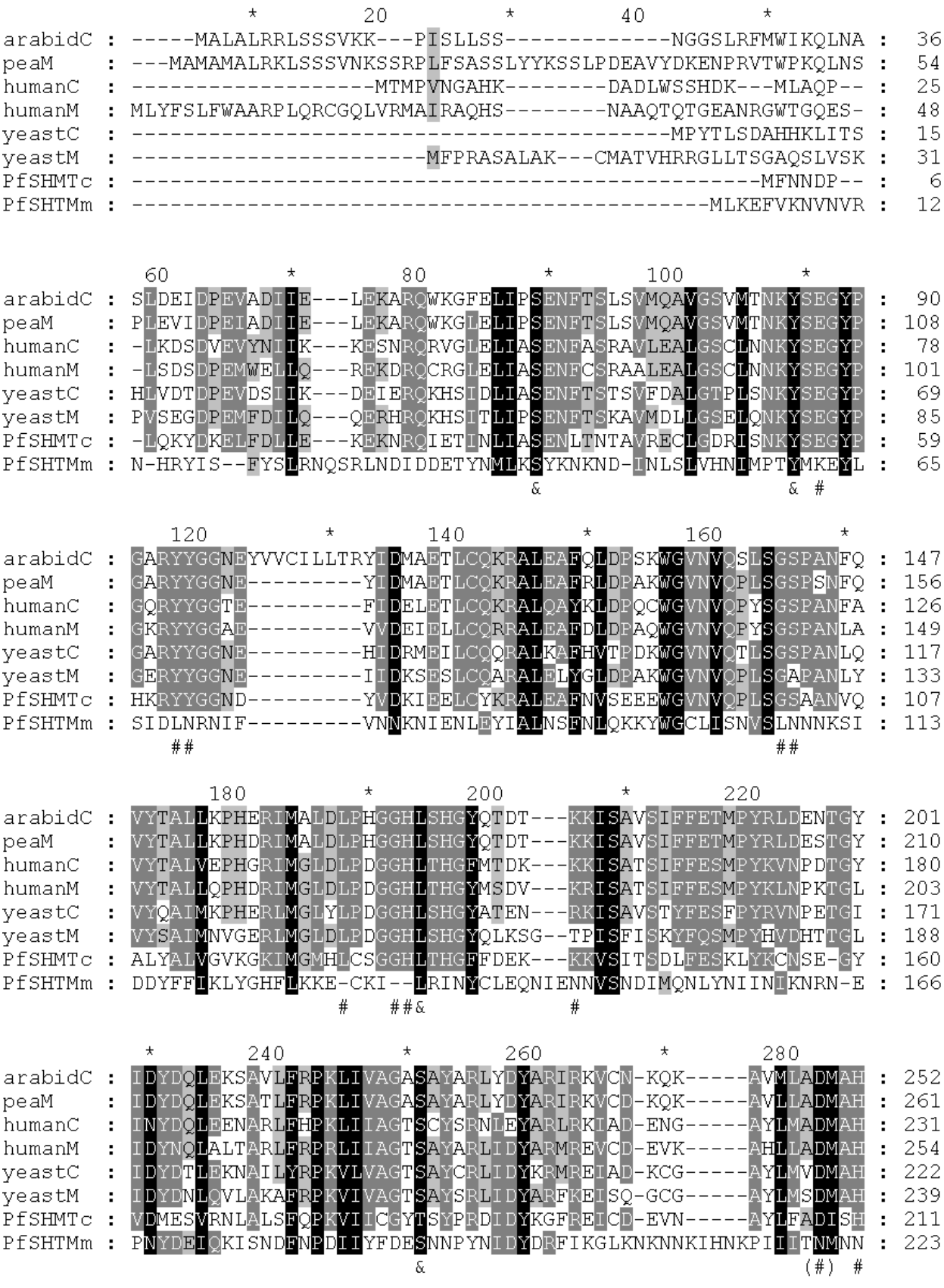
doi:10.1186/1475-2875-9-351
Cite this article as: Read *et al.*: Dynamic subcellular localization of isoforms of the folate pathway enzyme serine hydroxymethyltransferase (SHMT) through the erythrocytic cycle of *Plasmodium falciparum*. *Malaria Journal* 2010 **9**:351.

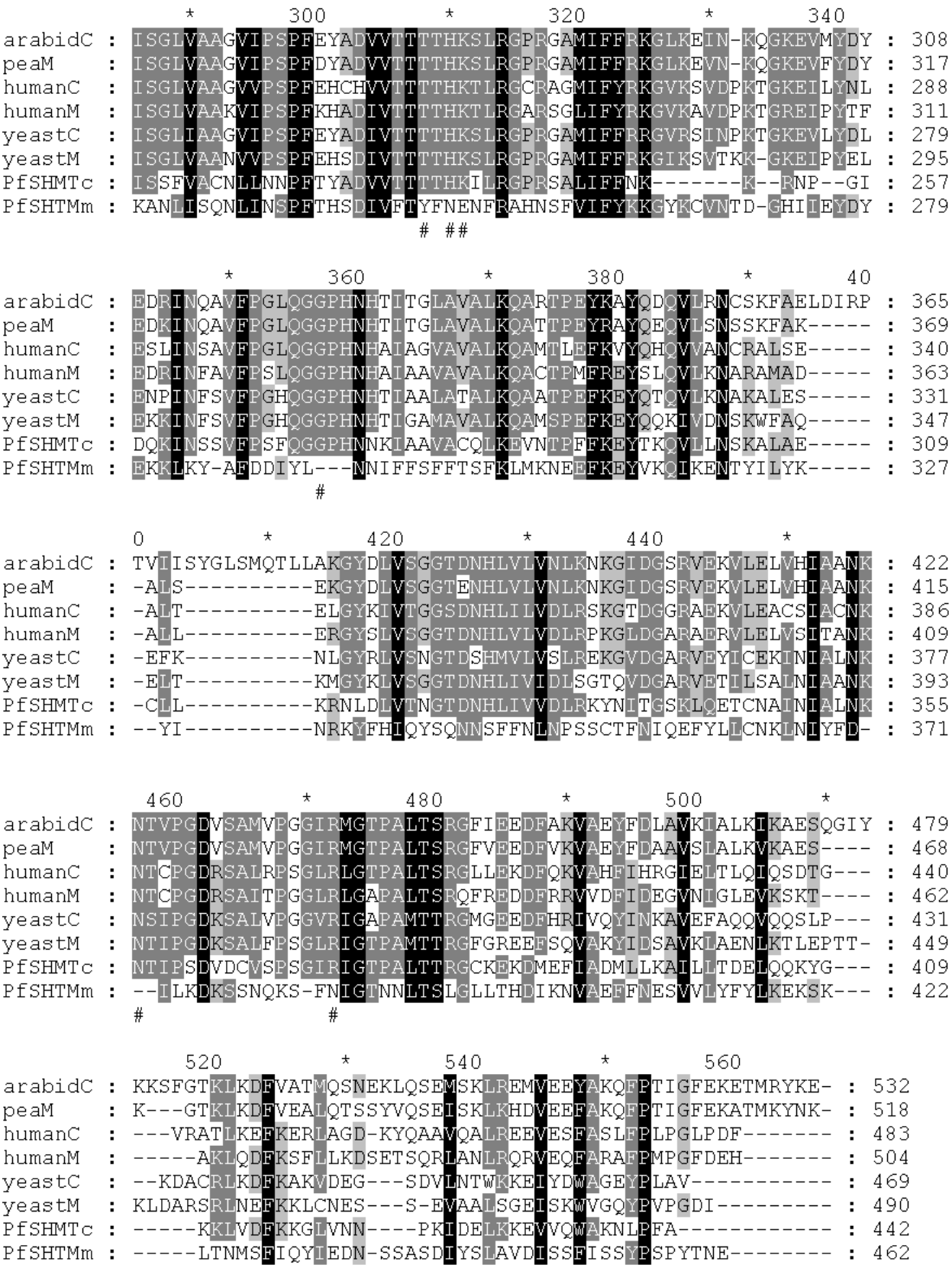


Supplementary Figure 1 - Negative control images for organellar staining.

A and **B.** anti-PfCS (chorismate synthase), **C.** anti-PfPK (cyclin-dependent protein kinase 5), both enzymes previously characterised as having a simple cytoplasmic distribution {Fitzpatrick, 2001 #618}. All parasites were expressing DsRED labelled PfACP. The control antibodies show little or no fluorescence coincident with the apicoplasts, despite the parasites being in the mitotic schizont phase where anti-PfSHMT fluorescence is most overt (scale bars **A** 3 μm, **B** and **C** 2 μm).



Supp. Fig. 2 part (i)



Supp. Fig. 2 part (ii)

Supplementary Figure 2 – Sequence alignments of the PfSHMT isoforms.

Alignment of PfSHMTc and PfSHMTm with cytoplasmic (C) and mitochondrial (M) SHMTs from other organisms, showing the absence in PfSHMTm of most of the highly conserved residues associated with the active site {Franca, 2005 #1015}. Ampersands (&) under the sequences indicate such residues that are conserved in PfSHMTm (4 in total), hashes (#) indicate such residues that are not conserved in PfSHMTc (16 in total) and ((#)) indicates the one conservative replacement.

Acknowledgements

Being able to work for over two decades in a single field of research is unusual in modern Biology, particularly for a bench scientist. I am very aware that I am fortunate to have been in such a position. John Hyde and Paul Sims are responsible for affording me, and indeed many other people, a very rewarding research experience in a supportive environment. For this I would like to express my sincere thanks.

After such a long time I think that I can, with some justification, say that my co-workers, who have helped in many ways and with whom I have made many friendships, are too numerous to name and thank individually, so I thank them collectively. However, I feel that I must individually acknowledge those colleagues who are co-authors on the papers contained in this thesis - namely my wife, Karen Read (nee Hicks), Wang Ping, Wang Qi, Vicia Hauser and Sarah Mitchell. My thanks are also due to external collaborators on two of the papers, Andrew Hanson for facilitating the work leading to the PCD paper and Ingrid Müller for undertaking the transformation studies on PfSHMTm and PfSHMTc.

I am grateful to my daughters, Laura and Fiona, for not interrupting the compilation of this thesis too much; a gratitude that I cannot extend to our over-affectionate cat, whose fascination with the world of malaria research impelled him to sit or lie on any piece of paper that he, uncannily, divined that I required.

Abbreviations

AAH	aromatic amino acid hydroxylase
BH_4	tetrahydrobiopterin
cDNA	complementary deoxyribonucleic acid
CHEF	contour-clamped-homogenous-electric-field
DNA	deoxyribonucleic acid
DHFR	dihydrofolate reductase
DHFR-TS	dihydrofolate reductase-thymidylate synthetase
DHNA	dihydroneopterin aldolase
DHPR	dihydropterin reductase
dTMP	deoxythymidine monophosphate
DHPS	dihydropteroate synthetase
dUMP	deoxyuridine monophosphate
et al.	*et alia* (Lat.) 'and others.'
FPGS	folate polyglutamate synthase
GTPCH	guanosine triphosphate cyclohydrolase
HPPPK-DHPS	hydroxymethylpterin pyrophosphokinase – dyhydropteroate synthetase
IC_{50}	half maximal inhibitory concentration
kD	kiloDalton
LDH	lactate dehydrogenase
L-DOPA	L-3,4-dihydroxyphenylalanine
PABA	*para*-aminobenzoic acid
PCD	pterin-4a-carbinolaminedehydratase
PCR	polymerase chain reaction
PFGE	pulsed field gel electrophoresis

PfMRP1	*P. falciparum* multidrug resistance protein 1
PfSHMTc	*P. falciparum* ‘cytoplasmic’ serine hydroxymethyltransferase
PfSHMTm	*P. falciparum* mitochondrial serine hydroxymethyltransferase
PhD	*philosophiae doctor* (Lat.) ‘doctor of philosophy’
PlasmoDB	database of the *Plasmodium falciparum* genome
PTPS	6-pyruvoyl-tetrahydropterin synthase
RACE	rapid amplification of cDNA ends
RNA	ribonucleic acid
rRNA	ribosomal ribonucleic acid
SHMT	serine hydroxymethyltransferase
TCA	tricarboxylic acid
THF	5,6,7,8-tetrahydrofolate
ToxoDB	database of the *Toxoplasma gondii* genome
TS	thymidylate synthase

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