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***Plasmodium falciparum*: a paradigm for alternative folate biosynthesis in diverse microorganisms?**

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Abstract

Folates have a key role in metabolism, and the folate-dependent generation of DNA precursors in the form of deoxythymidine 5'-phosphate is particularly important for the replication of malaria parasites. Although *Plasmodium falciparum* can synthesize folate derivatives *de novo*, a long-standing mystery has been the apparent absence of a key enzyme, dihydroneopterin aldolase, in the classical folate biosynthetic pathway of this organism. The discovery that a different enzyme, pyruvoyltetrahydropterin synthase, can produce the necessary substrate for the subsequent step in folate synthesis raises the question of whether this solution is unique to *P. falciparum*.

Bioinformatic analyses suggest otherwise and indicate that an alternative route to folate could be widespread among diverse microorganisms and could be a target for novel drugs.

The importance of folates

Folates are essential in almost all living organisms and provide the molecular vehicles that transfer one-carbon units such as $-\text{CH}_3$, $-\text{CH}_2$ and $-\text{CHO}$ to appropriate acceptor molecules. One of the most important one-carbon transfer reactions in malaria parasites is the methylation of the nucleotide deoxyuridine 5'-monophosphate (dUMP) to deoxythymidine 5'-monophosphate (dTMP), precursor for the deoxythymidine 5'-triphosphate (dTTP) needed for DNA synthesis, in the thymidylate cycle [1] – which is cyclical because the pterin ring of the folate cofactor involved (5,10-methylenetetrahydrofolate) is oxidized during this transfer and must then be reduced before it can be re-used. Humans and other metazoa cannot synthesize their own folate and must rely on dietary uptake to obtain this essential nutrient. By contrast, plants, most bacteria and many unicellular eukaryotes are equipped with a biosynthetic capacity, and – for reasons that are unclear – some parasitic protozoa such as *Plasmodium* and *Toxoplasma* are able to both synthesize folates *de novo* and salvage them from the host plasma [2-4]. The inhibition of reactions in folate metabolism has long been the basis of chemotherapeutic interventions against malaria and toxoplasmosis, in the form of combinations such as pyrimethamine with sulfadoxine or sulfadiazine, alongside common antibacterial formulations such as trimethoprim–sulfamethoxazole. In all of these cases, the dihydrofolate reductase (DHFR) and dihydropteroate synthase (DHPS) enzymes of the folate biosynthesis pathway are the respective activities targeted. However, in the apicomplexan parasites, none of the other

enzymes in this pathway has been exploited to date as a drug target. Here, we discuss evidence that one of the enzymes in the classical route of folate biosynthesis is missing in *Plasmodium falciparum* and that its function is, instead, provided by an enzyme that is normally associated in other organisms with a different metabolic pathway. We also explore the possibility that this alternative route to folate biosynthesis is found not only in *P. falciparum* and its close apicomplexan relatives but also across a wider range of microorganisms that include other important parasites and pathogens.

Dihydroneopterin aldolase is missing in *P. falciparum*

The ‘textbook’ biosynthetic route to tetrahydrofolate, the key folate to which one-carbon units are attached, involves a series of enzymic steps that starts with the opening, remodelling and reclosing of the purine ring system of guanosine triphosphate (GTP) to that of a pterin, dihydroneopterin triphosphate (DHNTTP). The triphosphate moiety is then removed, followed by the shortening of the side chain at the 6-position of the pterin ring from three carbons to one, giving 6-hydroxymethyl-7,8-dihydropterin (6HMDP). This reaction is mediated by dihydroneopterin aldolase (DHNA). 6HMDP is then activated by hydroxymethyldihydropterin pyrophosphokinase (HPPK or PPPK) [Figure 1a, scheme (i)]. Historically, before the completion of the genome sequence, most of the *P. falciparum* genes of folate biosynthesis and the thymidylate cycle had been cloned by exploiting sequence motifs conserved in well-established orthologues in other organisms, and then functionally characterized (reviewed in Ref. [5]). A notable exception was DHNA. Originally, the failure to clone a DHNA-encoding gene was ascribed to the low level of conservation apparent among known DHNAs, making this enzyme an elusive target for PCR primer design. However, the landmark 2002 *P. falciparum* genome paper explicitly stated that, at least in BLASTP searches, ‘all but one of the enzymes (dihydroneopterin aldolase) required for *de novo* synthesis of folate from GTP were identified’ [6]. This, of course, did not exclude there being a highly divergent DHNA that was missed by the BLASTP algorithm. Other possibilities were that the very long inserts found in the *P. falciparum* versions of the enzymes preceding and succeeding DHNA in the pathway carried out this activity, but the generally low complexity and poor sequence conservation of these inserts across different plasmodial species made this unlikely. However, it is well known that secondary and tertiary structure can be much more highly conserved than primary amino acid sequence in a protein family, an excellent example of which is the thymidylate synthase (TS) of the archaeon *Methanococcus jannaschii* that was found by structural prediction and database matching, despite extremely low sequence homology to the TSs of other organisms [7]. Yet, applying such an approach [8,9] to the predicted proteome of *P. falciparum* still failed to identify a credible candidate for a DHNA-encoding gene [10]. To complement the bioinformatic approaches, sensitive radiolabelling studies were carried out to detect the expected product, 6HMDP, from parasite extracts (demonstrably active for other folate pathway enzymes) incubated with the DHNA substrate, dihydroneopterin – but none was found [10].

An alternative source of 6HMDP

DHNA belongs to a structural family known as the tunnelling-fold or T-fold proteins, which comprise a small group of multimeric proteins that all bind to purine or pterin substrates and are characterized by a wide tunnel formed by a distinctive pattern of antiparallel β -sheet and antiparallel helices in each subunit [11]. Attention thus shifted to a protein that belongs to this family but shares no primary sequence similarity with DHNA. This enzyme, 6-pyruvoyltetrahydropterin synthase (PTPS), mediates a well-characterized step in the synthesis of tetrahydrobiopterin (BH₄), an essential cofactor for aromatic amino acid hydroxylases, glycerol ether monooxygenases and nitric oxide synthases, all of which are important in mammals and several other organisms but none of which seem to be present in

malaria parasites. What, then, is the function of a PTPS in *P. falciparum*? Compared to known orthologues, the *P. falciparum* protein is well conserved and possesses most of the residues that are known to be essential for PTPS function, such as a triad of His residues that coordinate the key Zn^{2+} ion in the active site (Figures 2 and 3a), and other residues involved in pterin positioning and catalysis [10]. However, a striking difference is the absence of the Cys residue in the active site in *P. falciparum*, other *Plasmodium* species and other apicomplexans (*Toxoplasma*, *Eimeria* and *Neospora*) (Figure 2). This Cys residue has been shown by biochemical and crystallographic studies of PTPSs from other organisms to provide the key nucleophilic centre (in the form of S^- on the side chain) that is necessary for abstraction of a proton from the DHNTP substrate in the first step of the reaction [12–14]. The comparison of X-ray structures of the *Caenorhabditis elegans*, rat and *P. falciparum* structures (see Ref. [14] and the RCSB Protein Data Bank at www.rcsb.org/pdb/home/home.do) showed that a Glu residue occupied the equivalent space in the *P. falciparum* protein (Figure 3a). This indicated that the *P. falciparum* PTPS might differ in specificity from PTPS enzymes characterized from other organisms. In parallel experimental comparisons with two such examples, from human and *Escherichia coli*, recombinant *P. falciparum* PTPS was found to uniquely produce 6HMDP [10], the necessary substrate for the subsequent enzyme in the folate biosynthesis pathway, HPPK [Figure 1a, scheme (ii)]. Interestingly, the reaction simultaneously produced a slightly lesser amount of the normal product, pyruvoyltetrahydropterin, which was the sole product from the human and bacterial recombinant enzymes (Figure 1b). The appearance of two products from the malarial protein can be ascribed to the presence of two centres of negative charge at the appropriate distance on the key active-site Glu residue, rather than the one associated with Cys in conventional PTPS molecules (Figure 3b). These centres of negative charge are thought to enable the abstraction of protons from two different sites on the pterin side chain, leading to two different outcomes. This, in turn, raises the interesting possibility that *P. falciparum* might be using its PTPS for two purposes, one of which is to provide the missing link in folate biosynthesis, and the other being unclear.

So, has the problem of the incomplete folate biosynthetic pathway in these parasites been definitively solved, or are there other possibilities? It is ultimately not possible to prove a negative, but extensive bioinformatic and biochemical work has yielded no hint of a *dhna* gene or gene product in *Plasmodium* and other apicomplexans, and it seems reasonable to conclude that their absence is real. As yet, the proven ability of *P. falciparum* PTPS to produce the substrate for HPPK, together with a plausible reaction mechanism, is the only alternative scenario. Furthermore, of the apicomplexan parasites with complete or near-complete genome sequences, those that have retained the folate biosynthetic pathway (*Plasmodium*, *Toxoplasma*, *Neospora* and *Eimeria*) all encode similar PTPS orthologues (Figure 2), whereas those that lack the pathway and must obtain all of their folate via salvage (*Cryptosporidium* and *Babesia*) do not. However, it is now becoming apparent from bioinformatic analysis of PTPS sequences and folate biosynthesis pathways in other organisms that this novel route is almost certainly not confined to apicomplexan parasites that are capable of making their own folate.

PTPS-like proteins might also replace DHNA in heterokonts and certain bacteria

Besides apicomplexans, other protists known to produce folates are the heterokonts (see Refs [15–17] and www.nefsc.noaa.gov/nefsc/publications/crd/crd0517/crd0517.pdf), a large group that includes oomycetes, various microalgae and brown algae. Because heterokonts are probably, phylogenetically, closely related to apicomplexans [18], heterokont genomes were searched for folate synthesis genes and *ptps*-like genes. *Leishmania*, an obligate folate auxotroph [19], and diverse plants (all folate prototrophs) were included in the analysis for

comparison. Six heterokont genomes were analysed: three species of the commercially important oomycete plant parasite *Phytophthora*, two diatoms (*Thalassiosira* and *Phaeodactylum*), and a pelagophyte (*Aureococcus*) (Figure 4a). None of these heterokont genomes encodes a protein with significant similarity to bacterial or plant DHNAs, although other folate synthesis genes are present. It is notable, however, that all the heterokonts have a *ptps*-like gene that is similar to that of *Plasmodium*, encoding a protein with a Glu residue but no Cys in the active-site region (Figure 4a). By contrast, the plant genomes encode a canonical DHNA but not PTPS. As expected from its folate dependence, *Leishmania* totally lacks folate synthesis genes; it also has no PTPS, in common with *Cryptosporidium* and *Babesia*. The opposite distribution patterns of DHNAs and PTPS-like proteins with an active-site Glu strongly imply that PTPS functionally replaces DHNA in heterokonts. Because heterokonts are diverse, numerous, and ecologically important, the initial finding for *Plasmodium* – if it were experimentally validated in heterokonts – would have wide implications for our understanding of folate biosynthesis in eukaryotes.

Such implications might not be confined to eukaryotes. A recent comprehensive survey of bacterial genomes revealed that DHNA is missing in many organisms with otherwise complete folate synthesis pathways [20]. A pilot survey indicates that many of the bacteria that lack *dhna* genes, and only these bacteria, have a gene specifying a PTPS-like protein with an active-site Glu residue. Moreover, *ptps* genes of this type are often clustered on the chromosome with folate synthesis genes, which indicates a functional relationship to the folate synthesis pathway [21]. A striking example occurs in the genus *Clostridium*, in which most species have DHNA, but *C. botulinum* does not (Figure 4b). Three members of this genus, *C. botulinum* included, have PTPS-like proteins with no active-site Glu; these proteins (QueD) are known to be involved in the synthesis of the modified nucleoside queuosine, which is found in certain transfer RNAs [22]. Only *C. botulinum* has a second PTPS-like protein with an active-site Glu. Further evidence of a role for this enzyme in folate biosynthesis is that the corresponding gene is clustered on the chromosome with two other folate synthesis genes (encoding HPPK and DHPS) whereas the *queD* gene clusters with other queuosine synthesis genes (Figure 4c). Interestingly, the PTPS-like protein with the active-site Glu from *C. botulinum* also has an immediately adjacent Cys residue in its active site, raising the possibility that it could have a broader catalytic specificity than either the classical PTPSs or the newly identified apicomplexan variants.

Concluding remarks

In conclusion, however diverse the microorganisms that are ultimately experimentally shown to carry out folate biosynthesis in a similar manner to *P. falciparum*, the more immediate question is whether PTPS represents a realistic antimalarial drug target, given that the inhibition of the folate pathway at other steps is clinically so well proven in this parasite. Unlike the subsequent enzymes of this pathway (HPPK, DHPS – the target of the sulfa drugs – and dihydrofolate synthase), PTPS has an essential counterpart in humans (in the BH₄ pathway). However, DHFR also has an essential counterpart in humans, and this enzyme has proven to be a highly effective target in many human pathogens, including *P. falciparum* and *Toxoplasma gondii*. Although no lead compounds for the inhibition of any version of PTPS have been reported yet, the structural differences around the active site of PfPTPS, in addition to the switch to a different nucleophilic side chain, indicate that it would be surprising if parasite-specific inhibitors could not eventually be identified, both for *Plasmodium* and for other important pathogens.

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Figure 1.

Folate biosynthesis and structures of pathway intermediates. (a) Conventional and alternative folate biosynthetic pathways. (i) The conventional folate biosynthetic scheme is found in plants, bacteria and lower eukaryotes that are capable of *de novo* folate synthesis. (ii) The alternative scheme is found in *P. falciparum*, in which PfPTPS provides a route to 6HMDP that bypasses the need for a DHNA activity. HPPK and DHPS activities are found on a single bifunctional protein in *P. falciparum* and related organisms (Figure 4). The asterisked metabolite in pathway (ii) was identified only from its oxidation product [10]. Enzymes are shown in boxes. Abbreviations: GCHI, GTP cyclohydrolase I; PP/P, sequential pyrophosphatase and phosphatase activities (the pyrophosphatase has been identified in several organisms [23,24]); DHNA, dihydroneopterin aldolase; HPPK, hydroxymethyldihydropterin pyrophosphokinase; DHPS, dihydropteroate synthase; DHFS, dihydrofolate synthase; PfPTPS, pyruvoyltetrahydropterin synthase orthologue from *P. falciparum*; pAB, *p*-aminobenzoate; L-Glu, L-glutamate. (b) Structures of the two products of the PfPTPS reaction in Figure 1a, scheme (ii). (i) 6-pyruvoyl-5,6,7,8-tetrahydropterin. (ii) 6HMDP.

<i>Plasmodium falciparum</i>	HFIAY-----NGFR-----E-TL--HGH
<i>Plasmodium vivax</i>	HFIAP-----KGFR-----E-TL--HGH
<i>Plasmodium gallinaceum</i>	HFIAY-----KGFR-----E-KL--HGH
<i>Plasmodium yoelii</i>	HFIAY-----KGFR-----E-TL--HGH
<i>Toxoplasma gondii</i>	HFIAY-----RGFR-----E-RL--HGH
<i>Eimeria tenella</i>	HFVAF-----KSFR-----E-RL--HGH
<i>Neospora caninum</i>	HFIAY-----RGFR-----E-RL--HGH
<i>Synechocystis sp</i>	HQLP-----HH-EG-KCRRL--HGH
<i>Streptococcus thermophilus</i>	HHLF-----NY-EG-KCKSL--HGH
<i>Haemophilus influenzae</i>	HLLD-----GH-DG-KCQNL--HGH
<i>Escherichia coli</i>	HRLP-----HVPEGHKCGRL--HGH
<i>Shigella flexneri</i>	HRLP-----HVPEGHKCGRL--HGH
<i>Yersinia enterocolitica</i>	HLLP-----HVPEGHKCGRL--HGH
<i>Homo sapiens</i>	HRLYSKFLSDEENLKLFQ-KCNNPNGHGH
<i>Rattus norvegicus</i>	HLHSPSLSAEENLKVFQ-KCNNPNGHGH
<i>Caenorhabditis elegans</i>	HLHSEKLSAENKETFG-KCNNPNGHGH
<i>Poecilia reticulata</i>	HLHSLRLSDEENKEVYG-KCNNPYGHGH
<i>Drosophila melanogaster</i>	*HLHSPQLSDAENLEVFG-KCNNFHHGH*

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Figure 2.

Aligned sequences of the active-site region of PTPS orthologues. These sequences lie between the three conserved histidine residues that coordinate the activesite Zn^{2+} ion (asterisks) in all PTPSs. Upper group, apicomplexans; middle group, bacteria; bottom group, metazoa. The active-site Cys residue conserved in the last two groups is marked by an arrow; the Glu residue that is proposed to act as nucleophile instead of Cys in the PTPS of *P. falciparum* and related organisms aligns structurally to the same position (Figure 3). Gaps (hyphens) have been introduced to optimize alignment among the diverse sequences, which exhibit variable lengths between the conserved histidines. UniProtKB/TrEMBL (www.uniprot.org/uniprot/) accession numbers for non-apicomplexan enzymes: metazoa: *Homo sapiens*, Q03393; *Rattus norvegicus*, P27213; *C. elegans*, O02058; *Poecilia reticulata*, Q90W95; *Drosophila melanogaster*, P48611. Bacteria: *Synechocystis*, Q55798; *Streptococcus thermophilus*, Q5M063; *Haemophilus influenzae*, P44123; *E. coli*, P65870; *Shigella flexneri*, P65872; *Yersinia enterocolitica*, A1JJS1. Gene loci for apicomplexans: malarial sequences are from PlasmoDB (www.plasmodb.org/): *P. falciparum*, PFF1360w; *P. vivax*, Pv114505; *Plasmodium gallinaceum*, BLAST searching of contigs; *Plasmodium yoelii*, MALPY00748. The *T. gondii*, *Eimeria tenella* and *Neospora caninum* sequences were also determined from BLAST searching of contigs in ToxoDB (www.toxodb.org/) and GeneDB (www.genedb.org/).

Figure 3.

Spatial comparisons of the active-site regions of PTPS from *C. elegans* and *P. falciparum*.

(a) The three His residues coordinating the essential Zn^{2+} ion (green sphere) are used as reference points to show the relative occupation in space of the active-site nucleophile Cys38 in *C. elegans* PTPS and the proposed nucleophile Glu38 in PfPTPS. Distances of the respective nucleophilic centres (the S atom of Cys38 and the two O atoms of Glu38) from the Zn^{2+} ion are measured as shown, together with the average (av) of the distances for the O atoms. The graphics are derived from the crystallographic coordinates of *C. elegans* PTPS (CePTPS) and the *P. falciparum* enzyme (PDB structures 2G64 and 1Y13, respectively; see www.rcsb.org/pdb/home/home.do). **(b)** Proposed nucleophilic attack of the Cys residue of CePTPS and the Glu residue of PfPTPS on the side chain of the DHNTP substrate (OPPP represents the triphosphate group thereof). This leads to two alternative products for PfPTPS, depending upon whether the proton from C1' or from the OH group on C2' is abstracted first. The loss of the C1' proton is predicted to give rise to 6-pyruvoyltetrahydropterin (PTP) as the product, and the loss of the OH proton on C2' will yield 6HMDP, by analogy with described PTPS [14] and DHNA [25] reaction mechanisms. For a Glu residue to act efficiently as a general base catalyst as shown, the pK of the side chain in its native environment would need to be raised above its level of approximately 4.3 in the free amino acid, as seen in other enzymes where Glu has this role [26].

Figure 4.

Comparative genomic evidence that PTPS-like proteins also replace DHNA in heterokonts and *Clostridia*. **(a)** Distribution of representative folate synthesis genes and *ptps*-like genes among protists and plants, and the active-site regions of the PTPS-like proteins of heterokonts, with the *P. falciparum* sequence for comparison. Glutamate residues are coloured in red, and other conserved residues are coloured in blue. Abbreviations: GCHI, GTP cyclohydrolase I; DHNA, dihydroneopterin aldolase; HPPK-DHPS, bifunctional hydroxymethyldihydropterin pyrophosphokinase-dihydropteroate synthase. Genomes were accessed at the following sites: *Phytophthora infestans*, Broad Institute (www.broad.mit.edu/annotation/genome/phytophthora_infestans); *P. falciparum* and *Leishmania major*, GeneDB (www.genedb.org/); *Arabidopsis thaliana*, GenBank (www.ncbi.nlm.nih.gov/GenBank/); others, JGI (<http://genome.jgi-psf.org/>). Protein IDs for PTPS gene products are: *Phytophthora sojae*, 139089; *Phytophthora ramorum*, 79196; *Phytophthora infestans*, PITG_02663; *Aureococcus anophagefferens*, 22298; *Thalassiosira pseudonana*, 173900; *Phaeodactylum tricornutum*, 18384; *P. falciparum*, PFF1360w. **(b)** Distribution of folate synthesis genes and two *ptps*-like genes among *Clostridium* species, and the active-site regions of the PTPS-like proteins. Note that one of the PTPS-like proteins is QueD, a biosynthetic enzyme for the modified nucleoside queuosine. GenBank accession numbers for the *Clostridium* species genomes are: *Clostridium acetobutylicum*, NC_003030; *Clostridium botulinum*, NC_009697; *Clostridium thermocellum*, NC_009012; *Clostridium kluyveri*, NC_009706; *Clostridium perfringens*, NC_003366; *Clostridium difficile*, NC_009089. The GenBank accession number of the *C. botulinum* PTPS protein with glutamate in the active site is YP_001383205. **(c)** Genomic context of the two *ptps*-like genes in *C. botulinum*, showing clustering of the QueD gene with genes for other queuosine synthesis enzymes (QueE and QueC) and clustering of the other *ptps*-like gene with HPPK- and DHPS-encoding genes.