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

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In higher eukaryotes, pteridine cofactors such as tetrahydro-biopterin (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I (GTPC; EC 3.5.4.16), an enzyme that is present in both *P. falciparum* [6] and *T. gondii* (Smith, Hyde and Sims, unpublished data).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-pyruvoyl-tetrahydropterin 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

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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 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].

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 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*.

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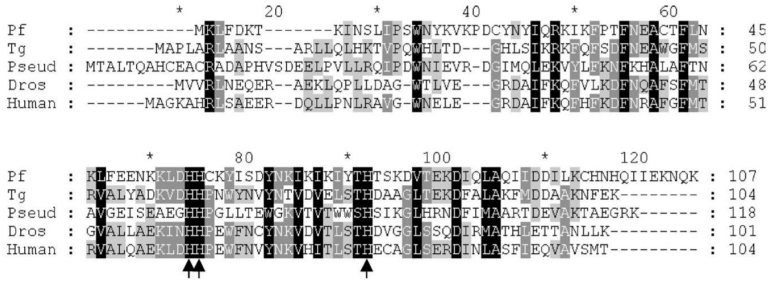

Fig. 1.

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].

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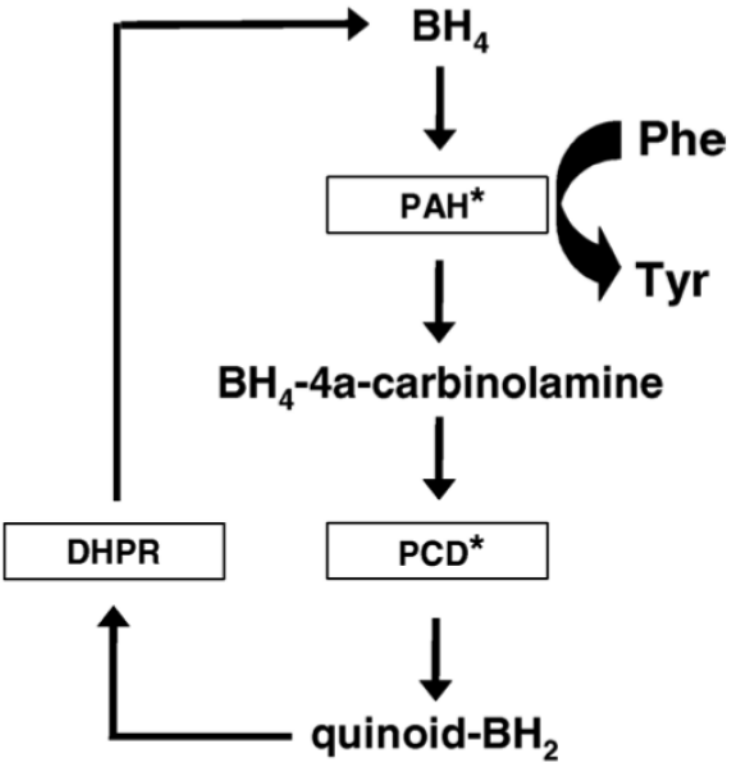

Fig. 2.

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*.

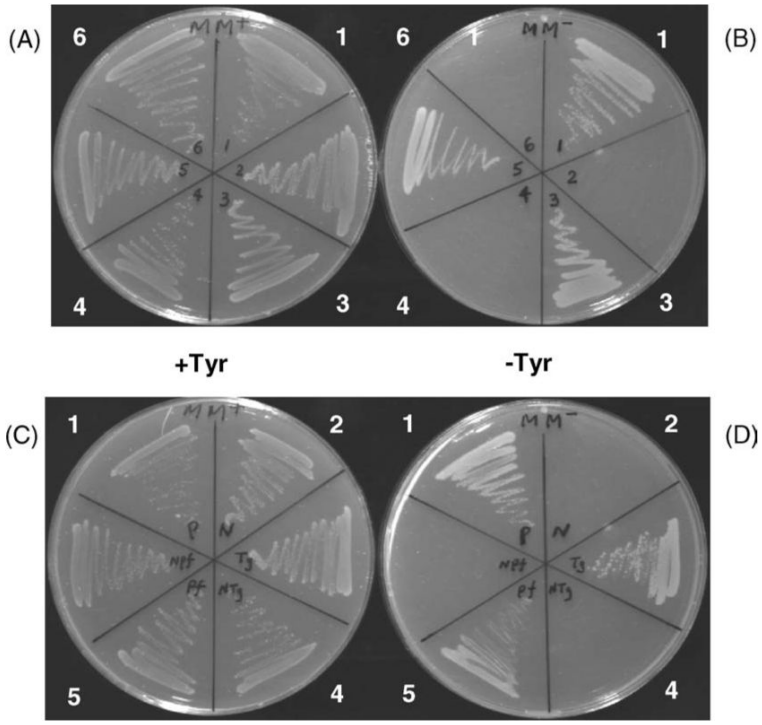

Fig. 3.

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 μg ml^{-1} streptomycin, 50 μg ml^{-1} phenylalanine, with (A and C) or without (B and D) 50 μg ml^{-1} tyrosine.

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