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A glycine-cleavage complex as part of the folate one-carbon metabolism of *Plasmodium falciparum*

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Abstract

The glycine-cleavage complex (GCV) and serine hydroxymethyltransferase represent the two systems of one-carbon transfer that are employed in the biosynthesis of active folate cofactors in eukaryotes. Although the understanding of this area of metabolism in *Plasmodium falciparum* is still at an early stage, we discuss evidence that genes and transcription products of the GCV are present and expressed in this parasite. The potential role of the GCV and its relevance to the life cycle and pathogenesis of the malaria erythrocytic stages are also considered. According to its expression profile, the GCV seems to be particularly active in gametocytes. The GCV enzyme dihydrolipoamide dehydrogenase has two isoforms encoded by two different genes. It has been demonstrated recently that both genes are functional, with one of them identified as being part of a pyruvate dehydrogenase complex that is present exclusively in the apicoplast of *Plasmodium* species. The other isoform probably forms part of the *Plasmodium* GCV. The GCV is the first enzyme complex involved in folate metabolism in this parasite that can be assumed, with a good degree of certainty, to be located in the mitochondria.

Synthesis and use of the one-carbon unit in folate

The glycine-cleavage complex (GCV) and serine hydroxymethyltransferase [SHMT, also called glycine hydroxymethyltransferase (EC 2.1.2.1)] form part of the folic acid biosynthesis pathway, generating one-carbon units from cleavage of the small amino acids glycine and serine, respectively. These one-carbon units are transferred onto folate coenzymes that carry and donate them, primarily for the synthesis of pyrimidines [e.g. thymidylate (5'-TMP)] and methionine in the malaria parasite [1]. SHMT can reversibly interconvert glycine and serine, and glycine itself is a precursor of the synthesis of glutathione, tryptophan and phospholipids in eukaryotes [2]. The importance of the ubiquitous GCV enzymatic complex is exemplified by its role in essential homeostatic processes. For instance, the synthesis of glutathione to maintain the redox balance of the erythrocyte plasma membrane needs an active but regulated glycine supply [3], the mitochondrial processing of high concentrations of phosphorespiration glycine in plants relies on plant GCVs [4], and a dys-functional GCV in humans caused by specific point mutations is related to an inherited condition known as nonketotic hyperglycinemia or glycine encephalopathy [5].

Whereas serine, through the reaction catalysed by SHMT, is the main source of one-carbon units in the cytoplasm, glycine is equally relevant in the mitochondrion, and the GCV

catalyses this reaction. SHMT and GCV have been characterized in most known organisms, with many eukaryotes (e.g. yeast, plants and mammals) expressing both cytoplasmic and mitochondrial isoforms of SHMT. So far, however, the GCV components have been found exclusively in the mitochondrion [4]. GCV catalyses the reversible oxidative cleavage of glycine into CO₂ and NH₃, with transfer of the remaining methylene (CH₂) moiety to tetrahydrofolate (THF) forming N⁵,N¹⁰-CH₂-THF (Figure 1).

The GCV is a multimeric assembly of four proteins known as P-protein [glycine decarboxylase (EC 1.4.4.2)], H-protein (which contains a lipoamide prosthetic group), T-protein [tetrahydrofolate aminomethyltransferase, also known as glycine synthase (EC 2.1.2.10)] and L-protein [dihydrolipoamide dehydrogenase (EC 1.8.1.4)]. In plants, in which the GCV has been studied extensively, the calculated molar stoichiometry indicates the presence of H-proteins, H-proteins, T-proteins and L-proteins to be 4: 27:9:2, respectively [4]. The activation and use of folate depend on covalent linkage to a one-carbon unit – a by-product of the balanced flux of carbon between serine and glycine – through SHMT and GCV, two independent but functionally related enzyme systems (Figure 1). The *de novo* production of fully reduced and glutamated (and, hence, functional) folate in *Plasmodium falciparum* is the result of the actions of six enzymatic activities, whose genes have been identified and characterized in this organism (with the notable exception of the gene encoding dihydroneopterin aldolase) [6-8]. The reduced folate product of this pathway – N⁵,N¹⁰-CH₂-THF – provides the one-carbon unit that is used principally for the synthesis of 5'-TMP, through dihydrofolate-reductase-thymidylate-synthase, and methionine after a further reduction of N⁵,N¹⁰-CH₂-THF to yield N⁵-CH₃-THF [1,9,10]. The cell compartments in which synthesis of the folate cofactors takes place are unknown in *Plasmodium*, although the products that they help to synthesize are needed in organelles in which synthesis of DNA and protein occurs. In *Plasmodium* species, the one-carbon synthesis aspect of folate biosynthesis and the compartmentalization of folate metabolism are just starting to be elucidated [8].

SHMT in *Plasmodium falciparum*

Two proteins encoded at different loci in *P. falciparum* are related to SHMT from other eukaryotes. One of them, encoded at PFL1720w, has been expressed and biochemically characterized [11]. It shows 87% similarity to *Plasmodium yoelii* PY00962 gene product (GenBank accession number EAA19589), 70% similarity to the *Toxoplasma gondii* protein with GenBank accession number AAT74582 and 65% similarity to the *Arabidopsis thaliana* protein with GenBank accession number CAB78435, and is considered to be the cytoplasmic form of the enzyme. A second gene (at locus PF14_0534) encodes a protein that shows 62% similarity to the *P. yoelii* PY00669 gene product (GenBank accession number EAA17740), which has been annotated as mitochondrial SHMT, 41% similarity with the mitochondrial SHMT of *A. thaliana* (GenBank accession number Q9SZJ5) and 46% similarity with *Saccharomyces cerevisiae* Shm1p (GenBank accession number NP_009822), which is the yeast mitochondrial isoform. Moreover, a mitochondrial transit peptide is present [MitoProt score 0.65/1.0, Box 1(iv)] in this second SHMT isoform of *P. falciparum* (encoded at locus PF14_0534), whereas no signal peptide is identified in the protein product of PFL1720w (Table 1). However, the assignment of the PF14_0534 gene product as an SHMT must be treated with caution at this stage because most of the known functional residues that are highly conserved in both cytoplasmic and mitochondrial SHMTs from other organisms are different in both the *P. falciparum* and the *P. yoelii* sequences.

The presence of fully active folate intermediates (reduced, glutamated and one-carbon unit added) has been demonstrated in yeast mitochondria and in plant mitochondria and chloroplasts [12]. A lack of active folates has been shown to result in defective mitochondria

in yeast [13]. Therefore, assuming the necessary presence of enzymes for the modifications of folate cofactors – including the condensation of one-carbon units – and the possible presence of a mitochondrial isoform encoded at PF14_0534 (putatively termed PfSHMTm), it is a reasonable working hypothesis that a one-carbon enzyme system comprising PfSHMT (encoded at PFL1720w) in the cytoplasm, and PfSHMTm and GCV in mitochondria is present in *Plasmodium* species (Figure 1).

Evidence of a GCV in *Plasmodium falciparum*

The genes encoding GCV enzymes, except the one encoding a homologue of the P-protein, have been identified in *P. falciparum* by sequence similarity, as described later. When the *P. falciparum* genome database [Box 1(ii)] was searched with each sequence of the four components of yeast and plant GCVs, two DNA fragments of significant similarity were identified by basic local alignment search tool (BLAST) as encoding potential homologues of the T-protein [PfGCVT, encoded at PF13_0345 (E-score 7e-23)] and the L-protein [PflPD1, encoded at PFL1550w (E-score 2e-92)]*. Homologues of an H-protein [PfGCVH, encoded at PF11_0339 (E-score 6e-18)] and, very interestingly, a second L-protein gene or isoform [PflPD2, encoded at PF08_0066 (E-score 7e-80)] were also discovered. Amplification of *gcv*-related gene fragments from *P. falciparum* genomic DNA was carried out for the genes *PfgcvT*, *PfgcvH*, *Pflpd1* and *Pflpd2*. Subsequently, the transcription and, therefore, expression of these genes were assessed by northern blotting and, thus far, mRNAs have been observed for all of them in the erythrocytic asexual stages (E. Salcedo, unpublished). Other basic characteristics of these genes and the proteins they are predicted to encode are provided in Table 1, including traits of primary protein structure that imply possible organelle compartmentalization (e.g. the presence of signal or transit peptides for the mitochondrion or apicoplast), in addition to transmembrane domains.

The primary structures of the GCV proteins identified so far in *P. falciparum* were compared with the structural domains compiled in Pfam [Box 1(iii)], and the following observations were made: (i) 33% of the T-protein PfGCVT sequence corresponds to the consensus domains reported (Pfam code 01571) for a GCV_T [glycine-cleavage T-protein (aminomethyltransferase)] flanked by a mitochondrial transit peptide [MitoProt score 0.82/1.0, Box 1(iv)] [14]; (ii) 55% of the H-protein PfGCVH sequence falls into recognizable domains [Pfam code 01597 for a GCV_H (glycine-cleavage H-protein)], outside of which there is an N-terminal peptide of 28 amino acids that includes a mitochondrial transit peptide (MitoProt score 0.98/1.0); (iii) the L-protein PflPD1 has 93% of its sequence within the consensus for this protein class (Pfam code COG1249), denoted as the dihydrolipoamide dehydrogenase (E3) component of the pyruvate-2-oxoglutarate dehydrogenase complex; and (iv) the L-protein PflPD2 has 68% of its sequence within the consensus for this protein class. A long N-terminal fragment of 122 amino acids and six internal loops of 6-21 residues lie outside the regions of high similarity.

On the basis of the assignments of these four proteins, it is reasonable to suppose that there is an equivalent of the GCV in *P. falciparum*. Additionally, the enzymes involved in the synthesis and salvage of lipoic acid, which is posttranslationally ligated to H-protein (Figure 1), have recently been characterized in *P. falciparum* [15]. According to its primary structure and compartmentalization in all known eukaryotes, the GCV represents the first enzyme complex involved in *P. falciparum* folate metabolism that, in principle, can be assigned to the mitochondria of this parasite. However, a homologue of the GCV P-protein remains to be identified. Considering the conserved nature of this protein in other organisms, it is possible that its gene could have been overlooked in the completed genome sequence of the

*E-scores are to the closest match from either yeast or plant homologue.

parasite owing to a high level of fragmentation by introns, although a divergent version of the P-protein in *P. falciparum* cannot be excluded at this stage.

Origin and segregation of two dihydrolipoamide dehydrogenases

The phylogenetic analysis of known dihydrolipoamide dehydrogenases (L-proteins) shows two clusters containing PfLPD1 and PfLPD2 separately, with the bacterial *Rickettsia prowazekii* and *Synechocystis* sp. (PCC6803) proteins in the first and second of these clusters, respectively (Figure 2). Because *Rickettsia* is regarded as the present-day species closest to the mitochondrial ancestor and because *Synechocystis* is closely related to plastid organelles, the result of the analysis suggests a separate origin for these two functionally equivalent proteins. In addition, PfLPD2 has a typical bipartite secretory-apicoplast signal peptide [PATS score 0.94/1.0, Box 1(v)] [16]. Both dihydrolipoamide dehydrogenase isoforms from *P. falciparum* have recently been characterized, substantiating the existence and functionality of the complexes in which these enzymes participate [17].

Box 1. Relevant links

The following databases and online tools are available, particularly for the analysis of protein primary structure domains that are of relevance in protein cell sorting:

- i. PlasMit (<http://gecco.org.chemie.uni-frankfurt.de/plasmit/>). Implements algorithms for prediction of mitochondrial transit peptides trained with *Plasmodium falciparum* proteins.
- ii. NCBI (http://www.ncbi.nlm.nih.gov/sutils/genom_tree.cgi?organism=euk). BLAST for eukaryotic genomes - useful queries for all Apicomplexa or single species can be performed.
- iii. Pfam (<http://www.sanger.ac.uk/Software/Pfam/>). Reference library for comparison of protein domain architectures.
- iv. MitoProt (<http://ihg.gsf.de/ihg/mitoprot.html>). Provides numerical scores for predicted mitochondrial-targeting fragments.
- v. PlasmoDB (<http://www.plasmodb.org>). Official source for annotated *Plasmodium* genomes.
- vi. TMAP (<http://bioweb.pasteur.fr/seqanal/interfaces/tmap.html>). Utility for the identification of membrane-spanning regions in proteins that is based at the Pasteur Institute.
- vii. TMHMM (<http://www.cbs.dtu.dk/services/TMHMM/>). Utility for the prediction of transmembrane helices in proteins that is based at the Centre for Biological Sequence Analysis of the Technical University of Denmark.
- viii. TMPred (http://www.ch.embnet.org/software/TMPRED_form.html). Utility for the prediction of transmembrane regions and orientation in proteins that is based at the Swiss Node of EMBnet.
- ix. TopPred (<http://bioweb.pasteur.fr/seqanal/interfaces/toppred.html>). Utility for topology prediction of membrane proteins that is based at the Pasteur Institute.

In plants, two different mitochondrial and chloroplast isoforms of dihydrolipoamide dehydrogenase have been characterized as part of the pyruvate dehydrogenase complexes (PDCs) present in both of these compartments [18]. Furthermore, a phylogenetic analysis of dihydrolipoamide dehydrogenases that includes known plant homologues [19] has also been

shown to follow the phylogenetic relationship that we have derived here for *Plasmodium* homologues (Figure 2). Overall, this supports the argument for separate ancestral lineages of the two dihydrolipoamide dehydrogenase isoforms found in both plants and *Plasmodium* species, and is in agreement with the proposed endosymbiotic origin of the *Plasmodium* apicoplast [20,21]. Thus, at least two dihydrolipoamide dehydrogenase genes would have been acquired and transferred to the nucleus, and their gene products selectively secreted to compartments in which decarboxylation for transfer of one-carbon units is undertaken.

Dihydrolipoamide dehydrogenase enzymes carry out equivalent reactions in up to four different dehydrogenation-decarboxylation complexes in eukaryotes; of these four complexes, the PDC [22], 2-oxoglutarate dehydrogenase, branched-chain α -keto acid dehydrogenase and the GCV seem to be present in *P. falciparum*. The GCV is localized in mitochondria in all known organisms and, in plants, the PDC is present in both mitochondria and chloroplasts [18]. If *Plasmodium* species also contain a PDC in plastids, as observed in plants, it could be inferred that the two separately encoded L-proteins, which are probably of different origins, are part of the GCV (PfLPD1) in mitochondria and the PDC (PfLPD2) in apicoplasts. In support of this view, both the α subunit of E1 [pyruvate dehydrogenase (EC 1.2.4.1), encoded at PF11_0256] and E2 [dihydrolipoamide *S*-acetyltransferase (EC 2.3.1.12), encoded at PF10_0407] have recognizable bipartite signal peptides and apicoplast transit peptides with PATS scores of 0.984 and 0.612, respectively [Box 1(v)]. Moreover, functional and subcellular location analyses have recently been reported for a *Plasmodium* PDC located in the apicoplast [23]. The results indicate that PfLPD2 is the third component (E3 dihydrolipoamide dehydrogenase) of the PDC in *Plasmodium* species, in which the experimentally determined location of this enzyme in the plastid is also consistent with the bioinformatic analysis [24]. In this respect, *Plasmodium* seems to differ from other eukaryotes, most of which have PDCs located in mitochondria or, as is the case for plants and algae, in both mitochondria and plastids [18].

The GCV and pathogenesis

The regulated flux of carbon units between serine and glycine is an essential component of one-carbon metabolism. In bacteria, there is a coordinated regulation of activity among the GCV, the PDC and the 2-oxoglutarate dehydrogenase complex. In yeast, such flux through the GCV–SHMT system is required for both the synthesis of serine from glycine and the use of glycine as the sole nitrogen source; in plants, the GCV is the enzymatic system that deals with the large quantities of glycine resulting from major metabolic reactions in illuminated leaves [25]. However, the importance of the GCV–SHMT system and the serine–glycine flux in pathogenic micro-organisms is largely unknown at present. Nonetheless, interesting conjectures are starting to appear. A highly syntenic region involved in the metabolic fitness of intracellular pathogens (*Mycobacterium* and *Rhodococcus* species) that invade hostile environments (e.g. phagocytic cells) contains *gcv* genes whose products could guarantee the maintenance of the NAD⁺ pool under O₂-limiting conditions [26]. Moreover, a specific *gcvB* locus of *Brucella abortus* has been shown to be necessary for sustained infection in a model host and is presumed to be part of an unknown mechanism for long-term persistence, particularly during states of non-replication [27].

Interestingly, *PfLPD1* and *PfLPD2* transcripts and protein products are differentially expressed in *P. falciparum* gametocytes compared with the asexual stages, as assessed by both proteomic approaches and oligonucleotide microarrays [28,29]. The mass spectrometry data suggest that the relative abundance of PfLPD1, as crudely measured by percent sequence coverage, is ~48% for gametocytes, 21% for trophozoites, 6% for sporozoites and 3% for merozoites. The maximal relative induction of its mRNA also occurs at the gametocyte stage [Box 1(v)]. Only transcriptional profiles are available for *PfLPD2* but they also show a

similar pattern of maximal induction in gametocytes. Although only circumstantial at this point, combined with the bacterial data, this evidence directs us to the possibility that the complexes in which these proteins are involved have pivotal roles in the gametocyte metabolism of *P. falciparum*. Relevant to this might be the behaviour of yeast cells when shifted from growth on medium that was rich in amino acids to growth on medium that was deficient in amino acids – the expression of lipoamide dehydrogenase was increased approximately twofold [30]. Of the different roles exhibited by the GCV in eukaryotes (e.g. serine synthesis, glycine degradation and NAD⁺/NADH+H⁺ electron transfer), amino acid metabolism and NADH+H⁺ synthesis would be prime candidates for the possible roles of a GCV in *Plasmodium*.

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The *Plasmodium falciparum* one-carbon enzyme systems SHMT and GCV, as deduced from enzymes found in other eukaryotes. The proteins for which genes have been identified are PfGCVH, PfGCVT, PfLPD1, PfSHMT [6,11] and the putative PfSHMTm. A candidate gene for the P-protein (P?) has not yet been identified in *P. falciparum*. Of the two L-protein isoforms that have been found, PfLPD1 is the enzyme that would be involved primarily in the mitochondrial GCV, catalysing the reoxidation of the lipoamide arm that is covalently bound to PfGCVH. The main use ascribed to folate derivatives in this parasite is the synthesis of thymidine 5'-monophosphate (5'-TMP) and methionine (Met). Both compounds are necessary in cell compartments, including mitochondria. The complex of PfGCV enzymes would carry out the decarboxylation of glycine, whereas SHMT would undertake the reversible conversion of serine to glycine. A dual presence of cytosolic and mitochondrial PfSHMT is proposed, whereas PfGCV is confined to mitochondria. This compartmentalization implies the transport of glycine, serine and one of several possible intermediate folate metabolites (Fol) through mitochondrial membranes, as has been shown for other eukaryotes [31].

Figure 2.

Origin of the dihydrolipoamide dehydrogenases (PflPD1 and PflPD2) in *Plasmodium* species. A tree originated with homologous proteins by Minimum Evolution, as implemented in Mega version 2.1, is shown [32]. Scores on branches are from bootstraps to 1000 repetitions. GenBank accession numbers are shown within square brackets after taxon unit names. The mitochondrial group contains *Rickettsia prowazekii* (an α -proteobacterium and the closest known descendent of the mitochondrial ancestor). *Synechocystis* sp. clusters with the plastid isoforms and supports a cyanobacterial origin for the PflPD2 protein. Names in bold indicate species with several isoforms of LPD proteins, except *Saccharomyces cerevisiae* (which encodes only one isoform and is highlighted as a model organism), and *Synechocystis* sp. and *Rickettsia prowazekii* (the importance of which is explained above). Scale bar shows proportion of amino acid differences.

Table 1

GCV and SHMT proteins of *Plasmodium falciparum*

Abbreviation	Gene locus ^a	Number of exons	Number of amino acids	Presence of signal or transit peptides ^b	Presence and number of transmembrane domains ^c
PIGCVT	PF13_0345	2	406	+	–
PIGCVH	PF11_0339	1	200	+	–
PLPD1	PFL1550w	1	498	–	2
PLPD2	PF08_0066	1	666	+	5
PISHMT	PFL1720w	3	442	–	1
PISHMTm?	PF14_0534	1	462	+	1

^a Nomenclature of gene loci is taken from release 4.2 of PlasmoDB [Box 1(v)].

^b Presence of signal or transit peptides for organelles (mitochondria or plastids - see text) as predicted by PlasMit [Box 1(i)] or PATS [Box 1(v)].

^c Predicted by TMAP [Box 1(vi)], TMHMM [Box 1(vii)], TMpred [Box 1(viii)], and TOPPED [Box 1(ix)].