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# The enzymes of the 10-formyl-tetrahydrofolate synthetic pathway are found exclusively in the cytosol of the trypanosomatid parasite *Leishmania major*

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ABSTRACT

In most organisms 10-formyl-tetrahydrofolate (10-CHO-THF) participates in the synthesis of purines in the cytosol and formylation of mitochondrial initiator methionyl-tRNA$^{Met}$. Here we studied 10-CHO-THF biosynthesis in the protozoan parasite *Leishmania major*, a purine auxotroph. Two distinct synthetic enzymes are known, a bifunctional methylene-tetrahydrofolate dehydrogenase/cyclohydrolase (DHCH) or formyl-tetrahydrofolate ligase (FTL), and phylogenomic profiling revealed considerable diversity for these in trypanosomatids. All species surveyed contain a *DHCH1*, which was shown recently to be essential in *L. major*. A second *DHCH2* occurred only in *L. infantum*, *L. mexicana* and *T. cruzi*, and as a pseudogene in *L. major*. DHCH2s bear N-terminal extensions and we showed a *Li*DHCH2-GFP fusion was targeted to the mitochondrion. FTLs were found in all species except *Trypanosoma brucei*. *L. major ftl*$^{-}$ null mutants were phenotypically normal in growth, differentiation, animal infectivity and sensitivity to a panel of pteridine analogs, but grew more slowly when starved for serine or glycine, as expected for amino acids that are substrates in C1-folate metabolism. Cell fractionation and western blotting showed that both *L. major* DHCH1 and FTL were localized to the cytosol and not the mitochondrion. These localization data predict that in *L. major* cytosolic 10-formyl-tetrahydrofolate must be transported into the mitochondrion to support methionyl-tRNA$^{Met}$ formylation. The retention in all the trypanosomatids of at least one enzyme involved in 10-formyl-tetrahydrofolate biosynthesis, and the essentiality of this metabolite in *L. major*, suggests that this pathway represents a promising new area for chemotherapeutic attack in these parasites.



## 1. Introduction

*Leishmania* are group of parasitic protozoa that pose substantial threats to health across a large part of the world. Different species of *Leishmania* cause a variety of pathologies that range from disfiguring skin lesions to fatal visceral infections. No vaccine is available for leishmaniasis and current drugs suffer from drawbacks such as toxicity, high cost or emerging resistance. Due to such problems, new drugs and new targets for drug development are needed. Historically, folate metabolism in parasitic protozoa has been a rich source of targets for chemotherapy, with folate biosynthesis and dihydrofolate reductase (DHFR) inhibitors showing good efficacy against malaria [1].

In this work we focus on the biosynthesis of 10-formyl-tetrahydrofolate (10-CHO-THF), which is used in cytosolic purine biosynthesis, or the formylation of the mitochondrial initiator methionyl-tRNA$^{Met}$ to make fMet-tRNA$^{Met}$ [2] (Fig. 1). There are two 10-CHO-THF biosynthetic pathways known (Fig. 1). One begins with 5,10-methylenetetrahydrofolate (5,10-$CH_2$-THF), which arises from serine via serine hydroxymethyltransferase (SHMT), or from glycine by the mitochondrial glycine cleavage complex (GCC). 5,10-$CH_2$-THF can be oxidized to 5,10-methenyltetrahydrofolate (5,10-CH=THF) by 5,10-$CH_2$-THF dehydrogenase (DH), and subsequently hydrolyzed by 5,10-CH=THF cyclohydrolase (CH) to yield 10-CHO-THF. These two sequential reactions are mediated by a single enzymatic site, so this bifunctional enzyme is termed DHCH [3]. In a second pathway, formate is added onto THF by an ATP-dependent formate-tetrahydrofolate ligase (FTL). In plants and prokaryotes, these enzymes are usually found as bifunctional DHCH and monofunctional FTL enzymes [2,4–7]. However, in humans and yeast the DHCH and FTL activities can be joined in a multienzyme polypeptide called the C1-synthase, which in yeast exists in two

*Abbreviations:* 10-CHO-THF, 10-formyl-tetrahydrofolate; 5,10-$CH_2$-THF, 5,10-methylenetetrahydrofolate; 5,10-CH=THF, 5,10-methenyltetrahydrofolate; FTL, formyl tetrahydrofolate ligase; DHCH, methylene tetrahydrofolate dehydrogenase/cyclohydrolase; PTR1, pteridine reductase 1; SHMT, serine hydroxymethyltransferase; GCC, glycine cleavage complex; THF, tetrahydrofolate; GFP, green fluorescent protein; WT, wild-type.

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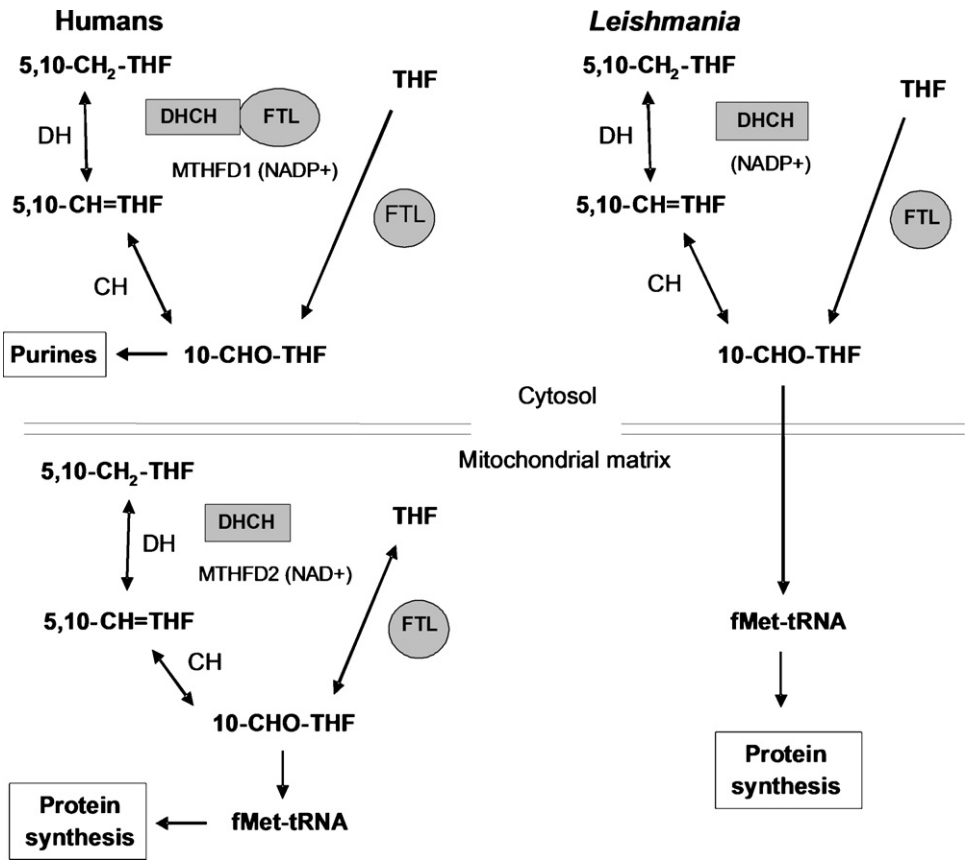


**Fig. 1.** 10-formyl-THF metabolism in humans and *L. major*. Two pathways of 10-CHO-THF synthesis are known; in the first pathway, formate is added onto THF by formate-tetrahydrofolate ligase (FTL). Alternatively, 10-CHO-THF is generated in two steps from 5,10-methylene-tetrahydrofolate (5,10-$CH_2$-THF): through oxidation to 5,10-methenyl-tetrahydrofolate (5,10-CH=THF) by a methylene-tetrahydrofolate dehydrogenase (DH), and then hydrolysis to 10-CHO-THF by methenyl-tetrahydrofolate cyclohydrolase (CH). In humans 10-CHO-THF is generated in the cytosol through either pathway by the trifunctional DHCH/FTL protein encoded by MTHFD1, often referred to as C1-THF synthase. In contrast the human mitochondrion contains a bifunctional DHCH and a monofunctional FTL. In *L. major*, 10-CHO-THF is generated in the cytosol by a bifunctional DHCH and a monofunctional FTL.

isoforms, one located in the cytosol and one in the mitochondrion [8,9]. In humans the trifunctional enzyme is cytosolic [10] while a bifunctional DHCH [11] and a monofunctional FTL are found in the mitochondria [12,13]. The coordination of 10-CHO-THF metabolism between these two compartments is thought to depend primarily on the shuttling of metabolites such as glycine, serine and formate [2]. However the situation may be more complex, as while initial studies suggested that reduced and polyglutamylated forms of tetrahydrofolate did not cross the inner mitochondrial membrane [14–16], recent studies suggest that trafficking of various C1-THF derivatives may occur in several species [17–19].

In the trypanosomatids the metabolic pathways using and producing 10-CHO-THF differ in several respects from those of their mammalian hosts. These parasites lack *de novo* purine biosynthesis and instead obtain purines through salvage pathways [20]. Therefore the major function for 10-CHO-THF that remains in these organisms is the production of fMet-$tRNA^{Met}$ [21]. While tRNA formylation has not been studied in *Leishmania*, it has been characterized in *Trypanosoma brucei*, where it shows a number of unusual properties. Firstly, the trypanosomatid mitochondrial genomes do not encode tRNAs, all of which must be imported from the cytosol [22]. Secondly, following import into the mitochondrion, the cytosolic elongator $tRNA^{Met\text{-}e}$ acts as either an elongator $tRNA^{Met}$, or is formylated and used as an initiator $tRNA^{Met}$; remarkably, the $tRNA^{Met\text{-}i}$ was not used in the trypanosome mitochondrion [23]. Lastly, methionyl-tRNA formylation is required for binding of the fMet-$tRNA^{Met}$ to initiation factor 2, and appears to be essential in trypanosomes [24]. In contrast, the requirement for initiator $tRNA^{Met}$ formylation appears highly variable in evolution; while initially considered essential in bacteria, later data suggested otherwise [25,26]. In some eukaryotic species it may be essential, in others it is dispensable, but aids growth in some circumstances [27–30].

Previous genomic predictions of trypanosomatid folate metabolism suggested that 10-CHO-THF is synthesized by a bifunctional DHCH (*DHCH1*) in *Leishmania*, *T. brucei* and *T. cruzi*, as well as by a monofunctional FTL in *Leishmania* and *T. cruzi* [21]. We showed previously that the *L. major DHCH1* is essential for parasite survival, although its loss could be rescued by *FTL* overexpression [31]. This identified 10-CHO-THF as an essential metabolite and established that its metabolism is a validated drug target. In this study we used database mining to fully catalog the DHCH and FTL gene families in the Trypanosomatidae, revealing the existence of a second family of DHCH homologues (*DHCH2*) in some *Leishmania* species and *T. cruzi*, but absent in *L. major* and *T. brucei*. Several of these DHCH2 proteins bear N-terminal extensions that resemble mitochondrial targeting sequences and the *L. infantum* DHCH2 was indeed shown to be a mitochondrial protein. We then studied the function, role in sensitivity to antifolates and compartmentalization of the DHCH1 and FTL in *Leishmania major*. We found that the *L. major* FTL exhibits the expected enzymatic activity *in vitro*, but this enzyme was not inhibited by any pteridine analogues tested. A null *ftl*$^{-}$ mutant *L. major* was produced by homologous replacement, with no detectable effect on growth, drug sensitivity, differentiation or infectivity in a susceptible mouse model, eliminating FTL as a unique drug target. However, nutritional studies