Biochemical and Genetic Analysis of Methylenetetrahydrofolate Reductase in Leishmania Metabolism and Virulence

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Methylenetetrahydrofolate reductase (MTHFR; EC 1.5.1.20) is the sole enzyme responsible for generation of 5-methyltetrahydrofolate, which is required for methionine synthesis and provision of methyl groups via 5-adenosylmethionine. Genome analysis showed that Leishmania species, unlike Trypanosoma brucei and Trypanosoma cruzi, contain genes encoding MTHFR and two distinct methionine synthases. Leishmania MTHFR differed from those in other eukaryotes by the absence of a C-terminal regulatory domain. Leishmania MTHFR differed from those in other eukaryotes by the absence of a C-terminal regulatory domain that infect mammals are methionine auxotrophs (3, 4). Methionine occupies a central place in metabolism because it is required for protein and S-adenosylmethionine (AdoMet)2 biosynthesis. AdoMet is particularly important in anabolism as it is the methyl donor in a wide variety of biosynthetic reactions, and decarboxylated AdoMet is used in polyamine biosynthesis. In Trypanosoma brucei, enzymes that consume and recycle AdoMet are well characterized chemotherapeutic targets (5–7), however, these pathways are less well studied in Leishmania. Homocysteine arising from AdoMet-dependent methylations is released by hydrolysis of S-adenosylhomocysteine, the product of methylation reactions. Homocysteine is then remethylated by methionine synthases using 5-methyltetrahydrofolate (5-CH3-THF) as the methyl donor. The sole route of 5-CH3-THF biosynthesis in eukaryotes is reduction of 5,10-methenyltetrahydrofolate (5-Ch2-THF) by methylenetetrahydrofolate reductase (MTHFR, EC 1.5.1.20) (8, 9). Thus here we focused on MTHFR as a key step leading to subsequent methionine metabolism.

MTHFR is either a NADH or NADPH-dependent flavoenzyme in eukaryotes and most prokaryotes (10–13). In mammals and yeast, NADPH-dependent reduction of 5,10-CH2-THF by MTHFR is physiologically irreversible and is inhibited by AdoMet (9, 14–17). Two major domains have been identified in the mammalian MTHFR: an N-terminal catalytic domain and a C-terminal allosteric regulatory domain that binds AdoMet (Fig. 1A)(9). Inhibition by AdoMet may prevent an NADPH-dependent and thus physiologically irreversible MTHFR from depleting 5,10-CH2-THF through the “methyl trap” effect (9, 18, 19). Under physiological conditions NADPH is strongly preferred by the mammalian enzymes (20), although at high phosphate concentrations porcine MTHFR is able to use NADH or NADPH with equal effectiveness. Dual cofactor

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2 The abbreviations used are: AdoMet, S-adenosylmethionine; THF, tetrahydrofolate; 5-Ch2-THF, 5-methyltetrahydrofolate; 5-Ch2-HF, 5-formyltetrahydrofolate; WT, wild type; 5,10-Ch2-THF, 5,10-methylenetetrahydrofolate; 5,10-Ch2-THF, 5,10-methylene tetrahydrofolate; MTHFR, methylenetetrahydrofolate reductase; DHFR, dihydrofolate reductase; P1, pteridine reductase; 1; Ches, 2-(cyclohexylamino)ethanesulfonic acid.

Footnotes

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† The on-line version of this article (available at http://www.jbc.org) contains supplemental methods, Table S1, and Figs. S1–S4.

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specificity is unusual as almost all pyridine nucleotide-dependent oxidoreductases are highly specific for one cofactor, with mammalian glutamate dehydrogenase being a well characterized exception (21–23). In contrast, reduction of 5,10-CH$_2$-THF by NADH-dependent MTHFR enzymes appears to be physiologically reversible and these proteins either contain a C-terminal domain that does not mediate allosteric regulation, as in plants (12, 17), or lack this domain entirely, as in *Escherichia coli* (24). A reversible MTHFR activity may also participate in salvage of host 5-CH$_3$-THF in *Plasmodium falciparum* (25).

The metabolism of reduced folate cofactors is of particular interest in drug discovery, because dihydrofolate reductase (DHFR) is commonly targeted by antimalarials and antibacterials. However, current therapeutic antifolates are ineffective because DHFR inhibition is by-passed by pteridine reductase 1 (PTR1), which is relatively insensitive to classical antifolates (26–28). Notably, a group of compounds derived from diaminopyrimidine, quinazoline, or pteridine scaffolds have been described that inhibit both DHFR and MTHFR, with specificity or activity or overexpression of these inhibitors appearing to additionally possess independent activity. This suggests that 5-CH$_3$-THF metabolizing enzymes such as MTHFR or methionine synthase may be additional targets of antifolates.

Here we show that *L. major* MTHFR (LmjMTHFR) is unique among characterized MTHFRs because it is AdoMet-insensitive and does not discriminate between NADH and NADPH. However, deletion of MTHFR and elimination of CH$_3$-THF biosynthesis in *L. major* had no effect on viability (under conditions where methionine was not limiting), on sensitivity to antifolates, on the ability to salvage 5-CH$_3$-THF, or on virulence.

**EXPERIMENTAL PROCEDURES**

Reagents—(6R,6S)-Tetrahydrofolate (THF) was obtained from Schircks Laboratories (Jona, Switzerland). [14C]Formaldehyde (55 mCi mmol$^{-1}$) was from PerkinElmer Life Sciences.

Parasite Culture and Infectivity—All studies used derivatives of *L. major* Friedlin (MHOM/IL/80/Friedlin) clone V1. Pro-mastigotes were routinely grown at 27 °C in M199 medium supplemented with 40 mM HEPES, pH 7.4, 62.5 μM adenosine, 2 μg ml$^{-1}$ bovine serum albumin as standard.

Parasite Culture and Infectivity—All studies used derivatives of *L. major* Friedlin (MHOM/IL/80/Friedlin) clone V1. Promastigotes were routinely grown at 27 °C in M199 medium supplemented with 40 mM HEPES, pH 7.4, 50 μM adenosine, 1 μg ml$^{-1}$ bovine serum albumin, 2 μg ml$^{-1}$ bovin serum albumin, 10% (v/v) heat-inactivated fetal calf serum. Null mutants and *mtthfr* /+ MTHFR cloning lines were generated as described in supplemental methods and Ref. 70. These lines were maintained in M199 containing 20 μg ml$^{-1}$ hygromycin and 30 μg ml$^{-1}$ puromycin for *mtthfr* and 12.5 μg ml$^{-1}$ G418 for *mtthfr* /+ MTHFR. Growth under semi-defined conditions used RPMI medium (Invitrogen) supplemented with 30 mM HEPES, pH 7.4, 62.5 μM adenosine, 2 μg ml$^{-1}$ bovin serum albumin, 5 μg ml$^{-1}$ hemin, and 1% (v/v) heat-inactivated fetal calf serum. RPMI medium was also prepared lacking methionine (RPMI-Met) or lacking folic acid and biotin (RPMI-FA-Biot).

Growth inhibition was determined by seeding parasites at 5 × $10^5$ cells ml$^{-1}$ at various concentrations of drug and counting cells using a model Z1 Coulter counter when control cultures reached late-log phase. The virulence of wild-type (WT) and *mtthfr* - cell lines were determined by infection of metacyclic parasites (purified by negative selection with peanut agglutinin as described (33)) into the footpad of susceptible BALB/c mice, followed by measurement of lesion growth, as described (34).

Cloning of *L. major* MTHFR—The MTHFR coding sequence was amplified with recombinant *Pfu* DNA polymerase (Stratagene) from *L. major* genomic DNA, which was prepared by the LiCl mini-prep method (35). PCR used the primers 5’-ATCATGCTCAAGCTTTATCAG and 5’-TCACCTCACTAGGCCGTTGTA. The amplified gene was cloned into pGEM-T (Promega), to create pGEM-T-MTHFR (B4775) and then subcloned into the Smal site of pXGneo (36) to create pXG-LmjMTHFR (B5128). All constructs were confirmed by DNA sequencing.

Expression of *L. major* MTHFR in Saccharomyces cerevisiae—The MTHFR coding sequence was amplified from the pGEM-T-MTHFR template using primers 5’-TGCAAGGATCCATGTCCAGCGCTATTCAGT (forward) and 5’-TGCAGTGTCATCTCACTAGGCGTT (reverse). This amplicon was digested with BamHI and PstI and cloned into the corresponding sites in pVT103-U (37). This plasmid contains *URA3* for selection and an *ADH1* promoter driving transgene expression. The construct and the empty pVT103-U vector were introduced into yeast strain RRY3, a met12 met13 double deletion (38). Control strains (RRY3 containing a cDNA encoding human MTHFR, and WT strain DAY4) were as previously described (12, 38).

Yeast Growth and Preparation of Extracts— Cultures of strain RRY3 expressing LmjMTHFR, human MTHFR, or the corresponding empty vector control were grown as described (12). Desalted protein extracts were obtained as previously described (12). These crude extracts were stored in aliquots at −80 °C after freezing in liquid N$_2$. Protein concentrations were determined by the method of Bradford (39), using bovine serum albumin as standard.

**Radiometric MTHFR Assay**—MTHFR activity was measured in the reductive direction by a NAD(P)H-5,10-CH$_2$-THF oxidoreductase radioassay (12) using 1 mM (6R,6S)-[14C]5,10-CH$_2$-THF and 800 μM NAD(P)H, unless otherwise indicated. The total protein content in each reaction mixture was 3 μg. Product formation was proportional to enzyme concentration and time.

Expression and Purification of Recombinant LmjMTHFR—An expression construct of LmjMTHFR was kindly provided by the Structural Genomics of Parasitic Protozoa consortium (clone Lmaj006157AAA, B5503). This used the expression vector pAVA0421 (40) that adds an N-terminal hexahistidine tag to the recombinant protein. Protein was expressed from this vector and purified by metal affinity chromatography, as described under supplemental methods.

**Spectrophotometric MTHFR Assays**—Spectrophotometric assays were carried out using a Beckman DU-640 spectrophotometer with 1-ml assays maintained at 37 °C. The NAD(P)H-menadione oxidoreductase activity of MTHFR was measured...
essentially as described (41). Assays contained 50 mM sodium phosphate, pH 7.2, 300 μM EDTA, 10 μM flavin adenine dinucleotide, or the same solution buffered by 50 mM Tris-Cl, pH 7.2. MTHFR was preincubated in the cuvette with NAD(P)H for 5 min before initiating reactions by adding menadione to a final concentration of 130 μM. Activity was monitored by following NAD(P)H oxidation at 343 nm (the isosbestic point of menadione), using the extinction coefficient of 6220 M⁻¹ cm⁻¹ (11). The concentration of menadione stocks was determined as described (14).

The NADH-5,10-CH₂-THF oxidoreductase activity of MTHFR was determined essentially as described (41) in deoxygenated phosphate assay buffer containing 10 mM formaldehyde. (6R,6S)-5,10-CH₂-THF was produced by anaerobic addition of varying concentrations of THF 10 min before initiation of the reaction by enzyme addition. Formaldehyde stocks were made daily from paraformaldehyde and THF stocks were made directly before use by dissolving THF to 5 mM in 250 mM triethanolamine-Cl, pH 7, containing 40 mM 2-mercaptoethanol. The suspensions were centrifuged (5,000 × g, 30 min, 4 °C) the clarified supernatants were decanted, sparged with nitrogen, and stored at −80°C. Samples were thawed and treated with 0.5 mL of dialyzed rat plasma at 37°C for 2 h to deglutamylate folates. The folate-binding column was scaled down from 5 to 1 mL and the volumes of wash and eluting buffers were reduced proportionately (42). The high-performance liquid chromatography column, mobile phase buffers, and elution program were as described (42). Detector response was calibrated using THF, 5-CH₂-THF, 5,10-methyl-THF (5,10-CH=THF), 5-formyl-THF (5-CHO-THF), and folic acid standards. Intracellular folate concentrations were calculated using an intracellular volume for *L. major* of 1.9 μL per 1 × 10⁶ cells (45).

**RESULTS**

**Identification of Leishmania MTHFR—** *L. major* MTHFR (*Lmj*F36.6390) was identified in searches of the *Leishmania* genome databases; orthologs were also detected in *Leishmania infantum* (LinJ36.0220) and *Leishmania braziliensis* (LbM35.6090). The *L. major* gene encoded a predicted protein of 309 residues with a mass of 34.8 kDa, which shows no obvious intracellular targeting sequences. *Lmj*MTHFR was 51 and 50% identical to *Zea mays* MTHFR and *Arabidopsis thaliana* MTHFR-2, respectively (12), but only 36% identical to *E. coli* (10) and 32% identical to human (46) MTHFRs. *Lmj*MTHFR is, however, much closer in length to the 296-residue *E. coli* enzyme than mammalian and plant MTHFRs, due to the absence of the C-terminal extension of ~300 residues present in these proteins (Fig. 1). *Lmj*MTHFR also lacked an N-terminal extension that is a site of regulatory phosphorylation in the human enzyme (47). Reanalysis of the *L. major* genome sequence data confirmed that these differences did not arise from assembly errors (not shown).

That *Lmj*MTHFR indeed has MTHFR activity *in vivo* was shown by complementation in a yeast methionine auxotroph strain. The *met12 met13* double deletion RRY3, which lacks MTHFR activity (38), grew in the absence of methionine after transformation with a *Lmj*MTHFR expression plasmid, but not a control plasmid (Fig. 2). The growth rate of these transformants was similar to that of the WT strain DAY4.

**Characterization of Leishmania MTHFR Activity—** *Lmj*MTHFR activity was studied both in crude yeast extracts and using purified enzyme obtained from engineered *E. coli*, with radiometric (yeast) or spectrophotometric assays (purified enzyme). Yeast expressing *Lmj*MTHFR showed high levels of activity with 5,10-CH₂-THF (about 80 nmol min⁻¹ mg⁻¹ protein), whereas none was detected in the *mthfr*⁻ RRY3 mutant containing vector alone (data not shown). A purification procedure was developed that yielded recombinant enzyme of >95% purity in a single chromatographic step (supplemental data Fig. S1). Purified *Lmj*MTHFR showed an absorbance spectrum typical of a flavoprotein, with prominent peaks at 381 and 454 nm. The 454-nm peak disappeared upon addition of excess NADH or NADPH, indicating reduction of a flavin cofactor (supplemental data Fig. S2).

When assayed in crude yeast extracts, *Lmj*MTHFR was found to use either NADH or NADPH as reductant (Fig. 3A), which was unexpected because previously characterized MTHFRs strongly prefer one or the other. In addition, *Lmj*MTHFR was insensitive to AdoMet, consistent with the absence of the C-terminal domain containing the AdoMet-binding site in mammalian and yeast MTHFRs (9, 17). As a control, extracts of yeast expressing human MTHFR were tested similarly (Fig. 3B). Here, the human enzyme strongly preferred NADPH and was AdoMet-sensitive, as previously reported (12), with about 50% inhibition by 1 mM AdoMet. That the activity of human MTHFR was only ~25% of that of *Lmj*MTHFR may reflect differences in stability or expression levels, because the specific activity of purified *Lmj*MTHFR was comparable with that of mammalian enzymes (see below).

Purified *Lmj*MTHFR tended to lose activity upon dilution, which was prevented by addition of flavin-adenine dinucleotide (data not shown), as with the *E. coli* enzyme (24). The pyridine nucleotide cofactor specificity of the purified *Lmj*MTHFR was identical to that shown when assayed in yeast extracts. Using menadione as the electron acceptor, kinetic constants were determined for NADH and NADPH in phosphate and Tris buffers (Table 1). The specificity constants for NADH and NADPH were the same in both buffers, showing that *Lmj*MTHFR was efficiently reduced by either nucleotide. 5,10-CH₂-THF was also an effective substrate, with a *kcat/Km* 1.3-
fold lower than the NADH/menadione reaction and $K_{m(app)}$ and $k_{cat}$ for 5,10-CH$_2$-THF within the range of previously determined values (10, 11). In contrast to the *E. coli* and pig liver enzymes (10, 11), substrate inhibition was not seen, up to a maximal concentration tested of 250 mM 5,10-CH$_2$-THF.

In addition, no folic acid, dihydrofolic acid, biopterin, or dihydrobiopterin reductase activities could be detected, in the pH range of 8 to 5 (sensitivity was $0.1$ mol min$^{-1}$ mg$^{-1}$).

$Lmj$MTHFR activity expressed in crude yeast extracts was tested for inhibition by a variety of pteridine analogs shown previously to inhibit the pteridine reductases PTR1 and DHFR (29), using NADH as reductant. None of these compounds significantly inhibited $Lmj$MTHFR activity (supplemental data Fig. S3). Using engineered *L. major* mutants described below, neither deletion nor overexpression of *MTHFR* had a significant effect on parasite sensitivity toward methotrexate or three of
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TABLE 1
Substrate specificity of LmjMTHFR

| Substrate | $k_{\text{cat}}$ | $k_{\text{cat}}/K_m$ |
|-----------|----------------|---------------------|
| NADPH-menadione oxidoreductase | 15.2 | 1.11×10⁶ |
| NADH (phosphate buffer) | 53±9 | 9.8 |
| NADPH (phosphate buffer) | 90±10 | 8.2 |
| NADPH (Tris buffer) | 170±40 | 8.2 |
| NADPH,5,10-CH₂-THF oxidoreductase | 5,10-CH₂-THF (phosphate buffer) | 7.4 |

TABLE 2
Folate contents of WT, mthfr⁻, and mthfr⁻/+MTHFR L. major FV1 cells

| Cell line | THF/5,10-CH₂-THF | 5-CH₂-THF | 10-CHO-THF/5,10-CH₂-THF | Total |
|-----------|-----------------|-----------|-------------------------|-------|
| WT        | 30±10           | 140±50    | 50±40                   | 230±100 |
| Mthfr⁻    | 35±6            | <0.6      | 70±30                   | 140±20 |
| mthfr⁻/+MTHFR⁻ | 30          | 230       | 0.2                     | 360   |

a Single sample analyzed.

an intracellular concentration of ~79 μM, similar to a previous estimate of 24 μM (49).

**Nutritional Phenotypes of mthfr⁻ Null Mutants**—The mthfr⁻ mutant grew normally in standard M199 medium, which contains 100 μM methionine. Amino acid analysis of supernatants of stationary phase cultures under these conditions showed little difference in methionine consumption between WT and mthfr⁻ cell lines (31 versus 28 nmol of methionine consumed per 10⁷ WT and mthfr⁻ cells, respectively). However, WT and mthfr⁻ L. major differed in their ability to grow in a minimal semidefined media (RPMI) at methionine concentrations below about 25 μM (Fig. 4), whereas at higher concentrations growth was similar (Fig. 4). In the absence of exogenous methionine, neither WT nor mthfr⁻ grew (Figs. 4 and 5), establishing L. major as a methionine auxotroph.

Homocysteine was able to support growth of WT, although at a rate lower than that seen with methionine. As expected, homocysteine did not support growth of mthfr⁻ cells (Fig. 5B). Interestingly, the add-back lines (mthfr⁻/+MTHFR⁻生长 well in the presence of homocystine, comparable with methionine or WT controls (Fig. 5C). This presumably reflects overexpression of MTHFR from the episomal expression vector and suggests that under some conditions, MTHFR activity could be limiting for growth. The growth of WT in RPMI-Met containing 200 μM homocystine and 200 μM glycine betaine was not significantly different from that in medium containing homocysteine alone and addition of betaine did not allow growth of the mthfr⁻ mutant on homocysteine (data not shown). Assuming betaine is taken up, this suggests that L. major lacks significant homocysteine/betaine methyltransferase activity.

Due to its ability to utilize NADH as a cofactor, LmjMTHFR could in principle operate in the reverse direction, and participate in 5-CH₂-THF salvage, as has been proposed in *Plasmodium falciparum* (25). The role of MTHFR in salvage of extracellular folates was therefore investigated by growing WT and mthfr⁻ parasites in media containing minimal folate levels (RPMI-FA-Biop). The only folates in this media come from the addition of 1% fetal calf serum, which will supply folate at an
approximate concentration of less than 0.1 nM, with 5-CH$_3$-THF as the major species (50). Neither line was able to grow past three passages in medium lacking L-biopterin (Fig. 6). This is consistent with previous studies, showing that _L. major_ requires unconjugated pterins for growth (51, 52). However, the WT and _mthfr$^{-}$_ lines showed similar growth in RPMI-FA supplemented with L-biopterin alone. Because _Leishmania_ require pteridines for growth (53–55), this suggests that under these conditions MTHFR is not essential for the utilization of folate in the form of 5-CH$_3$-THF.

**Virulence of _mthfr$^{-}$_ in a Susceptible Mouse Model**—Both WT and _mthfr$^{-}$_ _L. major_ were virulent in the BALB/c mouse footpad model infection, following inoculation with infective metacyclic promastigotes (34). In most experiments, the rate of lesion growth with _mthfr$^{-}$_ was comparable with that of WT (Fig. 7A). However, when smaller inocula were used, a lag in lesion growth was consistently observed, although the length of this delay was variable (Fig. 7B). Parasites were recovered from an _mthfr$^{-}$_ containing lesion and their genotype confirmed by testing for hygromycin and puromycin resistance (data not shown).

These data suggested that MTHFR was not essential for virulence in mouse infections, but its loss might produce a mild attenuation. Were this the case, restoration of MTHFR expression in _mthfr$^{-}$_ lines should result in full restoration of virulence. However, this was not found in several add-back lines, which showed only partial restoration of WT lesion formation (_mthfr$^{-}$+/MTHFR; Fig. 7B, or data not shown). It is well established that when cultured _in vitro_ over the periods of time required to generate null mutants (which requires two rounds of gene replacement for disomic loci), _Leishmania_ species can lose some or all virulence. Because MTHFR expression was restored in the _mthfr$^{-}$+/MTHFR lines (see above), these data argue that most of the loss of virulence in the _mthfr$^{-}$_ lines is due to culture-associated phenomena, rather than loss of MTHFR.

**DISCUSSION**

The data presented here establish that _L. major_ MTHFR is unique among characterized eukaryotic MTHFRs in that it lacks the C-terminal allosteric regulatory domain, and uses NADH and NADPH with equal efficiency. Although porcine liver MTHFR can use both cofactors _in vitro_, the use of NADH requires high concentrations of phosphate and the enzyme is predicted to be NADPH-dependent under physiological conditions (20). Interestingly, the mammalian bifunctional 5,10-CH$_2$-THF dehydrogenase/cyclohydrolase (EC 1.5.1.15, EC 3.5.4.9) also shows phosphate effects on cofactor specificity. For this enzyme, phosphate activates NAD$^+$-dependent activity and inhibits NADP$^+$-dependent activity (56). With _LmjMTHFR_, no such differential effects were seen, with the $k_{cat}/K_m$ values for NADH and NADPH being essentially unchanged when assayed in phosphate-free buffer. This surprising result indicates the _L. major_ enzyme could be reduced by either nucleotide _in vivo_. A few other oxidoreductases have been shown to have dual cofactor specificity, including aldose reductase from _Candida tenuis_ (57) and glucose-6-phosphate dehydrogenase from _Leuconostoc mesenteroides_ (58). However, in most cases one substrate is used more efficiently than the other, mammalian glutamate dehydrogenase being one of the very few exceptions (21–23).

The only published structure of an MTHFR is of the NADH-dependent _E. coli_ enzyme, which was determined with both NADH and 5-CH$_3$-THF as ligands (59). Although this protein is only 36% identical to _LmjMTHFR_, the majority of the _E. coli_ active-site residues are either conserved or conservatively substituted in the _L. major_ protein (Fig. 1). The determinants of the cofactor specificity of the _E. coli_ active site were explored by
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Pejchal et al. (59) by modeling a 2′ phosphate group onto the NADH ligand. In their model, no unfavorable contacts were predicted and long-range interactions between the 2′ phosphate and the basic residues Arg33 and Lys222 might be predicted to stabilize NADPH binding. They therefore noted that the structural basis for the NADH specificity of E. coli MTHFR is unclear. If LmjMTHFR shares the same fold as its E. coli homolog, it is possible that dual cofactor specificity could result from an active site very similar to that of E. coli MTHFR.

The lack of AdoMet inhibition of LmjMTHFR is also consistent with the ability of this enzyme to link 5,10-CH₂-THF/5-CH₃-THF interconversion to the NAD⁺/NADH redox couple. The reversibility of NADH-dependent MTHFRs under physiological conditions (12), which contrasts to the irreversibility of NADPH-dependent human MTHFR (60), probably limits AdoMet accumulation and makes feedback inhibition unnecessary (17). Another possible role of a reversible MTHFR activity is folate salvage through conversion of 5-CH₃-THF monoglutamate, the predominant form of folate in mammalian plasma (18), to other forms of folate. However, we found that mthfr⁻ L. major grew as well as WT in semidened media containing minimal levels of external serum-derived folate (presumably 5-CH₃-THF; Fig. 6). This suggests that exogenous 5-CH₃-THF can enter the Leishmania folate pool through an MTHFR-independent mechanism, most likely through the action of methionine synthases.

As anticipated from studies of other Leishmania species, we showed that L. major is auxotrophic for methionine (Figs. 4 and 5). Consistent with the presence of both MTHFR and two methionine synthases in the Leishmania genome, the methionine requirement could be satisfied through provision of homocysteine, albeit only partially (Fig. 5). Thus to probe the biological role of MTHFR, we generated null mutants (mthfr⁻) by serial gene replacement of both MTHFR alleles. The growth of the mthfr⁻ null mutant was unaffected in standard culture media, which typically contain high levels of methionine, also an essential amino acid for the host (Figs. 4 and 5). However, when external methionine levels were reduced sufficiently, growth of mthfr⁻ was impaired at concentrations below about 25 μM (Fig. 4). Presumably at these levels the contribution of homocysteine re-methylation via methionine synthase becomes limiting even in WT parasites, and this pathway is completely denied to mthfr⁻ due to the absence of 5-CH₃-THF. Correspondingly, whereas WT parasites were able to grow (albeit at a somewhat reduced rate) when homocysteine replaced methionine, as expected, the mthfr⁻ mutant was unable to grow on homocysteine alone (Fig. 5). Analysis of reduced folate levels showed that in fact the mthfr⁻ completely lacked 5-CH₃-THF (Table 2), and thus its survival could not be attributed to the presence of an unexpected novel MTHFR encoded elsewhere in the parasite genome.

These data show that homocysteine methylation and de novo methionine synthesis occur in L. major promastigotes, in contrast to T. brucei and T. cruzi, which lack MTHFR and methionine synthase activity and/or genes (61). It has been proposed that Leishmania might synthesize methionine by the reverse transsulfuration pathway (62), with cystathionine γ-lyase converting 2-oxobutyrate and cysteine to cystathionine, which could then be hydrolyzed to release homocysteine, the substrate for methionine synthases. However, cystathionine γ-lyase activity has not been detected in L. major (63) and even if this pathway were active, Leishmania methionine auxotrophy indicates that it cannot supply sufficient methionine for their metabolic needs.

The retention of MTHFR, despite the tendency for genome reduction in intracellular parasites (64, 65), suggests the ability to recycle homocysteine might confer a selective advantage to L. major under some conditions. Foremost among these would be the amastigote stage, which, unlike the promastigote stage...
residing extracellularly in sand flies and studied in most of our work here, multiplies within the macrophage phagolysosome. This site poses a challenge to obtaining nutrients, which must enter across the phagolysosomal membrane or through endocytic trafficking pathways. Whereas no direct measurements of amino acid levels within nor flux through the Leishmania parasitophorous vacuole have been reported, indirect evidence based upon studies of the effects of manipulation of parasite proteases or glucose metabolism suggest that amino acids are likely to be abundantly available (66–69).

The description of the phagolysosome as an amino acid-rich environment is consistent with the finding that the mthfr mutant showed little if any attenuation specifically attributable to loss of MTHFR in a mouse infection (Fig. 7). This supports the model that parasites can salvage from the host sufficient methionine for their metabolic needs. Whether MTHFR plays a critical role in Leishmania survival in parasite stages not studied here, for example, in promastigotes forms within the sand fly midgut (as opposed to culture vessels), or persistent parasites potentially residing in cells other than phagocytic macrophages, remains to be determined.

Because we have shown MTHFR is not an essential gene for in vitro or in vivo growth in L. major, this enzyme is unlikely to be a useful target for monochemotherapy. Potentially it could be exploited as a target in combination therapy with other anti-folates, as suggested by the role of 5-CH$_3$-THF and AdoMet metabolism in the acquisition of methotrexate resistance (32). Moreover, our studies also suggest that MTHFR is unlikely to be involved in the action of a group of hydrophobic pteridine analogues described previously. These compounds inhibit the Leishmania pteridine reductases PTR1 and DHFR and are highly toxic toward parasites, yet are insensitive to changes in PTR1 and DHFR levels and are thus likely to have additional targets (29).

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