THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 281, NO. 50, pp. 38150–38158, December 15, 2006
© 2006 by The American Society for Biochemistry and Molecular Biology, Inc. Printed in the U.S.A.

Biochemical and Genetic Analysis of Methylenetetrahydrofolate Reductase in *Leishmania* Metabolism and Virulence*[S]

Received for publication, August 31, 2006, and in revised form, October 10, 2006 Published, JBC Papers in Press, October 10, 2006, DOI 10.1074/jbc.M608387200

Tim J. Vickers[‡], Giuseppe Orsomando[§], Rocío Díaz de la Garza[§], David A. Scott[‡], Song O. Kang[‡], Andrew D. Hanson[§], and Stephen M. Beverley[‡1]

From the [‡]Department of Molecular Microbiology, Washington University School of Medicine, St. Louis, Missouri 63110 and the [§]Department of Horticultural Sciences, University of Florida, Gainesville, Florida 32611

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 *S*-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. *L. major* MTHFR was expressed in yeast and recombinant enzyme was produced in *Escherichia coli*. MTHFR was not inhibited by *S*-adenosylmethionine and, uniquely among folate-metabolizing enzymes, showed dual-cofactor specificity with NADH and NADPH under physiological conditions. *MTHFR* null mutants (*mthfr*$^-$) lacked 5-methyltetrahydrofolate, the most abundant intracellular folate, and could not utilize exogenous homocysteine for growth. Under conditions of methionine limitation *mthfr*$^-$ mutant cells grew poorly, whereas their growth was normal in standard culture media. Neither *in vitro* MTHFR activity nor the growth of *mthfr*$^-$ mutants or MTHFR overexpressors were differentially affected by antifolates known to inhibit parasite growth via targets beyond dihydrofolate reductase and pteridine reductase 1. In a mouse model of infection *mthfr*$^-$ mutants showed good infectivity and virulence, indicating that sufficient methionine is available within the parasitophorous vacuole to meet the needs of the parasite.

Protozoan parasites of the genus *Leishmania* are the causative agents of human diseases that range from lethal visceral infection to severely disfiguring mucocutaneous leishmaniasis. *Leishmania* are transmitted by phlebotomine sand flies, where they reside extracellularly within the digestive tract. Following infection of mammalian hosts, parasites multiply intracellularly, primarily within macrophage phagolysosomes. No approved vaccines are available against these pathogens and current chemotherapy relies on pentavalent antimony, which cannot be given orally, has significant toxicity, and is facing increasing resistance in the field (1, 2). New drugs and new drug targets are therefore urgently needed.

Leishmania 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-CH_3-THF) as the methyl donor. The sole route of 5-CH_3-THF biosynthesis in eukaryotes is reduction of 5,10-methylenetetrahydrofolate (5,10-CH_2-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-CH_2-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. 1*B*) (9). Inhibition by AdoMet may prevent an NADPH-dependent and thus physiologically irreversible MTHFR from depleting 5,10-CH_2-THF through the "methyltrap" 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

* This work was supported by National Institutes of Health Grants AI 21903 (to S. M. B.) and R01 GM071382 (to A. D. H.) and European Molecular Biology Organization long term fellowship ALTF 106–2005 (to T. V.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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

[1] To whom correspondence should be addressed: Box 8230, WA University School of Medicine, 660 S. Euclid Ave., St. Louis, MO 63110. E-mail: beverley@borcim.wustl.edu.

[2] The abbreviations used are: AdoMet, *S*-adenosylmethionine; THF, tetrahydrofolate; 5-CH_3-THF, 5-methyltetrahydrofolate; 5-CHO-THF, 5-formyltetrahydrofolate; WT, wild type; 5,10-CH_2-THF, 5,10-methylenetetrahydrofolate; 5,10-CH=THF, 5,10-methenyltetrahydrofolate; MTHFR, methylenetetrahydrofolate reductase; DHFR, dihydrofolate reductase; PTR1, pteridine reductase 1; Ches, 2-(cyclohexylamino)ethanesulfonic acid.

This is an Open Access article under the CC BY license.

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 against *Leishmania* 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 PTR1 and show nanomolar EC_{50} values in culture. However, these inhibitors appear to additionally possess independent mode(s) of action rendering their toxicity insensitive to deletion or overexpression of *PTR1* and/or *DHFR-TS* (29–31). Increased methionine adenosyltransferase activity and AdoMet facilitate acquisition of methotrexate resistance in *Leishmania major* (32), and MTHFR has been hypothesized to be involved in this process. 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 (*Lmj*MTHFR) 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—(6*R*,6*S*)-Tetrahydrofolate (THF) was obtained from Schircks Laboratories (Jona, Switzerland). [^{14}C]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. Promastigotes were routinely grown at 27 °C in M199 medium (U. S. Biological Corp.) supplemented with 40 mM HEPES, pH 7.4, 50 μM adenosine, 1 μg ml^{-1} biotin, 5 μg ml^{-1} hemin, 2 μg ml^{-1} biopterin, and 10% (v/v) heat-inactivated fetal calf serum. Null mutants and *mthfr*$^{-}$/+*MTHFR* clonal 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 *mthfr*$^{-}$ and 12.5 μg ml^{-1} G418 for *mthfr*$^{-}$/+*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} biopterin, 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 biopterin (RPMI-FA-Biop). 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 *mthfr*$^{-}$ 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′-ATCATGTCCAAGCTTATCAG and 5′-TCACTCCACTAGCGCGTTGA. The amplified gene was cloned into pGEM-T (Promega), to create pGEM-T.*MTHFR* (B4775) and then subcloned into the SmaI 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′-TGCA*GGATCC*ATGTCCAAGCTTATCAGT (forward) and 5′-TGCA*CTGCAG*TCACTCCACTAGCGCGTT (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 deletant (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 *Lmj*MTHFR, 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 (6*R*,6*S*)-[^{14}C]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-CH_2-THF oxidoreductase activity of MTHFR was determined essentially as described (41) in deoxygenated phosphate assay buffer containing 10 mM formaldehyde. (6*R*,6*S*)-5,10-CH_2-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. THF solutions were kept on ice under anaerobic conditions in the dark. Activity was monitored by following NADH oxidation at 340 nm, using the extinction coefficient of 6230 M^{-1} cm^{-1}. Calculations were based on the assumptions that *L. major* MTHFR resembles other MTHFRs in using only (6*R*)-5,10-CH_2-THF and that this isomer comprised 50% of the racemic mixture.

Folate Analysis—Folates were extracted and analyzed by high-performance liquid chromatography with electrochemical detection as described (42–44). Folates were extracted from WT, *mthfr*$^-$, and *mthfr*$^-$/+*MTHFR L. major* grown in M199 media to a density of 2–4 × 10^7 cells ml^{-1}. Cell pellets (2–4 × 10^{10} cells per sample) were resuspended in deoxygenated extraction buffer (50 mM HEPES, pH 7.9, 50 mM Ches, 2% (w/v) sodium ascorbate, 10 mM 2-mercaptoethanol). The suspensions were sparged with nitrogen and then boiled for 10 min. After centrifugation (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-CH_3-THF, 5,10-methenyl-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 × 10^8 cells (45).

RESULTS

Identification of Leishmania MTHFR—L. major MTHFR (LmjF36.6390) was identified in searches of the *Leishmania* genome databases; orthologs were also detected in *Leishmania infantum* (LinJ36.0220) and *Leishmania braziliensis* (LbrM35.6090). 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 deletant RRY3, which lacks MTHFR activity (38), grew in the absence of methionine after transformation with a *LmjMTHFR* 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-CH_2-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. 3*A*), 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. 3*B*). 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-CH_2-THF was also an effective substrate, with a k_{cat}/K_m 1.3-

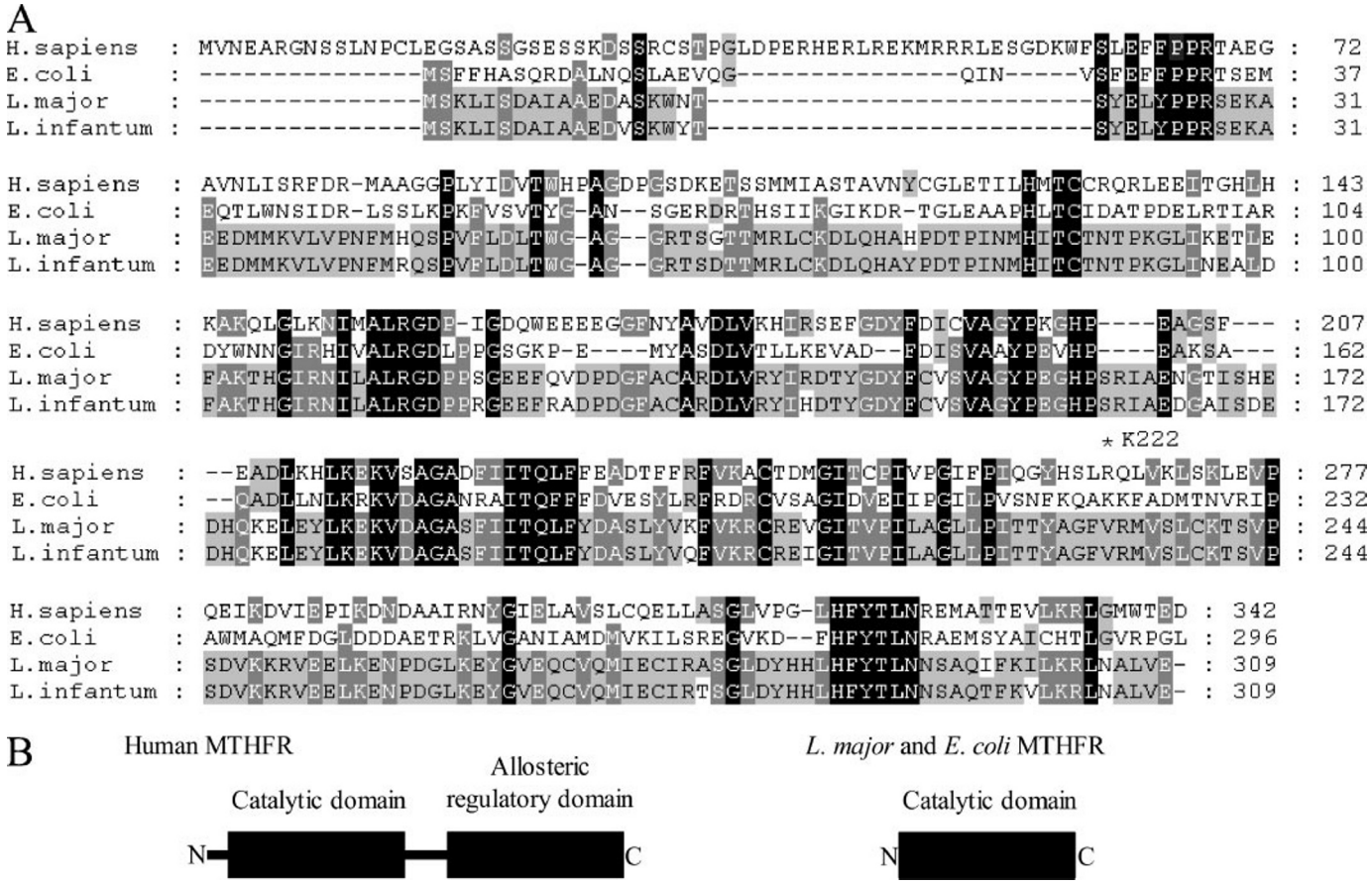

FIGURE 1. **Alignment of MTHFR amino acid sequences.** *A* shows *L. major* and *L. infantum* MTHFRs aligned with *E. coli* MTHFR (Swiss-Prot accession number P00394), the first 342 residues of human MTHFR (P42898) and the first 307 residues of the *Z. mays* MTHFR (Q9SE94). The alignment is *shaded* according to identity: *white text on black background*, complete identity; *white text on a dark-gray background*, 75% identity; and *black text on a light-gray background*, 50% identity. The positions of *E. coli* residues Arg^{33} and Lys^{222} discussed in the text are indicated *above* the alignment. *B* shows a comparison of the domain structures of the human, *L. major*, and *E. coli* MTHFR proteins.

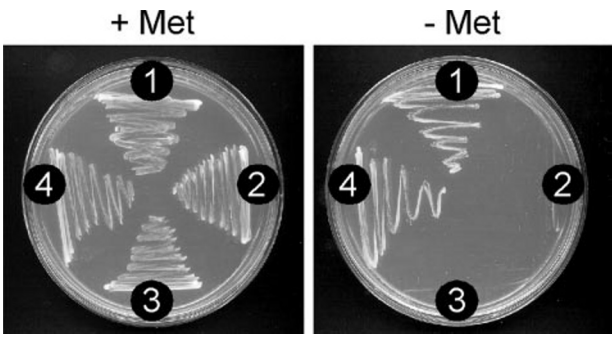

FIGURE 2. **Functional complementation of a yeast *mthfr*$^-$ mutant by *LmjMTHFR*.** *S. cerevisiae* strains DAY4 (WT) (*1*), RRY3 (null mutant, *met12 met13*) (*2*), or RRY3 transformed with the vector pVT103-U (*3*), or with pVT103-U expressing *LmjMTHFR* (*4*), were grown in minimal medium in the presence or absence of methionine. The data shown were obtained by plating a similar number of cells for each strain.

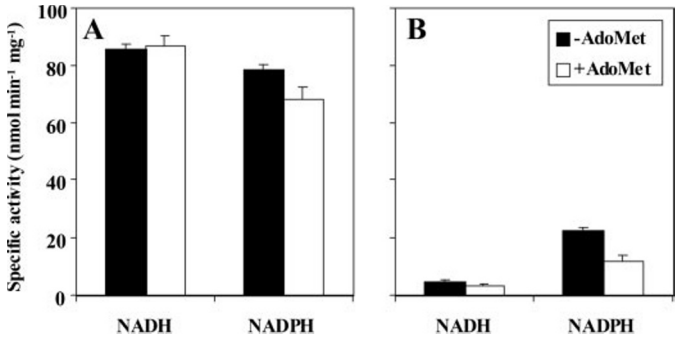

FIGURE 3. **Coenzyme preference and *S*-adenosylmethionine sensitivity of recombinant *Lmj*MTHFR in yeast extracts.** Extracts from yeast expressing *Lmj*MTHFR (*A*) or human MTHFR (*B*) were assayed using NADH or NADPH as reductant in the presence or absence of 1 mM *S*-adenosylmethionine. Before assay, protein extracts were preincubated for 1 h at 30 °C with buffer or *S*-adenosylmethionine. Data represent mean ± S.D. ($n \geq 3$).

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 μM (6*R*)-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 μmol 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 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

TABLE 1
Substrate specificity of *Lmj*MTHFR

Substrate	$K_{m(app)}$	k_{cat}[a]	k_{cat}/K_m
	μM	s^{-1}	$M^{-1} s^{-1} \times 10^5$
NAD(P)H-menadione oxidoreductase assay			
NADH (phosphate buffer)[b]	53 ± 9	52 ± 3	9.8
NADPH (phosphate buffer)[b]	90 ± 15	74 ± 6	8.2
NADH (Tris buffer)[b]	90 ± 10	87 ± 5	9.7
NADPH (Tris buffer)[b]	170 ± 40	140 ± 20	8.2
NADH-5,10-CH_2-THF oxidoreductase assay 5,10-CH_2-THF (phosphate buffer)[c]	15 ± 2[d]	11.1 ± 0.4	7.4

[a] Calculated assuming one active site per polypeptide.
[b] Measured with 130 μM menadione as the fixed substrate.
[c] Measured with 150 μM NADH as the fixed substrate.
[d] Concentration of (6*R*)-5,10-CH_2-THF.

TABLE 2
Folate contents of WT, *mthfr*$^-$, and *mthfr*$^-$/+*MTHFR L. major* FV1 cells

Data are means of three independent replicates, ±S.D. 5-formyl-THF was present in all samples, but could not be satisfactorily quantified.

Cell line	Folate species			
	THF/5,10-CH_2-THF	5-CH_3-THF	10-CHO-THF/5,10-CH=THF	Total
	pmol mg^{-1} *protein*			
WT	30 ± 10	140 ± 50	50 ± 40	230 ± 100
Mthfr$^-$	35 ± 6	<0.6	70 ± 30	140 ± 20
mthfr$^-$/+*MTHFR*[a]	30	230	20	360

[a] Single sample analyzed.

these pteridine analogues (25 (O/129), 70, or 34, see supplemental data Table S1).

Homozygous Replacement of MTHFR in L. major—A homozygous null *mthfr*$^-$ mutant was produced by serial replacement of the *MTHFR* open reading frame with puromycin and then hygromycin resistance gene open reading frames (supplemental methods). Gene replacement was confirmed by Southern analysis with a probe flanking the *MTHFR* locus (supplemental data Fig. S4). As discussed below, the null mutant grew well in standard M199 media. As a positive control, an episomal copy of *MTHFR* was reintroduced using the pXG expression vector to produce an "add-back" clonal line termed *mthfr*$^-$/+*MTHFR*. The absence of *MTHFR* in *mthfr*$^-$ and its presence in *mthfr*$^-$/+*MTHFR* was confirmed by PCR with primers to the *MTHFR* open reading frame (data not shown).

Lack of 5-CH_3THF in mthfr$^-$ *Cells*—Given the lack of an obvious phenotype in the null mutant, folates were analyzed in the WT, *mthfr*$^-$, and *mthfr*$^-$/+MTHFR lines to exclude the possibility that an alternative pathway could generate 5-CH_3-THF. In contrast to WT, where 5-CH_3-THF represents 60% of the intracellular folate pool, no 5-CH_3-THF was detectable in *mthfr*$^-$ cells (140 ± 50 *versus* less than 0.6 pmol mg^{-1}, respectively: Table 2). There were no other marked differences between the folate pools of WT, *mthfr*$^-$, and *mthfr*$^-$/+*MTHFR* lines. As expected, reintroduction of *MTHFR* in the *mthfr*$^-$/+*MTHFR* line restored 5-CH_3-THF production (Table 2), to a level of 230 pmol mg^{-1}, which exceeds that seen in WT. This may result from overexpression of *MTHFR* from the multicopy episomal vector, as seen with other proteins (36, 48). Total folate was estimated to be 230 pmol mg^{-1} protein in WT and 140 pmol mg^{-1} in *mthfr*$^-$ cells. The WT value corresponds to

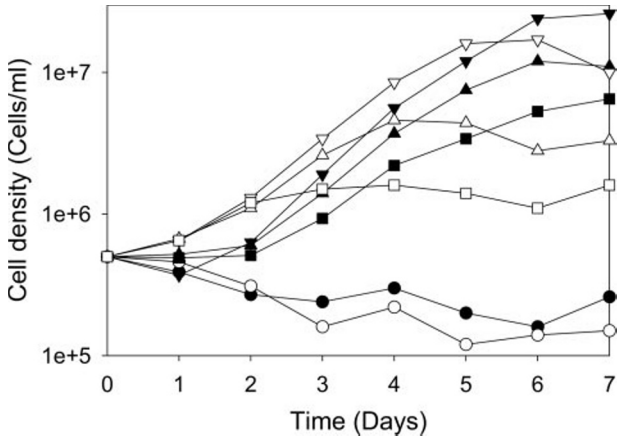

FIGURE 4. **Growth of WT and *mthfr*$^-$ clonal lines with varying methionine.** *Filled symbols* represent WT and *open symbols mthfr*$^-$. Parasites were grown in RPMI-Met media with no additions (●, ○), 12.5 (■, □), 25 (▲, Δ), or 100 μM (▼, ▽) methionine.

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 10^7 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.

Homocystine was able to support growth of WT, although at a rate lower than that seen with methionine. As expected, homocystine did not support growth of *mthfr*$^-$ cells (Fig. 5*B*). Interestingly, the add-back lines (*mthfr*$^-$/+*MTHFR*) grew well in the presence of homocystine, comparable with methionine or WT controls (Fig. 5*C*). 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 homocystine alone and addition of betaine did not allow growth of the *mthfr*$^-$ mutant on homocystine (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, *Lmj*MTHFR could in principle operate in the reverse direction, and participate in 5-CH_3-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

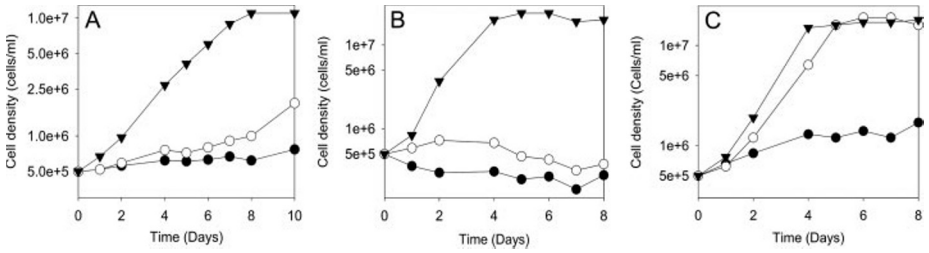

FIGURE 5. **Growth of WT, *mthfr*$^-$, and *mthfr*$^-$/+*MTHFR* clonal lines with methionine or homocystine.** *A* corresponds to WT; *B* to *mthfr*$^-$; and *panel C* to *mthfr*$^-$/+*MTHFR*. Symbols represent RPMI-Met (●), RPMI-Met plus 200 μM homocystine (○), and RPMI-Met plus 200 μM methionine (▼).

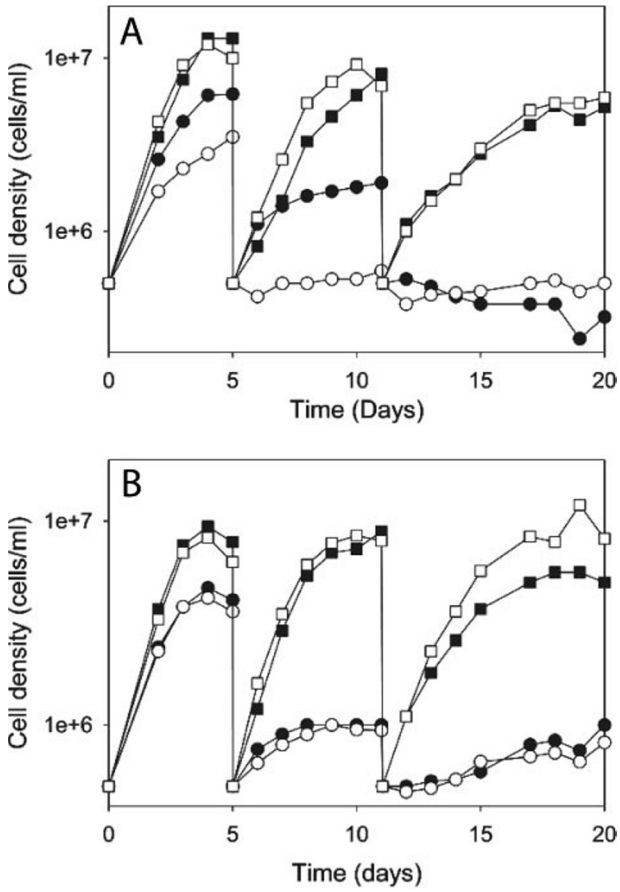

FIGURE 6. **Growth of WT and *mthfr*$^-$ clonal lines under low-folate conditions.** *A* corresponds to WT; *B* to *mthfr*$^-$. Symbols represent fdRPMI with no additions (●), fdRPMI plus 1 μM folic acid (○), fdRPMI plus 2 μg ml^{-1} L-biopterin (■), and fdRPMI plus 1 μM folic acid and 2 μg ml^{-1} L-biopterin (□).

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. 7*A*). However, when smaller inocula were used, a lag in lesion growth was consistently observed, although the length of this delay was variable (Fig. 7*B*). 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. 7*B*, 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 *Lmj*MTHFR, 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 *Lmj*MTHFR, 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

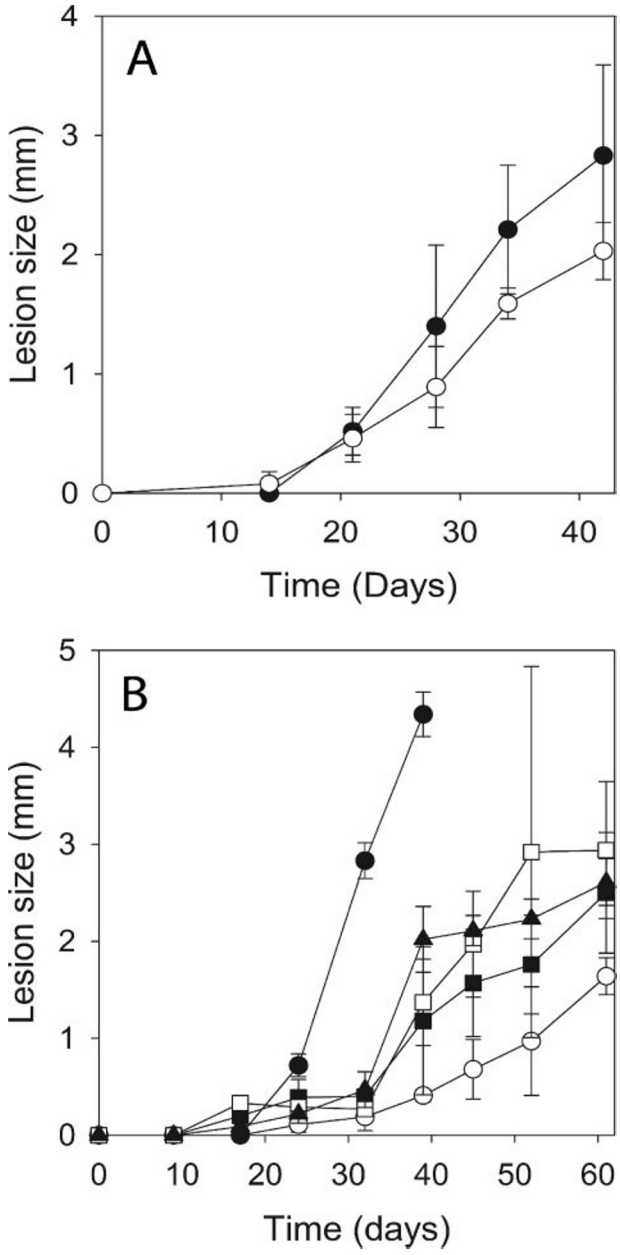

FIGURE 7. **Virulence of WT, *mthfr*$^-$, and *mthfr*$^-$/+*MTHFR* clonal lines in BALB/c mice.** Each panel shows lesion progression following footpad infections of BALB/c mice with metacyclic parasites. The average thickness and standard deviation of the lesion is shown. *A*, 5×10^5 metacyclic promastigotes per mouse (4 mice per group); ●, WT; and ○, *mthfr*$^-$. *B*, 1×10^5 metacyclic promastigotes per mouse. WT, ● (3 mice); ○, *mthfr*$^-$ lines (3 mice); ■, *mthfr*$^-$/+*MTHFR* clone 1 (2 mice); □, *mthfr*$^-$/+*MTHFR* clone 2 (3 mice); ▲, *mthfr*$^-$/+*MTHFR* clone 3 (2 mice).

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 Arg^{33} and Lys^{222} 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 *Lmj*MTHFR 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 *Lmj*MTHFR is also consistent with the ability of this enzyme to link 5,10-CH_2-THF/5-CH_3-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-CH_3-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 semidefined media containing minimal levels of external serum-derived folate (presumably 5-CH_3-THF; Fig. 6). This suggests that exogenous 5-CH_3-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 homocystine, 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-CH_3-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-CH_3-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 homocystine, 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 antifolates, 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).

Acknowledgments—We thank the Structural Genomics of Parasitic Protozoa consortium for materials, V. Stempliuk for assistance in molecular cloning, and Suzanne Hickerson for assistance in virulence tests.

REFERENCES

1. Croft, S. L., and Coombs, G. H. (2003) *Trends Parasitol.* **19,** 502–508
2. Ouellette, M., Drummelsmith, J., and Papadopoulou, B. (2004) *Drug Resist. Update* **7,** 257–266
3. Steiger, R. F., and Meshnick, S. R. (1977) *Trans. R. Soc. Trop. Med. Hyg.* **71,** 441–443
4. O'Daly, J. A., and Rodriguez, M. B. (1988) *Acta Trop.* **45,** 109–126
5. Fairlamb, A. H. (2003) *Trends Parasitol.* **19,** 488–494
6. Bacchi, C. J., Sanabria, K., Spiess, A. J., Vargas, M., Marasco, C. J., Jr., Jimenez, L. M., Goldberg, B., and Sufrin, J. R. (1997) *Antimicrob. Agents Chemother.* **41,** 2108–2112
7. Bacchi, C. J., Sufrin, J. R., Nathan, H. C., Spiess, A. J., Hannan, T., Garofalo, J., Alecia, K., Katz, L., and Yarlett, N. (1991) *Antimicrob. Agents Chemother.* **35,** 1315–1320
8. Lu, S. C. (2000) *Int. J. Biochem. Cell Biol.* **32,** 391–395
9. Matthews, R. G., Sheppard, C., and Goulding, C. (1998) *Eur. J. Pediatr.* **157,** S54–59
10. Sheppard, C. A., Trimmer, E. E., and Matthews, R. G. (1999) *J. Bacteriol.* **181,** 718–725
11. Daubner, S. C., and Matthews, R. G. (1982) *J. Biol. Chem.* **257,** 140–145
12. Roje, S., Wang, H., McNeil, S. D., Raymond, R. K., Appling, D. R., Shachar-Hill, Y., Bohnert, H. J., and Hanson, A. D. (1999) *J. Biol. Chem.* **274,** 36089–36096
13. Wohlfarth, G., Geerligs, G., and Diekert, G. (1990) *Eur. J. Biochem.* **192,** 411–417
14. Vanoni, M. A., Ballou, D. P., and Matthews, R. G. (1983) *J. Biol. Chem.* **258,** 11510–11514
15. Kutzbach, C., and Stokstad, E. L. (1967) *Biochim. Biophys. Acta* **139,** 217–220
16. Kutzbach, C., and Stokstad, E. L. (1971) *Biochim. Biophys. Acta* **250,** 459–477
17. Roje, S., Chan, S. Y., Kaplan, F., Raymond, R. K., Horne, D. W., Appling, D. R., and Hanson, A. D. (2002) *J. Biol. Chem.* **277,** 4056–4061
18. Stanger, O. (2002) *Curr. Drug Metab.* **3,** 211–223
19. Nijhout, H. F., Reed, M. C., Budu, P., and Ulrich, C. M. (2004) *J. Biol. Chem.* **279,** 55008–55016
20. Matthews, R. G., and Kaufman, S. (1980) *J. Biol. Chem.* **255,** 6014–6017
21. Olson, J. A., and Anfinsen, C. B. (1953) *J. Biol. Chem.* **202,** 841–856
22. Smith, T. J., Peterson, P. E., Schmidt, T., Fang, J., and Stanley, C. A. (2001) *J. Mol. Biol.* **307,** 707–720
23. McDaniel, H. G., Jenkins, R., Yeh, M., and Razzaque, A. (1984) *J. Mol. Cell. Cardiol.* **16,** 303–309
24. Guenther, B. D., Sheppard, C. A., Tran, P., Rozen, R., Matthews, R. G., and Ludwig, M. L. (1999) *Nat. Struct. Biol.* **6,** 359–365
25. Asawamahasakda, W., and Yuthavong, Y. (1993) *Parasitology* **107,** 1–10
26. Papadopoulou, B., Roy, G., and Ouellette, M. (1992) *EMBO J.* **11,** 3601–3608
27. Bello, A. R., Nare, B., Freedman, D., Hardy, L., and Beverley, S. M. (1994) *Proc. Natl. Acad. Sci. U. S. A.* **91,** 11442–11446
28. Callahan, H. L., and Beverley, S. M. (1992) *J. Biol. Chem.* **267,** 24165–24168
29. Hardy, L. W., Matthews, W., Nare, B., and Beverley, S. M. (1997) *Exp. Parasitol.* **87,** 157–169
30. Lye, L. F., Cunningham, M. L., and Beverley, S. M. (2002) *J. Biol. Chem.* **277,** 38245–38253
31. Ma, D., Beverley, S. M., and Turco, S. J. (1996) *Biochem. Biophys. Res. Commun.* **227,** 885–889
32. Drummelsmith, J., Girard, I., Trudel, N., and Ouellette, M. (2004) *J. Biol. Chem.* **279,** 33273–33280
33. Sacks, D. L., Hieny, S., and Sher, A. (1985) *J. Immunol.* **135,** 564–569
34. Cunningham, M. L., Titus, R. G., Turco, S. J., and Beverley, S. M. (2001) *Science* **292,** 285–287
35. Medina-Acosta, E., and Cross, G. A. (1993) *Mol. Biochem. Parasitol.* **59,** 327–329
36. Ha, D. S., Schwarz, J. K., Turco, S. J., and Beverley, S. M. (1996) *Mol. Biochem. Parasitol.* **77,** 57–64
37. Vernet, T., Dignard, D., and Thomas, D. Y. (1987) *Gene* (*Amst.*) **52,** 225–233
38. Raymond, R. K., Kastanos, E. K., and Appling, D. R. (1999) *Arch. Biochem. Biophys.* **372,** 300–308
39. Bradford, M. M. (1976) *Anal. Biochem.* **72,** 248–254
40. Alexandrov, A., Vignali, M., LaCount, D. J., Quartley, E., de Vries, C., De Rosa, D., Babulski, J., Mitchell, S. F., Schoenfeld, L. W., Fields, S., Hol, W. G., Dumont, M. E., Phizicky, E. M., and Grayhack, E. J. (2004) *Mol. Cell. Proteomics* **3,** 934–938
41. Matthews, R. G. (1986) *Methods Enzymol.* **122,** 372–381
42. Goyer, A., Collakova, E., Diaz de la Garza, R., Quinlivan, E. P., Williamson, J., Gregory, J. F., 3rd, Shachar-Hill, Y., and Hanson, A. D. (2005) *J. Biol. Chem.* **280,** 26137–26142
43. Gregory, J. F., 3rd, and Toth, J. P. (1988) *Anal. Biochem.* **170,** 94–104
44. Bagley, P. J., and Selhub, J. (2000) *Clin. Chem.* **46,** 404–411
45. Ellenberger, T. E., Wright, J. E., Rosowsky, A., and Beverley, S. M. (1989) *J. Biol. Chem.* **264,** 15960–15966
46. Goyette, P., Sumner, J. S., Milos, R., Duncan, A. M., Rosenblatt, D. S., Matthews, R. G., and Rozen, R. (1994) *Nat. Genet.* **7,** 195–200
47. Yamada, K., Strahler, J. R., Andrews, P. C., and Matthews, R. G. (2005) *Proc. Natl. Acad. Sci. U. S. A.* **102,** 10454–10459
48. Chakkalath, H. R., Siddiqui, A. A., Shankar, A. H., Dobson, D. E., Beverley,

S. M., and Titus, R. G. (2000) *Infect. Immun.* **68,** 809–814
49. Ellenberger, T. E., and Beverley, S. M. (1987) *J. Biol. Chem.* **262,** 10053–10058
50. Girard, C. L., Lapierre, H., Matte, J. J., and Lobley, G. E. (2005) *J. Dairy Sci.* **88,** 660–670
51. Cunningham, M. L., and Beverley, S. M. (2001) *Mol. Biochem. Parasitol.* **113,** 199–213
52. Nare, B., Hardy, L. W., and Beverley, S. M. (1997) *J. Biol. Chem.* **272,** 13883–13891
53. Beck, J. T., and Ullman, B. (1990) *Mol. Biochem. Parasitol.* **43,** 221–230
54. Scott, D. A., Coombs, G. H., and Sanderson, B. E. (1987) *Mol. Biochem. Parasitol.* **23,** 139–149
55. Petrillo-Peixoto, M., and Beverley, S. M. (1987) *Antimicrob. Agents Chemother.* **31,** 1575–1578
56. Yang, X. M., and MacKenzie, R. E. (1993) *Biochemistry* **32,** 11118–11123
57. Neuhauser, W., Haltrich, D., Kulbe, K. D., and Nidetzky, B. (1997) *Biochem. J.* **326,** 683–692
58. Vought, V., Ciccone, T., Davino, M. H., Fairbairn, L., Lin, Y., Cosgrove, M. S., Adams, M. J., and Levy, H. R. (2000) *Biochemistry* **39,** 15012–15021
59. Pejchal, R., Sargeant, R., and Ludwig, M. L. (2005) *Biochemistry* **44,** 11447–11457
60. Green, J. M., Ballou, D. P., and Matthews, R. G. (1988) *FASEB J.* **2,** 42–47
61. Avila, J. L., and Nosei, C. (1983) *Mol. Biochem. Parasitol.* **7,** 1–8
62. Chaudhary, K., and Roos, D. S. (2005) *Nat. Biotechnol.* **23,** 1089–1091
63. Walker, J., and Barrett, J. (1997) *Int. J. Parasitol.* **27,** 883–897
64. Lawrence, J. G. (2005) *Curr. Opin. Genet. Dev.* **15,** 584–588
65. Wernegreen, J. J. (2005) *Curr. Opin. Genet. Dev.* **15,** 572–583
66. Burchmore, R. J., and Barrett, M. P. (2001) *Int. J. Parasitol.* **31,** 1311–1320
67. Naderer, T., Vince, J. E., and McConville, M. J. (2004) *Curr. Mol. Med.* **4,** 649–665
68. Mottram, J. C., Souza, A. E., Hutchinson, J. E., Carter, R., Frame, M. J., and Coombs, G. H. (1996) *Proc. Natl. Acad. Sci. U. S. A.* **93,** 6008–6013
69. Selzer, P. M., Pingel, S., Hsieh, I., Ugele, B., Chan, V. J., Engel, J. C., Bogyo, M., Russell, D. G., Sakanari, J. A., and McKerrow, J. H. (1999) *Proc. Natl. Acad. Sci. U. S. A.* **96,** 11015–11022
70. Kapler, G. M., Coburn, C. M., and Beverley, S. M. (1990) *Mol. Cell. Biol.* **10,** 1084–1094