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S-Adenosylmethionine Decarboxylase from *Leishmania donovani*

MOLECULAR, GENETIC, AND BIOCHEMICAL CHARACTERIZATION OF NULL MUTANTS AND OVERPRODUCERS*

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Sigrid C. Roberts‡, Jerry Scott‡§, Judith E. Gasteier‡¶, Yuqui Jiang‡‖, Benjamin Brooks‡, Armando Jardim‡, Nicola S. Carter‡, Olle Heby‡‡, and Buddy Ullman‡§§**

From the ‡Department of Biochemistry and Molecular Biology, Oregon Health and Science University, Portland, Oregon 97201-3098, and the ‡‡Department of Molecular Biology, Umea University, Umea SE-901 87, Sweden

The polyamine biosynthetic enzyme, *S*-adenosylmethionine decarboxylase (ADOMETDC) has been advanced as a potential target for antiparasitic chemotherapy. To investigate the importance of this protein in a model parasite, the gene encoding ADOMETDC has been cloned and sequenced from *Leishmania donovani*. The Δ*adometdc* null mutants were created in the insect vector form of the parasite by double targeted gene replacement. The Δ*adometdc* strains were incapable of growth in medium without polyamines; however, auxotrophy could be rescued by spermidine but not by putrescine, spermine, or methylthioadenosine. Incubation of Δ*adometdc* parasites in medium lacking polyamines resulted in a drastic increase of putrescine and glutathione levels with a concomitant decrease in the amounts of spermidine and the spermidine-containing thiol trypanothione. Parasites transfected with an episomal *ADOMETDC* construct were created in both wild type and Δ*adometdc* parasites. *ADOMETDC* overexpression abrogated polyamine auxotrophy in the Δ*adometdc L. donovani*. In addition, ADOMETDC overproduction in wild type parasites alleviated the toxic effects of 5′-(((*Z*)-4-amino-2-butenyl)methylamino)-5′-deoxyadenosine (MDL 73811), but not pentamidine, berenil, or methylglyoxyl bis(guanylhydrazone), all inhibitors of ADOMETDC activities *in vitro*. The molecular, biochemical, and genetic characterization of ADOMETDC establishes that it is essential in *L. donovani* promastigotes and a potential target for therapeutic validation.

The protozoan parasite *Leishmania donovani* is the causative agent of visceral leishmaniasis, a devastating and often fatal disease in humans. The parasite exhibits a digenetic life cycle with the extracellular promastigote residing in the phlebotomine sandfly vector and the intracellular amastigote propagating within the phagolysosome of mammalian macrophages. Because no effective vaccine for leishmaniasis is available, chemotherapy offers the only means of disease treatment. However, the current arsenal of drugs for treating leishmaniasis is far from ideal because these compounds are moderately to highly toxic, the result of their lack of target specificity. Recently, the emergence of drug-resistant strains has exacerbated the need for more selective and efficacious drugs to treat or prevent leishmaniasis or, for that matter, many other parasitic diseases.

One pathway that has been exploited successfully in antiparasitic drug regimens is that for the synthesis of polyamines, organic cations that play indispensable roles in key cellular processes such as growth, differentiation, and macromolecular biosynthesis (1, 2). D,L-α-Difluoromethylornithine (DFMO),[1] an irreversible inhibitor of ornithine decarboxylase (ODC), the first enzyme in the polyamine biosynthetic pathway, can eradicate *Trypanosoma brucei* infections in both mice (3) and patients with late stage African sleeping sickness (4, 5). The selective window for drug efficacy is not achieved, however, by dissimilar DFMO binding affinities for the *T. brucei* and human ODC enzymes but rather to differences in enzyme stability (6–8). DFMO is also active against many other protozoan parasites, including the promastigote form of *L. donovani* (9–11).

Another inhibitor of the polyamine pathway, 5′-(((*Z*)-4-amino-2-butenyl)methylamino)-5′-deoxyadenosine (MDL 73811), is also effective in eradicating *T. brucei* infections in mice (12, 13). MDL 73811 is an irreversible inhibitor of ADOMETDC, the enzyme that catalyzes the irreversible decarboxylation of *S*-adenosylmethionine (AdoMet), generating the decarboxylated *S*-adenosylmethionine (dAdoMet) substrate for spermidine synthase (SPDSYN). In addition to MDL 73811, ADOMETDC is also inhibited *in vitro* by a battery of other trypanocidal drugs, including pentamidine, berenil, and methylglyoxyl bis-

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) U20091.

§ Present address: Community Blood Centers of Florida, 1700 N. State Road 7, Lauderhill, FL 33313.

¶ Present address: Institute for Hygiene, Dept. of Virology, University of Heidelberg, 69120 Heidelberg, Germany.

‖ Present address: Corixa Corporation, 1124 Columbia St., Seattle, WA 98104.

** Present address: McGill University, MacDonald Campus, 21,111 Lakeshore Rd., Ste. Anne Bellevue, Quebec, Canada, H9X 3V9.

§§ Burroughs Wellcome Fund scholar in molecular parasitology. To whom correspondence should be addressed: Dept. of Biochemistry and Molecular Biology, L224, Oregon Health Sciences University, 3181 S.W. Sam Jackson Park Rd., Portland, OR 97201-3098. Tel.: 503-494-8437; Fax: 503-494-8393; E-mail: ullmanb@ohsu.edu.

[1] The abbreviations used are: DFMO, D,L-α-difluoromethylornithine; ODC, ornithine decarboxylase; MDL 73811, 5′-(((*Z*)-4-amino-2-butenyl) methylamino)-5′-deoxyadenosine; ADOMETDC, *S*-adenosylmethionine decarboxylase; AdoMet, *S*-adenosylmethionine; dAdoMet, decarboxylated *S*-adenosylmethionine; SPDSYN, spermidine synthase; MGBG, methylglyoxyl bis(guanylhydrazone); MTA, 5-methylthioadenosine; DME-L, Dulbecco's modified Eagle-based medium designed for *Leishmania* cultivation; DME-L-CS, DME-L, in which the bovine serum albumin component was replaced with chicken serum; ORF, open reading frame; 5′F, 5′-flanking region; 3′F, 3′-flanking region.

(guanylhydrazone) (MGBG). Pentamidine is also the second most prescribed drug for treatment of visceral leishmaniasis. However, even though the *T. brucei* ADOMETDC enzyme is inhibited by each of these compounds, the intracellular target for these drugs has not been established. Indeed, other cellular targets for pentamidine and berenil have been proposed (14–17), and the potential lack of target specificity may also contribute to the host toxicity observed with these compounds.

To determine whether ADOMETDC inhibition is a valid therapeutic paradigm and to elucidate the cellular targets of inhibitors of ADOMETDC in a model and genetically tractable parasite, the *ADOMETDC* gene and its flanking sequences have been isolated from *L. donovani*. These DNAs were used to generate Δ*adometdc* knockouts employing double targeted gene replacement strategies (18–20) and *ADOMETDC* overexpressors. The Δ*adometdc L. donovani* were shown to be auxotrophic for spermidine, and incubation of these parasites in the absence of exogenous polyamines resulted in a marked depletion in the cellular pools of both spermidine and trypanothione, a spermidine-containing thiol conjugate. Furthermore, the ADOMETDC overproducers were resistant to MDL 73811 but not to the other ADOMETDC inhibitors, establishing that the enzyme is the primary intracellular target for only one of the four antiparasitic drugs that target the enzyme *in vitro*.

EXPERIMENTAL PROCEDURES

Materials, Chemicals, and Reagents—The pX63NEO, pX63HYG, pX63PAC, and pXNEO plasmids used in the transfection experiments were provided by Dr. Stephen M. Beverley (Washington University, St. Louis, MO). Spermidine, putrescine, MTA, berenil, pentamidine, and MGBG were obtained from Sigma. Hygromycin was procured from Roche Molecular Biochemicals, G418 was purchased from BioWhittaker, and MDL 73811 was a gift from the now defunct Marion Merrell Dow Research Institute.

Cell Culture—The wild type *L. donovani* clone, DI700, originally derived from the 1S Sudanese strain, was used for DNA isolation, library construction, and as a recipient strain in the initial transfections. *L. donovani* promastigotes were propagated in DME-L, a completely defined Dulbecco's modified Eagle-based medium especially designed for *Leishmania* cultivation (21). Transfected parasites were maintained in a modified medium, DME-L-CS, in which the bovine serum albumin component was replaced with 10% chicken serum to avoid polyamine oxidase-mediated toxicity (11). The *ADOMETDC*/*adometdc* heterozygote and the Δ*adometdc* null mutants were routinely maintained in DME-L-CS supplemented with either 50 μg/ml hygromycin or 50 μg/ml hygromycin, 20 μg/ml G418, and 100 μM spermidine, respectively, unless otherwise specified. The *ADOMETDC* overexpressor, DI700[p*ADOMETDC*], and DI700 parasites transfected with just pXNEO, DI700[pXNEO], were grown in DME-L-CS and 20 μg/ml G418. The Δ*adometdc* null mutants transfected with pX63PAC[*ADOMETDC*], designated Δ*adometdc*[p*ADOMETDC*], were propagated in DME-L-CS to which 50 μg/ml hygromycin, 20 μg/ml G418, and 100 μM puromycin were added. Growth rate experiments were conducted and parasites enumerated on a hemacytometer (Hausser Scientific Co.) or a Coulter Counter model ZF as described (11, 19).

Isolation of the ADOMETDC—A fragment of *ADOMETDC* was amplified from genomic DNA using PCR. The sense primer, 5′-CTCGGAATTCC-TT[CT]-GAG-GG[CGT]-[AC]C[CG]-GAG-AA-3′, was constructed with an 11-nucleotide leader encompassing an *Eco*RI site (underlined) followed by a degenerate sequence corresponding to amino acids 7–12, FEGTEK, of the human ADOMETDC, whereas the antisense primer, 5′-CTCGGGATCCC-CTC-[ACG]GG-[CG]GT-[AG]AT-GT-G-3′, was generated from residues 243–248, HITPEP of the human ADOMETDC and was preceded by an 11-nucleotide leader encompassing a *Bam*HI restriction site (underlined). Amplification of the *ADOMETDC* fragment was performed on a Coy Instruments thermocycler using the PCR conditions described by Hanson *et al.* (22). The resulting 720-bp fragment was sequenced according to the dideoxy termination method and used to screen an *L. donovani* EMBL3 genomic library (23) under stringent hybridization and washing conditions (22, 23). A 5.1-kb *Sal*I fragment that hybridized to the probe was subcloned from a plaque-purified phage into pBluescript KS+, and the entire nucleotide sequence of the *ADOMETDC* open reading frame (ORF) was obtained in both orientations. In addition, portions of the *ADOMETDC* 5′- and 3′-flanking regions were also sequenced. Multiple sequence alignments with ADOMETDC proteins from phylogenetically diverse organisms were performed using the Feng-Doolittle algorithm (24).

Antibody Generation and Characterization—To raise antibodies against ADOMETDC, the *ADOMETDC* gene was inserted into the pBAce prokaryotic expression vector and then induced in low phosphate induction medium as described (25). The protein was recovered in virtually pure form as inclusion bodies and employed as immunogen to generate polyclonal antibodies in rabbits (Cocalico Biologicals Inc.) using standard injection protocols. Immunoblot analysis was carried out according to standard protocols (26, 27).

Molecular Constructs for the Replacement of the ADOMETDC Alleles—To construct drug resistance cassettes to replace both *ADOMETDC* alleles, ~300 bp of 5′- and ~1 kb of 3′-flanking regions were subcloned into the appropriate sites of the pX63HYG and pX63NEO plasmids. The 5′-flanking region, 5′F, was amplified by PCR using the M13-20 oligonucleotide (containing pBluescript sequence) as a sense primer and an antisense primer, 5′-GGGAAAAGCGTCGACAA-GAGGCGATGAGC-3′, preceded by an *Sal*I site (underlined) and corresponding to a region ~120 nucleotides upstream from the *ADOMETDC* ORF. The ~1,200-bp PCR product was digested with *Sal*I and *Hin*dIII, a restriction site located ~300 bp upstream from the *Sal*I site, and 5′F was then subcloned into the *Hin*dIII/*Sal*I sites of pX63HYG and pX63NEO. The resulting plasmids were termed pX63HYG-5′F and pX63NEO-5′F, respectively. To amplify the 3′-flank, a sense primer, 5′-GTCCCCGGGCCGCCCCCCTTATCCCC-3′, and an antisense primer, 5′-CTCAGATCTCCCTTTAGTGAGGG-3′, containing *Sma*I and *Bgl*II restriction sites (underlined), respectively, were designed. The ~1-kb PCR product, 3′F, was then subcloned into the corresponding sites of pX63HYG-5′F and pX63NEO-5′F to yield the knockout constructs, pX63NEOΔ*adometdc* and pX63HYGΔ*adometdc*, respectively. The correct orientation of 5′F and 3′F within the two drug resistance cassettes was confirmed by restriction enzyme digestion and limited nucleotide sequencing.

ADOMETDC Overexpression Vectors—The ADOMETDC ORF was amplified by PCR and cloned into the pXNEO and pX63PAC vectors to create pXNEO[*ADOMETDC*] and pX63PAC[*ADOMETDC*], respectively. Oligonucleotides used for the construction of pXNEO[*ADOMETDC*] were 5′-TCTGGATCCAGCGGCTCTGAGGCG-3′ (*Bam*HI site underlined) for the sense primer and 5′-TCCTCTAGAGTGATGAC-CTTTGCG-3′ (*Xba*I site underlined) for the antisense primer. The PCR product was then subcloned into the *Bam*HI and *Xba*I sites of the leishmanial expression vector pXNEO to create pXNEO[*ADOMETDC*]. To amplify the *ADOMETDC* ORF for insertion into pX63PAC, both the sense primer, 5′-GCAGGATCCACCATGAATGTCGC-3′, and the antisense primer, 5′-CGCGGATCCAAGAACTAGTCGGGCC-3′, contained *Bam*HI restriction sites. The PCR product was then subcloned into the *Bam*HI site of pX63PAC to create pX63PAC[*ADOMETDC*], and the correct orientation was ascertained by further restriction enzyme digestion.

Transfections—The Δ*adometdc* null strains were generated from wild type *L. donovani* by double targeted gene replacement. The pX63HYGΔ*adometdc* and pX63NEOΔ*adometdc* plasmids were digested with *Hin*dIII and *Bgl*II, and the fragments containing the *ADOMETDC* flanking regions and drug resistance markers, designated HYGΔ*adometdc* and NEOΔ*adometdc*, respectively, were gel purified and transfected into wild type parasites and *ADOMETDC*/*adometdc* heterozygotes, respectively, using previously reported electroporation conditions (19, 28). Clonal *ADOMETDC*/*adometdc* heterozygotes were selected on plates in semisolid DME-L-CS medium containing 50 μg/ml hygromycin, and the genotypes were confirmed by Southern blotting. Clonal Δ*adometdc* knockout lines were then obtained from a heterozygote clone after a second round of transfection and plating in DME-L-CS medium supplemented with 100 μM spermidine, 100 μM MTA, 50 μg/ml hygromycin, and 20 μg/ml G418. The genotypes of the null mutants were again verified by Southern blotting.

ADOMETDC overproducers were also generated after transfection. DI700 parasites were transfected with pXNEO[*ADOMETDC*] and selected in DME-L-CS medium containing 200 μg/ml G418 to produce DI700[p*ADOMETDC*]. The Δ*adometdc* line was transfected with pX63PAC[*ADOMETDC*] and the Δ*adometdc*[p*ADOMETDC*] overexpressor isolated in DME-L-CS supplemented with 50 μg/ml hygromycin, 20 μg/ml G418, and 100 μM puromycin. ADOMETDC overproduction was ascertained by immunoblotting.

Polyamine, Thiol, and dAdoMet Pool Analysis—Polyamine, thiol, and dAdoMet pools were measured as reported previously (19, 20).

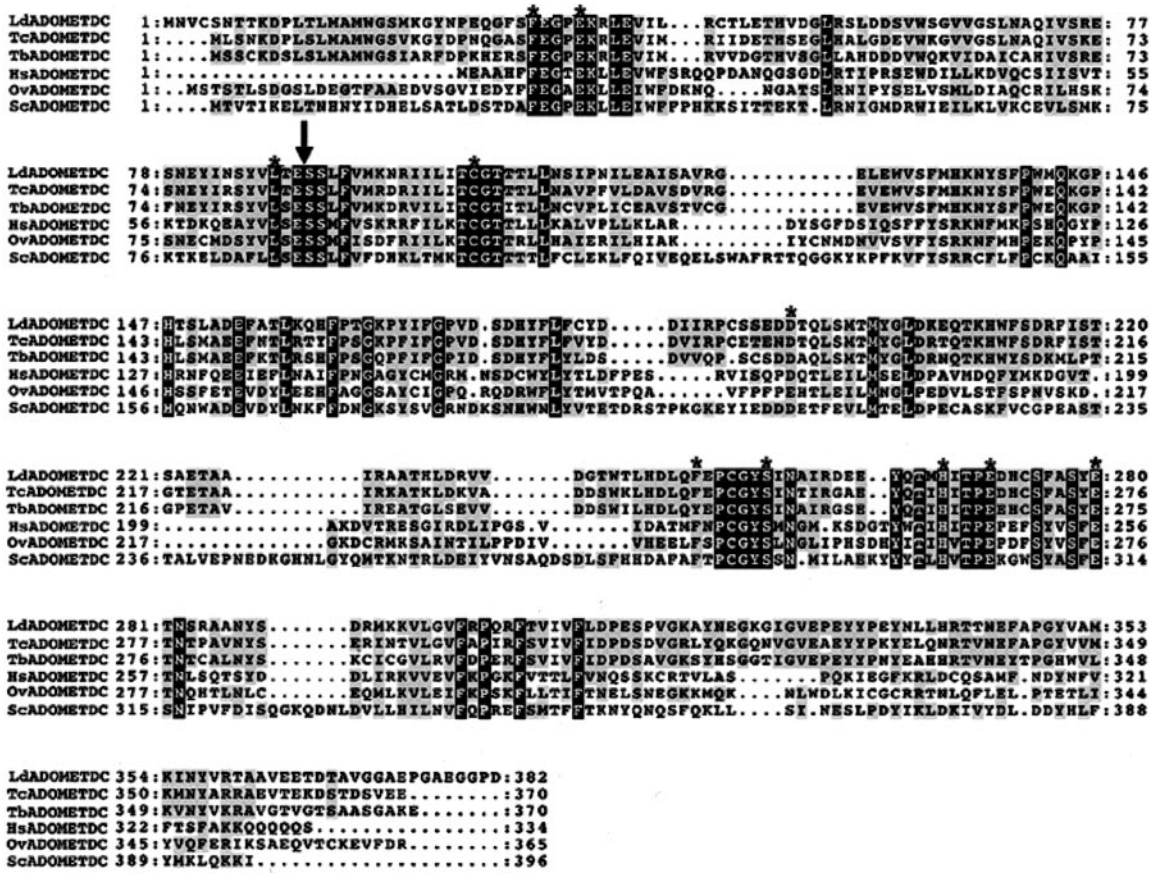

FIG. 1. **Multisequence alignment of phylogenetically diverse ADOMETDC proteins.** ADOMETDC proteins were aligned according to the Feng-Doolittle algorithm (44). ADOMETDC sequences shown include *L. donovani* (*LdADOMETDC*), *T. cruzi* (*TcADOMETDC*) (13, 17), *T. brucei* (*TbADOMETDC*) (accession number AAA61969), human (*hsADOMETDC*) (16), *O. volvulus* (*ovADOEMTDC*) (11), and *S. cerevisiae* (*scADOMETDC*) (12). Identical residues are *highlighted* in *dark boxes*, and similar residues are shown in *shaded boxes*. The proposed proenzyme cleavage site is shown by an *arrow*. Conserved residues that are implicated in catalysis in the human enzyme are marked with an *asterisk*.

RESULTS

Isolation of the ADOMETDC Gene—The gene encoding ADOMETDC was isolated on a 5.1-kb *Sal*I fragment using a 720-bp *ADOMETDC* PCR fragment as a hybridization probe. Nucleotide sequence analysis identified a 1,149-bp ORF encoding a protein of 382 amino acids with a molecular mass of 44.2 kDa for the proenzyme and 33.1 and 11.1 kDa for the α and β subunits, respectively. An in-frame termination codon was located 27 nucleotides upstream from the assigned initiation codon. In pairwise alignments, ADOMETDC exhibited amino acid identities of 70, 62, 30, 30, and 28% with the *Trypanosoma cruzi* (29, 30), *T. brucei* (accession number AAA61969), human (31), *Onchocerca volvulus* (32), and *Saccharomyces cerevisiae* (33) ADOMETDC proteins, respectively. A multisequence alignment revealed several regions of high homology, most notably in the putative proenzyme cleavage site between amino acids Glu-90 and Ser-91 of the *L. donovani* enzyme (34, 35) (Fig. 1). Southern blot analysis of *L. donovani* genomic DNA demonstrated that *ADOMETDC* is a single copy gene, and Northern blots revealed the existence of a single *ADOMETDC* 1.8-kb transcript in *L. donovani* promastigotes (data not shown).

Stability of the ADOMETDC Protein—Because the mammalian ADOMETDC is known to turn over very rapidly (the half-life is <1 h) (36–38), the stability of ADOMETDC in *L. donovani* promastigotes was examined. Unlike the mammalian enzyme, however, ADOMETDC is stable up to 24 h after treatment with cycloheximide at a concentration that obliterates protein synthesis in *L. donovani* (22). Indeed, the amount of ADOMETDC protein after 24 h of cycloheximide treatment is equivalent to that of exponentially growing parasites (Fig. 2).

Construction and Molecular Characterization of Δ*adometdc Null Mutants*—To disrupt the *ADOMETDC* locus in *L. donovani*, each gene copy was sequentially replaced with a drug resistance cassette. The first *ADOMETDC* copy was replaced with pX63HYGΔ*adometdc*, and clonal *ADOMETDC*/*adometdc* heterozygotes were selected in hygromycin. A second round of transfection with pX63NEOΔ*adometdc* replaced the second *ADOMETDC* allele, and clonal strains of Δ*adometdc* knockout parasites were selected in hygromycin, G418, MTA, and spermidine. The presence of hygromycin during the second round of gene targeting ensured that the pX63NEOΔ*adometdc* had replaced the wild type allele in the *ADOMETDC*/*adometdc* heterozygotes, whereas spermidine and MTA were added to the selective medium to bypass any potential auxotrophy. Approximately 16 colonies were picked from plates after the second round of gene targeting. After initial screenings, two of these clones, Δ*adometdc*13 and Δ*adometdc*14, were selected for further analysis.

Southern blot analysis of *Sal*I-digested genomic DNA prepared from wild type, *ADOMETDC*/*adometdc*, Δ*adometdc*13, and Δ*adometdc*14 parasites hybridized to the *ADOMETDC* ORF clearly demonstrated the loss of both *ADOMETDC* copies in the knockout lines (Fig. 3, *panel A*). Southern blot analysis of *Sac*I-cut DNA and probing with the *ADOMETDC* 5′- and 3′-flanking regions, 5′F and 3′F, respectively, revealed the precise nature of the rearrangements observed after the homologous recombination events that gave rise to the heterozygous and

homozygous genotypes. The creation of the novel and loss of the wild type alleles are unveiled by probing with either 5′F (Fig. 3, *panel B*) or 3′F (Fig. 3, *panel C*). Replacement of the wild type *ADOMETDC* copies by HYGΔ*adometdc* and NEOΔ*adometdc* created an additional 3.8-kb *Sac*I fragment that hybridized to 5′F (Fig. 3, *panel B*) and a 5.5-kb *Sac*I fragment recognized by 3′F (Fig. 3, *panel C*). The loss of the remaining wild type allele from the heterozygote is apparent after the second round of transfection with NEOΔ*adometdc* in both knockout lines (Fig. 3, *panels B* and *C*). Partial restriction maps of the wild type and replaced loci are depicted in Fig. 3, *panel D*. It is worth noting that 3′F also recognizes other sequences within the *L. donovani* genome and gives rise to the unexpected background signals in Fig. 3, *panel C*. These signals were unaffected by any of the recombination events at the *ADOMETDC* locus.

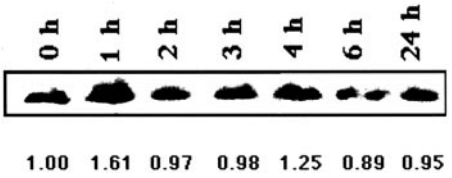

FIG. 2. **Stability of the *L. donovani* ADOMETDC protein.** Wild type parasites were treated with 500 mg/ml cycloheximide, and samples for Western blot analysis were taken before the addition of cycloheximide (0 h) and 1, 2, 3, 4, 6, and 24 h after the addition of cycloheximide. The Western blot was then probed with the polyclonal antibodies raised against *L. donovani* ADOMETDC. The bands were quantitated by scanning on a densitometer, and the signal intensities relative to the zero point control are indicated below the blot. This experiment was repeated one additional time with essentially equivalent results.

The loss of the wild type *ADOMETDC* copies was corroborated via Western blot analysis of ADOMETDC overproducer, wild type, Δ*adometdc*13 and Δ*adometdc*14 lysates (Fig. 4). Whereas the polyclonal antibodies recognize a single polypeptide of ~35 kDa, no band is observed in either of the knockout strains. This band presumably corresponds to the α subunit of ADOMETDC because it is consistent with the predicted molecular mass from the translated nucleotide sequence. The antibodies do not appear to recognize the β subunit of the enzyme with sufficient avidity for visualization by immunoblotting. Equal loading of protein lysates from wild type and knockout strains onto the gel was confirmed by normalization of the immunoblots to antisera raised against the purine salvage enzyme, hypoxanthine-guanine phosphoribosyltransferase.

Nutritional Requirements of the Δ*adometdc Strains*—To evaluate the nutritional requirements of the Δ*adometdc*

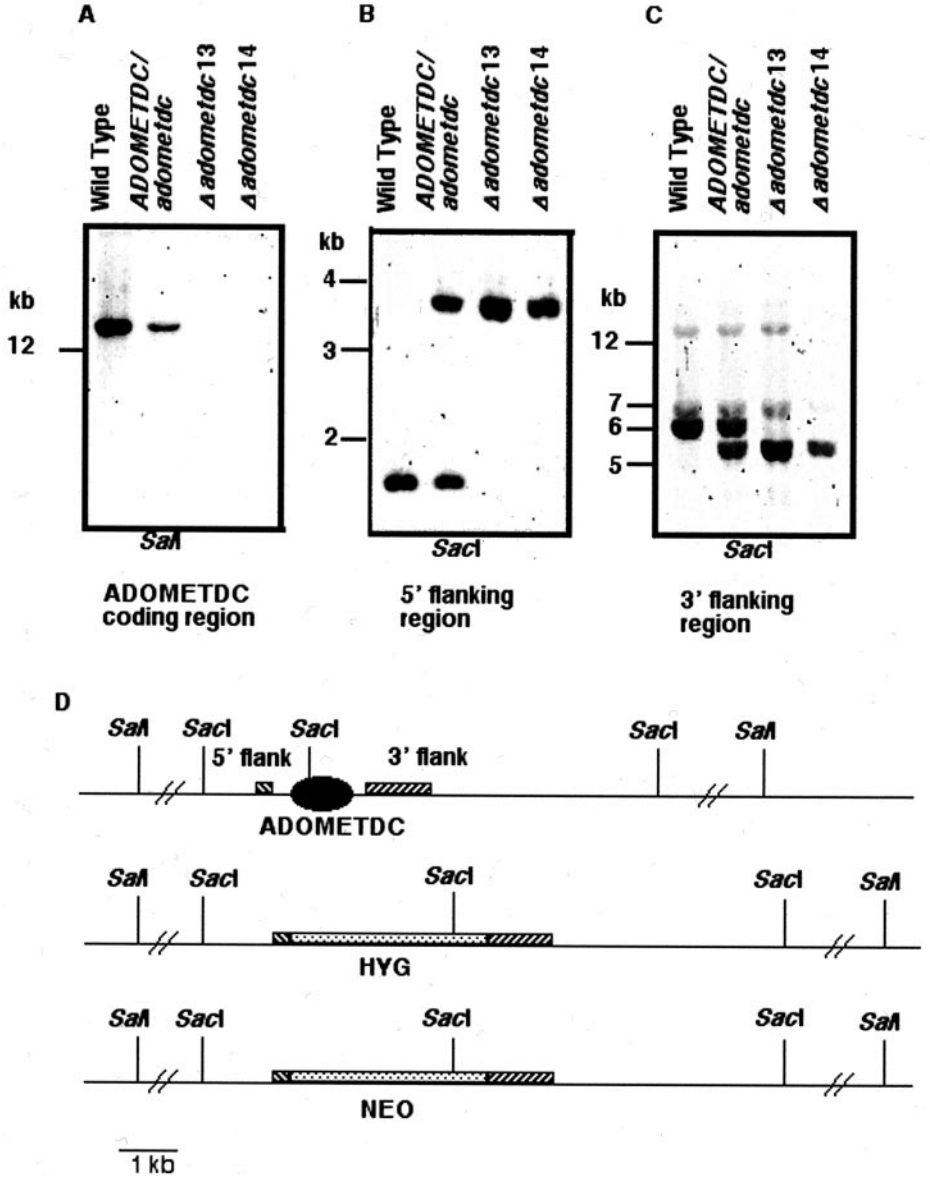

FIG. 3. **Southern blot analysis of wild type and mutant parasites.** *Panels A*, *B*, and *C*, 2 μg of genomic DNA from wild type, *ADOMETDC*/*adometdc*, Δ*adometdc*13, and Δ*adometdc*14 parasites was digested with the indicated restriction enzymes and hybridized to *ADOMETDC* probes from the coding region (*panel A*), the 5′F (*panel B*), or the 3′F (*panel C*). Molecular weight markers are indicated to the *left*. *Panel D*, restriction maps of the wild type *ADOMETDC* and novel loci are depicted. A *black oval* shows the *ADOMETDC* coding region probe, *hatched boxes* indicate both flanking regions, and a *dotted box* indicates the HYG or NEO locus.

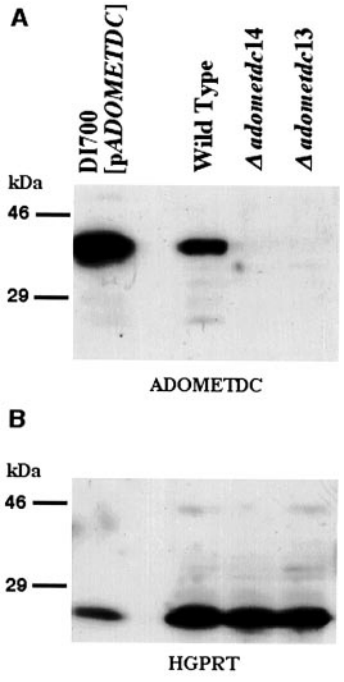

FIG. 4. **Western blot analysis of ADOMETDC protein in wild type and genetically altered *L. donovani*.** *Panel A*, polyclonal antiserum against *L. donovani* ADOMETDC was generated in rabbits and used to detect ADOMETDC protein in cell lysates prepared from 10^6 DI700[p*ADOMETDC*] overproducers, and 10^7 wild type, Δ*adometdc*14, and Δ*adometdc*13 parasites. *Panel B*, the same blot was reprobed with an antibody against the *L. donovani* hypoxanthine-guanine phosphoribosyltransferase (*HGPRT*) protein to normalize the loading onto each lane of the gel. Molecular mass markers are indicated to the *left*.

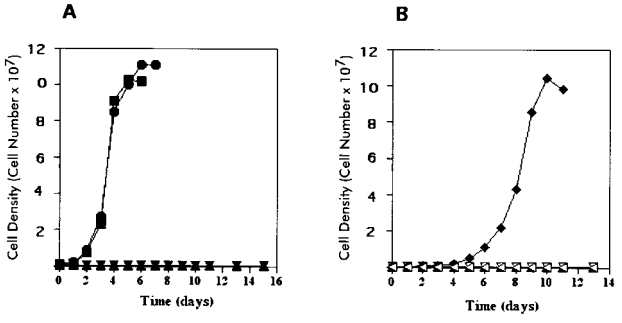

FIG. 5. **Growth of wild type and mutant parasites in DME-L-CS medium in the absence and presence of polyamine supplements.** *Panel A*, wild type (■), *ADOMETDC*/*adometdc* (●), Δ*adometdc*13 (▲), and Δ*adometdc*14 (▼) were grown in DME-L-CS medium with no polyamine additions. Parasites were enumerated every 24 h by counting in a hemacytometer. *Panel B*, the Δ*adometdc*14 parasites were removed from DME-L-CS medium that had been supplemented with 100 μM spermidine, washed twice in phosphate-buffered saline, and then incubated in medium without polyamine addition for 4 days and subsequently incubated in medium with no supplementation (△) or supplemented with either 100 μM spermidine (♦), MTA (▽), or putrescine (▲).

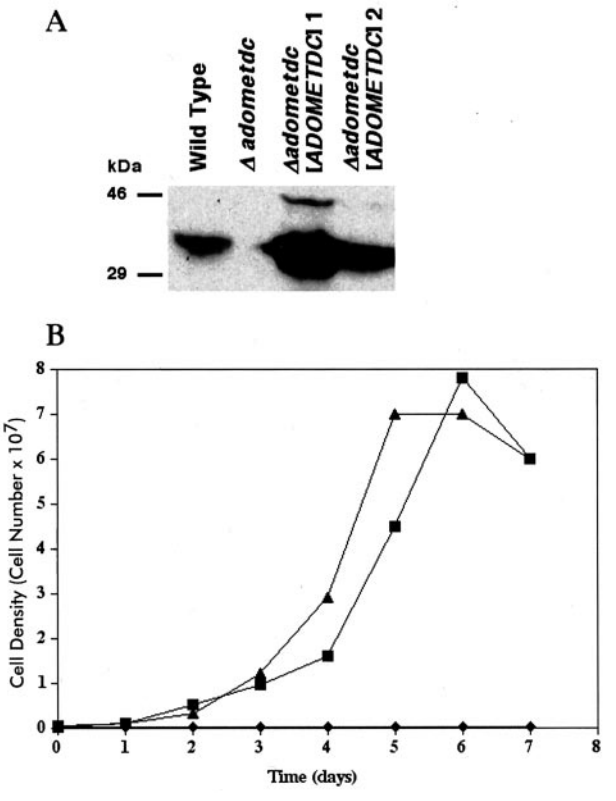

FIG. 6. **Genetic rescue of Δ*adometdc* parasites.** *Panel A*, Western blot analysis of wild type, Δ*adometdc* knockout, and Δ*adometdc* parasites transfected with pX63PAC[p*ADOMETDC*], Δ*adometdc*-[p*ADOMETDC*] 1, and Δ*adometdc*[p*ADOMETDC*] 2. Fractionated lysates were probed with polyclonal antisera against ADOMETDC. *Panel B*, ability of wild type (■), Δ*adometdc* (♦), and Δ*adometdc*-[p*ADOMETDC*] 1 (▲) to proliferate in DME-L-CS without spermidine addition was assessed by counting on a hemacytometer.

strains, the knockout parasites were incubated in growth medium with and without polyamines or MTA. As shown in Fig. 5*A*, wild type and *ADOMETDC*/*adometdc* grown in defined DME-L-CS without polyamine supplementation grew equally well, whereas the Δ*adometdc*13 and Δ*adometdc*14 cells did not proliferate at all. The null mutants incubated under these conditions arrested after three to four cell divisions, assumed a rounded morphology, and died after 4 weeks. The addition of 100 μM spermidine was able to circumvent the phenotypic consequences of the genetic lesion in Δ*adometdc* parasites, whereas an equivalent concentration of putrescine or MTA, a product of the *Leishmania* dADOMETC reaction, did not rescue the parasites (Fig. 5*B*). Finally, the growth defect of Δ*adometdc* parasites was also not restored by the addition of 100 μM spermine, a polyamine that is not found in *L. donovani* (19), to the culture medium (data not shown). The failure of these compounds to rescue the knockouts could not be imputed to their inherent toxicity because none of these compounds adversely affected the viability or growth of wild type *L. donovani* (data not shown). The ability of spermidine to circumvent the polyamine auxotrophy of Δ*adometdc* parasites demonstrates that the sole cellular function of dAdoMet is to serve as an aminopropyl donor in spermidine synthesis and that MTA production by this pathway is nonessential for *L. donovani* promastigotes.

Genetic Rescue of Δ*adometdc Mutants*—To establish that the auxotrophic phenotype observed in the knockout parasites could be ascribed to the genetic lesions at the *ADOMETDC* locus, an episomal copy of the *ADOMETDC* gene was introduced into the Δ*adometdc*14 line by transfection and amplified in the selective drug. As shown in Fig. 6*A*, two different clonal populations of Δ*adometdc*[p*ADOMETDC*] parasites contained levels of ADOMETDC protein significantly greater than that of wild type parasites (Fig. 6*A*). Furthermore, complementation of the knockout strain restored the ability of the parasites to proliferate in polyamine-deficient medium (Fig. 6*B*). In fact, the growth rate was slightly greater than that of wild type parasites.

Polyamine, Thiol, and dAdoMet Pools—To evaluate the consequences of polyamine starvation on the intracellular polyamine and reduced thiol pools, Δ*adometdc*14 parasites were

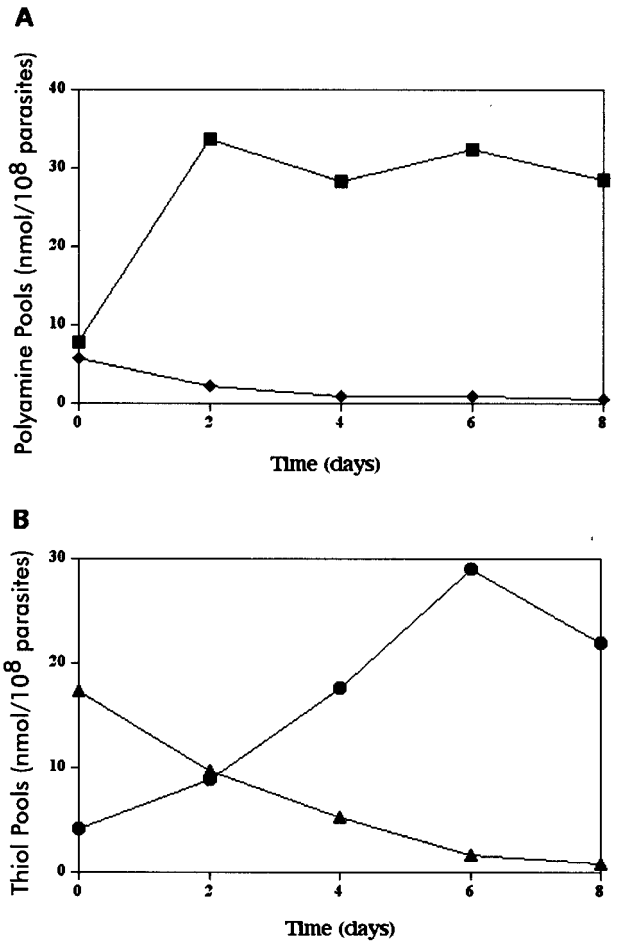

FIG. 7. **Polyamine and thiol pool measurements in Δ*adometdc* parasites.** The Δ*adometdc*14 parasites were incubated in DME-L-CS without polyamine addition, and aliquots were analyzed every 2 days for putrescine (■) and spermidine (♦) in *panel A*, and glutathione (●) and trypanothione (▲) in *panel B*.

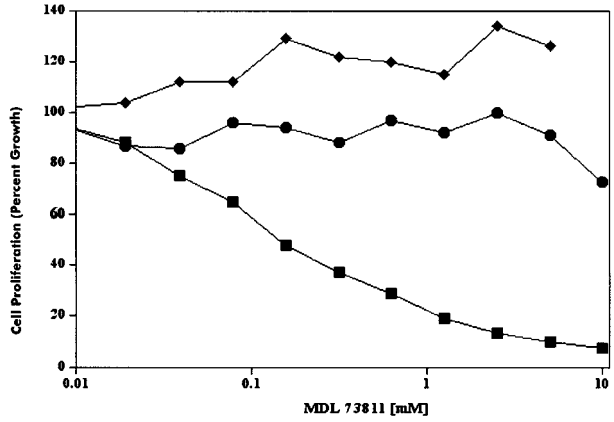

FIG. 8. **Proliferation of *L. donovani* parasites in medium containing the ADOMETDC inhibitor MDL 73811.** Wild type parasites (■), wild type parasites in 100 μM spermidine (●), and DI700[p*ADOMETDC*] (♦) parasites were incubated in medium in a serial dilution of MDL 73811. Parasites were inoculated at 5×10^5/ml and counted after 5 days in a Coulter Counter. The growth in the control well, containing no inhibitor, was taken as 100% cell proliferation. This is one example of several similar experiments.

incubated in polyamine-free medium for 8 days. During this incubation, when parasites were not dividing, the intracellular levels of spermidine decreased significantly with a concomitant increase in the intracellular putrescine pool (Fig. 7*A*). The putrescine:spermidine ratio increased from an initial 1.4 prior to the experiment to a final ratio of 60 after 8 days of incubation in the polyamine-deficient DME-L-CS. The absolute concentrations of both polyamines were similar in wild type (10 nmol of putrescine/10^8 cells and 10 nmol of spermidine/10^8 cells) and Δ*adometdc*14 parasites when the latter were maintained in spermidine (16 nmol of putrescine/10^8 cells and 6 nmol of spermidine/10^8 cells). Concurrent with the spermidine depletion was a diminution in the level of reduced trypanothione, a spermidine-containing thiol conjugate, and an increase in the pool of glutathione, the thiol precursor for trypanothione synthesis (Fig. 7*B*). Although trypanothione levels were 4-fold higher than glutathione levels in the Δ*adometdc*14 parasites that had been maintained in spermidine-supplemented growth medium, the levels of trypanothione and glutathione were equivalent after 2 days of polyamine starvation, and the glutathione pool was 27-fold greater than that of trypanothione after 8 days of incubation in the polyamine-deficient medium. The thiol pools of wild type and Δ*adometdc*14 parasites grown in spermidine-supplemented DME-L-CS were roughly equivalent (data not shown). Finally, as expected, dAdoMet was not detected in the Δ*adometdc*14 cells.

ADOMETDC Inhibition Profiles—To evaluate whether ADOMETDC was the intracellular target of a variety of known inhibitors of ADOMETDC enzymes *in vitro*, the effects of these enzyme inhibitors were compared on wild type and DI700-[p*ADOMETDC*] parasites that overexpressed *ADOMETDC* (Fig. 8 and Table I). As shown in Fig. 8, the ADOMETDC overproducer exhibited significant resistance to MDL 73811. Whereas the effective concentration of MDL 73811 which inhibited growth of wild type parasites by 50% (EC_{50} value) was ~40 μM, the parasites that overproduced ADOMETDC were virtually unaffected by the presence of 10 mM MDL 73811 in the culture medium. Addition of spermidine conferred refractoriness of wild type cells to MDL 73811 as well (Fig. 8). In contrast, the EC_{50} values of wild type and DI700[p*ADOMETDC*] parasites for berenil, pentamidine, and MGBG were equivalent (Table I). Spermidine addition did not influence the EC_{50} values of wild type parasites for any of the last three compounds.

DISCUSSION

The construction of Δ*adometdc* parasites by double targeted gene replacement established that *ADOMETDC* is an essential gene in *L. donovani* promastigotes. The Δ*adometdc* parasites were shown to be polyamine auxotrophs, and this auxotrophy could only be rescued by spermidine supplementation of the culture medium or by complementation with an episomal copy of *ADOMETDC*. The ability of spermidine to circumvent the polyamine auxotrophy of Δ*adometdc* parasites demonstrates that the sole cellular function of dAdoMet is to serve as an aminopropyl donor in spermidine synthesis. The growth phenotype and nutritional requirements of the Δ*adometdc* cells parallel those of previously described Δ*spdsyn L. donovani* (20). Conversely, a Δ*odc* knockout could also be rescued by spermidine, but only at high concentrations where growth rates were suboptimal (19). Thus, the growth phenotypes of the knockout lines demonstrate that spermidine is both essential and sufficient for promastigote survival. In contrast, putrescine, which is synthesized in both Δ*adometdc* and Δ*spdsyn*, is unable to meet the entire polyamine requirement of the parasite. However, Δ*odc* parasites grow better in medium supplemented with putrescine than with spermidine alone, suggesting that the diamine is essential for optimal growth and is, therefore, more than just a precursor for spermidine synthesis. The inability of spermine, a ligand for the polyamine transporter (39), to circumvent a genetic lesion in any of the three polyamine biosynthetic enzymes implies that *L. donovani* promastigotes lack a

TABLE I

EC_{50} values for several potential inhibitors of the polyamine pathway

The EC_{50} values for wild type parasites, wild type parasites grown in the presence of 100 μM spermidine, and ADOMETDC-overproducing parasites were established. Each value is a mean of three independent experiments.

Strain	Pentamidine	Berenil	MGBG	MDL 73811
	μM	μM	mM	mM
Wild type	1.9 ± 0.2	9.5 ± 3.9	1.8 ± 0.4	0.04 ± 0.02
Wild type + spermidine	2.0 ± 0.4	9.7 ± 2.4	1.8 ± 0.5	>10
DI700[p*ADOMETDC*]	1.7 ± 0.4	9.7 ± 2.4	1.5 ± 0.6	>10

mechanism to convert spermine to spermidine and spermidine to putrescine.

The precise mechanism by which a lesion in *ADOMETDC* precipitates a failure to thrive in the absence of spermidine is not clear. Although spermidine is essential for cell proliferation in higher eukaryotes that do not make trypanothione, the spermidine-conjugate is postulated to be vital for the maintenance of the redox potential in *Leishmania* (40, 41). Incubation of Δ*adometdc* cells in medium lacking spermidine resulted in a dramatic decrease in the intracellular pools of both spermidine and trypanothione with a coincident augmentation of putrescine and glutathione levels. The effects of the Δ*adometdc* lesion on polyamine and thiol pools mirror those obtained with Δ*spdsyn L. donovani*, although dAdoMet pools were undetectable in Δ*adometdc* but markedly elevated in Δ*spdsyn* cells (20).

The buildup of both putrescine and glutathione pools under polyamine starvation conditions (Fig. 7) may be the result of cellular overproduction of these two metabolites to compensate for the genetically induced spermidine and trypanothione deficiencies. In support of this hypothesis is the observation that Δ*adometdc* parasites supplemented with spermidine maintain putrescine and glutathione levels that are roughly equivalent to those of wild type parasites. Thus, spermidine and trypanothione levels somehow influence the synthesis of their precursors within the parasite by one or more unknown regulatory mechanisms. Obviously, however, the increases in putrescine and glutathione observed in the polyamine-starved Δ*adometdc* parasites do not ultimately compensate for the loss of spermidine and/or trypanothione.

ADOMETDC has received considerable attention as a potential antiparasitic drug target. Pentamidine, berenil, and MGBG, structural analogs of spermidine, and MDL 73811, a dAdoMet analog, are toxic toward *L. donovani* (Table I) and *T. brucei* (12, 42, 43), and each inhibits the mammalian and *T. brucei* ADOMETDC activities *in vitro* (42, 44, 45). However, all four inhibitors have a multitude of potential targets, and it remains unknown whether their cytotoxic effects can be ascribed to ADOMETDC inhibition (14, 15, 42, 46–48). To evaluate whether ADOMETDC was the primary intracellular target for these drugs in *L. donovani*, an ADOMETDC overproducer was generated. Comparisons of the drug profiles of wild type and the DI700[p*ADOMETDC*] overproducers revealed equal sensitivities of both lines to pentamidine, berenil, and MGBG, whereas the DI700[p*ADOMETDC*] parasites were dramatically resistant to the growth inhibitory and cytotoxic effects of MDL 73811 (Fig. 8 and Table I). The pharmacogenetic experiments are supported by the ability of spermidine to circumvent the toxicity of MDL 73811, but not of pentamidine, berenil, and MGBG, in wild type parasites. Thus, ADOMETDC is the primary cellular target for MDL 73811 in *L. donovani* promastigotes, whereas the other three drugs do not exert their toxicity via ADOMETDC inhibition.

A multisequence alignment of ADOMETDC proteins from phylogenetically diverse organisms revealed several regions of significant sequence homology. ADOMETDC maintains the conserved cleavage site between Glu-90 and Ser-91 which converts the apoenzyme into mature α and β subunits. Western blot analysis implies that the *L. donovani ADOMETDC* translation product is cleaved in the parasite and is present predominantly as the mature enzyme because the antibodies appear to recognize primarily the 33-kDa α subunit (Fig. 4). However, the 44-kDa apoenzyme can be observed as a very faint band in both Δ*adometdc*[p*ADOMETDC*] (Fig. 6) and DI700[p*ADOMETDC*] overexpressors (data not shown). In addition, virtually all of the residues that were identified by structural and genetic analyses to be critical for catalysis, ligand binding, and proenzyme processing of the human ADOMETDC are conserved in the *L. donovani* enzyme. These include (i) Cys-105, Ser-255, and His-268 of the *L. donovani* enzyme, which correspond to Cys-82, Ser-229, and His-243 of the human ADOMETDC and which are essential for catalysis; (ii) Phe-32, Leu-87, Phe-249, and Glu-271 of the *L. donovani* ADOMETDC, which match Phe-7, Leu-65, Phe-223, and Glu-247 of the human counterpart and which are necessary for proper ligand positioning and binding; and (iii) Glu-36, Asp-195, His-268, and Glu-280 of the parasite enzyme, which align with Glu-11, Asp-174, His-243, and Glu-256 of the human protein, which are crucial for ADOMETDC processing (49–52).

The mammalian ADOMETDC enzyme is regulated by several different mechanisms. One primary control mechanism is the rapid turnover rate (36–38), a feature not shared by the leishmanial counterpart (Fig. 2). The *L. donovani* ODC (22) and SPDSYN enzymes are also stable. In contrast, ODC of mammalian cells is among the most labile of cellular proteins (53, 54), whereas the mammalian SPDSYN is quite stable (55). The stability of the parasite ADOMETDC and the lability of the host equivalent may account for the ability of MDL 73811 to eradicate mouse infections of the related parasite *T. brucei*. Although both the mammalian and parasite ADOMETDCs are inhibited by MDL 73811 (56) (Fig. 8 and Table I), the ability of the host cell enzyme to recover rapidly from the pharmacological insult may account for the therapeutic selectivity of the drug (12, 43). A similar paradigm has been proposed and substantiated experimentally for the efficacy of DFMO on *T. brucei* infections (57). Although both mammalian and *T. brucei* ODC enzymes are equally sensitive to DFMO inhibition, the host cell, unlike the parasite, has the ability to regenerate ODC quickly after the drug has been eliminated.

These genetic studies have demonstrated that ADOMETDC is an essential protein in *L. donovani* promastigotes and that spermidine is an essential polyamine. The existence of ADOMETDC inhibitors that pharmacologically simulate a genetic deficiency in the enzyme, the disparate stabilities of the mammalian and parasite ADOMETDC proteins, and the previously determined biochemical discrepancies between the mammalian and leishmanial polyamine pathways imply that ADOMETDC may be a potential target for therapeutic manipulation of certain parasitic diseases.

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S-Adenosylmethionine Decarboxylase from*Leishmania donovani* : MOLECULAR, GENETIC, AND BIOCHEMICAL CHARACTERIZATION OF NULL MUTANTS AND OVERPRODUCERS

Sigrid C. Roberts, Jerry Scott, Judith E. Gasteier, Yuqui Jiang, Benjamin Brooks, Armando Jardim, Nicola S. Carter, Olle Heby and Buddy Ullman

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