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 Δadometdc null mutants were created in the insect vector form of the parasite by double targeted gene replacement. The Δadometdc 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 Δadometdc 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 Δadometdc parasites. ADOMETDC overexpression abrogated polyamine auxotrophy in the Δadometdc 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). 3-Aminoadipic acid (3-AAA), 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-
(guanilylhydrazone) (MGBG). Pentamidine is also the second most prescribed drug for treatment of visceral leishmaniasis. However, even though the \emph{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 benel 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 \emph{L. donovani}. These DNAs were used to generate \textit{Δadometdc} knockouts employing double targeted gene replacement strategies (18–20) and \textit{ADOMETDC} overexpressors. The \textit{Δadometdc} \emph{L. donovani} were shown to be auxotropic 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 depletion in the cellular pools of both spermidine and trypanothione, and incubation of these parasites in 5-flanking region, 5', was amplified by PCR using the M13-20 oligonucleotide (containing a Bluescript sequence) as a sense primer and an antisense primer, 5'-GGGAACCGTCGACAA-GAGCGGATGAGC-3', preceded by an Sulf site (underlined) and corresponding to a region ~120 nucleotides upstream from the \textit{ADOMETDC} ORF. The ~1,200-bp PCR product was digested with Sulf and HindIII, a restriction site located ~300 bp upstream from the Sulf site, and 5' was then subcloned into the HindIII/Sulf sites of p\textit{x63HYG} and p\textit{x63NEO}. The resulting plasmids were termed p\textit{x63HYG}-5'F and p\textit{x63NEO}-5'F, respectively. To amplify the 3'-flank, a sense primer, 5'-TCCTCCGGGCGGCGCCCTATCCCC-3', and an antisense primer, 5'-CTCAATTCCTCCGTATAGTGGG-3', containing Smal and BglII restriction sites (underlined), were designed. The 1-kb PCR product, 3'F, was then subcloned into the corresponding sites of p\textit{x63HYG}-5'F and p\textit{x63NEO}-5'F to yield the knockout constructs, p\textit{x63NEO}\textit{Δadometdc} and p\textit{x63HYG}\textit{Δadometdc}, 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.

\textbf{Molecular Constructs for the Replacement of the ADOMETDC Allele—}To construct drug resistance cassettes to replace both \textit{ADOMETDC} alleles, ~300 bp of 5' and ~1 kb of 3'-flanking regions were subcloned into the appropriate sites of the p\textit{x63HYG} and p\textit{x63NEO} plasmids. The 5'-flanking region, 5', was amplified by PCR using the \textit{M} spermidine, and 100 \muM spermidine, respectively, unless otherwise specified. The \textit{ADOMETDC} overexpressors, \textit{ADOMETDC} and \textit{DI700} parasites transfected with just \textit{p\textit{x63NEO}}, \textit{DI700[p\textit{x63NEO}]}, were propagated in DME-L-CS supplemented with 50 \muM hygromycin, 20 \mug/ml G418. The genotypes of the null mutants \textit{ADOMETDC} and \textit{DI700} parasites were transfected with \textit{p\textit{x63NEO}}\textit{Δadometdc} and \textit{p\textit{x63HYG}}\textit{Δadometdc}, respectively. The correct orientation was ascertained by immunoblotting.

\textbf{Antibody Generation and Characterization—}To raise antibodies against ADOMETDC, the \textit{ADOMETDC} gene was inserted into the \textit{pBac} prokaryotic expression vector and then induced in low phosphate 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).

\textbf{Experimental Procedures—}Materials, Chemicals, and Reagents—The \textit{p\textit{x63NEO}}, \textit{p\textit{x63HYG}}, \textit{p\textit{x63PAC}}, and \textit{p\textit{PNXO}} 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 Merrell Dow Research Institute.

\textbf{Cell Culture—}The wild type \emph{L. donovani} clone, \textit{DI700}, originally derived from the 1S Sudanese strain, was used for DNA isolation, library construction, and as a recipient strain in the initial transfections. \textit{L. donovani} promastigotes were propagated in DME-L, a completely defined Dulbecco's modified Eagle-based medium especially designed for \textit{Leishmania} cultivation (21). Transfected parasites were maintained in 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 \textit{ADOMETDC}/\textit{adometdc} heterozygote and the \textit{Δadometdc} null mutants were routinely maintained in DME-L-CS supplemented with either 50 \muM hygromycin or 50 \muM hygromycin, 20 \muM G418, and 100 \muM spermidine, respectively, unless otherwise specified. The \textit{ADOMETDC} overexpressors, \textit{ADOMETDC} and \textit{DI700} parasites transfected with just \textit{p\textit{x63NEO}}, \textit{DI700[p\textit{x63NEO}]}, were grown in DME-L-CS and 20 \muM G418.

The \textit{Δadometdc} null mutants transfected with \textit{p\textit{x63PAC}}\textit{ADOMETDC}, designated \textit{Δadometdc[p\textit{ADOMETDC}]}, were propagated in DME-L-CS to which 50 \muM hygromycin, 20 \muM G418, and 100 \muM 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).

\textbf{Isolation of the ADOMETDC—}A fragment of \textit{ADOMETDC} was amplified from genomic DNA using PCR. The sense primer, 5'-CTCGGAATTCC-CTT(CAT)-GAG-GG(CGC)-GAG-AA-3', was constructed with an 11-nucleotide leader encompassing an EcoRI site (underlined) followed by a degenerate sequence corresponding to amino acids 7–12, PGTEKE, of the human ADOMETDC, whereas the antisense primer, 5'-CTCGGGATCC-CTT(AAC)(GCG)-GAG-AATG-GT-G-3', was generated from residues 243–248, HITPEP of the human ADOMETDC, and was preceded by an 11-nucleotide leader encompassing a BamHI restriction site (underlined). Amplification of the \textit{ADOMETDC} fragment was performed on a Cycler 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 \textit{L. donovani} EMBL3 genomic library (23) under stringent hybridization and washing conditions (22, 23). A 5.1-kb Sulf site that hybridized to the probe was subcloned from a plaque-purified phage into pBluescript KS+, and the entire nucleotide sequence of the \textit{ADOMETDC} open reading frame (ORF) was obtained in both orientations. In addition, portions of the \textit{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).

\textbf{RESULTS—}
RESULTS

Isolation of the ADOMETDC Gene—The gene encoding ADOMETDC was isolated on a 5.1-kb SalI 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 /H9251 and /H9252 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 Δadometdc 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 pX63HYG/adometdc, and clonal ADOMETDC/adometdc heterozygotes were selected in hygromycin. A second round of transfection with pX63NEO/adometdc replaced the second ADOMETDC allele, and clonal strains of Δadometdc knockout parasites were selected in hygromycin, G418, MTA, and spermidine. The presence of hygromycin during the second round of gene targeting ensured that the pX63NEO/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, Δadometdc13 and Δadometdc14, were selected for further analysis.

Southern blot analysis of SalI-digested genomic DNA prepared from wild type, ADOMETDC/adometdc, Δadometdc13, and Δadometdc14 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 SacI-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′/H11032F (Fig. 3, panel B) or 3′/H11032F (Fig. 3, panel C). Replacement of the wild type ADOMETDC copies by HYG/adometdc and NEO/adometdc created an additional 3.8-kb SacI fragment that hybridized to 5′/F (Fig. 3, panel B) and a 5.5-kb SacI 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 NEO/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.

The loss of the wild type ADOMETDC copies was corroborated via Western blot analysis of ADOMETDC overproducer, wild type, Δadometdc13 and Δadometdc14 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.

**Nutritional Requirements of the Δadometdc Strains**—To evaluate the nutritional requirements of the Δadometdc
strains, the knockout parasites were incubated in growth medium with and without polyamines or MTA. As shown in Fig. 5A, wild type and ADOMETDC/adometdc grown in defined DME-L-CS medium without polyamine supplementation grew equally well, whereas the ADOMETDC13 and ADOMETDC14 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 Δadometdc parasites, whereas an equivalent concentration of putrescine or MTA, a product of the Leishmania dADOMETDC reaction, did not rescue the parasites (Fig. 5B). Finally, the growth defect of Δadometdc 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 Δadometdc 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 Δadometdc 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 Δadometdc14 line by transfection and amplified in the selective drug. As shown in Fig. 6A, two different clonal populations of Δadometdc[pADOMETDC] parasites contained levels of ADOMETDC protein significantly greater than that of wild type parasites (Fig. 6A). Furthermore, complementation of the knockout strain restored the ability of the parasites to proliferate in polyamine-deficient medium (Fig. 6B). 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, Δadometdc14 parasites were

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 10⁶ DI700[pADOMETDC] overproducers, and 10⁶ wild type, Δadometdc14, and Δadometdc13 parasites. Panel B, the same blot was reprobed with an antibody against the L. donovani hypoxanthine-guanine phosphoribosyltransferase (HGPR) 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 (●), Δadometdc13 (▲), and Δadometdc14 (▲) 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 Δadometdc14 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 Δadometdc parasites. Panel A, Western blot analysis of wild type, Δadometdc knockout, and Δadometdc parasites transfected with pX63PAC[pADOMETDC], Δadometdc[pADOMETDC]1, and Δadometdc[pADOMETDC]2. Fractionated lysates were probed with polyclonal antisera against ADOMETDC. Panel B, ability of wild type (□), Δadometdc (●), and Δadometdc[pADOMETDC]1 (▲) to proliferate in DME-L-CS without spermidine addition was assessed by counting on a hemacytometer.
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. 7A). 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 \( \Delta \)adometdc14 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. 7B). Although trypanothione levels were 4-fold higher than glutathione levels in the \( \Delta \)adometdc14 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 \( \Delta \)adometdc14 parasites grown in spermidine-supplemented DME-L-CS were roughly equivalent (data not shown). Finally, as expected, dAdoMet was not detected in the \( \Delta \)adometdc14 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-[pADOMETDC] 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 \(~40 \mu M\), 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-[pADOMETDC] 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 \( \Delta \)adometdc parasites by double targeted gene replacement established that ADOMETDC is an essential gene in \textit{L. donovani} promastigotes. The \( \Delta \)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 \( \Delta \)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 \( \Delta \)adometdc cells parallel those of previously described \( \Delta \)spdsyn \textit{L. donovani} (20). Conversely, a \( \Delta \)odc knock out 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 \( \Delta \)adometdc and \( \Delta \)spdsyn, is unable to meet the entire polyamine requirement of the parasite. However, \( \Delta \)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 \textit{L. donovani} promastigotes lack a...
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 in higher eukaryotes that do not make trypanothione, the spermidine-conjugate is postulated to be vital for the maintenance

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 Δadometdc 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 Δadometdc 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[pADOMETDC] overproducers revealed equal sensitivities of both lines to pentamidine, berenil, and MGBG, whereas the DI700[pADOMETDC] 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 a subunit (Fig. 4). However, the 44-kDa apoenzyme can be observed as a very faint band in both Δadometdc[pADOMETDC] (Fig. 6) and DI700[pADOMETDC] 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.

**Acknowledgments**—We thank Francy Peseck-Jardim and Birgitta Grahn for help with the cell culture and HPLC analyses, respectively.

### Table I

<table>
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<th>Strain</th>
<th>Pentamidine</th>
<th>Berenil</th>
<th>MGBG</th>
<th>MDL 73811</th>
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</thead>
<tbody>
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<td>Wild type</td>
<td>1.9 ± 0.2</td>
<td>9.5 ± 3.9</td>
<td>1.8 ± 0.4</td>
<td>0.04 ± 0.02</td>
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<td>Wild type + spermidine</td>
<td>2.0 ± 0.4</td>
<td>9.7 ± 2.4</td>
<td>1.8 ± 0.5</td>
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<td>DI700[pADOMETDC]</td>
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<td>9.7 ± 2.4</td>
<td>1.5 ± 0.6</td>
<td>&gt;10</td>
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S-Adenosylmethionine Decarboxylase from *Leishmania donovani*: MOLECULAR, GENETIC, AND BIOCHEMICAL CHARACTERIZATION OF NULL MUTANTS AND OVERPRODUCERS

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