Trypanosoma cruzi has not lost its *S*-adenosylmethionine decarboxylase: characterization of the gene and the encoded enzyme

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All attempts to identify ornithine decarboxylase in the human pathogen *Trypanosoma cruzi* have failed. The parasites have instead been assumed to depend on putrescine uptake and *S*-adenosylmethionine decarboxylase (AdoMetDC) for their synthesis of the polyamines spermidine and spermine. We have now identified the gene encoding AdoMetDC in *T. cruzi* by PCR cloning, with degenerate primers corresponding to conserved amino acid sequences in AdoMetDC proteins of other trypanosomatids. The amplified DNA fragment was used as a probe to isolate the complete AdoMetDC gene from a *T. cruzi* genomic library. The AdoMetDC gene was located on chromosomes with a size of approx. 1.4 Mbp, and contained a coding region of 1110 bp, specifying a sequence of 370 amino acid residues. The protein showed a sequence identity of only 25 % with human AdoMetDC, the major differences being additional amino acids present in the terminal regions of the *T. cruzi* enzyme. As expected, a higher sequence identity (68–72 %) was found in comparison with trypanosomatid AdoMetDCs. When the coding region was expressed in *Escherichia coli*, the recombinant protein underwent autocatalytic cleavage, generating a 33–34 kDa α subunit and a 9 kDa β subunit. The encoded protein catalysed the decarboxylation of AdoMet (K_m 0.21 mM) and was stimulated by putrescine but inhibited by the polyamines, weakly by spermidine and strongly by spermine. Methylglyoxal-bis-(guanylhydrazone) (MGBG), a potent inhibitor of human AdoMetDC, was a poor inhibitor of the *T. cruzi* enzyme. This differential sensitivity to MGBG suggests that the two enzymes are sufficiently different to warrant the search for compounds that might interfere with the progression of Chagas' disease by selectively inhibiting *T. cruzi* AdoMetDC.

INTRODUCTION

Chagas' disease, also known as American trypanosomiasis, is a severely debilitating infection widely distributed in rural zones of Latin America. About 16–18 million people are infected and about 90 million are at risk [1]. The causative agent is a parasitic protozoan, *Trypanosoma cruzi*, transmitted by blood-sucking insects of the subfamily Triatominae. Other routes of transmission include blood transfusions or organ transplants from infected donors, as well as congenital transmission from infected mothers to the foetus.

T. cruzi parasites, like almost all other unicellular and multicellular organisms, are likely to require an adequate level of the polyamines spermidine and spermine, and their diamine precursor putrescine, for growth and survival. It is particularly interesting to note that *T. brucei brucei*, another trypanosomatid, seems to be more sensitive to depletion of these amines than its mammalian host [2]. Consequently, African sleeping sickness caused by *T. brucei gambiense* infection can be cured by treatment with α-difluoromethylornithine (DFMO) [3,4], an enzyme-activated irreversible inhibitor of ornithine decarboxylase (ODC), the initial enzyme in the polyamine biosynthetic pathway. Remarkably, even patients with central nervous system involvement are cured by DFMO treatment [3,4], and the drug seems to have no serious side effects.

There are many possible reasons for the remarkable effectiveness of DFMO treatment against infections with *T. brucei*. One reason might be that the ODC of the parasite is a stable enzyme ($t_{\frac{1}{2}} > 6$ h), whereas that of the mammalian host has a rapid turnover ($t_{\frac{1}{2}} < 1$ h) [5]. This implies that the irreversible ODC inhibitor causes persistent inhibition of ODC in the parasite but not in the host cells because the latter can transiently escape the drug's inhibitory action by synthesizing enzymically active ODC molecules at a much higher rate than the parasites can. Another reason might be that the DFMO-mediated depletion of spermidine prevents the synthesis of trypanothione, a bis(glutathionyl)-spermidine conjugate that is truly unique to the trypanosomatids and acts as a free radical scavenger [6,7]. Yet another reason for the effectiveness of DFMO might be its perturbation of AdoMet metabolism, because the antitrypanosomal effect correlates better with the increase in AdoMet than with the decrease in polyamines [8]. Massive accumulation of AdoMet and its decarboxylated product might cause aberrant methylation in those reactions in which AdoMet acts as the methyl group donor [9,10].

To determine whether DFMO would have a similar effect on *T. cruzi*, trypomastigote-stage parasites were treated with the ODC inhibitor [11]. Surprisingly, DFMO exerted no significant antiparasitic effect, apparently owing to a lack of the target enzyme, ODC [11–13]. We and others have been unable to detect

Abbreviations used: AdoMetDC, *S*-adenosylmethionine decarboxylase; DFMO, α-difluoromethylornithine; MGBG, methylglyoxal-bis(guanylhydrazone); Ni-NTA, Ni^{2+}-nitrilotriacetic acid; ODC, ornithine decarboxylase; ORF, open reading frame; PFGE, pulsed-field gel electrophoresis; RT–PCR, reverse transcriptase-mediated PCR; SL, spliced leader; UTR, untranslated region.

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The nucleotide sequence data reported will appear in DDBJ, EMBL and GenBank Nucleotide Sequence Databases under the accession number (BankIt151525) AF032907.

any ODC activity or any ODC gene in *T. cruzi*. Nevertheless the parasites can synthesize spermidine and spermine, because radiolabelled putrescine is incorporated into both polyamines [12]. Therefore *S*-adenosylmethionine decarboxylase (AdoMetDC; EC 4.1.1.50), the rate-limiting enzyme in the biosynthesis of spermidine and spermine [14], should be present in *T. cruzi*, although no direct evidence has been presented.

AdoMetDC catalyses the production of decarboxylated AdoMet [14]. This is the aminopropyl group donor both in the conversion of putrescine to spermidine (catalysed by spermidine synthase) and of spermidine to spermine (catalysed by spermine synthase). Under physiological conditions, decarboxylated AdoMet is a limiting factor in polyamine synthesis. Although ubiquitous in prokaryotic and eukaryotic cells, AdoMetDC constitutes only a minor fraction of the intracellular proteins. This is due partly to its very short half-life and partly to the fact that AdoMetDC expression is highly regulated, at multiple levels: transcriptional and translational as well as post-translational [14–16]. Interestingly, there is evidence suggesting that the polyamines act as feedback regulators at all these levels. AdoMetDC expression is induced by hormones, growth factors, tumour promoters and other stimuli affecting growth [14].

Mammalian AdoMetDC is synthesized as a 38 kDa proenzyme (333–334 residues) [14,16]. The protein has no catalytic activity unless it is autocatalytically cleaved into a 31 kDa α subunit (265–266 residues) and an 8 kDa β subunit (67 residues), generating a pyruvate prosthetic group at the N-terminus of the α subunit by serinolysis [17]. Putrescine stimulates both processing and catalytic activity [18,19]. The mammalian enzyme contains two pairs of these non-identical subunits ($\alpha_2\beta_2$), and probably two catalytic centres [14]. Both subunits seem to be necessary for catalytic activity. The amino acid sequence of the protein is highly conserved (more than 90% identical) among mammalian species [16].

AdoMetDC genes have been cloned and sequenced from *Leishmania donovani* (GenBank accession number U20091), *T. brucei* (GenBank accession number U20092) and many other eukaryotes, including man [20]. The objective of the present study was to isolate the *T. cruzi* AdoMetDC gene, to determine its primary structure and to analyse the characteristics of the encoded protein compared with the human counterpart, i.e. that of the host. The ultimate goal of our work is to determine whether polyamine depletion, accomplished by AdoMetDC inhibition, can be as effective in the early treatment of Chagas' disease as is DFMO in the case of African sleeping sickness.

The *T. cruzi* AdoMetDC gene that was isolated in the present study specified a protein with a somewhat larger size than that of the mammalian counterpart. It proved to be 68–72% identical with the *L. donovani* and *T. brucei* enzymes. When the *T. cruzi* AdoMetDC gene was expressed in bacteria it encoded a proenzyme that was cleaved autocatalytically. The encoded protein was purified and was shown to exhibit enzymic activity that was stimulated by putrescine but inhibited by spermidine and by spermine. Methylglyoxal-bis(guanylhydrazone) (MGBG), a strong inhibitor of mammalian AdoMetDC [21], was found to be a very weak inhibitor of the *T. cruzi* enzyme.

EXPERIMENTAL

Materials

S-Adenosyl-L-[*carboxy*-^{14}C]methionine (59 mCi/mmol; 1 Ci = 37 GBq) and all other isotopes were purchased from Amersham. Restriction endonucleases were from Promega. All ligations were performed with Ready-To-Go T4 DNA Ligase from Pharmacia Biotech.

Source and preparation of *T. cruzi* genomic DNA

Because of striking genetic variability of the parasite, a reference clone (CL Brener) selected for the WHO *T. cruzi* Genome Initiative [22] was used as the main source of DNA and RNA in the present study. This reference clone was derived from the CL strain, which presents all the important characteristics of *T. cruzi*. Historically, the CL strain was isolated from a specimen of *Triatoma infestans*, the main domestic insect vector. Clone CL Brener was in turn isolated from the blood of a mouse chronically infected with the CL parental strain.

Genomic DNA was prepared from freeze-dried epimastigotes by incubation of parasites in 4% sarkosyl/1 mg/ml proteinase K/10 mM Tris/1 mM EDTA buffer (pH 7.9) at 37 °C for at least 1 h. The DNA was isolated by extracting the lysate 3 times with phenol and chloroform/3-methyl-butan-1-ol (24:1) and was then precipitated with ethanol.

Source and preparation of *T. cruzi* RNA

Total RNA, isolated from epimastigotes of the CL Brener reference clone, was kindly provided by Dr. Carlos Frasch and was used in reverse transcriptase-mediated PCR (RT–PCR). The RNA was prepared with the guanidinium method described by Ausubel et al. [23]. It was isolated in a CsCl-gradient step and was subsequently precipitated with ethanol.

PCR amplification and cloning of an AdoMetDC-specific DNA fragment from *T. cruzi* genomic DNA

A DNA probe for isolation of the complete AdoMetDC gene from a *T. cruzi* cosmid library was obtained by amplifying an AdoMetDC-specific DNA fragment by using degenerate primers corresponding to conserved regions in known trypanosomatid AdoMetDC proteins. The sense primer 5′-MGNRTNATHC-TNATHACNTGYGGNAC-3′ (PCR primer 1; see Figure 1) and the anti-sense primer 5′-ATNGCRTTDATNSWRTANC-CRCANGG-3′ (PCR primer 2; see Figure 1) were derived from regions within AdoMetDC genes of *Leishmania donovani* (GenBank accession number U20091) and *T. brucei* (GenBank accession number U20092) having conserved amino acids (see Figure 2). The PCR was performed in a volume of 100 μl with 0.1 μg of genomic *T. cruzi* DNA, 2 μM each of the sense and the anti-sense primer, 2.5 units of AmpliTaq DNA polymerase (Perkin Elmer), 200 μM dATP, 200 μM dCTP, 200 μM dGTP and 200 μM dTTP, and a buffer [10 mM Tris/HCl (pH 8.3)/ 50 mM KCl/1.5 mM $MgCl_2$/0.001% gelatin] supplied with AmpliTaq. The amplification was performed in a DNA thermal cycler (Perkin Elmer) under the following conditions: initial denaturation (94 °C for 5 min), then 35 cycles of annealing (43 °C for 40 s), extension (72 °C for 60 s) and denaturation (94 °C for 40 s), with a final cycle (43 °C for 40 s; 72 °C for 10 min).

An amplified DNA fragment of 482 bp was isolated and cloned into a pT7Blue T-vector (Novagen). This construct was used for transformation of competent *Escherichia coli* strain JM 109 and then isolated for sequencing. Sequence comparison with *T. brucei* AdoMetDC showed the DNA fragment to represent a portion of the AdoMetDC gene.

DNA sequencing

All DNA sequencing reactions were performed with the linear amplification sequencing method described by Murray [24] and Craxton [25] by using Thermo Sequenase DNA polymerase (Amersham) and a DNA thermal cycler (Perkin Elmer).

Screening of a *T. cruzi* cosmid library for the complete AdoMetDC gene

For the isolation of the complete *T. cruzi* AdoMetDC gene we used a large-fragment genomic library with DNA from the CL Brener reference clone of *T. cruzi* [26]. The PCR fragment generated from the AdoMetDC gene was labelled with [α-^{32}P]dCTP by random priming (Megaprime; Amersham) and used to probe high-density filters of the *T. cruzi* cosmid library [26]. The hybridization was performed in 0.5 M sodium phosphate buffer, pH 7.2, containing 7 % (w/v) SDS, 1 mM EDTA and 100 μg/ml tRNA at 65 °C. The filters were washed in 40 mM sodium phosphate buffer, pH 7.2, at 65 °C. The screening procedure yielded 16 positive clones; of these, one, cSAMtc, was selected and analysed further. The cSAMtc cosmid clone was grown in Luria–Bertani medium containing 30 μg/ml kanamycin. Cosmid DNA was prepared in accordance with the QIAGEN Midi Plasmid Kit protocol, and the AdoMetDC gene was sequenced as described above.

Mapping the 5′ splice acceptor site in the *T. cruzi* AdoMetDC transcript

The 5′ splice acceptor site in the 5′ untranslated region (UTR) of the *T. cruzi* AdoMetDC mRNA was revealed by amplification and sequencing of the 5′ terminus of two cDNA species generated by RT–PCR of total *T. cruzi* RNA. The synthesis of first-strand cDNA was generated with oligo(dT) primer by using approx. 5 μg of total RNA at 37 °C for 1 h. The RT–PCR reactions were performed essentially in accordance with the suggestions of the manufacturer of the RT–PCR kit (Stratagene).

For subsequent amplification of the 5′ terminus, we used one sense primer (5′-AACGCTATTATTGATACAGTTTCTGTACTATATTG-3′), representing a 35 nt sequence in the mini-exon, or spliced leader (SL), of *T. cruzi* [27], and two anti-sense primers (5′-ATTAAGTAGAGTCGTCGTGC-3′ or 5′-ATGCTTCGTCTGCGTACG-3′) complementary to sequences (nt 302–321 and nt 604–621 respectively) in the open reading frame (ORF) (see Figure 1). The concentrations of these two anti-sense primers were 0.65 μM and 0.73 μM respectively. The experimental conditions for the amplifications were: 30 cycles of 91 °C for 1 min, 54 °C for 1 min, and 72 °C for 2 min, with a final extension at 72 °C for 10 min. The amplified products were cloned into the pGEM-T Vector (Promega) and used for transformation of competent *E. coli* strain JM 109. Both products generated with the separate anti-sense primers were sequenced.

Size fractionation of *T. cruzi* chromosomal bands by pulsed-field gel electrophoresis (PFGE) and AdoMetDC gene mapping

Agarose plugs containing *T. cruzi* epimastigotes of three cloned stocks (CL Brener, CA-1/72 and Sylvio X10/7) were kindly provided by Dr. Juan José Cazzulo and were prepared as described previously [28–30]. PFGE separations of the *T. cruzi* chromosomes were made in a Bio-Rad CHEF Mapper with 0.5 × TBE (1 × TBE = 45 mM Tris/borate/1 mM EDTA) buffer maintained at 14 °C. A standard separation (350–1800 kbp) was done in 1 % (w/v) Beckman LE agarose, by using linear ramping with a pulse time of 58–169 s at 6 V/cm. PFGEs, optimized for the separation of larger chromosomes, were performed in 0.5 % agarose (Bio-Rad agarose, chromosomal grade) at 2 V/cm by using linear ramping with pulse times of 6–12 min and 10–15 min respectively. Amounts of DNA, corresponding to approx. (5–10) × 10^6 epimastigotes, were used in each well. The DNA was transferred to nylon membranes (Hybond; Amersham) as described previously [29,30]. The AdoMetDC PCR fragment was hybridized overnight to a filter in 0.5 M sodium phosphate buffer, pH 7.2, containing 7 % (w/v) SDS, 1 mM EDTA and 100 μg/ml tRNA at 65 °C. The filter was washed in 40 mM sodium phosphate buffer, pH 7.2, at 65 °C for 2 h.

Determination of the AdoMetDC gene copy number in the *T. cruzi* genome by Southern blot analysis

Genomic high-molecular-mass *T. cruzi* DNA (3 μg) was digested to completion with either *Eco*RI or *Nco*I. The digested samples were loaded on a 0.7 % agarose gel, separated by electrophoresis and then transferred to a Hybond-N+ nylon membrane (Amersham) with a Vacu-Blot XL (Pharmacia). After vacuum blotting, the membrane was subjected to UV cross-linking (Stratagene). Prehybridization was perfomed in 4 × SSC [standard saline citrate; 20 × SSC = 3 M NaCl/0.3 M trisodium citrate (pH 7.0)]/0.1 M sodium phosphate buffer (pH 6.8)/10 mM sodium pyrophosphate/0.2 % SDS/10 × Denhardt's solution [50 × Denhardt's solution = 1 % (w/v) Ficoll (type 400; Pharmacia)/1 % (w/v) polyvinylpyrrolidone/1 % (w/v) BSA (fraction V, Sigma) in water]/0.2 mg/ml salmon sperm DNA for 3 h at 65 °C. The hybridization was performed overnight at 65 °C in 4 × SSC/0.1 M sodium phosphate buffer (pH 6.8)/10 mM sodium pyrophosphate/0.2 % SDS/1 × Denhardt's solution/0.2 mg/ml salmon sperm DNA, with a ^{32}P-labelled *T. cruzi* AdoMetDC DNA probe spanning the entire ORF. Post-hybridization washes were performed with 2 × SSPE [20 × SSPE = 3 M NaCl/0.18 M NaH_2PO_4/0.02 M EDTA (pH 7.2)] for 2 min at room temperature, and then 0.5 × SSPE twice for 1 h at 65 °C. All hybridization steps were performed in a Hybridiser HB-2D (Techne) hybridization oven.

Generation of a recombinant His-tagged *T. cruzi* AdoMetDC protein

The coding region of the *T. cruzi* AdoMetDC gene was fused at its 3′ end to a six-histidine tag coding sequence, and the construct was subcloned into the pQE-70 bacterial expression vector (Qiagen) and expressed in *E. coli*. AdoMetDC gene-specific primers were designed to make possible the insertion of the construct into the pQE-70 expression vector. Thus in the sense primer (5′-GTTATGCATG↓**CTA**AGCAATAAGGACCC-3′) the underlined *Sph* I recognition sequence (↓ indicates the cleavage site) was introduced by changing the second codon of the ORF from TTA to **CTA** (see Figure 1). This mutation does not change the encoded protein because both triplets specify leucine. For the anti-sense primer (5′-ATTATG↓GATCC**CT**-**C**TTCCACAGAATCTGTG-3′) the underlined *Bam*HI recognition sequence (↓ indicates the cleavage site) was introduced after the last codon (**GAG**) specifying Glu^{370} (see Figure 1). The PCR reaction was performed as outlined above with the exception that 0.1 μg of cSAMtc DNA and 0.25 μM final primer concentrations were used. The experimental conditions were: 94 °C for 5 min; three cycles of 49 °C for 40 s, 72 °C for 1 min and 94 °C for 40 s; then 35 cycles of 61 °C for 40 s, 72 °C for 1 min and 94 °C for 40 s. The final step was 61 °C for 40 s; 72 °C for 10 min. The product was verified as above and purified with a PCR clean-up column (Qiagen). A portion of the amplifed product was cut with *Sph*I and *Bam*HI, then purified and ligated into *Sph*I- and *Bgl*II-digested pQE-70 vector. Thus the final construct (ending with -GGA-TCC-CAT-CAC-CAT-CAC-CAT-CAC-3′) should translate into a recombinant AdoMetDC protein having an extension of the C terminus with -Gly-Ser-His_6. The *Sph*I digestion described above was designed to remove the endogenous ATG, and the expression construct instead used

the ATG of the pQE-70 vector as the start codon. The plasmid was used to transform competent *E. coli* strain JM 109. Plasmid DNA was extracted and the nucleotide sequence of the construct was confirmed by sequencing. The construct was maintained in *E. coli* strain JM 109. Competent *E. coli* M15[pREP4] (Qiagen) were transformed with the construct and used for expressing the protein.

Expression of the *T. cruzi* AdoMetDC gene construct in *E. coli* and purification of the encoded His-tagged protein

E. coli, transformed with the pQE-70 *T. cruzi* AdoMeDC construct, were grown overnight at 37 °C in Luria–Bertani medium containing 100 μg/ml carbenicillin and 25 μg/ml kanamycin. They were then diluted 1:50 in the same medium and grown for 3 h. To induce the expression of *T. cruzi* AdoMetDC, isopropyl β-D-thiogalactoside was added to a final concentration of 2 mM. The bacteria were then harvested by centrifugation for 10 min at 4000 ***g*** and 4 °C, and the cell pellets were stored at −70 °C until used for AdoMetDC purification.

Frozen cell pellets were resuspended and lysed at 4 °C for 30 min in buffer A [10 mM Tris/HCl (pH 7.4)/300 mM NaCl/2.5 mM putrescine/10 % (v/v) glycerol/0.025 M imidazole] containing 1 mg/ml lysozyme. The lysates were agitated periodically and finally sonicated. After centrifugation for 20 min at 10000 ***g*** and 4 °C, the supernatant was decanted and passed through a Sartorius non-pyrogenic, hydrophilic 0.45 μm pore-size filter.

The His-tagged *T. cruzi* AdoMetDC protein was purified by metal chelate-affinity chromatography with Ni^{2+}-nitrilotriacetic acid resin (Ni-NTA agarose; Qiagen). Columns were equilibrated with buffer A. After the material had been loaded, non-specific components were removed by washing the column with buffer A, and subsequently with 0.05 M imidazole in buffer A. The His-tagged protein was eluted under mild conditions by adding imidazole (0.1 M in buffer A) as a competitor. To remove endogenous (*E. coli*) putrescine, the purified protein was passed through a Sephadex G-25 column (PD-10; Pharmacia Biotech). The purified *T. cruzi* AdoMetDC was analysed by SDS/PAGE [6 % (w/v) stacking gel/12 % (w/v) separating gel], and its migration compared with coloured molecular mass markers (Rainbow; Amersham). Proteins in the gel were revealed by staining with Coomassie Brilliant Blue G-250.

Removal of the His tag from the recombinant AdoMetDC protein by digestion with carboxypeptidase A

The His-tag was removed from the recombinant *T. cruzi* AdoMetDC protein by treatment with carboxypeptidase A (Boehringer Mannheim), which causes successive cleavage of amino acids from the C-terminus. Mixtures of *T. cruzi* AdoMetDC and carboxypeptidase A (50:1, 25:1, 10:1 and 5:1, w/w) in 0.05 M Tris/HCl buffer, pH 8.0, containing 2.5 mM putrescine were incubated for 1 h at room temperature. The digest was separated by SDS/PAGE [6 % (w/v) stacking gel/12 % (w/v) separating gel, or 5 % (w/v) stacking gel/8 % (w/v) separating gel]. One gel was stained with Coomassie Brilliant Blue G-250 and another gel was subjected to Western blot analysis with an anti-His monoclonal antibody (Invitrogen) recognizing an oligohistidine sequence at the C-terminus of proteins. The secondary antibody used was horseradish peroxidase-conjugated anti-(mouse IgG) (Santa Cruz Biotechnology). Digested samples were also analysed for AdoMetDC activity before and after passage through a Ni-NTA agarose column.

Assay of AdoMetDC activity

Enzyme activities of recombinant AdoMetDC preparations (of *T. cruzi* and human origins) were determined by measuring the release of $^{14}CO_2$ from *S*-adenosyl-L-[*carboxy*-^{14}C]methionine. The standard reaction mixture contained 50 mM Tris/HCl, pH 7.4, 1.25 mM dithiothreitol, 2.5 mM putrescine, 0.2 mM AdoMet, 0.1 μCi of *S*-adenosyl-L-[*carboxy*-^{14}C]methionine (specific radioactivity 2 mCi/mmol) and 100 μl of the enzyme preparation (equivalent to 1–10 μg of protein) in a total volume of 250 μl. The total protein content of purified *T. cruzi* AdoMetDC preparations was determined with the Bio-Rad protein assay, originally described by Bradford [31], and was used to calculate the specific activity of each enzyme preparation.

Determination of the K^{AdoMet}_m for *T. cruzi* AdoMetDC

The K_m for the recombinant *T. cruzi* AdoMetDC was established by varying the substrate (AdoMet) concentration in the reaction mixture between 0.01 and 0.6 mM. The specific radioactivity of the *S*-adenosyl-L-[*carboxy*-^{14}C]methionine substrate was kept constant at 2 mCi/mmol.

Effects of putrescine and polyamines on *T. cruzi* AdoMetDC activity

The effects of putrescine, spermidine and spermine on the activity of the recombinant *T. cruzi* AdoMetDC was determined by assaying for AdoMetDC activity in the presence of 0.5–5.0 mM putrescine, spermidine or spermine (instead of 2.5 mM putrescine). Endogenous polyamines had previously been removed from the enzyme preparation by gel filtration through a PD-10 column.

Effect of MGBG on *T. cruzi* AdoMetDC activity

The inhibitory effects of MGBG on recombinant human and *T. cruzi* AdoMetDC preparations were compared by assaying for AdoMetDC activity in the presence of 0.1 μM to 1.2 mM concentrations of the drug. The inhibitory effect of MGBG on recombinant *T. cruzi* AdoMetDC was also analysed after removal of the C-terminal His tag by digestion with carboxypeptidase A.

RESULTS

Isolation and sequencing of the AdoMetDC gene from *T. cruzi*

A 482 bp AdoMetDC-specific DNA fragment was amplified from genomic *T. cruzi* DNA by PCR, by using degenerate primers based on conserved amino acid sequences within the AdoMetDC genes of *L. donovani* and *T. brucei*. This fragment was cloned and sequenced to verify that it represented a portion of AdoMetDC sequence. After being labelled with ^{32}P, the DNA fragment was used as a probe for the isolation of the entire AdoMetDC locus from high-density filters of a large-fragment genomic *T. cruzi* library [26]. Screening of the filters yielded 16 positive cosmid clones. Assuming a diploid genome size of approx. 90 Mbp, as estimated for the CL Brener clone ([32], and J. Swindle, personal communication), the cosmid library [26] would represent approx. 16 genome equivalents. The corresponding figures indicate that the AdoMetDC gene locus in *T. cruzi* most probably contains a single-copy gene, which is not tandemly duplicated in the genome as are some other genes. This view is also supported by Southern blot analyses of genomic high-molecular-mass *T. cruzi* DNA digested with restriction

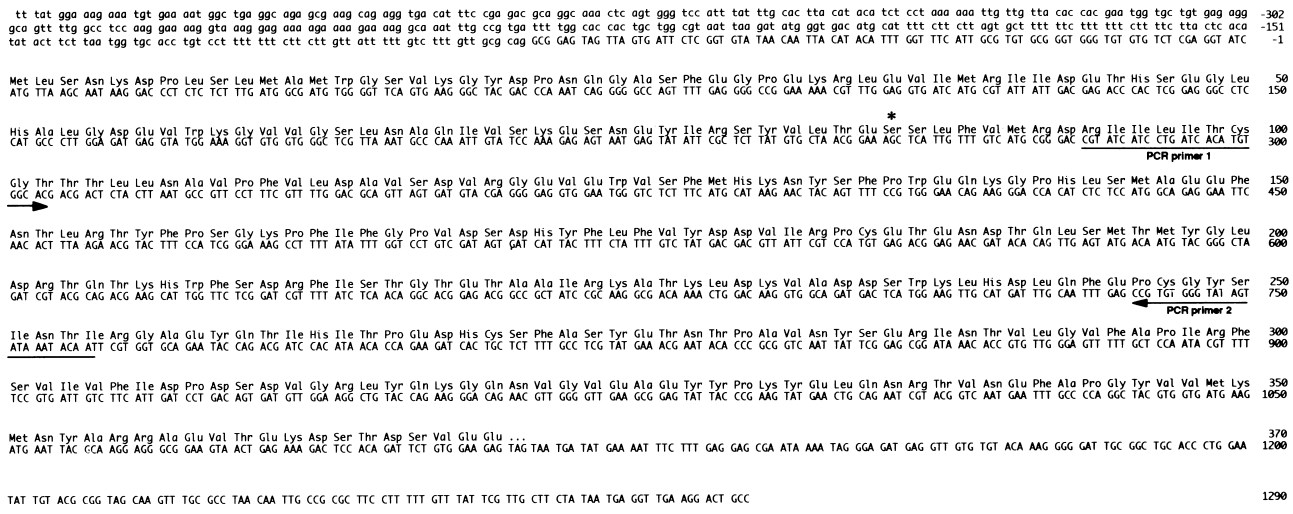

Figure 1 Nucleotide and amino acid sequences of the *T. cruzi* AdoMetDC gene and its predicted protein product

The nucleotide sequence of the protein coding region of the *T. cruzi* AdoMetDC gene and adjacent 5′ and 3′ regions were determined as described in the Materials and methods section. Numbering of the nucleotides begins with the A (+1) in the first ATG (the putative translation start codon) and ends with nt 1290, 177 nt downstream of the TAG stop codon. The predicted amino acid sequence begins with the initiating amino acid (Met^{1}) and ends with Glu^{370} juxtaposed to the termination signal TAG. The 1110 nt ORF specifies a sequence of 370 residues, which corresponds to a proenzyme of AdoMetDC. As in other prokaryotes and eukaryotes, the proenzyme is likely to be processed to a large α subunit and a small β subunit. In *T. cruzi* AdoMetDC, the bond split is likely to occur between Glu^{85} and Ser^{86} (*). In this process the serine residue is converted to pyruvate, which becomes the N-terminus of the large α subunit and acts as the prosthetic group of the enzyme. The glutamic residue becomes the C-terminus of the small β subunit. The two degenerate PCR primers employed in isolating the 482 bp AdoMetDC-specific probe, which was then used in the screening of the *T. cruzi* genomic library, are shown (arrows). For the determination of the SL junction site, three different primers (not shown in the figure) were used; one sense primer (the characteristic 35 nt sequence of the *T. cruzi* SL) and two anti-sense primers (complementary to nt 302–321 and nt 604–621 in the ORF). Nucleotides 5′ to the splice acceptor site are printed in lower-case letters. In the processed transcript the 126 nt 5′ UTR (generated by splicing the 39 nt mini-exon to the 87 nt 5′ UTR sequence shown) contains no small ORF corresponding to the *cis*-acting MAGDIS sequence found in mammals. The end of the 3′ UTR cannot be deduced from the nucleotide sequence because there are no traditional polyadenylation signals in *T. cruzi* genes.

endonucleases having a single recognition site in the coding sequence of the AdoMetDC gene (see below).

One of the 16 AdoMetDC-positive clones, designated cSAMtc, was propagated and subjected to linear amplification sequencing. Our sequencing strategy produced the entire nucleotide sequence of the *T. cruzi* AdoMetDC gene as well as several hundred nucleotides of 5′ and 3′ flanking sequences (Figure 1). The entire nucleotide sequence was consolidated by sequencing of the complementary strand.

In accordance with other trypanosomatid genes, the *T. cruzi* AdoMetDC gene contained no introns. Its uninterrupted coding region of 1110 nt predicts a protein of 370 residues, with a calculated molecular mass of 42 107 Da. The amino acid sequence corresponds to the proenzyme form of AdoMetDC, observed in all prokaryotic and eukaryotic species studied [16,19]. On the basis of the similarity of a sequence (LTESS^{87}) in the *T. cruzi* AdoMetDC and a sequence (LSESS) that is highly conserved in all other known eukaryotic AdoMetDCs [33], the *T. cruzi* AdoMetDC proenzyme is likely to be processed to an α subunit and a β subunit with calculated molecular masses of 32 682 and 9443 Da respectively. The bond split is expected to occur between Glu^{85} and Ser^{87}, in a process in which the serine is converted to pyruvate. The pyruvate should become the N-terminus of the large α subunit and act as the prosthetic group of the enzyme [14,17]. The glutamic acid should become the C-terminus of the small β subunit. Together they might form an $\alpha_2\beta_2$ complex with two catalytic centres, as demonstrated for the mammalian enzyme [14].

At variance with many other trypanosomatid genes (including the *L. donovani* ODC gene), in which the third codon position is extremely biased to G/C [34], only 192 of the 370 codons in the *T. cruzi* AdoMetDC gene, i.e. 52 %, contained a G or C residue in the third position. In the first and second codon positions the frequency of G/C was 51 % and 37 % respectively.

The predicted amino acid sequence for the *T. cruzi* AdoMetDC is of the same length as that of *T. brucei* (Figure 2) but 22 residues shorter than that of *L. donovani* (Figure 2) and 36–37 residues longer than that of mammals [14,16]. The overall sequence identity is 68 % between the primary structure of the *T. cruzi* AdoMetDC and the corresponding AdoMetDC enzymes from *T. brucei* and *L. donovani*. The sequence comparisons (Figure 2) indicate that the *L. donovani* enzyme contains extensions in the N-terminal and C-terminal regions. Therefore, when aligning comparable sequences (370 residues), a somewhat greater similarity is seen between the *T. cruzi* and the *L. donovani* AdoMetDCs (72 %) than between the *T. cruzi* and the *T. brucei* enzymes (68 %).

The isoelectric points for the *T. cruzi* and the human AdoMetDCs, as calculated on the basis of their amino acid sequences, differed significantly. However, pI values of 4.67 for the *T. cruzi* enzyme and 5.58 for the human enzyme show that both are acidic proteins.

Determination of the position of the SL junction site in the 5′ UTR of the primary transcript of the *T. cruzi* AdoMetDC gene

Synthesis of mRNA in *T. cruzi* involves the process of *trans*-splicing, in which a 39 nt mini-exon, which is identical for all mRNA species, is joined to a splice site in the 5′ UTR of a presumably polycistronic mRNA [35]. Two cDNA species were amplified from total *T. cruzi* RNA by RT–PCR. An oligo(dT) primer was used in the reverse transcriptase reaction. In the subsequent amplification reaction, the 39 nt SL sequence served as the sense primer, and two internal primers [located in the first

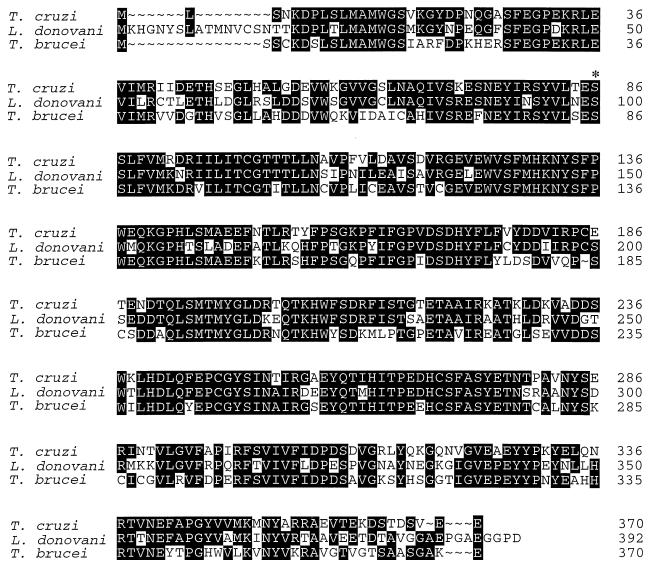

Figure 2 Comparison of the amino acid sequence of the *T. cruzi* AdoMetDC protein with its counterpart in other trypanosomatids

An optimized sequence alignment of the predicted amino acid sequences of the AdoMetDC proteins from *T. cruzi*, *L. donovani* and *T. brucei* is shown. The latter sequences were obtained through GenBank accession numbers U20092 and U20091 respectively. Amino acids identical in all three species are shown as white letters on black background. In the *T. cruzi* sequence, the asterisk denotes Ser^{86}, which is split from Glu^{85} and converted to pyruvate. The pyruvate becomes the N-terminus of the large α subunit and acts as the prosthetic group of the enzyme. The corresponding residues in *L. donovani* and *T. brucei* AdoMetDC are Ser^{100} and Ser^{86} respectively. The program SEQPUP was used for making the sequence alignment and MACBOXSHADE for shading the areas of identity.

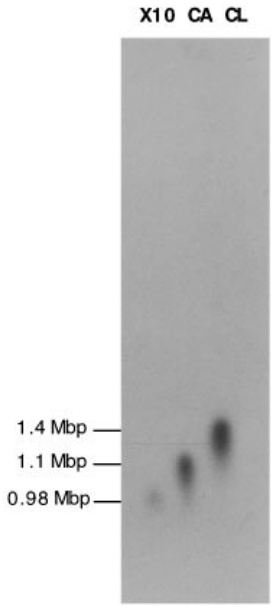

Figure 3 Chromosomal localization of the *T. cruzi* AdoMetDC gene

The 482 bp PCR fragment, comprising part of the AdoMetDC gene, was hybridized to Southern blots of *T. cruzi* chromosomes separated by PFGE. The blot shown represents a separation of larger chromosomes from three different strains of *T. cruzi*. The AdoMetDC gene is associated with chromosomal bands with sizes of approx. 1.4, 1.1 and 0.98 Mbp in the strains CL Brener (CL), CA-1/72 (CA) and Sylvio X10/7 (X10) respectively.

third (nt 300–321) and in the middle (nt 604–621) of the ORF respectively] served as individual anti-sense primers.

PCR cloning and linear amplification sequencing revealed a nucleotide sequence in which the SL junction site of the AdoMetDC transcript was identical for both cDNAs. Figure 1 shows the location of the splice site, at nt -87 from the predicted translation initiation site (nt -1), with the nucleotides 5′ to the splice acceptor site printed in lower-case letters. Consensus sequences are present for 5′ and 3′ splice sites conforming to the GU/AG rule at the appropriate positions in the SL RNA [27] and in the AdoMetDC gene sequence (Figure 1) respectively.

5′ UTR sequence

The 5′ UTR of the *T. cruzi* AdoMetDC mRNA is composed of the 39 nt SL RNA sequence (including the 'cap 4 structure', i.e. the 7-methylguanosine cap and the adjacent four nucleotides, which are also modified with methyl groups [35,36]) and an 87 nt sequence derived from the AdoMetDC gene. In the 5′ UTR there is no upstream ORF corresponding to that found in mammalian AdoMetDC mRNA species, which encodes a peptide with the sequence MAGDIS [37]. The splice site in the *T. cruzi* 5′ UTR (Figure 1) exhibits a great resemblance to the 3′ end of introns in eukaryotes with two long stretches of pyrimidines (22 and 16 nt long) preceding the consensus sequence NCAG↓G (↓ indicating the splice site), where AG↓G is invariant.

Mapping of the AdoMetDC gene in the *T. cruzi* genome

The chromosomes of *T. cruzi* cannot be studied by conventional cytogenetics because they do not condense during mitosis. However, they can be partly separated by PFGE, which allows the identification of up to 42 distinct bands representing chromosomes ranging in size from 0.45 to 4.0 Mbp [30,38]. Most of the chromosomal bands are suggested to represent pairwise homologous chromosomes, although large differences between several homologous chromosome pairs are present in a number of *T. cruzi* strains. The maximal number of chromosomes in the CL Brener clone was estimated to be at least 64 [32,38] and the total nuclear DNA size approx. 90 Mbp excluding the kinetoplast DNA ([32], and John Swindle, personal communication). In the present study, a fragment of the AdoMetDC gene was hybridized to Southern blots of PFGE-separated *T. cruzi* chromosomes. In the cloned stock CL Brener, the gene was located on chromosome(s) with a size of approx. 1.4 Mbp (Figure 3). In the clones CA-1/72 and Sylvio X10 the AdoMetDC gene is located on chromosomes of slightly smaller sizes, approx. 1.1 and 0.98 Mbp respectively (Figure 3).

Determination of the AdoMetDC gene copy number in the *T. cruzi* genome by Southern blot analysis

Protozoan parasites have some of their genes organized in tandemly repeated arrays. To determine whether the *T. cruzi* AdoMetDC gene locus exhibits a similar organization, genomic high-molecular-mass *T. cruzi* DNA was analysed by Southern blotting. The DNA was digested to completion, with either *Eco*RI or *Nco*I, which cut only once within the AdoMetDC gene. After agarose gel electrophoresis and blotting, the restriction fragments were hybridized to a radiolabelled probe spanning the entire AdoMetDC coding region. Two bands were generated (Figure 4), indicating the presence of a single copy, and no tandem repeats, of the AdoMetDC gene in the *T. cruzi* genome. The same result was obtained for the cSAMtc cosmid clone after digestion with *Eco*RI (results not shown).

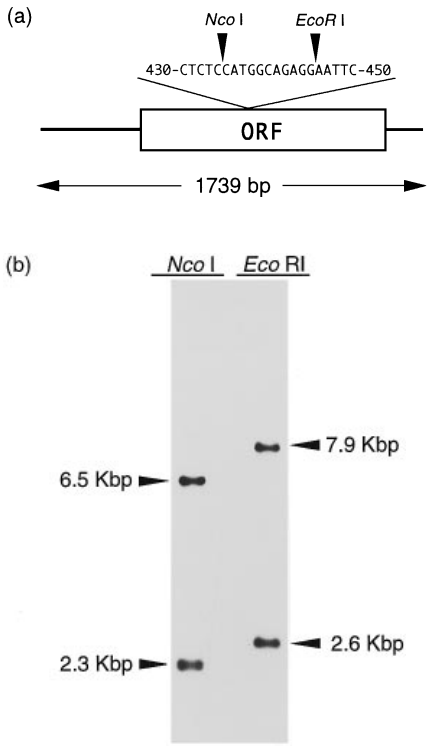

Figure 4 Copy number of the AdoMetDC gene in the *T. cruzi* genome

(**a**) Schematic drawing showing the only restriction sites for *Eco*RI and *Nco*I within a sequence of 1739 bp of the *T. cruzi* AdoMetDC gene locus. Digestion with either restriction endonuclease should cleave the ORF in half. A single-copy gene should generate two bands, for both digests, when probing a Southern blot with a *T. cruzi* AdoMetDC PCR fragment spanning the entire ORF. (**b**) Southern blot analysis of *Eco*RI and *Nco*I digests of genomic high-molecular-mass *T. cruzi* DNA, separated by agarose gel electrophoresis. The presence of two bands demonstrates that the *T. cruzi* AdoMetDC gene is a single-copy gene. Identical results were found when digesting the SAMtc cosmid clone with *Eco*RI (results not shown).

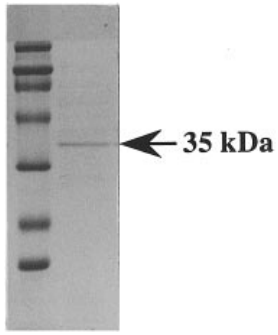

Figure 5 Electrophoretic mobility of the protein encoded by the *T. cruzi* AdoMetDC gene

His-tagged *T. cruzi* AdoMetDC protein, expressed in *E. coli* strain M15[pREP4], was purified by metal chelate-affinity chromatography with a Ni-NTA resin, separated by SDS/PAGE [12% (w/v) gel] and stained with Coomassie Brilliant Blue G-250. The migration of His-tagged AdoMetDC protein (contained in one of the fractions eluted from the Ni-NTA column after the addition of 0.1 M imidazole as competitor; right lane) was compared with protein standards with molecular masses of 200, 97.4, 69, 46, 30, 21.5 and 14.3 kDa (top to bottom; left lane). When the -Gly-Ser-His_6 tag is taken into account, the estimated molecular mass of 35 kDa for the purified, enzymically active, protein corresponds well with the molecular mass of 34 kDa for the α subunit of the protein, calculated on the basis of its amino acid sequence. This implies that the enzyme has been autocatalytically cleaved and that the catalytic activity resides with the α subunit of the protein.

Expression of the *T. cruzi* AdoMetDC gene in *E. coli* and purification of the recombinant protein

To facilitate the purification and characterization of the protein encoded by the *T. cruzi* AdoMetDC gene, an affinity tag (His_6) was fused to the coding sequence of AdoMetDC. The tag was placed at the C-terminus mainly because the presence of a C-terminal tag demonstrates complete translation of the tagged protein. After expression of the *T. cruzi* AdoMetDC gene construct in *E. coli*, the recombinant protein was purified by metal chelate-affinity chromatography and separated by SDS/PAGE (Figure 5). The electrophoretic mobility indicated a molecular mass of 35 kDa (Figure 3) for the purified protein, including the C-terminal extension (-Gly-Ser-His_6) with its calculated molecular mass of 966 Da. Therefore the molecular mass of the enzyme proper should be approx. 34 kDa. This figure corresponds well to the α subunit of the enzyme with a calculated molecular mass of 33 kDa (Figure 1).

Taken together, our sequence and electrophoretic mobility results suggest that *T. cruzi* AdoMetDC first appears as a proenzyme (with a deduced molecular mass of approx. 42 kDa), which is then autocatalytically cleaved at the Glu^{85}-Ser^{86} peptide bond to form a 33–34 kDa α subunit (identified by SDS/PAGE) and a β subunit (with a deduced molecular mass of approx. 9 kDa). Together these subunits might form one or two catalytic centres, as shown for the mammalian enzyme [14]. The prosthetic group required for enzymic activity is contained in the α subunit, where a pyruvoyl moiety is probably generated by conversion of Ser^{86}, as in the corresponding site of mammalian AdoMetDCs. The fact that the purified recombinant AdoMetDC exhibited enzymic activity is in itself evidence of cleavage of the proenzyme, because this process generates the prosthetic group of the enzyme and is thus required for catalytic activity.

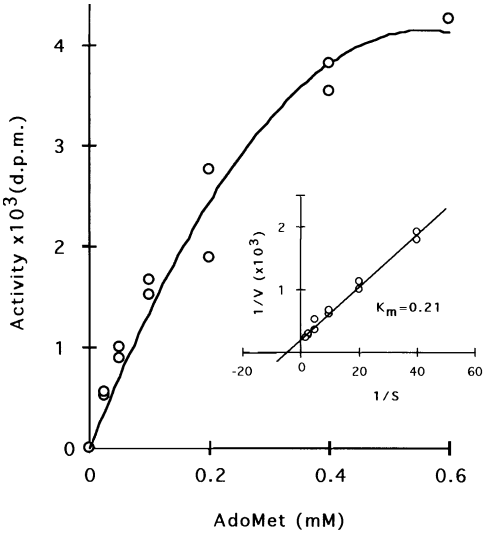

Figure 6 Effect of substrate (AdoMet) concentration on *T. cruzi* AdoMetDC activity

AdoMetDC activity was assayed under standard conditions in the presence of 2.5 mM putrescine, with variations in the concentration of AdoMet. Purified recombinant *T. cruzi* AdoMetDC was used as enzyme source. The inset depicts a Lineweaver–Burk double-reciprocal plot of reaction velocity against substrate concentration in one representative experiment. The apparent K_m for AdoMet is 0.21 ± 0.04 mM (S.E.M., $n = 5$).

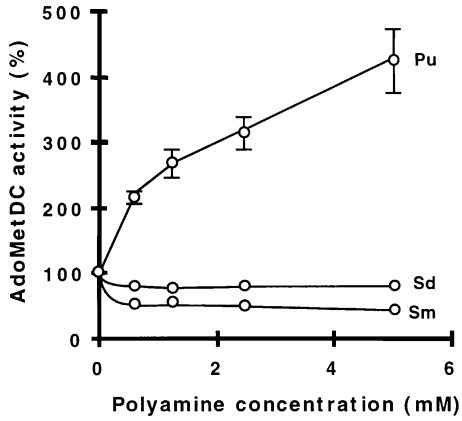

Figure 7 Effects of putrescine, spermidine and spermine on *T. cruzi* AdoMetDC activity

AdoMetDC activity was assayed under standard conditions in the presence of various concentrations of putrescine (Pu), spermidine (Sd) or spermine (Sm). Purified recombinant *T. cruzi* AdoMetDC was used as enzyme source. Points are means ± S.D. ($n = 3$). For the effects of spermidine and spermine on the AdoMetDC activity, the error bars were all within the limits of the points drawn.

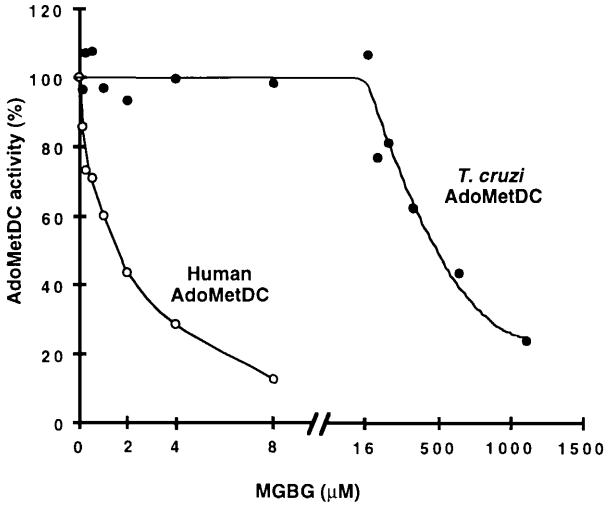

Figure 8 Effects of MGBG on the activity of human and *T. cruzi* AdoMetDCs

AdoMetDC activity was assayed under standard conditions in the presence of 2.5 mM putrescine, and MGBG was added to a final concentration of 0.1 μM to 1.2 mM. Purified recombinant AdoMetDCs of human and *T. cruzi* origin were used as enzyme sources.

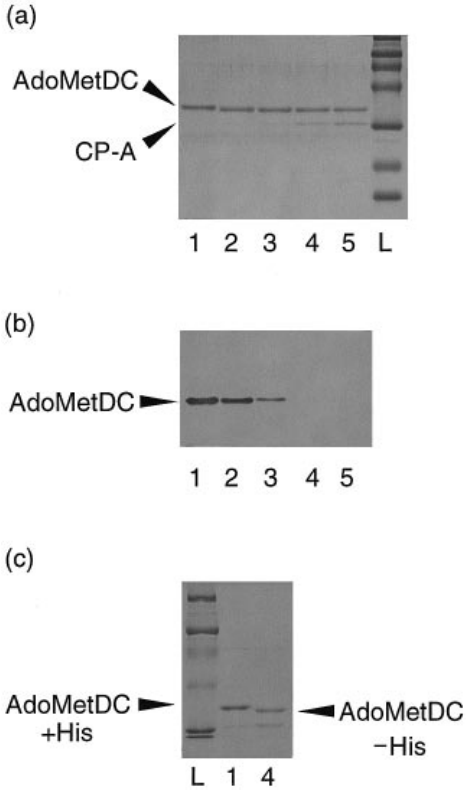

Figure 9 Removal of the C-terminal His tag of the recombinant *T. cruzi* AdoMetDC by digestion with carboxypeptidase A

(**a**) His-tagged *T. cruzi* AdoMetDC was treated with carboxypeptidase A (CP-A) at room temperature for 1 h, separated by SDS/PAGE [12% (w/v) gel] and stained with Coomassie Brilliant Blue G-250. The amount of His-tagged *T. cruzi* AdoMetDC was kept constant (2.6 μg), whereas the amount of CP-A was varied. Lane 1, untreated AdoMetDC; lane 2, AdoMetDC treated with 0.052 μg of CP-A; lane 3, AdoMetDC treated with 0.104 μg of CP-A; lane 4, AdoMetDC treated with 0.260 μg of CP-A; lane 5, AdoMetDC treated with 0.520 μg CP-A. The migration of the proteins was compared with protein standards with molecular masses of 200, 97.4, 69, 46, 30, 21.5 and 14.3 kDa (top to bottom; lane L). (**b**) A gel, identical with that in (**a**), was subjected to Western blot analysis, with an anti-His monoclonal antibody. The gradual removal of the His tag with increasing concentration of CP-A is revealed in lanes 1–5 [see (**a**) for identification]. (**c**) Evaluation of the extent of C-terminal truncation by comparison of the migration in an 8% (w/v) gel of untreated *T. cruzi* AdoMetDC (lane 1) with that of AdoMetDC treated with 0.4 μg of CP-A (lane 4). It is estimated that only the C-terminal extension including the His tag, corresponding to a molecular mass of 966 Da, was removed by this treatment. The migration of the proteins was compared with protein standards with molecular masses of 200, 97.4, 69, 46, 30, 21.5 and 14.3 kDa (top to bottom; lane L).

The fractions collected from the affinity column during elution with 0.1 M imidazole were assayed for AdoMetDC activity, and those containing the highest activities were pooled and used for characterization of the enzyme. The specific activity of the purified enzyme when assayed in the presence of 0.2 mM AdoMet and 2.5 mM putrescine was 1.5 units/mg of protein, with 1 unit of enzyme activity being defined as that quantity releasing 1 nmol of CO_2/min.

Kinetic properties of the *T. cruzi* AdoMetDC enzyme

The AdoMetDC reaction exhibited classic Michaelis–Menten kinetics with regard to changes in substrate (AdoMet) concentration, in the presence of putrescine (2.5 mM) (Figure 6). The K_m for AdoMet was calculated to be 0.21 ± 0.04 mM (mean ± S.E.M., $n = 5$) from Lineweaver–Burk double-reciprocal plots of the rate of catalysis against AdoMet concentration. This value is significantly higher than those reported for AdoMetDCs isolated from various mammalian species [19].

Effects of diamines and polyamines on *T. cruzi* AdoMetDC activity

The *T. cruzi* AdoMetDC activity was stimulated more than 4-fold at a putrescine concentration of 5 mM (Figure 7) but was inhibited by spermidine and spermine. Spermidine was only slightly inhibitory, whereas spermine, at concentrations exceeding 0.5 mM, decreased the AdoMetDC activity by approx. 50%.

Table 1 Effects of MGBG on the activity of *T. cruzi* AdoMetDC with or without a C-terminal His tag

	AdoMetDC with His tag		AdoMetDC without His tag	
[MGBG] (μM)	(units/mg of protein)	(%)	(units/mg of protein)	(%)
0	0.576	100	0.153	100
32	0.523	91	0.147	96
320	0.448	78	0.093	61
992	0.239	41	0.041	27

Effects of MGBG on *T. cruzi* and human AdoMetDC activities

In accordance with another investigation [39], MGBG was found to be a potent inhibitor of human AdoMetDC activity, with an IC_{50} of approx. 1.5 μM (Figure 8). AdoMetDC, isolated from another trypanosomatid, *T. brucei brucei*, was reported to be significantly less sensitive to MGBG, with an IC_{50} of 32 μM [39]. Notably, the *T. cruzi* enzyme was even less sensitive (Figure 8). It was completely unaffected by MGBG concentrations up to 16 μM, and the IC_{50} was somewhat less than 500 μM, i.e. more than 300-fold that of human AdoMetDC.

Effects of MGBG after removal of the His tag from the recombinant *T. cruzi* AdoMetDC protein

When the C-terminal affinity tag was removed from the recombinant *T. cruzi* AdoMetDC by digestion with carboxypeptidase A (Figure 9), the truncated recombinant enzyme exhibited the same degree of inhibition as the His-tagged enzyme when treated with MGBG (Table 1). Therefore it can be concluded that the lower sensitivity to MGBG of the *T. cruzi* AdoMetDC, 1/300 that of the human enzyme, is an inherent property of the enzyme and not an artifact associated with the C-terminal His tag used for purification.

DISCUSSION

Putrescine, spermidine and spermine are ubiquitous in Nature and are required in many growth and differentiation processes [40]. Most organisms synthesize their putrescine from ornithine by the action of ODC, but synthesis from arginine by the action of arginine decarboxylase and agmatinase has also been observed in bacteria and plants. In *T. cruzi*, however, there is no evidence of ODC activity, and although trace amounts of arginine decarboxylase activity have been reported [41,42], it remains unclear to what extent the agmatine pathway contributes to putrescine formation [13]. The findings that enzyme-activated irreversible inhibitors of ODC and arginine decarboxylase exert no effect, or only a slight effect, on *T. cruzi* multiplication [11,12,43] are also consistent with the view that this organism has no capacity to synthesize its own putrescine. Instead these parasites seem to depend on putrescine uptake for their synthesis of spermidine and spermine. In fact, *T. cruzi* epimastigotes (the insect stage) have been shown to take up exogenous putrescine by a rapid, high-affinity, transport system, showing high specificity for putrescine but low specificity for spermidine and spermine [44].

The fact that *T. cruzi* parasites are deficient in enzyme activities responsible for putrescine synthesis *de novo* makes AdoMetDC a prime target for antiparasitic agents. AdoMetDC catalyses an essential step in the synthesis of spermidine and spermine, the formation of decarboxylated AdoMet [14]. The latter is the actual donor of aminopropyl groups to putrescine and spermidine, thus forming spermidine and spermine by the action of spermidine synthase and spermine synthase respectively. To facilitate the development and analysis of drugs that specifically and selectively inhibit the AdoMetDC activity of *T. cruzi* in a human host, we have cloned, sequenced and mapped the *T. cruzi* AdoMetDC gene. The amino acid sequence of the enzyme was deduced from the nucleotide sequence of the gene and compared with that of human AdoMetDC. It was found to correspond closely to the proenzyme form of AdoMetDC, which has to go through autocatalytic cleavage to generate the prosthetic group essential for catalytic activity.

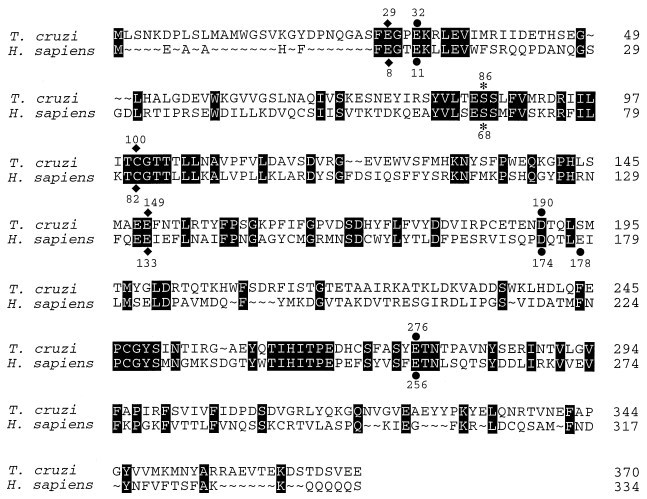

Figure 10 Comparison of the amino acid sequences of the *T. cruzi* and the human AdoMetDC proteins

An optimized sequence alignment of the predicted amino acid sequences of the AdoMetDC proteins from *T. cruzi* and its human host is shown. Amino acids identical in the proteins from both species are shown as white letters on a black background. The asterisk denotes the serine residue (Ser^{86} and Ser^{68} in the *T. cruzi* and human proenzymes respectively) that is converted to pyruvate. Acidic sites to which putrescine might bind are indicated with black dots and numbers, and other sites that might be of importance in processing or activity are indicated with black diamonds and numbers. SEQPUP was used for making the sequence alignment and MACBOXSHADE for shading the areas of identity.

Studies on AdoMetDC proenzymes of other eukaryotes, from yeast [45] to human [17], have shown that the cleavage site is contained in the sequence LSESS, with the underlined serine [Ser^{68} in the human enzyme; Ser^{86} in the *T. cruzi* enzyme (Figure 10)] forming the pyruvate. Among trypanosomatids the corresponding amino acid sequence differs in the second position (shown in bold type), which is a threonine residue (Thr^{84}) in *T. cruzi* (Figure 10), asparagine (Asn^{98}) in *L. donovani* and serine (Ser^{84}) in *T. brucei* (as in higher eukaryotes). Because *T. cruzi* AdoMetDC is cleaved autocatalytically, it can be concluded that at least a serine to threonine substitution in the second position is compatible with cleavage. So far, cleavage of the *L. donovani* AdoMetDC proenzyme has not been studied experimentally. However, studies with the use of site-directed mutagenesis indicate that replacement of the corresponding serine ($Ser^{66} \rightarrow$ Ala) in the human AdoMetDC enzyme has no effect on proenzyme cleavage [17].

AdoMetDC processing and activity are stimulated by putrescine [18,19]. This has obvious physiological significance because of the resulting formation of decarboxylated AdoMet, the aminopropyl group donor in the reaction in which putrescine is converted to spermidine. Site-directed mutagenesis studies of

the human proenzyme have shown that the stimulation of processing mediated by putrescine requires an interaction with four acidic residues, Glu^{11}, Asp^{174}, Glu^{178} and Glu^{256} [18,19,33]. Therefore it has been proposed that the binding to these acidic sites of two molecules of putrescine, each having two positive charges, might cause the change in conformation that is a prerequisite for the processing reaction [33]. On identification of the equivalent amino acids in *T. cruzi* AdoMetDC, i.e. Glu^{32}, Asp^{190}, Ser^{194} and Glu^{276} (Figure 10), it becomes obvious that one (Ser^{194}) of the four amino acids is not acidic. Interestingly, both *L. donovani* and *T. brucei* have a serine residue in the equivalent position, i.e. Ser^{208} and Ser^{193} respectively (Figure 2). This finding indicates that the binding of putrescine to the trypanosomatid enzymes might engage another amino acid.

In human AdoMetDC there is yet another glutamic residue (Glu^{133}) that is of importance in proenzyme processing [19]. As seen in Figure 10, the *T. cruzi* AdoMetDC also contains a glutamic residue in the corresponding position (Glu^{149}).

In addition to the sequence containing the proenzyme cleavage site, another very highly conserved sequence is the TCGTT sequence, in which the underlined cysteine residue is essential [18]. In the human AdoMetDC, this cysteine (Cys^{82}), which is equivalent to Cys^{100} in the *T. cruzi* enzyme (Figure 10), is part of the active site [18]. This region is one of the very few that show any similarity between the eukaryotic AdoMetDCs and the *E. coli* enzyme [16,19]. Thus the first three residues TCG (Cys^{140} in *E. coli* AdoMetDC [46]) are identical.

To be able to characterize the *T. cruzi* AdoMetDC protein, the gene was expressed in *E. coli* and its encoded product was purified. The purified AdoMetDC exhibited catalytic activity and a molecular mass of approx. 34 kDa, on the basis of its migration in a 12% (w/v) polyacrylamide gel. From the amino acid sequence of the *T. cruzi* AdoMetDC protein it can be concluded that this protein corresponds to the α subunit, the larger of the two subunits formed by the intramolecular cleavage of the proenzyme. The mechanism of the cleavage reaction, not only forming the two subunits but also generating the pyruvate at the N-terminus of the α subunit from a serine residue, has been described in detail by Xiong et al. [33]. Conceivably, a detailed analysis of the possible differences between the AdoMetDC proenzyme cleavage reaction in trypanosomes compared with the human host might provide a basis for the design of a new class of drugs. Having a mechanism of action based on interference with proenzyme cleavage, drugs would prevent the appearance of catalytic activity, because the prosthetic group cannot form without cleaving the proenzyme at a specific site.

When comparing the properties of the *T. cruzi* AdoMetDC with those of the human enzyme, it becomes evident that they are different in some aspects but similar in other. The K_m (AdoMet) for the *T. cruzi* enzyme was calculated to be 0.21 mM, which is significantly higher than that reported for mammalian AdoMetDCs [19]. Activation by putrescine, and inhibition by spermidine and spermine, as seen for the *T. cruzi* AdoMetDC, were also observed with the mouse enzyme [47]. However, very slight or no inhibition by spermidine and spermine was observed in studies of the human enzyme [39].

MGBG, which is a potent inhibitor of mammalian AdoMetDC, proved to be a poor inhibitor of the *T. cruzi* enzyme, precluding its use as an inhibitor of polyamine synthesis with possible chemotherapeutic effect against Chagas' disease. Another AdoMetDC inhibitor, 5′-([(*Z*)-4-amino-2-butenyl]-methylamino)-5′-deoxyadenosine, has been shown to affect *T. cruzi* parasites adversely in their ability to infect and to multiply within rat heart myoblasts [48]. It seems that the mechanism of action does not involve a decrease in production of spermidine and spermine caused by inhibition of AdoMetDC, because the addition of either polyamine did not reverse the inhibition of infectivity exerted by treatment with 5′-([(*Z*)-4-amino-2-butenyl]methylamino)-5′-deoxyadenosine. Instead the massive accumulation of AdoMet is a more plausible explanation in view of the fact that exogenous AdoMet significantly inhibited the infectivity and intracellular growth of *T. cruzi* [48]. The fact that African trypanosomes have a unique transporter of AdoMet, whereas mammalian cells apparently do not, indicates a selective route for delivery of AdoMet analogues with trypanocidal action [49]. Obviously, even if a similar AdoMet transporter were present in *T. cruzi*, the effects of AdoMet analogues in Chagas' disease would be limited to extracellular forms of the parasite.

In view of the fact that safe and efficient chemotherapeutic approaches to *T. cruzi* infections are non-existent, it is important that the search for alternative drugs is intensified. The remarkable effectiveness of polyamine synthesis inhibitors against African trypanosomiasis, and their limited toxicity to the patient, suggests that targeting of polyamine biosynthetic enzymes in *T. cruzi* parasites might provide an approach to the effective treatment of Chagas' disease, at least in its acute phase. With the present demonstration of an AdoMetDC in *T. cruzi*, we have identified the initial and rate-limiting enzyme in polyamine synthesis, and a possible target for therapeutic approaches. Further characterization of the *T. cruzi* AdoMetDC compared with that of the human host might provide a basis for selective interference with polyamine synthesis in the parasite, while sparing the host.

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