Allosteric regulation of an essential trypanosome polyamine biosynthetic enzyme by a catalytically dead homolog

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African sleeping sickness is a fatal disease that is caused by the protozoan parasite Trypanosoma brucei. Polyamine biosynthesis is an essential pathway in the parasite and is a validated drug target for treatment of the disease. S-adenosylmethionine decarboxylase (AdoMetDC) catalyzes a key step in polyamine biosynthesis. Here, we show that trypanosomatids uniquely contain both a functional AdoMetDC and a paralog designated prozyme that has lost catalytic activity. The T. brucei prozyme forms a high-affinity heterodimer with AdoMetDC that stimulates its activity by 1,200-fold. Both genes are expressed in T. brucei, and analysis of AdoMetDC activity in T. brucei extracts supports the finding that the heterodimer is the functional enzyme in vivo. Thus, prozyme has evolved to be a catalytically dead but allosterically active subunit of AdoMetDC, providing an example of how regulators of multimeric enzymes can evolve through gene duplication and mutational drift. These data identify a distinct mechanism for regulating AdoMetDC in the parasite that suggests new strategies for the development of parasite-specific inhibitors of the polyamine biosynthetic pathway.

gene duplication | S-adenosylmethionine decarboxylase | prozyme | Trypanosoma brucei

African sleeping sickness is a neglected disease that is caused by the protozoan parasite Trypanosoma brucei. It is epidemic in subSaharan Africa, where 60 million people are at risk, and tens of thousands die annually of this disease (1). Without treatment, the disease is always fatal. Current drug therapies are limited by toxicity and the requirement for complex treatment regimes (2). Polyamines are essential for cell growth in all organisms. They influence chromatin structure, modify translation initiation factor eIF5A, and regulate gene expression through a number of mechanisms (3–6). In trypanosomes, the polyamine spermidine is conjugated with glutathione to form a unique cofactor termed trypanothione that functions in cellular redox reactions (7). Excess polyamines lead to cancer in mammalian cells (4), and inhibitors of polyamine biosynthesis have been widely studied for their potential as antiproliferative agents (6, 8). The most successful clinical application of these inhibitors is the treatment of African sleeping sickness with the ornithine decarboxylase suicide inhibitor α-difluoromethylornithine (efornithine) (9). Efornithine is the only therapy for sleeping sickness with a known mechanism of action, and its effectiveness demonstrates the importance of the polyamine pathway to the parasite.

S-adenosylmethionine decarboxylase (AdoMetDC) is required for the formation of the precursor used for the synthesis of spermidine and spermine from putrescine, and in mammalian cells it is a rate-limiting step in polyamine formation (10). Knockout of the AdoMetDC gene in the trypanosomatid Leishmania donovani led to spermidine auxotrophy (11). The suicide inhibitor of AdoMetDC, MDL73811, has demonstrated efficacy in animal models of both T. brucei (12) and a related parasite Trypanosoma cruzi (13), which is the causative agent of Chagas disease. Thus, AdoMetDC is considered a promising, but as yet unexploited target for the development of new anti-trypanosomal drugs.

AdoMetDC is a pyruvoyl-dependent enzyme and uses this cofactor to stabilize the carbanion intermediate formed during the decarboxylation reaction. The pyruvoyl-moiety derives from an autocatalytic cleavage reaction that generates the active enzyme consisting of two chains (βα), with the pyruvoyl group formed at the N terminus of the α-chain (14, 15). Human AdoMetDC is a homodimer, and both the processing reaction and decarboxylation of AdoMet are stimulated by putrescine (10, 16). The x-ray structure shows that the active sites sit in a large cleft between β-sheets distal from the dimer interface and that the putrescine-binding sites are formed by a group of acidic residues in the β-sandwich core ~15 Å from the active sites (14, 17). This site is eliminated in the structure of the monomeric plant enzyme, which is fully active without putrescine (18). The putrescine-binding site is partially conserved in the trypanosomatid enzymes. Putrescine stimulates the activity of the recombinant T. cruzi enzyme, but it is not required for processing (19–23). Perplexingly, the putrescine-activated T. cruzi enzyme has significantly lower catalytic efficiency than the enzyme from mammals and plants, thus suggesting the possibility that other regulatory factors are necessary for enzyme function.

The polyamine biosynthetic and catabolic enzymes are tightly regulated in animals, plants, and yeast (3, 4, 24). Unusually, in the trypanosomatid parasites, analogous regulatory mechanisms for the control of polyamine biosynthesis have not been identified. Here, we show that T. brucei AdoMetDC is activated by formation of a heterodimer with a catalytically inactive regulatory subunit termed prozyme that arose in the trypanosomatids as a gene duplication of the ancestral enzyme. The regulation of AdoMetDC by an inactive homolog is unique to the trypanosomatid parasites. The finding has implications for both the regulation of polyamines in the parasite and for the development of enzyme inhibitors that will block this essential pathway.

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This article is a PNAS Direct Submission. A.E.P. is a guest editor invited by the Editorial Board. Abbreviations: AdoMetDC, S-adenosylmethionine decarboxylase; BF, blood stream form trypanosomes; PF, procyclic form trypanosomes; MDL 73811, 5′-[(Z)-4-amino-2-butenyl] methylaminio]-5′-deoxyadenosine; AdoMetDC/prozyme refers to the purified complex between AdoMetDC and prozyme.

Data deposition: The sequences reported in this paper have been deposited in the GenBank database [accession nos. EF545594 (Prozyme) and EF545595 (AdoMetDC)].

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Genomic Analysis of the Trypanosomatid AdoMetDC Family. Evolutionary analysis of the AdoMetDC family indicates that the trypanosomatids, *T. brucei*, *T. cruzi*, and *Leishmania major*, encode two types of AdoMetDC genes: an ortholog to the functional enzyme in other species, AdoMetDC, and a paralog defined herein as prozyme [Fig. 1 and see supporting information (SI) Fig. 4], named in analogy to the ornithine decarboxylase inhibitory protein, antizyme (25).

AdoMetDC and prozyme are found in close proximity in the genome; however, they have diverged significantly; prozyme shares only ~30% amino acid sequence identity with the AdoMetDC from the same trypanosomatid species. Sequence analysis suggests that prozyme is not present outside of the trypanosomatid lineage. Thus, it appears to have arisen by gene duplication of the ancestral enzyme after the divergence of the trypanosomatids from other eukaryotes.

Northern blot analysis demonstrates that both AdoMetDC and prozyme are expressed in blood form and procyclic *T. brucei* parasites, suggesting that they both will have a functional role in the parasites (Fig. 2A). However, the prozyme is missing several critical residues required for processing and catalytic activity (10, 14, 15, 17, 20). Therefore the prozyme is unlikely to display AdoMetDC activity and must have a unique function.

Recombinant *T. brucei* AdoMetDC Has Low Catalytic Efficiency. *T. brucei* AdoMetDC was expressed and purified from *Escherichia coli* to evaluate its kinetic properties. Recombinant *T. brucei* AdoMetDC is processed to generate the pyruvoyl cofactor (Fig. 3A), and it catalyzes the decarboxylation of AdoMetDC (Table 1 and SI Fig. 5). Like the *T. cruzi* enzyme (19–23), *T. brucei* AdoMetDC is stimulated by putrescine but not sufficiently to generate a catalytically efficient enzyme. The *kcat* of the putrescine-stimulated enzyme remains 320-fold lower than for human AdoMetDC. To determine whether the low activity could be caused by a change in activator specificity, several other polyamines were tested as activators. Norspermidine, spermidine, and cadaverine also activate *T. brucei* AdoMetDC; however, the activation was similar to what was observed for putrescine, suggesting that activator-specificity differences do not account for the low activity of the trypanosome enzymes (SI Fig. 5).

Characterization of AdoMetDC Activity in Blood Stream Form *T. brucei* Parasites. The kinetic data on the recombinant trypanosomatid AdoMetDCs suggested that these enzymes either have intrinsically lower activity or they are activated by a previously uncharacterized mechanism. To address this question, the specific activity of AdoMetDC in the blood form *T. brucei* parasites was determined. Decarboxylation was followed by the standard assay using [14C]-AdoMet as the substrate, and the concentration of AdoMetDC protein in the cell extract was estimated first by titrating the number of active sites with the AdoMetDC suicide inhibitor MDL73811 (26, 27) and second by Western blot (Fig. 2B). Both methods of determining AdoMetDC concentration in the extracts yielded similar results and demonstrated that the
specific activity of AdoMetDC in blood stream form *T. brucei* parasites is $3 \text{s}^{-1}$ (4 $\mu$mol/min/mg), a value that is $\sim 400$-fold higher than the $k_{\text{cat}}$ measured for the putrescine-stimulated recombinant enzyme (Table 1). These data provide evidence that the trypanosomatid enzymes in vivo have activities similar to those reported for AdoMetDC from other species [e.g., human AdoMetDC (23)].

**Prozyme Is a Regulatory Subunit of *T. brucei* AdoMetDC.** The finding that AdoMetDC in *T. brucei* cell extracts has higher activity than the recombinant enzyme suggested that an unaccounted for factor is present in the parasites that regulates the activity of the trypanosomatid AdoMetDCs. These results led us back to question the role of the prozyme. To determine whether prozyme might regulate the activity of AdoMetDC, recombinant *T. brucei* prozyme was expressed and purified from *E. coli*. The recombinant protein is a single polypeptide chain of 38 kDa that is not processed by the self-cleavage reaction (Fig. 3A). Consistent with the predictions from the sequence analysis, the purified recombinant prozyme is inactive and unable to decarboxylate AdoMet. However, when purified recombinant AdoMetDC is mixed with prozyme, the AdoMetDC activity is stimulated 1,200-fold, with maximum activity observed at a ratio of 1:4 AdoMetDC/prozyme (Fig. 3B). The requirement for excess prozyme to fully activate AdoMetDC can be attributed to the observation that prozyme is partially aggregated when purified in the absence of AdoMetDC (as demonstrated by gel-filtration analysis; SI Fig. 6).

**The AdoMetDC/Prozyme Complex Is the Catalytically Functional Enzyme.** The stability and function of the AdoMetDC/prozyme complex was assessed by copurification of the recombinant His-tagged AdoMetDC with Flag-tagged prozyme. The proteins were expressed separately and then copurified by Ni$^{2+}$-agarose and anion-exchange column chromatography. The two subunits in the purified complex elute as a single peak on gel...
filtration (SI Fig. 6), demonstrating that the complex remains associated through three successive column chromatography steps (e.g., Ni²⁺-agarose, anion exchange, and gel filtration). SDS/Page analysis suggests that prozyme and AdoMetDC are present in the purified complex at a 1:1 molar ratio and Western blot analysis using either antibody to AdoMetDC or the Flag-tag confirms the presence of both subunits (Fig. 3A). Sedimentation equilibrium analysis by analytical ultracentrifugation demonstrates that the complex is a heterodimer of AdoMetDC and prozyme, which is formed at high affinity ($K_d < 0.5 \mu M$; Fig. 3C). In contrast, the AdoMetDC and prozyme homodimers form with weaker affinity ($K_d = 50 \pm 6$ and $4 \pm 0.5 \mu M$, respectively; SI Fig. 7).

The rate constant for the decarboxylation of AdoMet by the copurified heterodimer was determined by steady-state kinetic analysis, and the $k_{cat}$ of 1.4 s⁻¹ (Table 1) is in good agreement with the activity estimated in the $T. brucei$ extracts. Unlike for the $T. brucei$ AdoMetDC homodimer, putrescine does not affect the activity of the heterodimeric enzyme (Table 1 and SI Fig. 5). In summary, AdoMetDC is activated 1,200-fold by formation of a functional heterodimer with prozyme. The AdoMetDC/prozyme complex has equivalent activity to orthologs from other species, and, like the monomeric plant enzyme, heterodimeric $T. brucei$ AdoMetDC/prozyme is not activated by polyamines.

### Discussion

The polyamine pathway is tightly regulated in many species, including mammals, plants, and yeast, where the biosynthetic enzymes are controlled by transcriptional and translational regulation, and by several posttranslational mechanisms, including regulation of protein stability (4). AdoMetDC from the trypanosomatid parasites has evolved to be regulated by a mechanism not found in any other species. Our data demonstrate that the functional AdoMetDC in the trypanosomatid parasites is a heterodimer between AdoMetDC and prozyme. The prozyme arose through gene duplication of the ancestral AdoMetDC gene after the trypanosomatids diverged from other eukaryotes. AdoMetDC and prozyme then apparently coevolved such that one subunit was subject to selective pressure to remain catalytically active, and the other was selected for its regulatory function while losing catalytic activity.

Our data suggest that regulators of enzyme function can evolve through gene duplication, followed by mutational drift that results in loss of catalytic function. In addition to catalyzing chemistry, many enzymes also form functional interactions with macromolecules. The interaction binding surfaces may be maintained in these “pseudoenzymes,” providing a perfect scaffold for the evolution of novel regulatory functions. A reported analysis of the genomes of metazoan species found that inactive homologs are common and that they are present in a large variety of enzyme families (28). Few biochemical studies demonstrating the function of pseudoenzymes have been published, however several examples suggest that they function as regulators of the active enzymes. Our discovery of a pseudoenzyme in a protozoan parasite suggests that the hijacking of inactive homologs to regulate function is a general mechanism that will be found throughout evolution.

The structural basis for the regulation of AdoMetDC by prozyme is likely to arise from the induction of an allosteric transition. Previous data on the $T. cruzi$ enzyme suggested that putrescine regulates the activity by an allosteric mechanism (23). The current observations support a model whereby putrescine induces a partial conformational change from the inactive structure toward the active one; however, the fully active conformation is realized only upon binding to the prozyme. The unusual requirement for two gene products to generate AdoMetDC activity provides a means to regulate the production of polyamines in the cell by regulating the expression level of prozyme. It remains to be determined whether the parasite utilizes this regulation in a dynamic way to change the flux through the polyamine pathway under different environmental challenges.

AdoMetDC provides an alternative and likely very effective target for the development of new antitrypanosomal drugs within a proven pathway. Our discovery of the mechanism by which AdoMetDC is activated is a key finding that will aid in the identification of potent inhibitors of this enzyme and, thus, in the generation of lead compounds that can exploit this target. Inhibitors developed against the heterodimeric enzyme may be more fully complimentary to the active site than inhibitors of the homodimer alone. In addition, our data suggest new approaches for inhibiting AdoMetDC in trypanosomes, either by blocking formation of the AdoMetDC–prozyme complex and/or by stabilizing the inactive conformation of AdoMetDC. Successful strategies for these approaches have recently been described for other proteins (29–31).

Although the evolution of the prozyme occurred by a unique gene duplication event in the trypanosomatids, gene duplication has played a key role in the evolution of the polyamine pathway and its regulation. Structural data suggests that the eukaryotic AdoMetDCs themselves arose from a gene duplication and gene fusion of the bacterial enzymes (32). Substrate-specificity changes have evolved by gene duplication in both the spermine/spermidine synthase family (33) and in the group IV decarboxylase family (34). Ornithine decarboxylase in mammalian and yeast cells is regulated by a protein inhibitor termed antizyme, which appears to be a duplication of a catalytic enzyme in the pathway, spermine–spermidine N²-acetyltransferase (35). Antizyme is, in turn, regulated by antizyme inhibitor, which is itself a pseudoenzyme, having arisen by gene duplication of ornithine decarboxylase, followed by loss of catalytic activity (36). The polyamine pathway thus provides a paradigm for the evolution of metabolic pathways and their regulation by gene duplication and divergence.

### Materials and Methods

#### Multiple Sequence Alignment and Phylogenetic Tree Generation

AdoMetDC sequences were compiled by using the National Center for Biotechnology Information program Blast with the $T. brucei$ AdoMetDC protein sequence as the search query. The prozyme sequence was found in the genome databases of the trypanosomatids (www.genedb.org/genedb/tryp), where it is annotated as putative AdoMetDC-like. Blast analysis with the $T. brucei$ AdoMetDC prozyme as the query identified the prozyme

### Table 1. Steady-state kinetic analysis of purified recombinant $T. brucei$ AdoMetDC and the copurified AdoMetDC/prozyme complex

<table>
<thead>
<tr>
<th></th>
<th>No putrescine</th>
<th>5 mM putrescine</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>$k_{cat}$ s⁻¹</td>
<td>$K_m$, mM</td>
</tr>
<tr>
<td>AdoMetDC</td>
<td>0.0013 ± 0.0004</td>
<td>0.38 ± 0.15</td>
</tr>
<tr>
<td>AdoMetDC/prozyme</td>
<td>1.4 ± 0.1</td>
<td>0.11 ± 0.02</td>
</tr>
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Data were collected in triplicate, and errors are the standard error of the mean. For human AdoMetDC, the steady-state kinetic parameters in the presence of putrescine were previously reported to be $k_{cat} = 2.6$ s⁻¹; $k_{cat}/K_m = 4.4 × 10^4$ M⁻¹ s⁻¹ (23).
and AdoMetDC were cloned and sequenced (SI Fig. 4B). The splice leader for prozyme and AdoMetDC is inserted 70 and 161 base pairs, respectively, upstream of the ATG start sites. These data confirm that the full-length ORFs were correctly predicted by the annotation of the genes in GeneDB.org.

**Northern Blot Analysis.** mRNA (1 μg) was separated by denaturing 1% agarose gel electrophoresis, transferred to a positively charged nylon membrane (BrightStar-Plus, Ambion) and cross-linked. Probe templates were prepared from plasmid DNA and radiolabeled ([32P]dATP; MP Biomedicals, Irvine, CA) probes were prepared by using the Strip-EZ PCR kit (Ambion).

**Steady-State Kinetic Analysis.** Steady-state kinetics were performed by trapping liberated 14C-O2 on a filter paper soaked in saturated barium hydroxide as described (19, 23). For homodimeric AdoMetDC, reactions were performed over a range of enzyme (1–4 μM) and 1,14C-CO2-AdoMet (Amersham) concentrations (10–160 μM monomer) with or without saturating putrescine (5 mM) or higher order polyamines (0.05 to 5 mM) at 37°C in buffer [100 mM Hepes (pH 8.0)/50 mM NaCl/l mM DTT]. Reactions were allowed to proceed for various times (5–40 min) before quenching with 6 M HCl. For assay of the heterodimeric, the AdoMetDC/prozyme copurified complex (25–400 nM, based on monomer concentration) was incubated with 14C-AdoMet (25 μM) and unlabeled AdoMet (0–975 μM) for various time points (2.5–10 min) before quenching as above. Data were fitted to the Michaelis–Menten equation to determine the steady-state kinetic parameters by using Prism (GraphPad, San Diego, CA).

**Determination of Molecular Weight of the AdoMetDC/Prozyme Complex.** The molecular weights of the complex, and the individual subunits were determined by equilibrium sedimentation analysis using a XL1 analytical ultracentrifuge (Beckman, Fullerton, CA) equipped with an AN60 Ti rotor. Samples in buffer [50 mM Hepes (pH 8.0)/50 mM NaCl/l mM 2-mercaptoethanol] were loaded into a six-sector equilibrium centrifuge and equilibrated for data collection at 15,000 and/or 20,000 rpm. After equilibrium was reached (~24 h), absorption data were collected at 280 nm through sapphire windows by using a radial step size of 0.001 cm. Baseline absorbance readings for each cell were acquired by over speed at 42,000 rpm. Data sets were analyzed by using equations (SI Fig. 7) describing a single ideal species model or a monomer–heterodimer model as described (45) or, for the AdoMetDC/prozyme complex, globally fitted to a single ideal species model by using the Beckman XL-A/XL-I Data Analysis Software version 6.0. Both analyses gave similar results.

**Synthesis of 5′-[(Z)-4-aminobutyl][methylamino]-5′-deoxyadenosine (MDL 73811).** MDL 73811 was synthesized by a previously undescribed method (SI Scheme 1) that improved yield over the published method (26, 27).

(Z)-1-BOCamine-4-chloro-2-butene: (Z)-1-amino-4-chloro-2-butene was obtained as described (28).
2-butene (10.0 g, 70.4 mmol) was dissolved in tetrahydrofuran (50 ml), di-tert-butyl dicarbonate (16.13 g, 73.9 mmol) was added as a solid, and the reaction was stirred overnight at room temperature. THF was removed, and the solid was dissolved in CH2Cl2 and extracted three times with deionized water. The CH2Cl2 layer was dried with anhydrous MgSO4 and filtered. The CH2Cl2 layer was then used to remove starting material and impurities, then MeOH/Et3N in MeOH (96/4) was used to elute the product. Fractions were collected and the solvent removed to yield a tan solid (0.34 g). LC/MS [an 1100 Series Chromatograph (Agilent Technologies, Palo Alto, CA)] confirmed purity and identity. 1H NMR spectra were equipped with a PE API2000 mass spectrometer (Sciex, South San Francisco, CA) confirmed purity and identity. The 1H NMR spectra were used to identify the compound. 

The 5’deoxy-5’-methylaminoadenosine (0.5 g) was added to a microwave vial with 20 ml of the methylene/methanol solution (2 M). The reaction vial was sealed and heated in a Biotage Initiator 60 microwave to 120 °C for 2 h. After cooling, the methanol and methylamine were removed. The resulting yellow syrup was purified by column chromatography on silica gel. First a mobile phase of CH2Cl2/MeOH/Et2N (66/30/4) was used to remove starting material and impurities, then MeOH/Et2N (96/4) was used to elute the product. Fractions were collected and the solvent removed to yield a tan solid (0.34 g). LC/MS [an 1100 Series Chromatograph (Agilent Technologies, Palo Alto, CA)] confirmed purity and identity. The 1H NMR spectra were used to identify the compound. 

The 5’deoxy-5’deoxy-5’-chloroadenosine (0.5 g) was added to a microwave vial with 20 ml of the methylene/methanol solution (2 M). The reaction vial was sealed and heated in a Biotage Initiator 60 microwave to 120 °C for 2 h. After cooling, the methanol and methylamine were removed. The resulting yellow syrup was purified by column chromatography on silica gel. First a mobile phase of CH2Cl2/MeOH/Et2N (66/30/4) was used to remove starting material and impurities, then MeOH/Et2N (96/4) was used to elute the product. Fractions were collected and the solvent removed to yield a tan solid (0.34 g). LC/MS [an 1100 Series Chromatograph (Agilent Technologies, Palo Alto, CA)] confirmed purity and identity. The 1H NMR spectra were used to identify the compound.

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