THE ROLE OF S-ADENOSYLMETHIONINE DECARBOXYLASE ON REGULATION OF POLYAMINE AND TRYPANOTHIONE METABOLISM IN *TRYPANOSOMA BRUCEI*

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DEDICATION

I dedicate this to my family, who I thank for their love and encouragement. I especially thank my husband and best friend, Andy Willert, for his constant love, understanding and support through the years.

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THE ROLE OF S-ADENOSYLMETHIONINE DECARBOXYLASE ON REGULATION OF POLYAMINE AND TRYPANOTHIONE METABOLISM IN *TRYPANOSOMA BRUCEI*

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The University of Texas Southwestern Medical Center at Dallas, 2008

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Trypanosoma brucei is the causative agent of Human African Trypanosomiasis (HAT), a fatal and neglected disease affecting Sub-Saharan Africa. Current therapeutics are limited for several reasons, underscoring the need for new and improved drugs. The polyamine/trypanothione pathway is essential for *T. brucei*, and the biosynthetic enzymes in this pathway are potential drug targets. We have characterized *T. brucei* S-adenosylmethionine decarboxylase (AdoMetDC), a key enzyme required for the synthesis of spermidine and trypanothione, and examined the role of AdoMetDC on the regulation of polyamine and trypanothione metabolism.

The recombinant *T. brucei* AdoMetDC enzyme displays low catalytic efficiency as compared to the human enzyme (1000 fold lower). Also, the specific activity in trypanosome cell lysates is about 400 fold higher than that of the recombinant enzyme. We have discovered that the product of a second gene, which we have named prozyme, is required for full activity. Prozyme arose through gene duplication and mutational drift, and has no intrinsic decarboxylase activity. AdoMetDC and prozyme form a tight heterodimer, and have a catalytic efficiency that is 1,200 fold higher than AdoMetDC alone. The heterodimeric organization may be a means for polyamine regulation in *T. brucei*, and the differences between host and parasite enzymes suggest that AdoMetDC is an intriguing drug target.

In order to better understand the role of AdoMetDC, we created a stable *T. brucei* cell line that can be induced to knockdown AdoMetDC expression by RNAi. AdoMetDC knockdown cells are auxotrophic for spermidine. In these cells, putrescine, the precursor of spermidine, is increased five fold, and spermidine levels drop to about 50% of uninduced cells. Levels of glutathionyl-spermidine and trypanothione are almost completely abolished, indicating that the trypanosomes are maintaining spermidine levels at the expense of trypanothione. Protein levels of prozyme, ornithine decarboxylase and trypanothione synthetase are increased during AdoMetDC knockdown. Therefore AdoMetDC has a central role in the biosynthesis and metabolism of polyamines and trypanothione.

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PRIOR PUBLICATIONS

1. Erin K. Willert, Richard Fitzpatrick and Margaret A. Phillips
Allosteric regulation of an essential trypanosome polyamine biosynthetic enzyme by a catalytically dead homolog.
PNAS, 2007 vol. 104, no. 20, pp 8275-8280

2. Tracy C. Beswick, Erin K. Willert, and Margaret A. Phillips
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LIST OF ABBREVIATIONS

AdoMet – S-adenosylmethionine

AdoMetDC – S-adenosylmethionine Decarboxylase

B – Blasticidin

BBB – Blood Brain Barrier

BF – Blood stream form trypanosome

cDNA – complementary DNA

cKO– conditional knock out

CNS – Central Nervous System

DEV – double expression vector

DFMO – difloromethylornithine

DNA – deoxyribonucleic acid

DNDI – Drug for Neglected Disease Initiative

FP – Flag-tagged prozyme

γ-GCS – Gamma glutamyl-cysteine synthetase

GSH – glutathione

GSP – glutathonyl-spermidine

H – hygromycin

HAT- Human African Trypanosomiasis

*Hs*AdoMetDC- Homo sapiens S-adenosylmethionine Decarboxylase

KO – knock out

MDL 73811 – 5'-([(Z)-4-amino-2-butenyl]methylamino)-5'-deoxyadenosine

mRNA – messenger RNA

N – G418

ODC – ornithine decarboxylase

ORF – open reading frame

PAO – N^1-acetylpolyamine oxidase

PF – procyclic (insect midgut) form trypanosome

Put – putrescine

PVDF – polyvinylidene difluoride

RNA – ribonucleic acid

RNAi – RNA interference

rRNA – ribosomal RNA

RT – room temperature

RT-PCR – reverse transcriptase polymerase chain reaction

SKO-H – single knock out- hygromycin resistance

SKO-N – single knock out- G418 resistance

SKO-N-FP – single knock out with conditional Flag prozyme- G418 and blastscidin resistance

SMO – spermidine oxidase

Spd – spermidine

SpdSyn – spermidine synthetase

SpmSyn – spermine synthetase

SSAT – Spermidine/Spermine N^1-acetyl transferase

*Tb*AdoMetDC – *Trypanosoma brucei* S-adenosylmethionine Decarboxylase

*Tc*AdoMetDC – *Trypanosoma cruzi* S-adenosylmethionine Decarboxylase

tet – tetracycline

TLF – trypanosome lytic factor

tRNA –transfer RNA

TSH – trypanothione

TrypRed – trypanothione reductase

TrypSyn – trypanothione synthetase

uORF – upstream open reading frame

UTR – untranslated region

WHO – World Health Organization

CHAPTER ONE

INTRODUCTION

A. TRYPANOSOMIASIS

Overview

The trypanosomatid parasites, *Trypanosoma brucei*, *Trypanosoma cruzi* and *Leishmania* species cause significant human and veterinary disease worldwide. *Trypanosoma brucei* is the causative agent of Human African Trypanosomiasis (HAT), also known as African Sleeping Sickness, and Nagana, a wasting disease in animals. *T. brucei* is transmitted by the tsetse fly (*Glossina* species). In sub-Saharan Africa, tens of thousands of people die annually of HAT, and more than 60 million people are at risk for infection ((WHO) 2004). The threat of Nagana infection of livestock has rendered a large part of the African continent unsuitable for beef and dairy production, contributing to the poverty of the area. *Trypanosoma cruzi* is responsible for the Central and South American Chagas disease, and is transmitted by the reduviid bug. Both trypanosome diseases are fatal if untreated, and current therapeutics are very limited, underscoring the need for the development of new and improved anti-trypanosomal drugs. Species of *Leishmania* are found in Central and South America, Southern Europe, Asia, the Middle East, and Africa. *Leishmania* parasites are transmitted by some sandfly species, and cause a range of disease: visceral, cutaneous and mucocutaneous leishmaniasis, which can lead to painful disease, disfigurement and fatality.

Trypanosomes are flagellated protozoan parasites of the Kinetoplastida order, which are grouped together by the presence of a kinetoplast, a mass of mitochondrial

DNA that is found within the mitochondria. Trypanosomes lead a divalent life, with stages in the insect vector and the mammalian host; parasites have morphological and metabolic differences in these life stages. Though *Leishmania* species and *T. cruzi* are intracellular parasites, *T. brucei* parasites are extracellular, surviving in the bloodstream of their mammalian host. The trypanosomes are covered by a dense surface coat made up of the variable surface glycoprotein (VSG) on bloodstream form (BF) cells. *T. brucei* encodes more than 1000 VSG genes or pseudogenes, though only one is active in an individual at a time. Though the immune system can mount an effective antibody response to the VSG, there is always a sub-population that has switched expression to another VSG gene, and can evade this immune response (Taylor and Rudenko 2006). This effective form of immune evasion brings about the requirement for chemotherapeutic intervention for parasite clearance, and also severely thwarts the development of effective vaccinations.

Human African Trypanosomiasis

Disease

In 2000, the World Health Organization (WHO 2004) estimated that there were 300,000 to 500,000 cases of HAT, and that 60 million people were at risk for contraction of the disease. Additionally, reduced farming and grazing land due to risk of Nagana adds to socioeconomic problems in the region. More recently, the number of reported cases has decreased. However, most cases of HAT occur in rural areas without adequate healthcare and surveillance, making it difficult to determine accurate disease burden.

HAT is a disease that exhibits early and late stages of illness. The early, hemolymphatic stage occurs when the parasites are multiplying in the blood and lymph.

Symptoms of the early stage include flu-like symptoms: fever, headaches, joint pain and general malaise. The late (neurological) stage occurs after the trypanosomes have crossed the blood-brain barrier (BBB) to enter the central nervous system (CNS). Hallmarks of the late stage include confusion/disorientation, motor skill deficiency, extreme fatigue and lethargy, and disrupted sleep patterns (WHO 2007). Erratic sleeping patterns include extended day sleeping and bouts of insomnia and gave rise to the common name of African Sleeping Sickness for this disease. If untreated, late stage disease results in coma and death. Some patients that are treated after the disease has progressed to the CNS may be left with irreversible neurological damage.

Two forms of HAT are caused by African trypanosomes, depending on the subspecies of the parasite present in the patient. *T. brucei gambiense* is the most common subspecies (more than 90 % of reported cases) and causes a chronic disease in western and central Africa. *T. brucei gambiense* has a slower presentation of disease, with symptoms arising after months to years, and is often diagnosed after the disease has progressed to the late stage. *T. brucei rhodesiense* causes a rapid and acute form of the disease, with invasion of the CNS occurring in weeks to months. This subspecies is less common (less than 10% of reported cases) and is mostly found in eastern and southern Africa, though the region of risk is expanding (Picozzi, Fevre et al. 2005). The trypanosome subspecies that are infectious to humans can also be transmitted through domestic and wild animals, providing a reservoir for disease propagation. A third subspecies, *Trypanosoma brucei brucei,* causes veterinary trypanosomiasis; however this subspecies is killed by the trypanosome lytic factor (TLF) in human serum and therefore is not infectious to humans (Hajduk, Hager et al. 1992). Other trypanosome species cause

a variety of animal trypanosomiasis. Recent identification of a human infection with *Trypanosoma evansi* was determined to arise in an individual who was lacking TLF activity, and is not likely to pose a risk to the general population (Vanhollebeke, Truc et al. 2006).

Current Therapeutics

Infection with *T. brucei* requires drug treatment for the clearance of the parasite and relief from the disease state. Ideal drugs to treat HAT would be inexpensive, nontoxic, orally active, and effective against late stage disease. Also, the ideal drugs would not be prone to the development of resistance, and should be able to be easily stored in tropical regions. Current drug treatments are insufficient for several reasons including toxicity, high cost, difficult dosing requirements and limitations on efficacy for different stage and/or subspecies. Three of the four drugs registered to treat HAT were discovered more than 50 years ago.

Pentamidine isethionate is an antifungal compound discovered in 1941 that shows good clinical anti-trypanosomal activity, though the mechanism of action and drug target are unknown. Pentamidine is given as an injection and is generally well tolerated. Side effects include allergic reaction, liver and pancreatic damage and GI effects. This drug treats only early stage disease, as it cannot cross the BBB. Pentamidine is only affective against the *T. brucei gambiense* subspecies (WHO 2007).

Suramin sodium is a urea based compound with anti-protozoal activity and was discovered in 1921. It is an injected therapy that has several undesirable side effects including nausea and vomiting, urinary tract infections and allergic reactions. Suramin

cannot reach the CNS and therefore only treats early stage HAT. This drug is used to treat *T. brucei rhodesiense* infections (WHO 2007).

For many years, the only effective treatment for late stage trypanosomiasis was melarsoprol, which was discovered in 1949. This is an arsenic containing drug that can cross the BBB to enter the CNS and is effective against late stage trypanosomiasis caused by both subspecies. However, melarsoprol is a particularly undesirable treatment. It must be administered via IV in a solution in propylene glycol, which is a very painful process. The most serious side affect of melarsoprol treatment is a reactive encephalophathy that occurs in 5 to 10 % of treated patients, leading to death in about half of those affected. Resistant clinical isolates have also been identified (WHO 2007).

The newest compound for the treatment of late state HAT, eflornithine, has had clinical success in treating *T. brucei gambiense* infections, though it is not effective against the *rhodesiense* subspecies. Eflornithine crosses the BBB and has been called "the resurrection drug" for the ability to awaken patients from a comatose state. Eflornithine is the chemical difloromethylornithine (DFMO) and is the only anti-trypanosomal with a know mechanism of action (Bacchi, Nathan et al. 1980). DFMO is an irreversible inhibitor of ornithine decarboxylase (ODC), a key enzyme required for the biosynthesis of polyamines. A major limitation of eflornithine treatment is the dosing regime, which requires an IV infusion of 400 mg/kg for 14 days, with 4 infusions per day.

Most of the development, distribution and publicity of treatment for neglected diseases is pushed forward by non-profit groups, such as the WHO and Drug for Neglected Disease Initiative (DNDI). Currently, DNDI has a new formulation for the

treatment of late stage HAT in clinical trials. This treatment involves a simplified dosing of eflornithine (2 infusions per day for 7 days) in combination with an oral dosing of nifurtimox, a drug that has been used to treat Chagas disease (DNDI 2007). The current state of available chemotherapeutics to treat trypanosomiasis underscores the need for the validation of promising drug targets and the development of new drugs.

Chagas Disease (South American Trypanosomiasis)

Trypanosoma cruzi is the causative agent of South American trypanosomiasis, also called Chagas disease. *T. cruzi* is most commonly transmitted by the bite of the reduviid bug (kissing bug), but can also be passed on by blood transfusions or congenitally from mother to child. Unlike *T. brucei,* the *T. cruzi* parasites are intracellular. Similar to HAT, Chagas disease has two stages. The early stage is often asymptomatic, or may have swelling around the infection site or more generalized symptoms of fever and swollen lymph nodes. As such, the early stage is not often diagnosed. The chronic stage can take years to develop, involving the invasion of parasites into the cells of the heart, intestine and other organs, and is often fatal. The current chemotherapy available to treat Chagas disease is even bleaker than for African trypanosomiasis, as currently there are no drugs available for treating the chronic stage. There are treatments for the early stage, nifurtimox and benznidazole, which are effective, though they do have side effects.

B. POLYAMINES

Overview

Polyamines are small, cationic molecules that are required for growth and proliferation of both prokaryotes and eukaryotes. They are linear, flexible compounds that are positively charged at physiological pH. The charges are separated by fixed distances. The major polyamines found in most organisms are putrescine (1,4-diaminobutane), spermidine (N-(3-aminopropyl)butane-1,4-diamine) and spermine (N,N'-bis(3aminopropyl) butane-1,4-diamine) (Figure 1.1). Polyamines can be synthesized within cells from an arginine precursor, and many cells also have polyamine transporters on the cell membrane. Polyamines must be maintained at the proper abundance to ensure cell viability. Cells with decreased polyamine content exhibit severe growth retardation or cessation, whereas high polyamine content is found in rapidly growing cells, including many cancer cells. Some of the enzymes involved in polyamine metabolism have been associated with tumorgenesis. Polyamine metabolism and transport are highly regulated in most cells, and this regulation occurs on several levels.

Though the requirement for polyamines is certain, the exact molecular roles are not completely understood. Polyamines have multiple positive charges spaced by a flexible hydrocarbon backbone, and are able to bind to nucleic acids. Polyamines can bind to and stabilize DNA, and they also can bind to RNA and affect the protein synthesis in several ways. Polyamines can alter secondary structure of messenger RNA (mRNA), transfer RNA (tRNA) and ribosomal RNA (rRNA), and have been shown to associate with the ribosome and regulate translation. There is also a requirement for polyamines to form the functional eukaryotic initiation factor 5A (EIF-5A). Active EIF-

5A requires the post-translational modification of a lysine moiety into hypusine, which is formed from spermidine. Polyamines have also been shown to associate with some receptor channels, and may have a role in metabolite and/or ion flux (reviewed in (Igarashi and Kashiwagi 2000)).

Enzymes involved in polyamine biosynthesis have been highlighted as potential anti-proliferative drug targets. Since polyamine metabolism and function are often aberrant in cancer progression, inhibitors of this pathway are attractive drug targets. DFMO treatment of HAT is the most successful clinical application of a polyamine inhibitor to date. Several other chemicals have been shown to interfere with polyamine homeostasis, and polyamine analogs may be promising candidates for therapeutics (reviewed in (Casero and Marton 2007)).

Polyamine Regulation in Mammals

In mammalian cells, the polyamines putrescine, spermidine and spermine are synthesized from the amino acids arginine and methionine in several steps (Figure 1.2). Ornithine decarboxylase (ODC) catalyzes the conversion of ornithine to putrescine. S-adenosylmethionine decarboxylase (AdoMetDC) produces dcAdoMet, which is then able to donate an aminopropyl group to putrescine to generate spermidine, a reaction that is catalyzed by spermidine synthetase (SpdSyn). Spermine synthase (SpmSyn) can conjugate a second aminopropyl group from dcAdoMet to spermidine to form spermine. In mammalian cells, ODC and AdoMetDC are the rate limiting enzymes in this pathway, and both enzymes are highly regulated on many levels. The other biosynthetic enzymes, SpdSyn and SpmSyn, appear to be stable enzymes, regulated only by product availability.

Regulation of polyamines in mammalian cells occurs on several levels (Figure 1.2; reviewed in (Casero and Marton 2007)). There is a pathway for the back conversion of the higher polyamines to putrescine which can be initiated by exposure to high amounts of polyamines. Spermine and spermidine are N^1-acetylated by spermidine/spermine N^1-acetlytransferase (SSAT) by transfer of the acetyl group from acetyl coenzyme-A. SSAT activity is stimulated in the presence of high polyamine levels. The acetylated polyamines can then be oxidized to spermidine and putrescine respectively by N^1-acetylpolyamine oxidase (PAO). PAO appears to be regulated by substrate availability. Spermine can also be converted to spermidine by spermine oxidase (SMO). SMO can be induced by tumor necrosis factor (TNF) and produces H_2O_2 as a byproduct; this has been proposed as potential mechanism for inflammation-induced carcinogenesis. The N-acetylated polyamines are a better substrate for export out of the cell than unaltered polyamines; however, they are poor substrates for influx into the cell. The back-catabolism pathway is therefore able to reduce the amount of polyamines in the cell.

The biosynthesis of polyamines is controlled by the regulation of the two rate limiting enzymes: ODC and AdoMetDC; this regulation occurs at the level of transcription, translation and protein turnover (review in (Shantz and Pegg 1999; Pegg 2006; Casero and Marton 2007)). ODC and AdoMetDC promoters contain regulatory elements that allow for response to extracellular signals; for example AdoMetDC translation can be stimulated by insulin, and ODC is a target of the *MYC* oncogene.

Both AdoMetDC and ODC have long 5' untranslated regions (5'UTRs) that have strong predicted secondary structure in mammals. The translation of AdoMetDC is

specifically inhibited by excess polyamines. AdoMetDC contains a small upstream open reading frame (uORF) encoding a 6 amino acid (MAGDIS) peptide that is a polyamine responsive element. Increased levels of spermidine and spermine lead to inhibition of AdoMetDC translation by ribosomal stalling at the termination codon of the uORF (Raney, Baron et al. 2000; Law, Raney et al. 2001; Raney, Law et al. 2002). Translational repression mediated by polyamine-responsive uORFs has also been demonstrated in yeast and plants (Mize and Morris 2001; Hanfrey, Elliott et al. 2005). Translation of ODC is greatly enhanced by eIF-4E, a translational initiator that recognizes the 5'cap of mRNA. There is also cap-independent translational regulation of ODC, and ODC translation may be regulated by polyamine responsive elements in the 5'UTR (reviewed in (Pegg 2006)).

Both AdoMetDC and ODC can be regulated post-translationally in mammals. AdoMetDC and ODC are two of the shortest lived proteins in mammals, and are degraded by the 26S proteasome. Though AdoMetDC is ubiquitinated prior to degradation (Yerlikaya and Stanley 2004), the mechanism of ODC turnover is unique and ubiquitin-independent (reviewed in (Pegg 2006)). The protein inhibitor antizyme forms a tight heterodimer with ODC, abolishes activity of ODC, and promotes the interaction of ODC with the proteasomal degradation machinery. Antizyme is synthesized in response to high levels of polyamines by promotion of a +1 frameshifting event that allows for translation of the protein; high polyamines also inhibit antizyme degradation. The antizyme mediated depletion of ODC can be abolished by antizyme inhibitor (AZI). AZI is similar to ODC but has no catalytic activity, binds to antizyme tighter than ODC does, and leads to degradation of antizyme and subsequent stabilization of the ODC protein. Antizyme is also involved in transport of polyamines; it acts to inhibit the import of

extracellular polyamines and can increase efflux out of the cell (Mitchell, Judd et al. 1994; Suzuki, He et al. 1994; Sakata, Kashiwagi et al. 2000). Therefore, antizyme is an important part of the polyamine regulation system in mammals.

AdoMetDC is synthesized as a pro-enzyme; and the autoprocessing reaction that generates two non-identical subunits is required. Putrescine stimulates the processing of mammalian AdoMetDC. Putrescine binds to the enzyme in a site distal from the active site and also acts as an allosteric activator of the enzymatic activity (Pegg 1986). However, in plants, AdoMetDC is active as a monomer and its processing and activity are not stimulated by putrescine. Structural studies have shown that the putrescine binding site in plants is already occluded with a positively charged arginine molecule, obviating the need for putrescine (Xiong, Stanley et al. 1997; Bennett, Ekstrom et al. 2002).

Polyamine metabolism in trypanosomes

Putrescine and spermidine are the polyamines synthesized from the arginine precursor in *T. brucei*. The *T. brucei* genome encodes genes for ODC, AdoMetDC and SpdSyn. *T. brucei* does not encode a SpmSyn gene and therefore does not produce spermine. Polyamines in *T. cruzi* differ from *T. brucei* in several ways. *T. cruzi* does not have ODC and therefore cannot synthesize putrescine, but relies on uptake to obtain this polyamine. *T.cruzi* also can uptake and utilize cadaverine to some extent. (Reviewed in (Heby, Persson et al. 2007).)

In the trypanosomatid parasites, there is a very unique role for spermidine (Figure 1.3 A). Spermidine is used to conjugate two molecules of glutathione (GSH) to form trypanothione (TSH). Trypanothione is a kinetoplast-unique, essential cofactor that

is involved in cellular redox reactions. GSH is generated in two steps from cysteine, glutamate and glycine by means of gamma glutamyl-cysteine synthetase (γ-GCS) and glutathione synthetase (GS). The formation of both the precursor, glutathionylspermidine (GSP) and TSH is catalyzed by trypanothione synthetase (TrypSyn). TrypSyn is a dual-function enzyme with two separate domains: the C terminal synthetic domain and an N terminal CHAP (cysteine, histidine-dependent amidohydrolases/peptidases) domain that is able to catalyze the conversion of trypanothione back to spermidine and glutathione (Oza, Ariyanayagam et al. 2003). Oxidized TSH is reduced by trypanothione reductase (TrypRed), and the TSH/TrypRed system acts analogously to the GSH/GSH reductase system found in most eukaryotic cells. (Figure 1.3 B, Reviewed in (Muller, Liebau et al. 2003).)

Polyamines are essential for parasite proliferation; inhibition of ODC with DFMO can kill *Trypanosoma brucei* (Bacchi, Nathan et al. 1980), and AdoMetDC inhibitors are also curative of mouse models of trypanosomiais (Bacchi, Nathan et al. 1992). Studies using RNA interference or genetic knockouts have demonstrated that other biosynthetic enzymes are essential (Krieger, Schwarz et al. 2000; Huynh, Huynh et al. 2003; Ariyanayagam, Oza et al. 2005). However, regulation of the polyamine/trypanothione pathway is largely unknown. Though the genome is published (Berriman, Ghedin et al. 2005), there is no evidence that *T. brucei* (or the other trypanosomatids) encodes SSAT or PAO, the enzymes for back conversion of polyamines. The trypanosomes also do not have the antizyme gene and therefore lack that regulatory arm.

There are also several important differences in ODC and AdoMetDC regulation between trypanosomes and their mammalian host. ODC and AdoMetDC are long lived proteins in trypanosomes. The *T.brucei* ODC sequence lacks the C-terminal region which is required for rapid degradation of the mammalian protein. Also, *T. brucei* does not have the uORF required for translational inhibition by polyamines.

Polyamine transporters have been shown to be present in trypanosomes, though their molecular identity and regulation have not yet been fully described. There are differences in polyamine transport between *T. cruzi* and *T. brucei,* because the intracellular environment that the *T. cruzi* parasites live in is different from the bloodstream where *T. brucei* resides. The blood is a poor source of polyamines, but there are millimolar quantities of polyamines available inside of most mammalian cells. Consequently, *T. cruzi* has a robust transport system to bring polyamines into the parasite (Gonzalez, Ceriani et al. 1992), though the transport is scant in *T. brucei*. *T. cruzi* is able to import and use putrescine, spermidine and cadaverine. Though *T. brucei* can synthesize polyamines *de novo*, *T. cruzi* lacks ODC and is auxotrophic for putrescine (Ariyanayagam and Fairlamb 1997). The *T. cruzi* parasites encode an AdoMetDC gene, and inhibitors of this enzyme have shown to be effective against mouse models of the disease. This requirement for AdoMetDC may be due to buildup of AdoMet, which can impact methylation reactions in the cell.

Trypanosome protein levels are usually regulated on the level of message stability, translational efficiency, and protein modification and/or turnover. There is little transcriptional regulation, since open reading frames are transcribed into polycistronic RNAs and processed by trans- splicing. The regulation of polyamines in trypanosomes is

an open question, though it is different from the mechanisms in other cells. Regulation of the biosynthetic enzymes post-transcriptionally, as well as unknown factors, may be involved.

C. S-ADENOSYLMETHIONINE DECARBOXYLASE (ADOMETDC)

Overview

As previously mentioned, AdoMetDC is a key enzyme in the biosynthesis of polyamines, is highly regulated in mammals, and is required for trypanothione production in trypanosomatids. AdoMet is also the substrate for methylation of proteins, DNA and phospholipids, and AdoMet is also involved in trans-sulfuration reactions that lead to cysteine and glutathione production. Once AdoMet has been decarboxylated by AdoMetDC, it can only be used for the production of polyamines. Dysregulation of AdoMetDC can therefore also have other effects on the intracellular environment in addition to disruption of polyamine homeostasis.

Processing of AdoMetDC

All known AdoMetDCs are synthesized as proenzymes and undergo an internal serinolysis in order to generate the essential pyruvate cofactor. This autoprocessing reaction generates two non-identical subunits, an N-terminal β-subunit and a C-terminal α-subunit; the α-subunit contains the pyruvate moiety as a modified N-terminal (Figure 1.4). The α- and β-subunits remain closely associated, and residues in both subunits are critical for processing and activity of AdoMetDC.

The cleavage site of AdoMetDC from mammals was found to be at serine 68 (in the human sequence), the underlined residue in the processing site sequence Val-Leu-Ser-

Glu-Ser-Ser (Stanley, Pegg et al. 1989). The processed human enzyme contains residues 1-67 as the β-subunit and 68-329 as the α-subunit, with pyruvate as the modified N-terminus. Structural studies with mutant human AdoMetDCs that cannot process (S68A) or process slowly (H243A) have helped explain the reaction (Ekstrom, Tolbert et al. 2001; Tolbert, Zhang et al. 2003). The cleavage occurs by an internal serinolysis, where the hydroxyl side chain of Ser 68 attacks the carbonyl carbon of the adjacent Glu 67. This leads to the formation of an ester intermediate, which is cleaved by β-elimination; this is facilitated by proton extraction by a basic residue in the enzyme. The ester intermediate can be trapped by in the H243A mutant, implicating this residue as the base involved in β-elimination. This results in the formation of the β-subunit and the α-subunit with a dehydroalanine residue at the N-terminus. Nucleophilic attack by a water molecule leads to the formation of a carbinolamine intermediate, and the subsequent loss of ammonia produces the mature α-subunit with an N-terminal pyruvate (Figure 1. 4).

Catalytic mechanism of action

The generation of the pyruval moiety by the processing reaction is essential for activity. The pyruvate binds the substrate AdoMet to form a Schiff's base by interaction of the AdoMet amine with the ketone. The pyruvate then acts as an electron sink to facilitate the cleavage of the carbon-carboxyl bond, leading to release of CO_2. Product release is accomplished by protonation of dcAdoMet (Figure 1. 5). This proton is probably provided by the highly conserved active site cysteine (Cys 82 in the human sequence). Aberrant protonation leads to substrate mediated transamination, which permanently inhibits the activity of the enzyme (Xiong, Stanley et al. 1999). Ubiquitination and degradation of AdoMetDC is accelerated by transamination

(Yerlikaya and Stanley 2004). The only role for dcAdoMet is to provide the aminopropyl group for the formation of spermidine and spermine from the putrescine precursor. Since SpdSyn and SpmSyn are regulated by substrate availability, AdoMetDC and ODC catalyze rate limiting steps in this pathway.

The mammalian AdoMetDC is functionally active as a $(\alpha\beta)_2$ homodimer. Structural analysis of the human enzyme (Ekstrom, Mathews et al. 1999) shows that the (αβ) monomer consists of two central β sheets with antiparallel arrangement with helices on either side of the sheet (Ekstrom, Mathews et al. 1999). Though the peptide bond is cleaved during the processing reaction, the α- and β-subunits do not form separate domains but are interwoven in the structure. The dimer interface involves the association of β-sheets and is maintained through hydrogen bonds. The active site of the protein is far from the dimer interface, and the two pyruvoyl groups are more than 50 Ä apart in the homodimer. Some important catalytic residues in the active site are Glu8, Glu11, Ser68, Cys82 (probable proton donor used in Schiff base hydrolysis), Ser229 and His 243.

Of the published inhibitors of AdoMetDC, some compounds, such as CGP 40215, have been shown to be very potent inhibitors of human AdoMetDC (6 to 10 nM). This inhibitor is much less potent on AdoMetDC from *T.cruzi* (3.2 to 32 uM), demonstrating that species-specific inhibitors can be discovered (Beswick, Willert et al. 2006). Hopefully, inhibitors with the opposite specificity can be uncovered and used in the treatment of trypanosomiasis.

Allosteric Activation of AdoMetDC

In the structure of a mutated human enzyme (H243A), the AdoMetDC enzyme is trapped in the ester intermediate of the processing reaction (Ekstrom, Tolbert et al. 2001).

This structure also contains a bound putrescine molecule, which defines the binding site for the allosteric activator. The binding of putrescine to the human enzyme is very tight, and putrescine is observed in human AdoMetDC structure, often when it is not part of the crystallization conditions. The mutant structure is highly similar to an earlier wildtype structure (Ekstrom, Mathews et al. 1999); however, the ester bond and associated structural changes are seen. The putrescine binding site was found to be buried between two β-sheets. Unlike the interior of most proteins, this site contains several acidic residues. Putrescine binds in this site by forming hydrogen bonds either directly (E15, T176 and D174) or indirectly through ordered water molecules (E15, E178, E256, S113) and by hydrophobic contacts with several residues (F111, F285 and Y318). A hydrogen bonding network that stretches from putrescine to the active site E11 residue (through water molecules, E256, K80, S254) connects the allosteric site with the active site, which enables putrescine to stimulate auto-processing and catalysis.

This network is conserved in the structure of potato AdoMetDC, even though the plant enzyme is not stimulated by putrescine (Bennett, Ekstrom et al. 2002). Instead of bound putrescine, the potato enzyme contains three positively charged residues (R18, R114 and H294) along with water molecules to facilitate the hydrogen binding in the buried charge pocket. These positive charges are not present in the human enzyme and explain how the two AdoMetDCs are differentially regulated by putrescine. Though there is no structure for the *T. cruzi* enzyme, sequence and mutational analysis demonstrates that this enzyme has characteristics of both the plant and human allosteric binding site, which correlates with the finding that putrescine stimulates activity but does not affect processing of the *T.cruzi* enzyme. (Clyne, Kinch et al. 2002). The replacement of the

hydrophobic Leu13 in the human enzyme with an Arg is seen in both plants and *T. cruzi*. In *T. cruzi,* Arg13 is required for processing, and D174 (conserved with human but differing from plant) plays a role in putrescine stimulation of catalysis. The apparent K_d for putrescine binding is about 50 fold higher for the trypanosome enzyme than the human (Kinch, Scott et al. 1999).

The requirement for allosteric activation of AdoMetDC is species specific. *E.coli* AdoMetDC uses a divalent cation, such as Mg^{2+}, for activation. Putrescine stimulates the processing and activity of mammalian and yeast enzymes, though plant enzymes are not activated by putrescine. The kinetic parameters for several AdoMetDCs, including yeast, human, *T. cruzi* and plant have been determined (Table 1.1, (Poso, Sinervirta et al. 1975; Schroder and Schroder 1995; Park and Cho 1999; Beswick, Willert et al. 2006)). Though most AdoMetDCs have k_{cat}/K_m in the same area, the catalytic efficiency for *T. cruzi* AdoMetDC is much lower than that seen in the other enzymes, even in the presence of putrescine. The trypanosomatid AdoMetDC appears to have evolved to have very low activity in the basal state, and to be regulated by putrescine levels.

There are two classes of AdoMetDC enzymes. Class 1 AdoMetDCs are found in bacteria: 1A enzymes form $(\alpha\beta)_4$ homotetramers and are activated by divalent cations, and 1B enzymes make $(\alpha\beta)_2$ homodimers and do not require any activator molecules. The class 2 AdoMetDCs are found in eukaryotes and have different oligomeric states and allosteric activators. Plant AdoMetDC is an αβ monomer and requires no activator, and the mammalian enzyme is a $(\alpha\beta)_2$ homodimer that is stimulated by putrescine at the level of processing and allosteric activation (Stanley, Shantz et al. 1994; Xiong, Stanley et al. 1997). Putrescine was found to have no effect on the processing of AdoMetDC from *T.*

cruzi but does activate the enzyme allosterically (Clyne, Kinch et al. 2002). There is no sequence identity observed between class 1 and class 2 enzymes, and very little sequence homology between class 1A and 1B enzymes. However, structural studies have demonstrated that there is a high degree of structural similarity between the classes. Comparison of a class 1B AdoMetDC from *Thermatoga maritime* with the human structure indicates that there was a gene duplication and fusion of the ancestral AdoMetDC to bring about the eukaryotic class 2 enzymes (Toms, Kinsland et al. 2004).

D. DISSERTATION SCOPE

African trypanosomiasis, caused by the parasitic protozoa *T. brucei*, is an important but neglected disease, and new drugs are urgently required. In particular, the polyamine pathway has been implicated as a source for drug targets to combat trypanosomiasis, as inhibition of the polyamine biosynthetic enzyme ODC by DFMO is clinically utilized. Another biosynthetic enzyme, AdoMetDC, has good potential as a drug target, as inhibitors have been able to cure mouse models of the disease. Several differences between AdoMetDC in the human host and the parasite are already known, which may facilitate the creation of species-specific inhibitors for trypanosomiasis.

ODC and AdoMetDC catalyze the two regulated, rate limiting steps of polyamine biosynthesis in mammalian cells. Polyamine homeostasis must be maintained to prevent cell death due to depletion or hyperproliferation/carcinogenesis by excess levels. The regulation of polyamines is unknown in trypanosomes.

In this work, I will be focusing on AdoMetDC as a key enzyme in the polyamine and trypanothione biosynthetic pathways in *T. brucei*. The activity of recombinant

AdoMetDC from the related parasite, *T. cruzi,* is very low in comparison to the human homolog. I have characterized the kinetics of *T. brucei* AdoMetDC, and show why this low activity is seen in the *in vitro* reaction. I describe the active form of the enzyme, and illustrate how allosteric activation of the *T. brucei* enzyme differs from the human. These findings are also are also relevant for other trypanosomatid AdoMetDCs.

By depleting AdoMetDC protein in *T. brucei* I confirm that AdoMetDC is an essential parasite enzyme, providing the genetic validation of this target. Using this system, I will also discuss the regulation of the polyamine and trypanothione biosynthetic pathways. AdoMetDC plays a central role in the regulation of these metabolites, and depletion of the AdoMetDC protein levels show that trypanosomes do indeed regulate polyamine homeostasis, but by mechanisms that are completely novel.

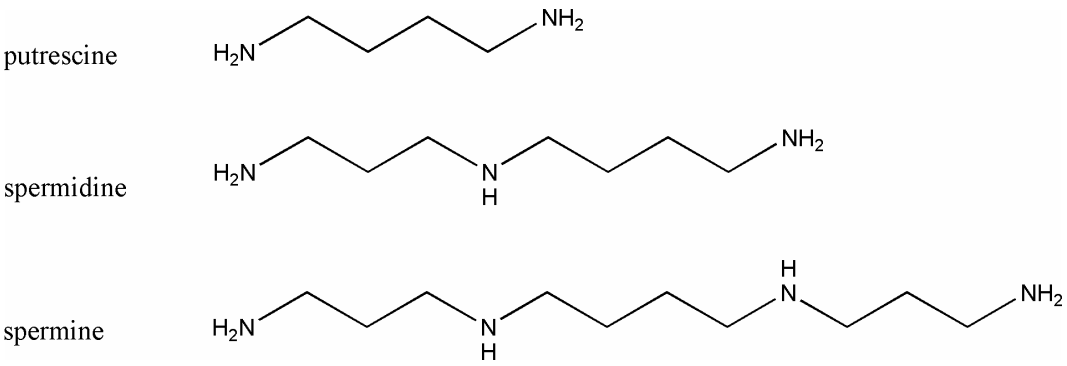


Figure 1.1 Polyamine structures: Structures of putrescine, spermidine and spermine are shown.

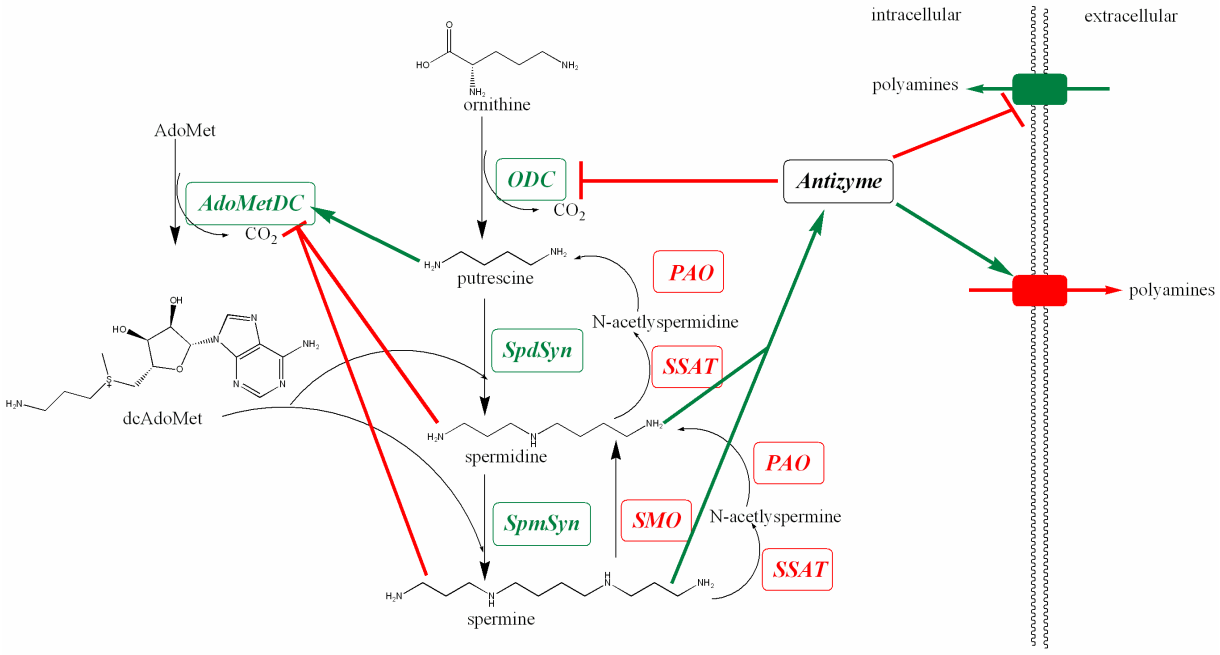


Figure 1.2 Polyamine regulation in mammals. A model of the biosynthesis, catabolism and transport of the putrescine, spermidine and spermine demonstrates the complexity of polyamine regulation. Green arrows indicate stimulation and red lines indicate inhibition. Biosynthetic enzymes are in green, and back-converting enzymes are in red. Abbreviations: *AdoMetDC* S-adenosylmethionine decarboxylase; *dcAdoMet* decarboxylated S-adenosylmethionine decarboxylase (S-adenosylmethioniamine); *ODC* ornithine decarboxylase; *SpdSyn* spermidine synthetase; *SpmSyn* spermine synthetase; *SMO* spermine oxidase; *SSAT* spermidine/spermine N^1-acetyltransferase, *PAO* N^1-acetylpolyamine oxidase.

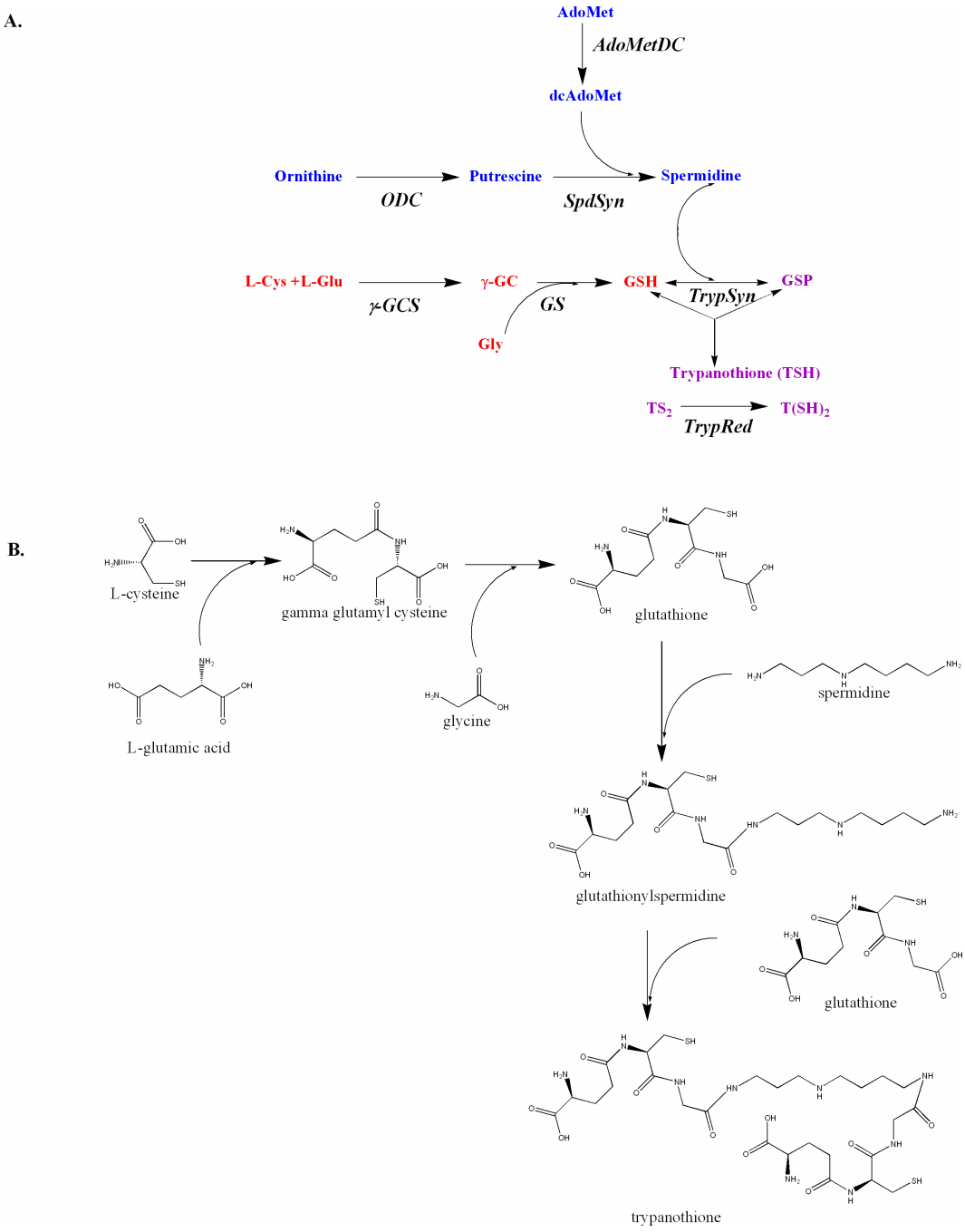


Figure 1.3 The polyamine and trypanothione pathway in trypanosomatids. The polyamine and glutathione pathway converge (A) resulting in the formation of trypanothione (B).

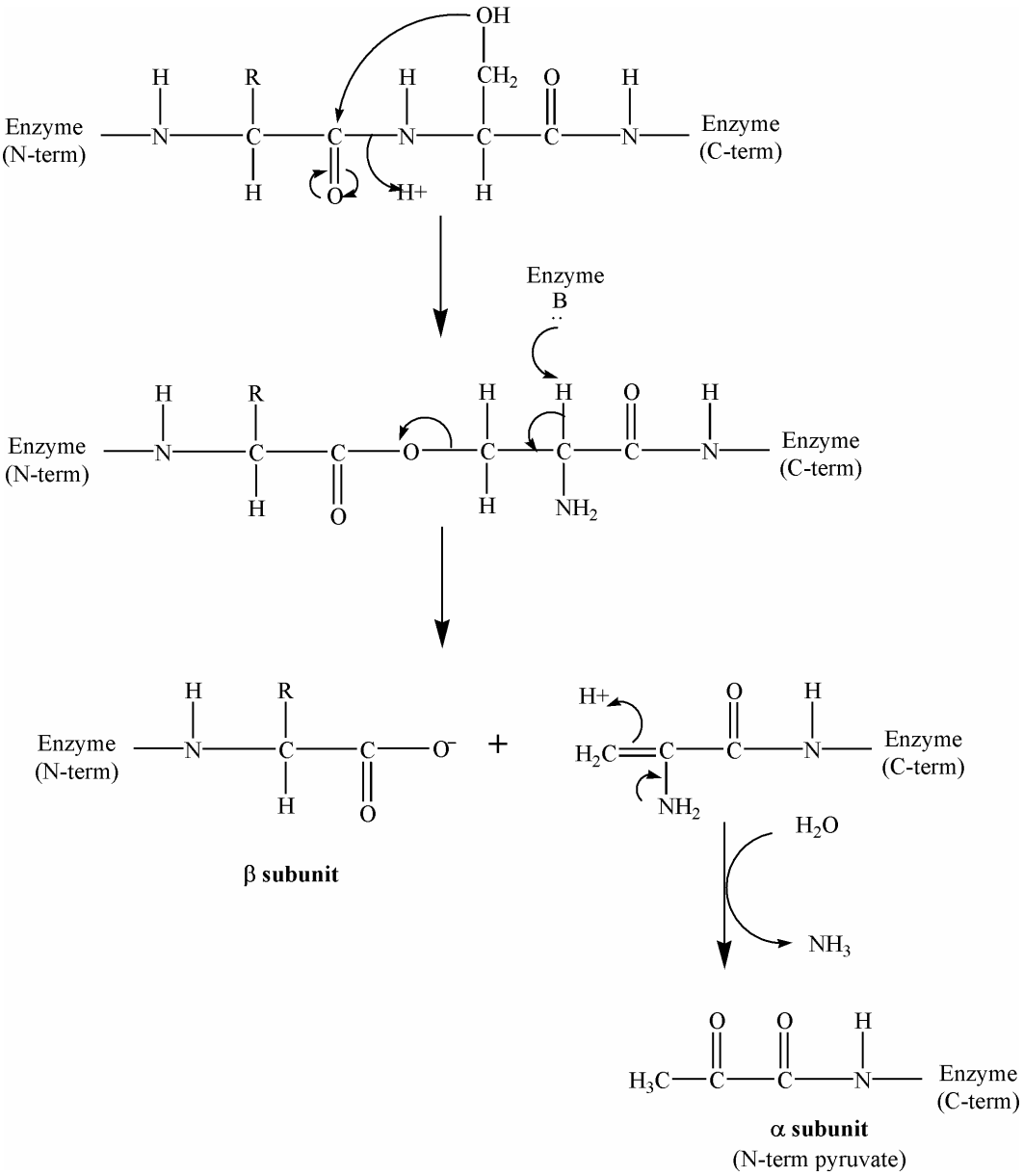


Figure 1.4 Processing of AdoMetDC AdoMetDC is synthesized as a proenzyme. An internal serinolysis converts the enzyme to the mature form, with a N-terminal β-subunit and a C-terminal α-subunit with a covalently bound pyruvate as the modified N-terminus.

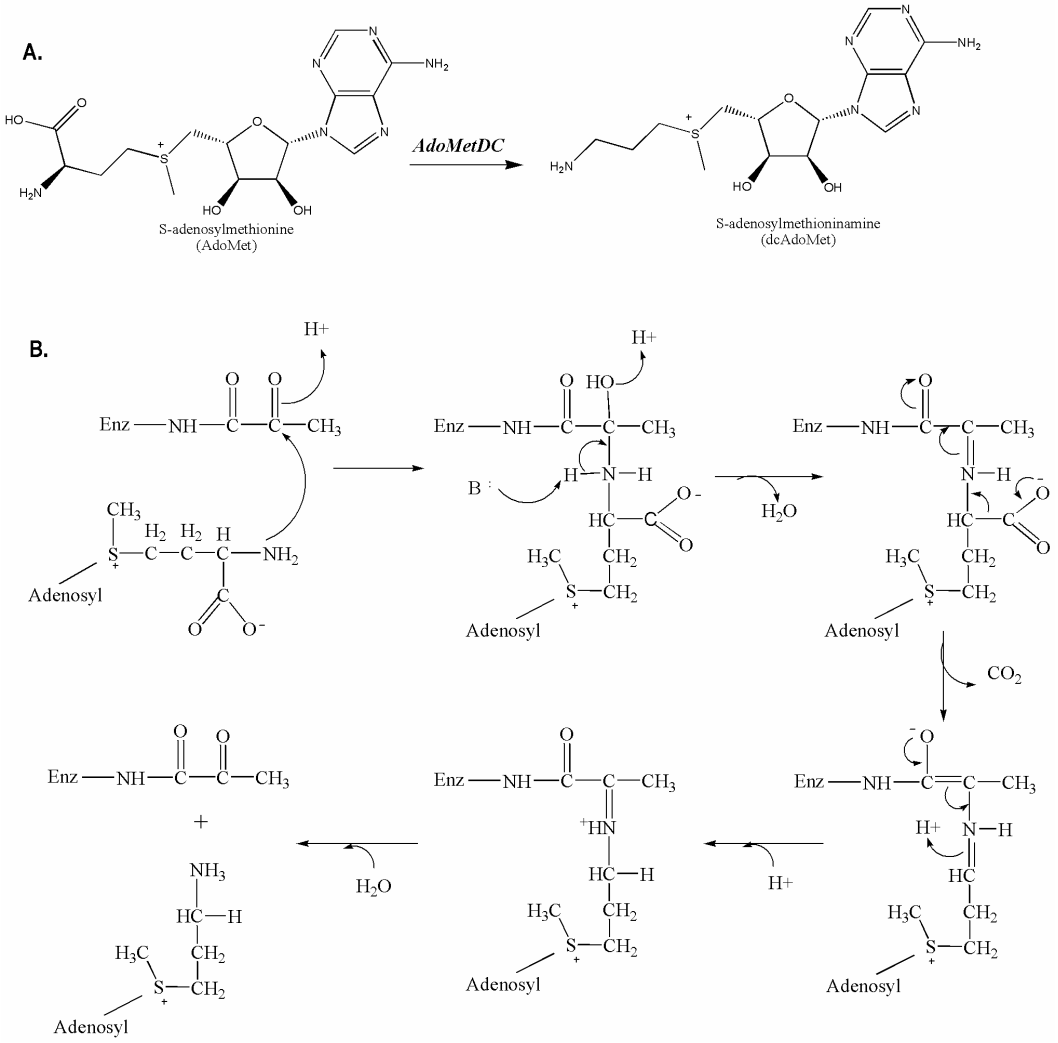


Figure 1.5 Pyruvoyl-dependant AdoMetDC decarboxylation reaction The overall reaction (A) and the mechanism of catalysis (B) are shown.

	No putrescine			+ putrescine		
	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m ($M^{-1}s^{-1}$)	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m ($M^{-1}s^{-1}$)
T. cruzi	0.0009	0.26	3.5	0.024	0.25	100
S. Cerevisaie				0.9	0.09	$1. x10^4$
Homo Sapiens	1.9	0.074	$2.5 x10^4$	2.6	0.059	$4.4 x10^4$
Various Plants	3-46	0.008-0.04		3-46	0.008-0.04	

Table 1.1 Comparison of AdoMetDC activity The comparison of AdoMetDCs from different species demonstrates that *T. cruzi* AdoMetDC has low activity, even after stimulation by putrescine.

CHAPTER TWO

T. BRUCEI ADOMETDC IS ALLOSTERICALLY REGULATED BY A CATALYTICALLY INACTIVE PARALOG

A. INTRODUCTION

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 enzyme in polyamine formation (Pegg, Xiong et al. 1998). Knockout of the AdoMetDC gene in the trypanosomatid *Leishmania donovani* led to spermidine auxotropy (Roberts, Scott et al. 2002). The suicide inhibitor of AdoMetDC, MDL73811, has demonstrated efficacy in animal models of infection with both *T. brucei* (Bacchi, Nathan et al. 1992) and a related parasite *Trypanosoma cruzi* (Yakubu, Majumder et al. 1993), 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, utilizing 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 (Ekstrom, Tolbert et al. 2001; Tolbert, Zhang et al. 2003). Human AdoMetDC is a homodimer, and both the processing reaction and decarboxylation of AdoMet are stimulated by putrescine (Pegg, Xiong et al. 1998; Ekstrom, Mathews et al. 1999). 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 ~15Å from the active sites (Ekstrom, Tolbert et al. 2001; Tolbert, Ekstrom et al. 2001). This site is filled in with positively charged residues in the structure of the monomeric plant enzyme, which is fully active without putrescine (Bennett, Ekstrom et al. 2002). 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 (Persson, Aslund et al. 1998; Kinch, Scott et al. 1999; Kinch and Phillips 2000; Clyne, Kinch et al. 2002; Beswick, Willert et al. 2006). 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 (Childs, Mehta et al. 2003; Hanfrey, Elliott et al. 2005; Pegg 2006). 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.

B. MATERIALS AND METHODS

Multiple Sequence Alignment and Phylogenic Tree Generation

AdoMetDC sequences from listed species were compiled using the NCBI Blast with the *T. brucei* AdoMetDC protein sequence as the search query. The prozyme sequence was found on the *T. brucei* gene database (http://www.genedb.org/genedb/tryp/) where was annotated as S-adenosylmethionine decarboxylase proenzyme-like, putative (since updated to prozyme). The alignment was created using clustalW (http://www.ebi.ac.uk/clustalw/index.html?). Sequences can be accessed from PubMed or the trypanosome database. (From PubMed: *Homo sapiens* gi:178518; *Drosophila melanogaster* gi:6166112; *Caenorhabditis elegans* gi:17508719; *Neurospora crassa* gi:4929540; *Saccharomyces cerevisiae* gi:6324521; *Dictyostelium discoideum* gi:66819483; *Arabidopsis thaliana* gi:15232910; *Solanum tuberosum* gi:416883; gi:4929540. From the *T. brucei* geneDB: *Trypanosoma brucei*: Tb927.6.4460; Trypanosoma brucei AdoMetDC prozyme: Tb927.6.4470; *Trypanosoma cruzi*: Tc00.1047053504257.30 ; *Trypanosoma.cruzi* AdoMetDC prozyme: Tc00.1047053509167.110; *Leishmania major*: LmjF30.3110 ; *Leishmania major* AdoMetDC prozyme: LmjF30.3120; *Plasmodium falciparum*: PF10_0322.)

The phylogenetic tree was generated using ProtML and Njdist of the MOLPHY package (http://bioweb.pasteur.fr/seqanal/interfaces/prot_nucml.html) with default settings (Saitou and Nei 1987; Adachi and Hasegawa 1992; Jones, Taylor et al. 1992). The reliability of the tree was determined by the RELL method of MOLPHY (Kishino and Hasegawa 1990).

Determination of the AdoMetDC and prozyme 5' start site

Reverse Transciption polymerase chain reaction (RT-PCR) and DNA sequencing were used to determine the 5' starting sequence of the AdoMetDC and prozyme genes. cDNA to prozyme and AdoMetDC was cloned and amplified by RT-PCR as follows. 10 ng mRNA was used as a template to create cDNA with the SuperScript III One-Step RT-PCR System with Platinum® *Taq* DNA Polymerase (Invitrogen) according to manufacturer instructions. A primer to the spliced leader sequence common to all *T. brucei* mRNA was used to prime the 5'end and a prozyme or AdoMetDC specific primer from the 3'UTR was used to prime the 3'end (Table 2.1). The cDNA was then cloned into the PCR-Blunt II- Topo vector (Invitrogen) according to manufacturer instructions and sequenced in its entirety. Sequencing was performed at the McDermott Center for Human Growth and Development Center for Human Genetics DNA Sequencing Core Facility at UT Southwestern Medical Center, using core facility primers to the T7 and SP6 sequences, and the results were translated by the Expasy translate tool (http://us.expasy.org/tools/dna.html).

***Trypanosoma brucei* cell growth and preparation of lysates**

Trypanosome parasites were cultured as described (Brun and Schonenberger 1979; Hirumi and Hirumi 1989; Wirtz, Leal et al. 1999). 90-13 bloodstream form (BF) trypanosomes were cultured in HMI-9 media supplemented with 10% fetal bovine serum and the appropriate antibiotics (G418, 2.5 μg/ml; hygromycin 5 μg/ml) at 37 °C with 5% CO_2. 29-13 procyclic form (PF) trypanosomes were cultured in SDM-79 with 10% FBS and the appropriate antibiotics (G418, 15 μg/ml; hygromycin 50 μg/ml) at 25°C. 90-13

and 29-13 cells express the tet repressor and T7 polymerase (for regulation of tetracycline (tet) responsive elements).

To collect trypanosome lysates for anaylsis, cells were pelleted by centrifugation, washed with cold phosphate-buffered saline (PBS, pH= 7.4), and resuspended in lysis buffer (50 mM HEPES pH 8, 100 mM NaCl, 5 mM 2-mercaptoethanol, 2 mM phenylmethylsulfonyl fluoride, 1 µg/ml leupeptin, 2 µg/ml antipain, 10 µg/ml benzamidine, 1 µg/ml pepstatin, 1 µg/ml chymostatin). Cells were lysed by freeze/thaw cycles and the lysate clarified by centrifugation.

Expression and purification of AdoMetDC and prozyme

The *T. brucei* AdoMetDC and prozyme genes were amplified by PCR from genomic DNA and were cloned into the pET15b vector (Novagen) to generate N-terminal His_6-tag fusions. Additionally, prozyme was cloned into the pT7-Flag1 (Sigma) vector to generate an N-terminal Flag-tag. Cloning primers are described in Table 2.1.

AdoMetDC or prozyme were expressed individually in *Escherichia coli* BL21/DE3 cells containing the respective construct, and purified in two steps by Ni^{2+}-agarose (Qiagen) and anion exchange column chromatography (Amersham Mono Q 5/50 GL column) as described (Kinch, Scott et al. 1999), except that the buffer was exchanged after elution from Ni^{+2} agarose and Mono Q by a HiPrep 26/10 desalting column (Amersham) equilibrated in storage buffer (50 mM HEPES pH 8.0, 50 mM NaCl, 1 mM dithiothreitol). Flag-tagged prozyme was co-purified with His_6-tagged AdoMetDC from BL21 cells that contained the individual constructs. Cell pellets were resuspended in lysis buffer, the lysates were mixed together and the proteins were co-purified as above. Purified proteins were quantified using their respective extinction coefficients (measured

at 280 nm): AdoMetDC 65.3 mM^{-1} cm^{-1}; prozyme, 35.9 $mM^{-1}cm^{-1}$; AdoMetDC/prozyme complex, 102.7 mM^{-1} cm^{-1}. SDS-PAGE and analytical gel filtration were used to assess purity.

Preparation of protein samples and Western Blot Analysis

Total protein from lysates (40 μg/lane) and purified recombinant *T. brucei* AdoMetDC, prozyme or co-purified AdoMetDC/prozyme complex (10 ng/lane) were separated by SDS-PAGE and transferred to a polyvinylidene difluoride (PVDF) membrane (Hybond-P, Amersham). Membranes were blocked by standard methods and incubated with either rabbit polyclonal antibody to *T. brucei* AdoMetDC at 1:2,500, or with monoclonal anti Flag-M2 (Sigma) at 1:1,000. Horseradish peroxidase linked donkey anti-rabbit or anti-mouse IgG secondary antibodies (Amersham Biosciences) were used at 1:10,000. Antigen was visualized using the ECL Western Blotting Analysis system (Amersham).

Preparation of mRNA and Northern Blot Analysis

Messenger RNA (mRNA) was isolated from at least $3x10^8$ trypanosome cells using the micro polyA purist kit (Ambion) according to the manufacturer protocol. Northern blot analysis was facilitated by the NorthernMax kit (Ambion). RNA was quantified spectrophotometrically, and 1 μg/lane was separated by denaturing 1% agarose gel electrophoresis. The mRNA was transferred to a positively charged nylon membrane (BrightStar-Plus, Ambion) and cross-linked. Probe templates were prepared from plasmid DNA, and radiolabeled ([^{32}P]dATP) probes were prepared using the Strip-EZ PCR kit (Ambion). Probe hybridization was visualized on film (Kodak biomax XAR).

Steady-state kinetic analysis

Steady state kinetics were performed by trapping liberated $^{14}CO_2$ on a filter paper soaked in saturated barium hydroxide as described (Kinch, Scott et al. 1999; Beswick, Willert et al. 2006). For homodimeric AdoMetDC, reactions were performed over a range of enzyme (1 - 4 μM) and 1-[^{14}C]-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, and 1 mM DTT). Reactions were allowed to proceed for various times (5 – 40 min) before quenching with 6 M HCl. For assay of the heterodimer, the AdoMetDC/prozyme co-purified complex (25 - 400 nM based on monomer concentration) was incubated with 1-[^{14}C]-AdoMet (25 μM) and unlabeled AdoMet (0 - 975 μM) for various time points (2.5 - 10 minutes) before quenching as above. Substrate titration data were fitted to the Michaelis-Menten equation to determine the steady-state kinetic parameters using Prism (GraphPad).

Measuring the concentration of AdoMetDC in *T. brucei* cell lysates

AdoMetDC active site titration with MDL 73811 in T. brucei cell lysates

MDL 73811 (0-100 nM) was incubated with 0.01 ml (40 μg total protein) *T. brucei* blood form cell lysate at 37 °C for 30 min – 2 hours in assay buffer, which allowed the reaction to proceed to completion. After incubation, the enzymatic reaction was initiated by the addition of 40 μM 1-[^{14}C]-AdoMet substrate followed by incubation for 30 minutes prior to quenching with 6 M HCl. The remaining enzyme activity was determined (v_i) and compared to the uninhibited activity (v_o) in the lysate. The concentration of AdoMetDC in lysates was estimated from this data using standard

protocols for the estimation of active site concentration by titration with tight-binding or irreversible inhibitors (Copeland 1996).

Western blot comparison with purified, recombinant AdoMetDC

As a second method, we analyzed the amount of AdoMetDC in PF and BF trypanosome cell lysates (0.04 mg total protein/lane) by western blot. Intensity of the 34 kDa band was compared to that measured for a titration of recombinant AdoMetDC (0.25 to 3 ng protein) providing an estimate of the AdoMetDC protein concentration in the BF lysates.

Determination of molecular weight of the AdoMetDC /prozyme complex

Gel filtration column chromatography of T. brucei AdoMetDC proteins

The Superdex 200 HR 10/30 (Amersham) size exclusion column was equilibrated with buffer (50 mM HEPES pH = 8, 150 mM NaCl) and calibrated with standards (1.35 to 670 kDa, BioRad) using a flow rate of 0.5 ml/min. Purified AdoMetDC, AdoMetDC prozyme and co-purified AdoMetDC /AdoMetDC prozyme complex (50 μM protein in 50 mM HEPES pH= 8, 50 mM NaCl, 1 mM DTT) were loaded on to the column individually, and the apparent molecular weight was estimated from the standard curve.

Sedimentation Equilibrium by Analytical Ultracentrifugation

The molecular weights of the complex and the individual subunits were determined by equilibrium sedimentation analysis using a Beckman XLI analytical ultracentrifuge equipped with an AN60 Ti rotor. Samples in buffer (50 mM HEPES pH 8.0, 50 mM NaCl, and 1 mM β-mercaptoethanol) were loaded into a six-sector equilibrium centerpiece and equilibrated for data collection at 15,000 and/or 20,000 rpm.

After equilibrium was reached (approximately 24 hours), absorption data were collected at 280 nm through sapphire windows using a radial step size of 0.001 cm. Base-line absorbance readings for each cell were acquired by over speed at 42,000 rpm. Data sets were analyzed using equations describing a single ideal species model (equation 2.1), or a monomer-homodimer model (equation 2.2), or a monomer-heterodimer model (equation 2.3), as previously described (Lebowitz, Lewis et al. 2002), or for the AdoMetDC/prozyme complex globally fitted to a single ideal species model using the Beckman XL-A/XL-I Data Analysis Software version 6.0. Both analyses gave similar results.

Equation 2.1. $A = c*\varepsilon*d*\exp[(M*(1-v_{bar}*\rho)*\omega^2*(r^2-r_o^2)/2*R*T] + \delta$

Equation 2.2. $A = c*\varepsilon*d*\exp[(M*(1-v_{bar}*\rho)*\omega^2*(r^2-r_o^2)/2*R*T] + 2* c^2*\varepsilon*d*\exp[2* M*(1-v_{bar}*\rho)*\omega^2*(r^2-r_o^2)/2*R*T] + \delta$

Equation 2.3. $A = c*\varepsilon_A*d*\exp[(M_A*(1-v_{barA}*\rho)*\omega^2*(r^2-r_o^2)/2*R*T] + c*\varepsilon_B*d*\exp[(M_B*(1-v_{barB}*\rho)*\omega^2*(r^2-r_o^2)/2*R*T] + c^2*(\varepsilon_A + \varepsilon_B)*d*K_{a,AB}*\exp[((M_A*(1-v_{barA}*\rho) + (M_B*(1-v_{barB}*\rho))*\omega^2*(r^2-r_o^2)/2*R*T] +\delta$

Where: A = Absorbance at 280; c = concentration at the meniscus; ε= extinction coefficient; d= pathlength; M = Molar mass; V_{bar} = partial specific volume; ρ = buffer density; ω = (2*π*rpm)/60; r = radius; r_o= reference radius; R = gas constant; T = temperature; δ = offset; K_a = association constant.

C. RESULTS

Genomic analysis of the trypanosomatid AdoMetDC family

Evolutionary analysis of the AdoMetDC family indicates that the trypanosomatids, *T. brucei*, *T. cruzi* and *L. major*, encode two types of AdoMetDC genes: an ortholog to the functional enzyme in other species, AdoMetDC, and a paralog defined herein as prozyme (Figures 2.1 and 2.2), named in analogy to the ornithine decarboxylase inhibitory protein, antizyme (Heller, Fong et al. 1976). 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. Phylogenetic 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 (Figure 2.2). 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 (Figure 2.3). However the prozyme is missing several critical residues required for processing and catalytic activity (Pegg, Xiong et al. 1998; Kinch and Phillips 2000; Ekstrom, Tolbert et al. 2001; Tolbert, Ekstrom et al. 2001; Tolbert, Zhang et al. 2003). Therefore the prozyme is unlikely to display AdoMetDC activity and may have a novel function.

Recombinant *T. brucei* AdoMetDC has low catalytic efficiency

T. brucei AdoMetDC was expressed and purified from *E. coli* to evaluate its kinetic properties. Recombinant *T. brucei* AdoMetDC is processed to generate the pyruvoyl-cofactor, and it catalyzes the decarboxylation of AdoMetDC (Table 2.2 and

Figure 2.4A) Like the *T. cruzi* enzyme (Persson, Aslund et al. 1998; Kinch, Scott et al. 1999; Kinch and Phillips 2000; Clyne, Kinch et al. 2002; Beswick, Willert et al. 2006), *T. brucei* AdoMetDC is stimulated by putrescine but not sufficiently to generate a catalytically efficient enzyme. The k_{cat} of the putrescine-stimulated enzyme remains 320-fold lower than for human AdoMetDC (figure 2.4B). In order to determine if 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 (Figure 2.4C).

Characterization of AdoMetDC activity in *T. brucei* parasites

The kinetic data on the recombinant trypanosomatid AdoMetDCs suggested that these enzymes either have intrinsically lower activity or are activated by a novel mechanism. To address this question the specific activity of AdoMetDC in the blood form *T. brucei* parasites was determined. 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 (Casara, Marchal et al. 1989; Danzin, Marchal et al. 1990) and second by Western blot (Figure 2.5). Both methods of determining AdoMetDC concentration in the extracts yielded similar results of ~ 5 nM. Decarboxylation was followed by the standard assay using 1-[^{14}C]-AdoMet as the substrate. Based on the MDL 73811 titration and the western analysis, the specific activity of AdoMetDC in the BF lysate can be estimated to be 0.8 s^{-1} (0.9 μmole/min/mg) at 0.04 mM AdoMet, which would correspond to ~3 s^{-1} (4 μmol/min/mg), at saturating substrate (assuming the K_m

reported in Table 2.2). This specific activity is approximately ~ 400-fold higher than the k_{cat} measured for the putrescine-stimulated recombinant enzyme (Table 2.2 and Figure 2.6). 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 (Beswick, Willert et al. 2006)).

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 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. In order to determine if 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 (Figure 2.7). 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 (Figure 2.8). Maximum activity is observed at a ratio of 1:4 AdoMetDC:prozyme. This 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 (Figure 2.9).

AdoMetDC and prozyme form a high affinity heterodimer

The stability and function of the AdoMetDC/prozyme complex was assessed by co-purification of the recombinant His-tagged AdoMetDC with Flag-tagged prozyme.

The proteins were expressed separately and then co-purified by Ni^{+2}-agarose and anion exchange column chromatography. The two subunits in the purified complex elute as a single peak on gel filtration (Figure 2.9), demonstrating that the complex remains associated through three successive column chromatography steps (e.g. Ni^{+2}-agarose, anion exchange and gel filtration). The individual subunits were also examined by gel filtration analysis. AdoMetDC eluted from the column as predominately a single peak (apparent MW = 92 kDa, predicted MW = 87.8 kDa) corresponding to the expected molecular weight of the homodimer, while AdoMetDC prozyme eluted as two equal peaks, one corresponding to an apparent molecular weight slightly larger than the homodimer (apparent MW = 97 kDa, predicted MW = 81.4kDa) and one that is consistent with an aggregated, high MW species. AdoMetDC/prozyme complex eluted from the column at a higher apparent molecular weight (apparent MW = 130 kDa) than either of the homodimers, consistent with an elongated dimer. The larger apparent size of the heterodimer by gel filtration may indicate that the two subunits self associate into a more asymmetric heterodimer than then the individual homodimers or may reflect a conformational change in one or both of the subunits.

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 (Figure 2.10).

To get an accurate determination of molecular weight, we used sedimentation equilibrium analysis by analytical ultracentrifugation (Figure 2.11). By fitting the data to a single species model, the molecular weight of the complex was found to be 80.4 kDa, in good agreement with the predicted heterodimer of 81.4 kDa. We have demonstrated that

the complex is a heterodimer of AdoMetDC and prozyme, which is formed at high affinity. (The affinity constant is below the limit of detection for this method, so the K_d is < 0.5 μM.)

In contrast, the AdoMetDC and prozyme homodimers form with weaker affinity (Figure 2.12). The single species fit of *T .brucei* AdoMetDC (monomer MW = 43.9 kDa) gives a molecular weight of 53.1 kDa, and the K_d of dimerization was found to be 50 ± 6 μM, indicating that there is a significant portion in the monomeric form. The non-aggregated prozyme (monomer MW = 38.4 kDa) sample was analyzed, and fit to a single species model at 63.4 kDa, with a dimerization K_d of 4 ± 0.5 μM. Therefore the AdoMetDC/prozyme heterodimer forms a higher affinity complex than either heterodimer by at least 10 fold.

AdoMetDC/prozyme heterodimer is the catalytically functional enzyme

The rate constant for the decarboxylation of AdoMet by the co-purified heterodimer was determined by steady-state kinetic analysis (Figure 2.13 and Table 2.2). The k_{cat} of 1.4 s^{-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. The velocity of *T. brucei* AdoMetDC is activated 1,200-fold by formation of a functional heterodimer with prozyme. The catalytic efficiency (k_{cat}/K_m) of the heterodimer is 3000 fold higher than the unstimulated homodimer (and 300 fold higher than putrescine activated AdoMetDC). The AdoMetDC/prozyme complex has equivalent activity to orthologs from other species, and forms a higher affinity complex than the homodimer, indicating that the AdoMetDC/prozyme heterodimer is the functional species in the parasite.

D. CONCLUSIONS

The polyamine pathway is tightly regulated in many species. In mammals, plants, and yeast, the biosynthetic enzymes are controlled by transcriptional and translational regulation as well as by several posttranslational mechanisms, including protein stability (Pegg 2006). AdoMetDC from the trypanosomatid parasites has evolved to be regulated by a novel mechanism not found in any other species. Our data demonstrate that the functional AdoMetDC in the trypanosomatid parasites is a heterodimer formed between AdoMetDC and prozyme. The prozyme arose through gene duplication of the ancestral AdoMetDC enzyme after the trypanosomatids diverged from other eukaryotes. AdoMetDC and prozyme then apparently co-evolved 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.

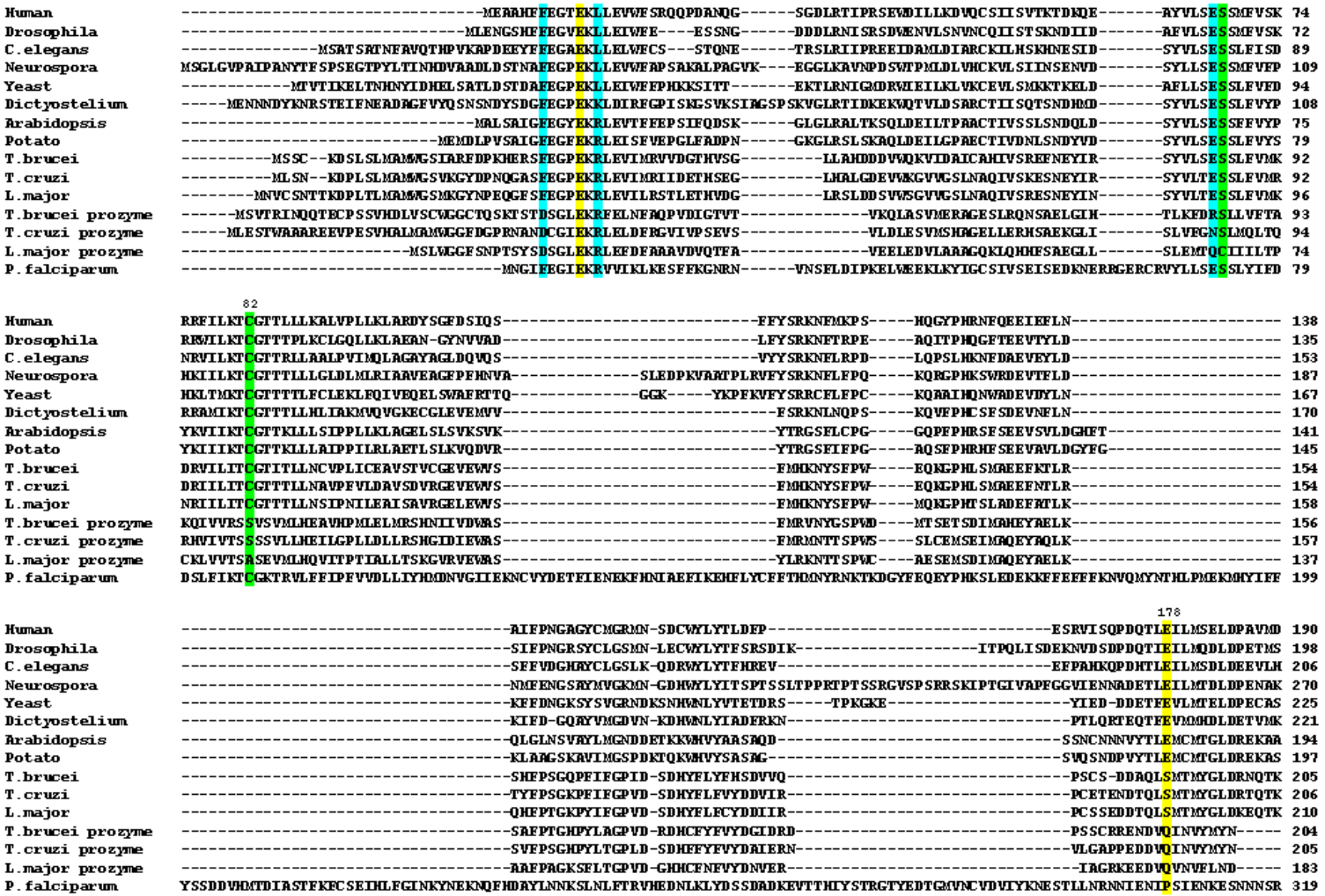
Our data suggest that regulators of enzyme function can evolve through gene duplication followed by mutational drift that results in loss of catalytic function. In addition to catalyzing chemistry, many enzymes also form functional interactions with macromolecules. The interaction binding surfaces may be maintained in these “pseudo-enzymes”, providing a perfect scaffold for the evolution of novel regulatory functions. A reported analysis of the genomes of metozoan species found that inactive homologs are common, and that they are present in a large variety of enzyme families (Pils and Schultz 2004). Few biochemical studies demonstrating the function of “pseudo-enzymes” have been published; however, several examples suggest that they function as regulators of the active enzymes. Our discovery of a “pseudo-enzyme” 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 (Beswick, Willert et al. 2006). The current observations support a model whereby putrescine induces a partial conformational change from the inactive structure towards the active one. However the fully active conformation is only realized 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.

AdoMetDC provides an alternative and likely very effective target for the development of new anti-trypanosomal 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 novel 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 (Arkin and Wells 2004; Noble, Endicott et al. 2004; Scheer, Romanowski et al. 2006).

While 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 (Toms, Kinsland et al. 2004). Substrate specificity changes have evolved by gene duplication in both the spermidine/spermine synthase family (Korhonen, Halmekyto et al. 1995) and in the group IV decarboxylase family (Shah, Akella et al. 2007). Ornithine decarboxylase in mammalian and yeast cells is regulated by a protein inhibitor termed antizyme, which appears to be a duplication of a catabolic enzyme in the pathway, spermine-spermidine N^1-acetyltransferase (Hoffman, Carroll et al. 2005). Antizyme is in turn regulated by antizyme inhibitor, which is itself a pseudo-enzyme having arisen by gene duplication of ornithine decarboxylase followed by loss of catalytic activity (Mangold 2006). The polyamine pathway thus provides a paradigm for the evolution of metabolic pathways and their regulation by gene duplication and divergence.


Human
Drosophila
C.elegans
Neurospora
Yeast
Dictyostelium
Arabidopsis
Potato
T.brucei
T.cruzi
L.major
T.brucei prozyme
T.cruzi prozyme
L.major prozyme
P.falciparum
82
178

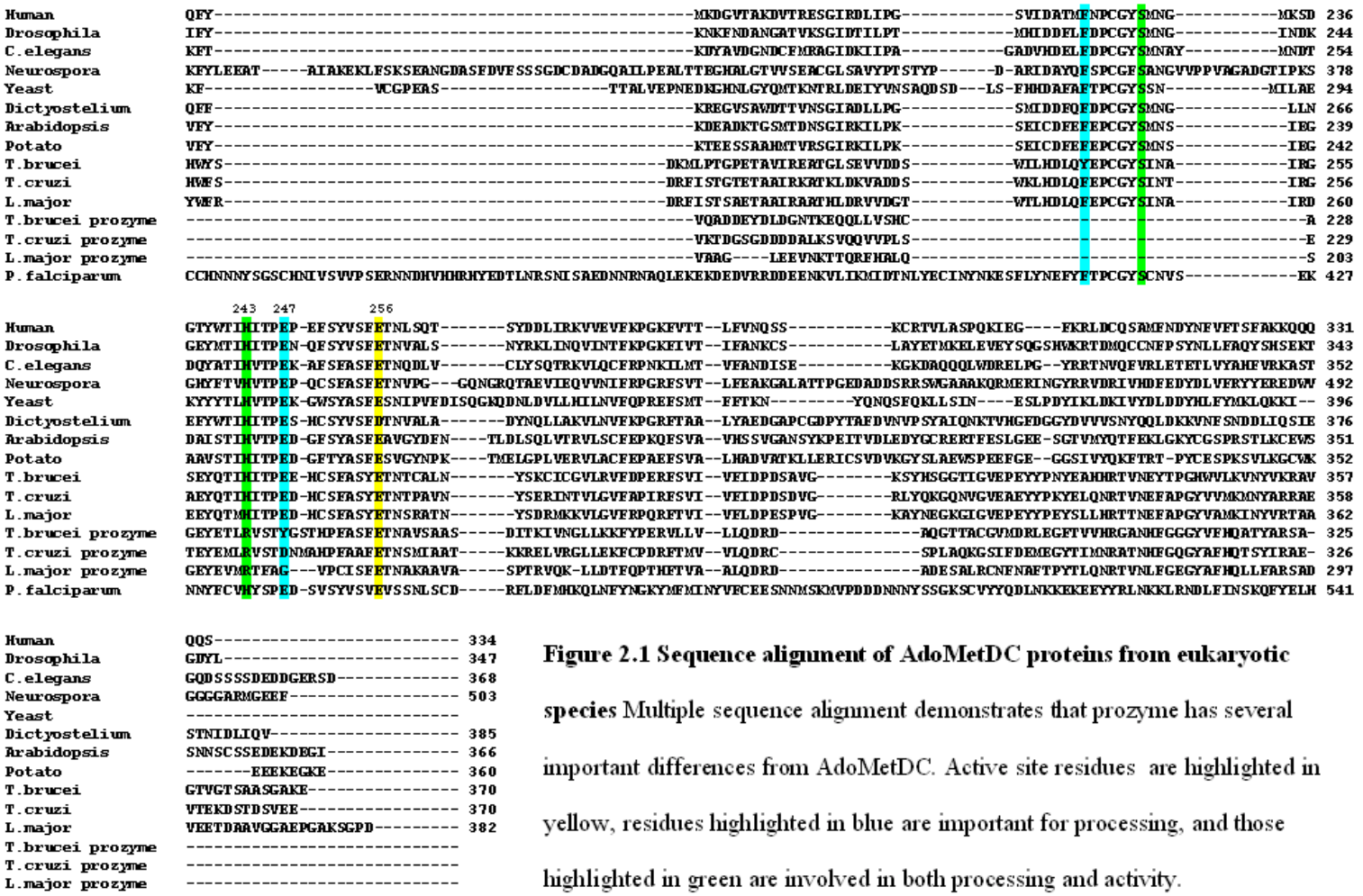


Figure 2.1 Sequence alignment of AdoMetDC proteins from eukaryotic species Multiple sequence alignment demonstrates that prozyme has several important differences from AdoMetDC. Active site residues are highlighted in yellow, residues highlighted in blue are important for processing, and those highlighted in green are involved in both processing and activity.

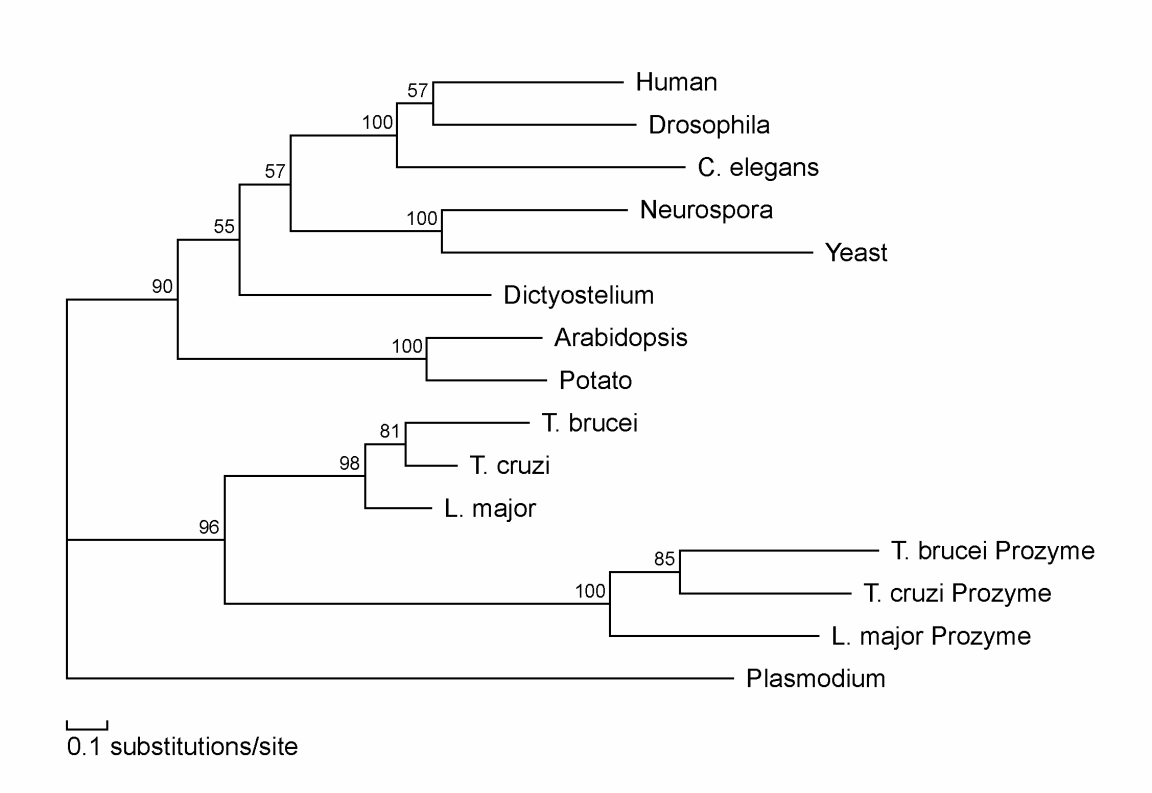


Figure 2.2 Phylogenetic analysis of AdoMetDCs from eukaryotic species The phylogenetic tree generated by multiple sequence alignment of eukaryotic AdoMetDCs shows that prozyme after the divergence of trypanosomatids from other eukaryotes.

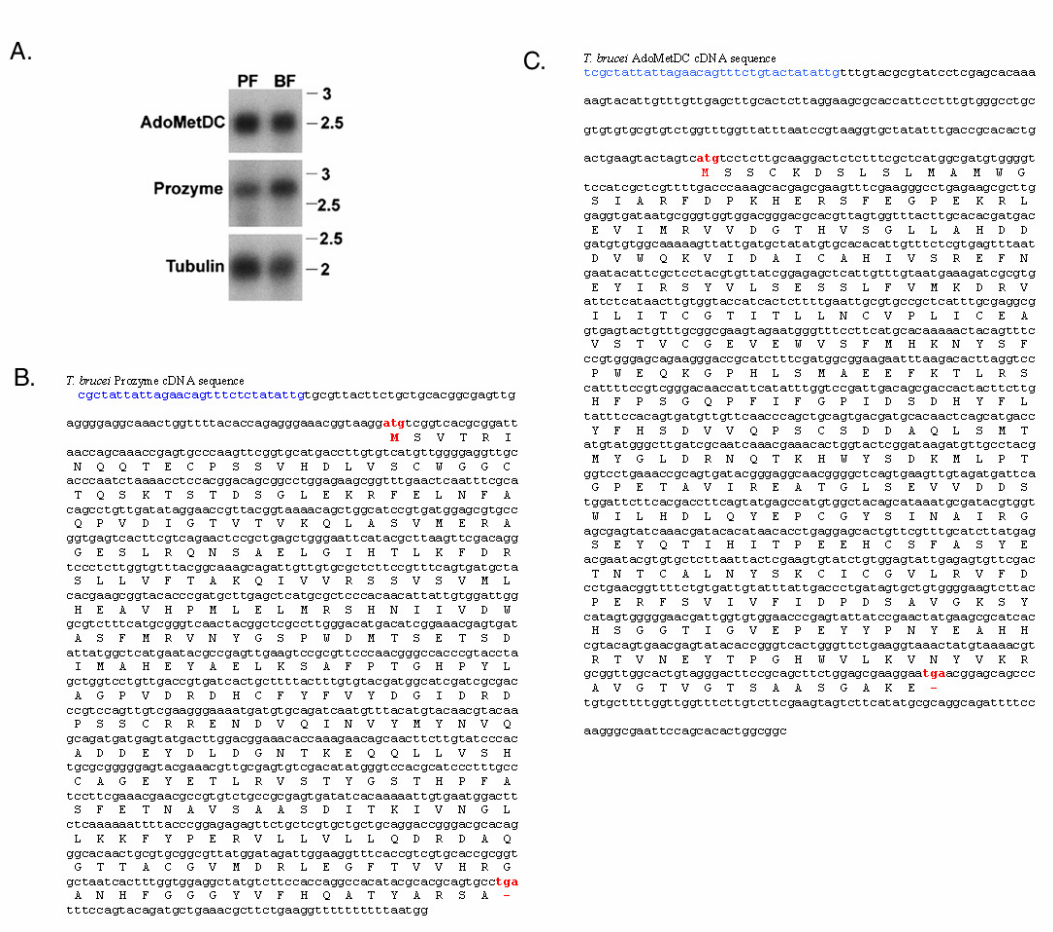


Figure 2.3 Analysis of the AdoMetDC and prozyme genes mRNA from bloodstream form (BF) and procyclic form (PF) trypanosomes was probed for AdoMetDC and prozyme (and tubulin as a loading control) by Northern blot analysis, demonstrating that both genes are present in both life cycle stages (A), determination of the 5'start site of AdoMetDC(B) and prozyme (C) mRNA.

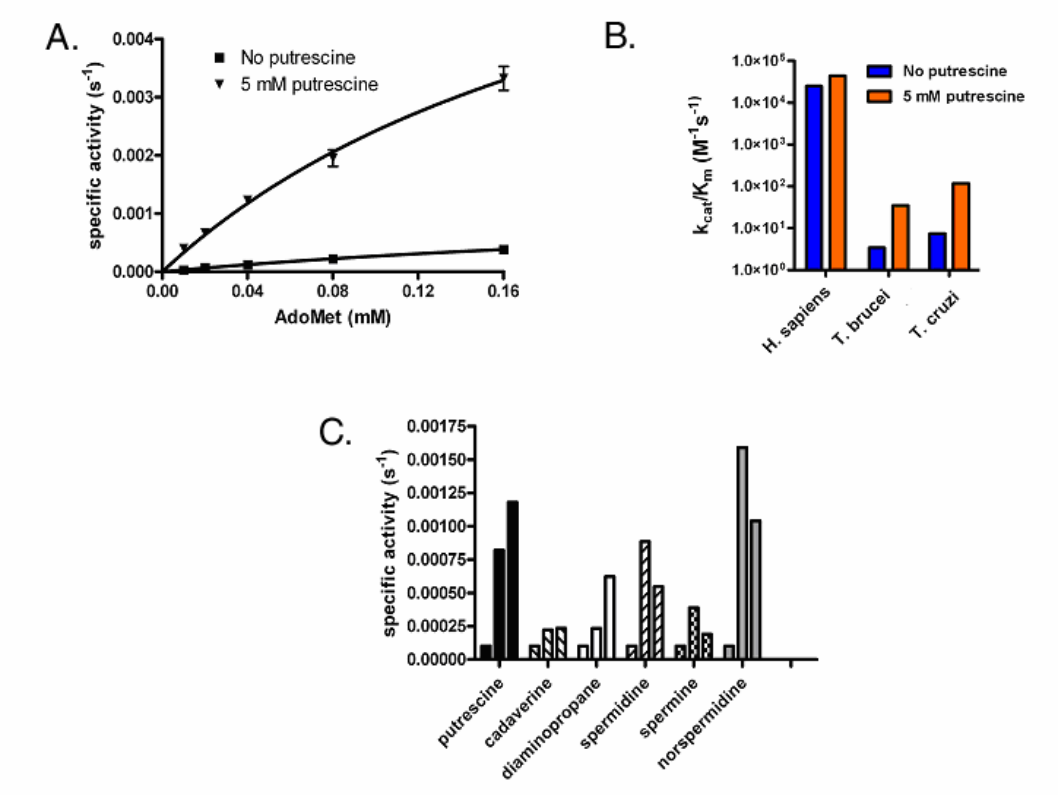


Figure 2.4. Kinetic analysis of *T. brucei* AdoMetDC Substrate titration of *T.brucei* AdoMetDC in the presence (triangles) and absence (squares) of putrescine (A), comparison of AdoMetDCs from human, *T.brucei* and *T.cruzi* in the presence (blue bars) and absence (orange bars) of putrescine (B) as well as activation of AdoMetDC by other polyamines (C). Bars (left to right in C) are 0, 0.5 and 5.0 mM polyamine in assay.

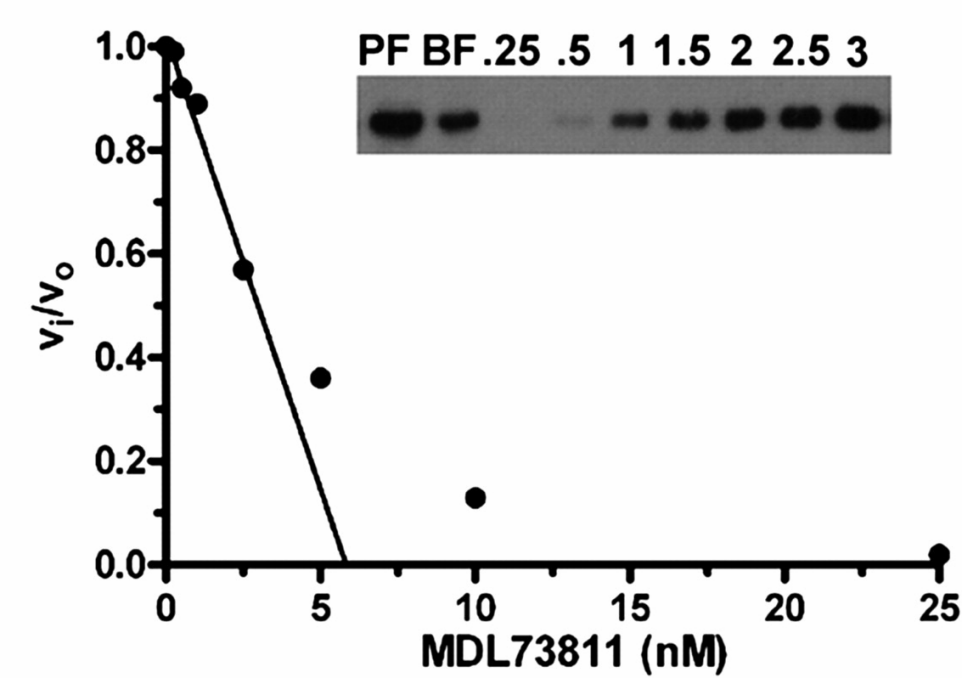


Figure 2.5 Concentration of AdoMetDC in BF lysates Active site titration of AdoMetDC in trypanosome BF cell lysates: the concentration of AdoMetDC in lysates was estimated from the linear portion of the graph by the x-intercept (AdoMetDC = 6 nM). Inset: Western blot of *T. brucei* AdoMetDC in PF and BF trypanosome cell lysates in comparison to a series of purified protein (amount shown in ng) estimates the AdoMetDC protein concentration in the BF lysates (AdoMetDC = 5 nM).

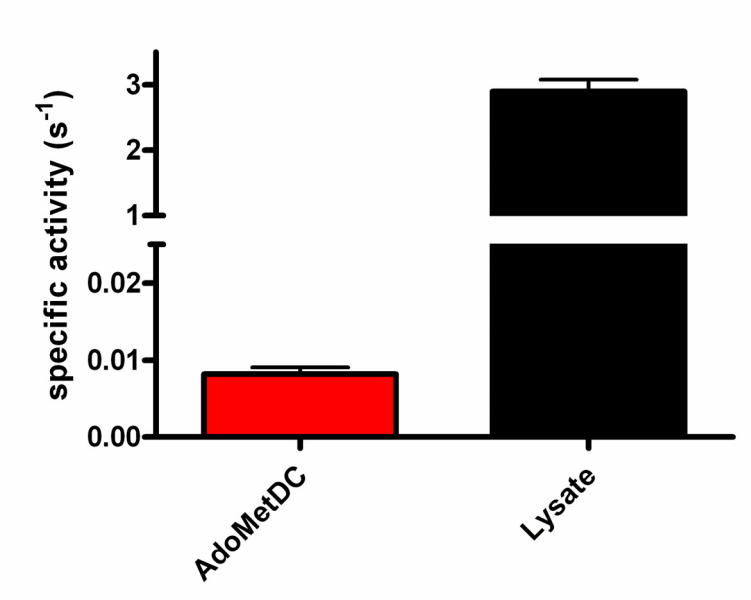


Figure 2.6 Specific activity of recombinant AdoMetDC and BF lysate. Assays measuring the AdoMetDC activity were carried out in the presence of 5 mM putrescine.

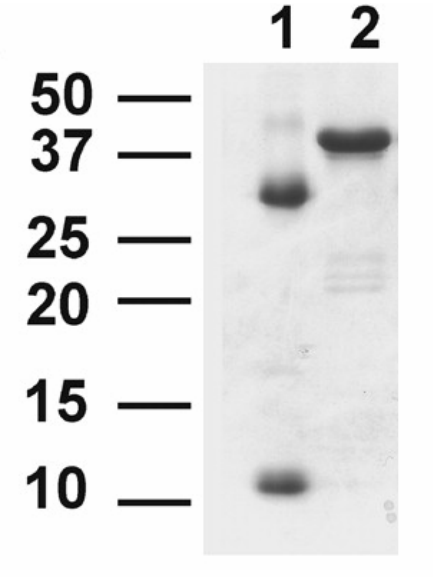


Figure 2.7 Expression of recombinant AdoMetDC and prozyme His_6-taged AdoMetDC and prozyme were expressed in *E. coli*, and then purified by Ni^{2+} and anion exchange. Both the α-and β-subunit of AdoMetDC are seen; prozyme does not process into an active enzyme.

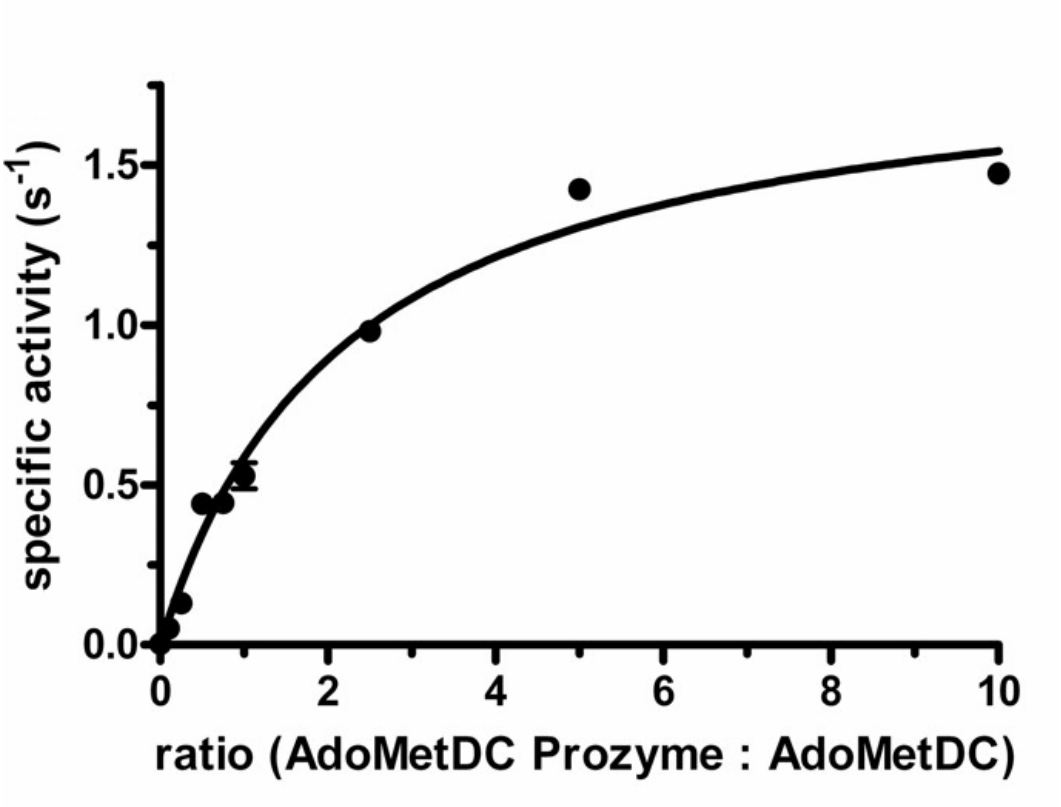


Figure 2.8 Prozyme activation of AdoMetDC The activation of AdoMetDC catalysis is demonstrated by titration of purified recombinant prozyme (0.2 to 2 μM) into purified, recombinant AdoMetDC (0.2 μM) at saturating AdoMetDC (1 mM).

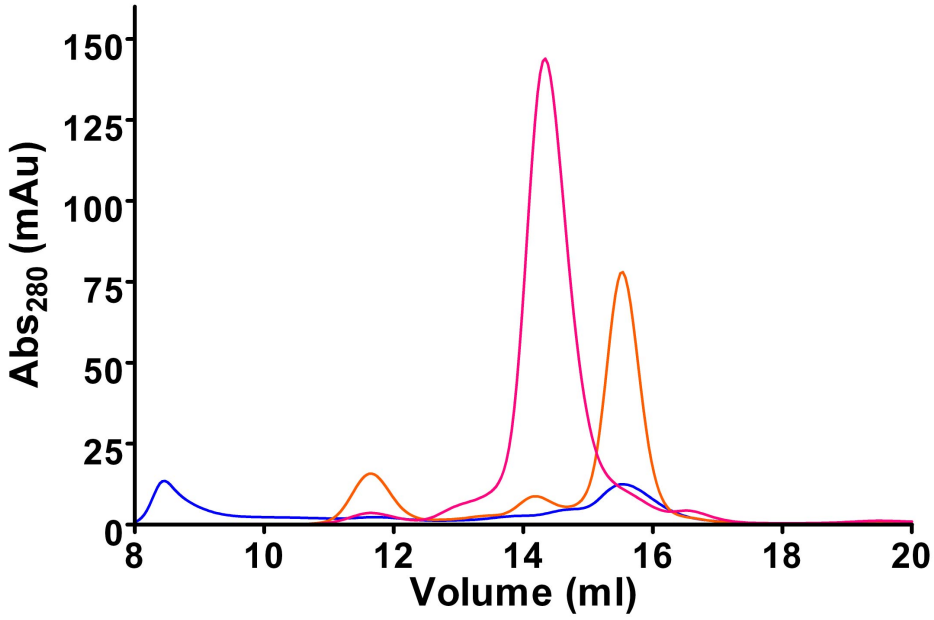


Figure 2.9 Gel filtration analysis of AdoMetDC, prozyme, and the co-purified complex. The figure shows an overlay of the chromatograms for 50 μM purified recombinant AdoMetDC (orange), AdoMetDC prozyme (blue) and AdoMetDC/prozyme complex (pink). The apparent molecular weight of each species was estimated based on standard molecular weight markers (BioRad) used to calibrate the column.

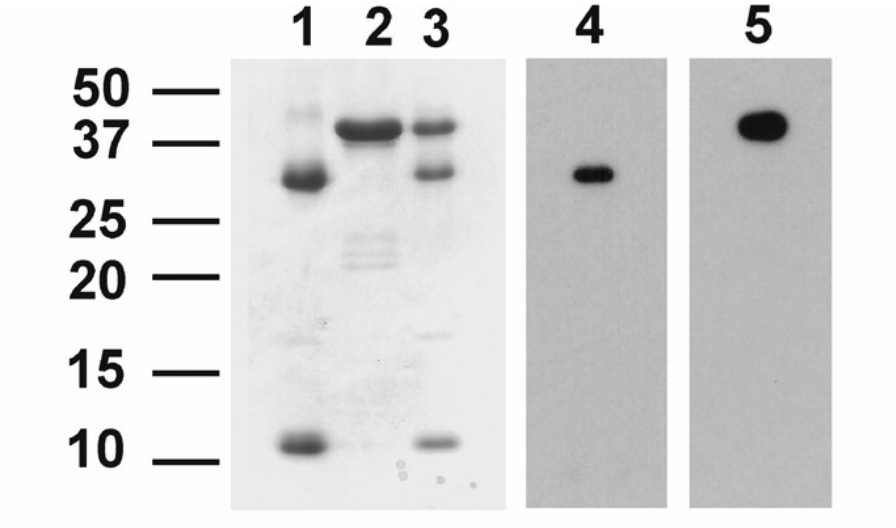


Figure 2.10 Co-purification of AdoMetDC and prozyme Recombinant His_6 tagged AdoMetDC (α- and β-subunits; 1) and Flag-tagged prozyme (2) were purified individually and also co-purified (3) over nickel, anion exchange and gel filtration columns. Western blot analysis of the co-purified complex was probed with α-*T. brucei* AdoMetDC (4) and α-FLAG (recognizing prozyme, 5).

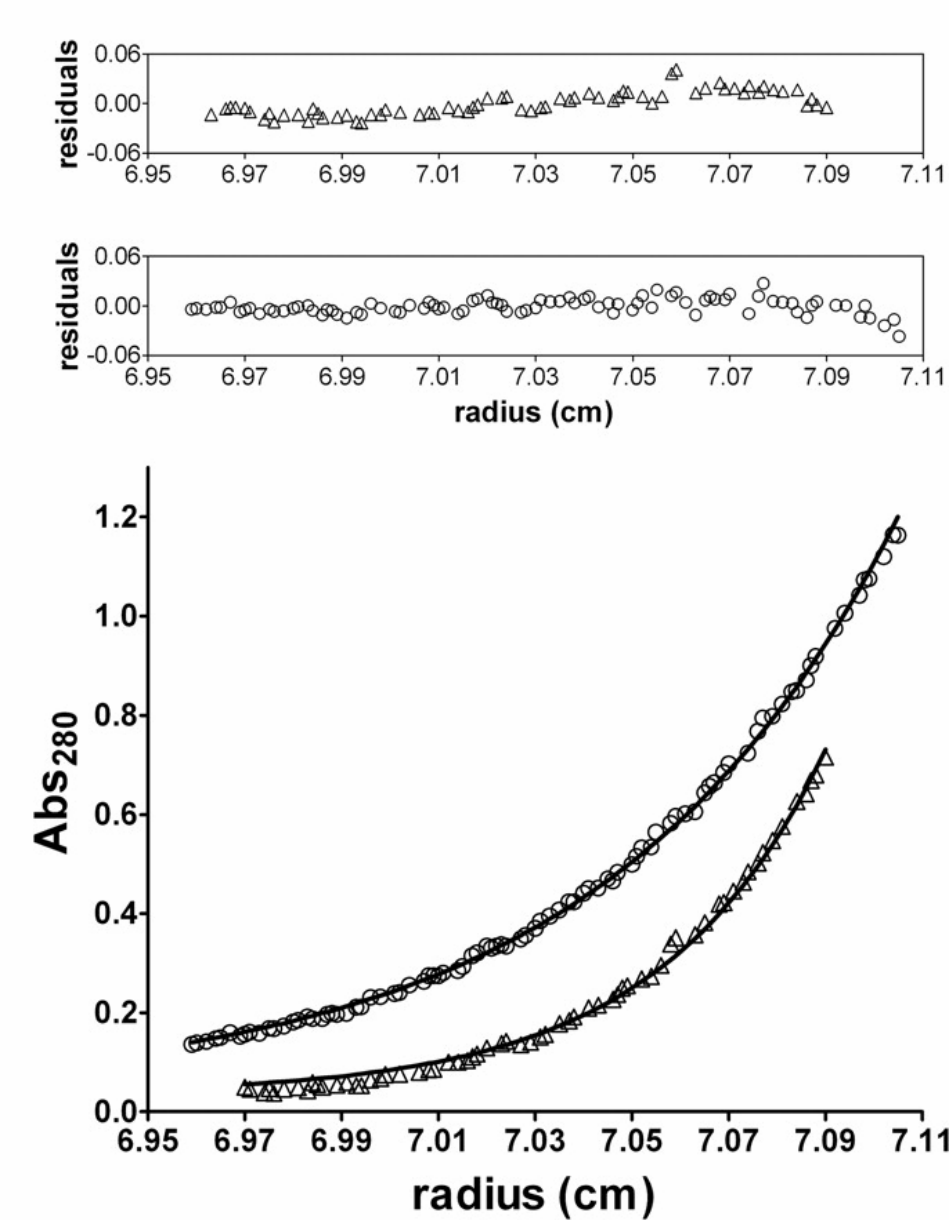
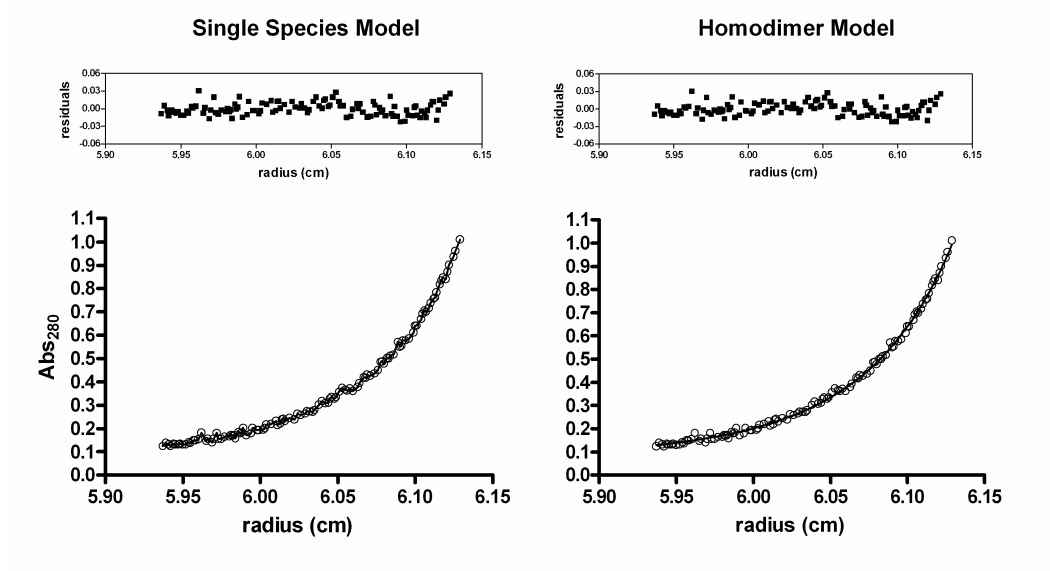


Figure 2.11 Analytical Ultracentrifugation of the AdoMetDC/prozyme heterodimer

Sedimentation equilibrium data were collected at 15,000 (o) and 20,000 (Δ) rpm at 6 μM complex. The data was globally fit to a single species model, and the molecular weight was found to be 80.4 kDa.

A.



B.

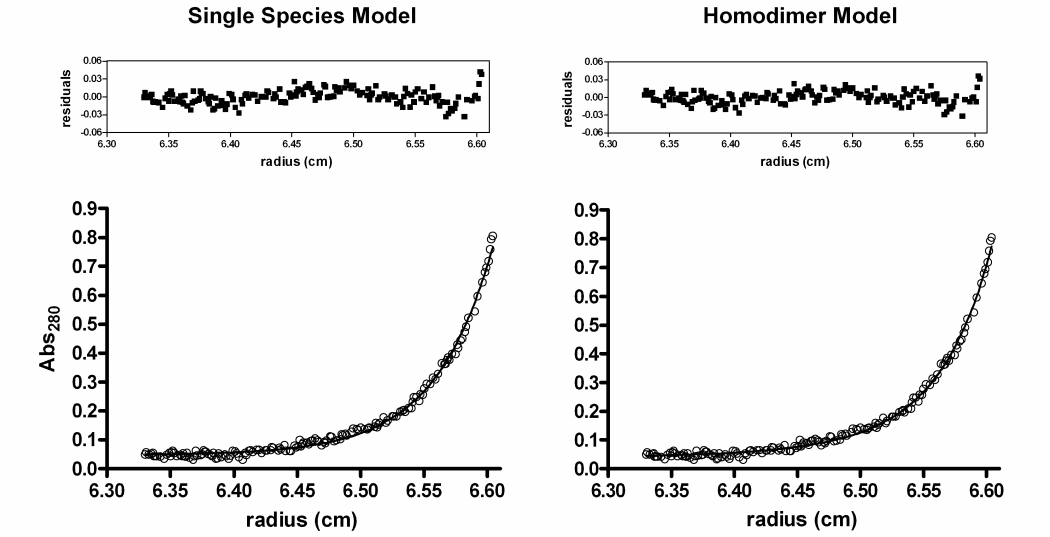


Figure 2. 12 Analytical Ultracentrifugation of AdoMetDC and prozyme homodimers

Sedimentation equilibrium analysis of AdoMetDC homodimer (A) and prozyme homodimer (B) was fit to a single species model (left) and a homodimer model (right).

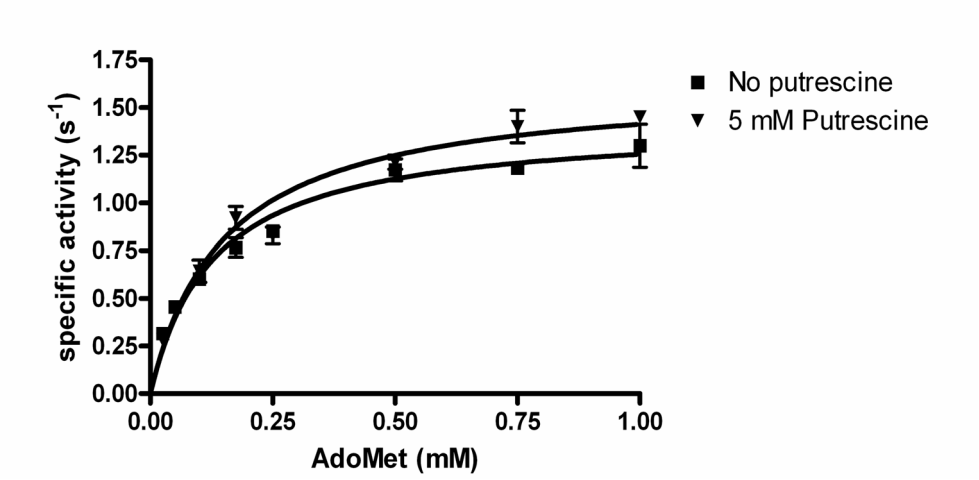


Figure 2.13 Kinetic Analysis of the AdoMetDC/prozyme heterodimer. Substrate titration of AdoMet to the AdoMetDC/prozyme heterodimer was fit to the Michaelis-Menton equation to determine kinetic parameters (see Table 2.2).

Gene	Vector	Site	Primer
AdoMetDC-Forward	pET 15b	NdeI	5'-CCATGCACAAGCACATGGCCTCTTGC-3'
AdoMetDC- Reverse	pET 15b	BamHI	5'-CGATAAGCTTGGATCCTCAGTGATGGTG-3'
Prozyme-Forward	pET 15b	XhoI	5'-CGCCTCGAGATGTCGGTCACGCGGATTAACCAGC-3'
Prozyme- Reverse	pET 15b	BamHI	5'-CGCGGATCCTCGGCACTGCGTGCGTATGTGG-3'
Prozyme-Forward	pT7-Flag	HindIII	5'-CGCAAGCTTATGTCGGTCACGCGGATTAACCAGC-3'
Prozyme- Reverse	pT7-Flag	KpnI	5'-CCACATACGCACGCAGTGCCTGAGGTACCGCG-3'
Spliced Leader			5'-CGCTATTATTAGAACAGTTTCTGTACTATATTG-3'
Prozyme 3' UTR-Reverse			5' GCTGAGGTGTGTTTTCGCGCTGACCAGGGTGACC 3'
AdoMetDC 3' UTR-Reverse			5' GGAAAATCTGCCTGCGCATATGAAGACTACTTCG- 3'

Table 2.1. Oligonucleotide primers Primers used to create protein expression constructs and for determination of the mRNA 5' start site are shown.

	No putrescine			5 mM putrescine		
	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m ($M^{-1}s^{-1}$)	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m ($M^{-1}s^{-1}$)
AdoMetDC	0.0013 ± 0.0004	0.38 ± 0.15	3.4 ± 0.5	0.0082 ± 0.0015	0.24 ± 0.06	35 ± 3
AdoMetDC/ prozyme	1.4 ± 0.1	0.11 ± 0.02	1.2 ± .1 x10^4	1.7 ± 0.1	0.17 ± 0.02	1.1 ± .05 x10^4

Table 2.2 Kinetic activity of recombinant AdoMetDC and AdoMetDC/ prozyme complex. Data were collected in triplicate and errors are the standard error of the mean. Steady-state kinetic parameters for human AdoMetDC in the presence of putrescine were previously reported to be k_{cat} = 2.6 s^{-1}; k_{cat}/K_m = 4.4 x 10^4 $M^{-1}s^{-1}$ (Beswick, Willert et al. 2006).

CHAPTER THREE

ACTIVATION OF ADOMETDC BY PROZYME FROM OTHER TRYPANOSOMATIDS

A. INTRODUCTION

T. cruzi AdoMetDC has been well characterized (Kinch, Scott et al. 1999; Kinch and Phillips 2000; Clyne, Kinch et al. 2002; Beswick, Willert et al. 2006). The *T. cruzi* protein shares 31% identity to the human protein, and 68% identity to *T. brucei* AdoMetDC. Processing of the enzyme into the mature form is not stimulated by putrescine, though putrescine is an allosteric activator of decarboxylation. Though putrescine stimulates the activity about 30 fold, the *T. cruzi* enzyme has a much lower activity than the human enzyme. In contrast to the human enzyme, *T. cruzi* AdoMetDC can be activated by other amines, such as cadaverine and CPG 48664A (a chemical inhibitor that activates *T. cruzi* AdoMetDC at low concentrations, then inhibits at higher amounts). Through mutational analysis, a mutant *T. cruzi* enzyme was discovered that had higher catalytic efficiency than the wild type protein. This suggested that the *T. cruzi* AdoMetDC has evolved to have sub-optimal catalytic efficiency.

We have demonstrated that the functional form of AdoMetDC in *T. brucei* is a heterodimer with prozyme (Willert, Fitzpatrick et al. 2007). The other trypanosomatids, *T. cruzi* and *Leishmania* species, also have a prozyme gene. Prozyme homologs from the three species share more than 65 % identity, even though the AdoMetDC and prozyme from the same species only have about 30% identity. Here we describe how AdoMetDC

and prozyme work in *T. cruzi*. Since the proteins are quite similar, we tested the ability of prozyme from one trypanosome species to activate AdoMetDC from the other species. We found that the *T. cruzi* prozyme can form a heterodimer with *T. cruzi* AdoMetDC. The AdoMetDC/prozyme heterodimer is much more active than the AdoMetDC homodimer, and in *T. cruzi* this activity is further stimulated by putrescine. We have also found that the *T. cruzi* prozyme can activate *T. brucei* AdoMetDC, and vice versa, to some degree.

B. MATERIALS AND METHODS

Cloning of *T. cruzi* prozyme and AdoMetDC

The expression construct (TcAdoMetDC) for *T. cruzi* AdoMetDC has been described previously (Beswick, Willert et al. 2006). The gene for *T. cruzi* prozyme was cloned and amplified by PCR from genomic DNA, and ligated into the pET 15b vector to make an N-terminal His_6-tagged construct (Tcprozyme). To ease co-purification, we created a double expression vector (DEV) that allows for expression of both His-tagged AdoMetDC and untagged prozyme in *E.coli*. First, the *T. cruzi* prozyme gene was introduced into the pET22b with its stop codon to produce an untagged protein. Then, the ribosomal binding site (rbs), the T7 promoter and the His_6-AdoMetDC coding region was amplified by PCR from the TcAdoMetDC construct and ligated into this vector to create the DEV. (Table 3.1 contains the primers used.)

Expression and purification of *T. cruzi* prozyme and AdoMetDC/prozyme complex

T. cruzi AdoMetDC or prozyme were expressed individually in *Escherichia coli* BL21/DE3 cells containing the respective construct, and purified in two steps by Ni^{2+}-

agarose (Qiagen) and anion exchange column chromatography (Amersham Mono Q 5/50 GL column) as described (Beswick, Willert et al. 2006), except that the buffer was exchanged after elution from Ni^{+2} agarose and Mono Q by a HiPrep 26/10 desalting column (Amersham) equilibrated in storage buffer (50 mM Hepes pH 8.0, 50 mM NaCl, 1 mM dithiothreitol). For co-purification, the *T. cruzi* DEV construct was expressed in *E. coli,* and the proteins were purified as above. Purified proteins were quantified using their respective extinction coefficients (at 280 nm): AdoMetDC, 61.3 mM^{-1} cm^{-1}; prozyme, 36.9 $mM^{-1}cm^{-1}$; AdoMetDC/prozyme complex, 98.2 mM^{-1} cm^{-1}. SDS-PAGE was used to assess purity.

Steady-state kinetic analysis

Steady state kinetics were performed by trapping liberated $^{14}CO_2$ on a filter paper soaked in saturated barium hydroxide as described (Kinch, Scott et al. 1999; Beswick, Willert et al. 2006). For homodimeric AdoMetDC, Reactions were performed over a range of enzyme (1 - 4 μM homodimer, 25-400 nM heterodimer) and were incubated with 1-[^{14}C]-AdoMet (25 μM) and unlabeled AdoMet (0 - 975 μM) with or without saturating putrescine (5 mM) at 37 °C in buffer (100 mM HEPES pH 8.0, 50 mM NaCl, and 1 mM DTT). Reactions were allowed to proceed for various times (2.5 – 20 min) before quenching with 6 M HCl. Substrate titration data were fitted to the Michaelis-Menten equation to determine the steady-state kinetic parameters using Prism (GraphPad).

To analyze the activation of AdoMetDC by prozyme, the proteins were incubated together at various ratios (1:0.25 to 1:20 (AdoMetDC: prozyme) during the AdoMetDC assay. To understand the kinetics of activation, the recombinant AdoMetDC and prozyme

were mixed at a ratio of 1:10, a substrate titration was performed as described above, and the data was analyzed by Michaelis-Menton kinetics.

Analytical Ultracentrifugation

The molecular weight of the *T.cruzi* AdoMetDC/prozyme complex was determined by equilibrium sedimentation analysis using a Beckman XLI analytical ultracentrifuge, as described in chapter 2. Samples (4, 6 and 8 μM) were equilibrated for data collection at 14,000, 20,000 and/or 28,000 rpm. Data sets were analyzed using equations describing a single ideal species model (equation 2.1), or a or a monomer-heterodimer model (equation 2.3), as previously described (Lebowitz, Lewis et al. 2002). Predicted molecular weights for the *T. cruzi* proteins are: AdoMetDC monomer 44 kDa; prozyme monomer 36.6 kDa and heterodimer 80.6 kDa.

C. RESULTS

T. cruzi AdoMetDC is activated by prozyme

Recombinant, His-tagged *T. cruzi* AdoMetDC and prozyme were expressed individually and activity was determined by the standard ^{14}C assay, in the presence and absence of putrescine (Figure 3.1A). As previously reported, the activity of *T. cruzi* AdoMetDC is stimulated by putrescine; however, it displays low activity compared to other eukaryotic orthologs, such as the human enzyme (Beswick, Willert et al. 2006). Similar to the protein in *T. brucei* (Willert, Fitzpatrick et al. 2007), the *T. cruzi* prozyme has no intrinsic decarboxylase activity. When we added *T. cruzi* prozyme to AdoMetDC, we saw an increase in specific activity (Figure 3.1B). The recombinant prozyme

aggregates during purification, and therefore the amount needed to activate AdoMetDC can not be determined accurately from this experiment.

***T. cruzi* AdoMetDC/prozyme complex is stimulated by putrescine**

Since the *T. brucei* prozyme aggregation problem is relieved when it is co-purified together with AdoMetDC, we co-expressed and co-purified the *T. cruzi* AdoMetDC and prozyme proteins. The proteins remained associated through Ni^{2+}, anion exchange and gel filtration column chromatography (Figure 3.2). This co-purified complex was used to determine the kinetic parameters by substrate titration (Figure 3.3 and Table 3.2). In the absence of putrescine, the catalytic efficiency of the *T. cruzi* heterodimer is increased by more than 100 fold over the unstimulated homodimer. In contrast to the situation in *T. brucei,* the *T. cruzi* heterodimer is further stimulated by putrescine by an additional 5 fold (Figure 3.3). Putrescine has affects on both kinetic parameters; it lowers the substrate K_m and increases the k_{cat}. The catalytic efficiency of *T. cruzi* AdoMetDC/prozyme complex (5×10^3 $M^{-1}s^{-1}$) is about 2 fold lower than that for the *T. brucei* complex ($11 \times 10^3 M^{-1}s^{-1}$) in the presence of putrescine.

***T. cruzi* AdoMetDC/prozyme form a heterodimer**

To determine the oligomeric state of the *T. cruzi* AdoMetDC/prozyme complex, we used sedimentation equilibrium analysis by analytical ultracentrifugation. The samples were run at three concentrations (4, 6 and 8 μM) at three speeds (14, 20 and 28 krpm). The data was fit to a single species model and a model for heterogeneous association of two proteins. The average molecular weight determined by fitting to the single species model is 71.8 ± 4 kDa. This value indicates that there is more heterodimer (predicted mass of 81 kDa) than monomer (prediceted masses of 37 kDa for prozyme and

44 kDa for AdometDC) present. The dissociation constant for the heterodimer was found to be 0.55 ± 0.2 μM and is significantly lower than that measured for the *T. cruzi* AdoMetDC homodimer (Kd = 15-30 μM) (Clyne, Kinch et al. 2002).

AdoMetDC from one trypanosome species can be stimulated by prozyme from the other

The AdoMetDC and prozyme proteins are well conserved in the trypanosomatid family; each enzyme is approximately 65% similar to the orthologs in the other species. Though prozyme arose by a gene duplication event from AdoMetDC, there was considerable mutational drift, so that the AdoMetDC and prozyme paralogs from the same species have less than 30 % similarity. We wanted to test the ability of prozyme from one species to activate the AdoMetDC from another. These experiments may also show why *T. cruzi* requires putrescine for full activation.

By titrating in *T. cruzi* prozyme to *T. brucei* AdoMetDC and vice versa, we saw that the prozyme from one trypanosome species was able to activate AdoMetDC from the other. In order to compare the activation properties, the subunits were mixed in a ratio of 1:10 AdoMetDC: prozyme, and assayed. The data from a substrate titration were then fit to the Michaelis-Menton equation to determine the k_{cat}, K_m and k_{cat}/K_m values (Figure 3.5 and Table 3.2). As seen for the co-purified complex, *T. cruzi* AdoMetDC is activated by its own prozyme and is further stimulated in the presence of putrescine. The catalytic efficiency of *T. brucei* AdoMetDC is stimulated by its prozyme in the presence and absence of putrescine. However, the activity of each AdoMetDC was stimulated by the homologous prozyme from the other species only in the presence of putrescine.

D. CONCLUSIONS

Evolutionary considerations suggest that the functional form of *T. cruzi* AdoMetDC would be similar to the enzyme in *T. brucei* and form a heterodimer with prozyme to make an active enzyme. We have shown that this is the case; however, there are a few differences between the species. The *T cruzi* AdoMetDC/prozyme heterodimer is much more active than the AdoMetDC homodimer. In both species, the heterodimer forms with a higher affinity than the AdoMetDC homodimer. Unlike the enzyme from *T. brucei, T. cruzi* AdoMetDC/prozyme heterodimer is stimulated by putrescine. This stimulation reflects both increased k_{cat} and decreased K_m values, and the catalytic efficiency is increased by 5 fold. The overall activity of the *T. cruzi* complex is also slightly lower than that for *T. brucei.*

Since *T. cruzi* cannot synthesize putrescine *de novo,* this additional regulation by putrescine may be a way for the parasite to control polyamine levels in response to the extracellular environment. In situations where *T.cruzi* has abundant polyamine pools to import, it can convert this to spermidine, which can then go on to make trypanothione. Putrescine may have a distinct role separate from being a precursor to spermidine, and in times of low putrescine, the slower enzyme may allow for the maintenance of putrescine pools. Though *T. cruzi* can also import spermidine, AdoMetDC inhibitors have been shown to be effective against mouse models of disease (Yakubu, Majumder et al. 1993). Once AdoMet is converted to dcAdoMet, it can no longer be used for trans-methylation reactions. Having functional AdoMetDC activity may help protect the cell from hypermethylation.

The similarity of trypanosome AdoMetDCs and prozymes on the protein level suggested that the homologous prozyme may be able to activate AdoMetDC from the other species. Prozyme from either species, which has no intrinsic activity, was mixed in 10 fold excess with AdoMetDC and assayed. Since the enzymes show differences in putrescine activation, activity was tested in the presence and absence of putrescine. For comparison, we looked at the activation of each AdoMetDC by its own prozyme. *T. brucei* and *T. cruzi* AdoMetDCs are activated by their own prozymes, and the activity of the *T. cruzi* mixture is further stimulated by putrescine, with effects seen in both kinetic parameters. The catalytic efficiency of the mixed complex is lower than that for the co-purified heterodimer by about 2 fold.

The activity of *T. brucei* AdoMetDC was activated by *T. cruzi* prozyme only in the presence of putrescine. This stimulation consisted a slightly increased k_{cat} (~2 fold) and a decreased K_m (~10 fold), resulting in a catalytic efficiency increase of about 18 fold. Similarly, the *T. cruzi* AdoMetDC was stimulated by *T. brucei* prozyme only in the presence of putrescine. The catalytic efficiency in the absence of putrescine is still much higher than unstimulated AdoMetDC homodimers, but increases another 7 fold in the presence of putrescine. This affect is due to an almost 17 fold increase in k_{cat}. Therefore, both subunits of the *T. cruzi* AdoMetDC/prozyme complex require putrescine for full activity. This result also shows that the prozyme and AdoMetDC proteins are somewhat interchangeable in the trypanosome species *T. brucei* and *T. cruzi.*

In *T. cruzi*, the current drugs available for treatment are only effective against the early stage of the disease. This stage of the disease is often asymptomatic, and therefore is not often diagnosed and treated. The development of new drugs is urgently required,

and since inhibitors of AdoMetDC have proven to be effective in mouse models of disease, AdoMetDC is a valid drug target. We have shown that the functional form of the enzyme in *T. cruzi* is the AdoMetDC/prozyme heterodimer. The knowledge of the active complex may facilitate the development of species-specific inhibitors.

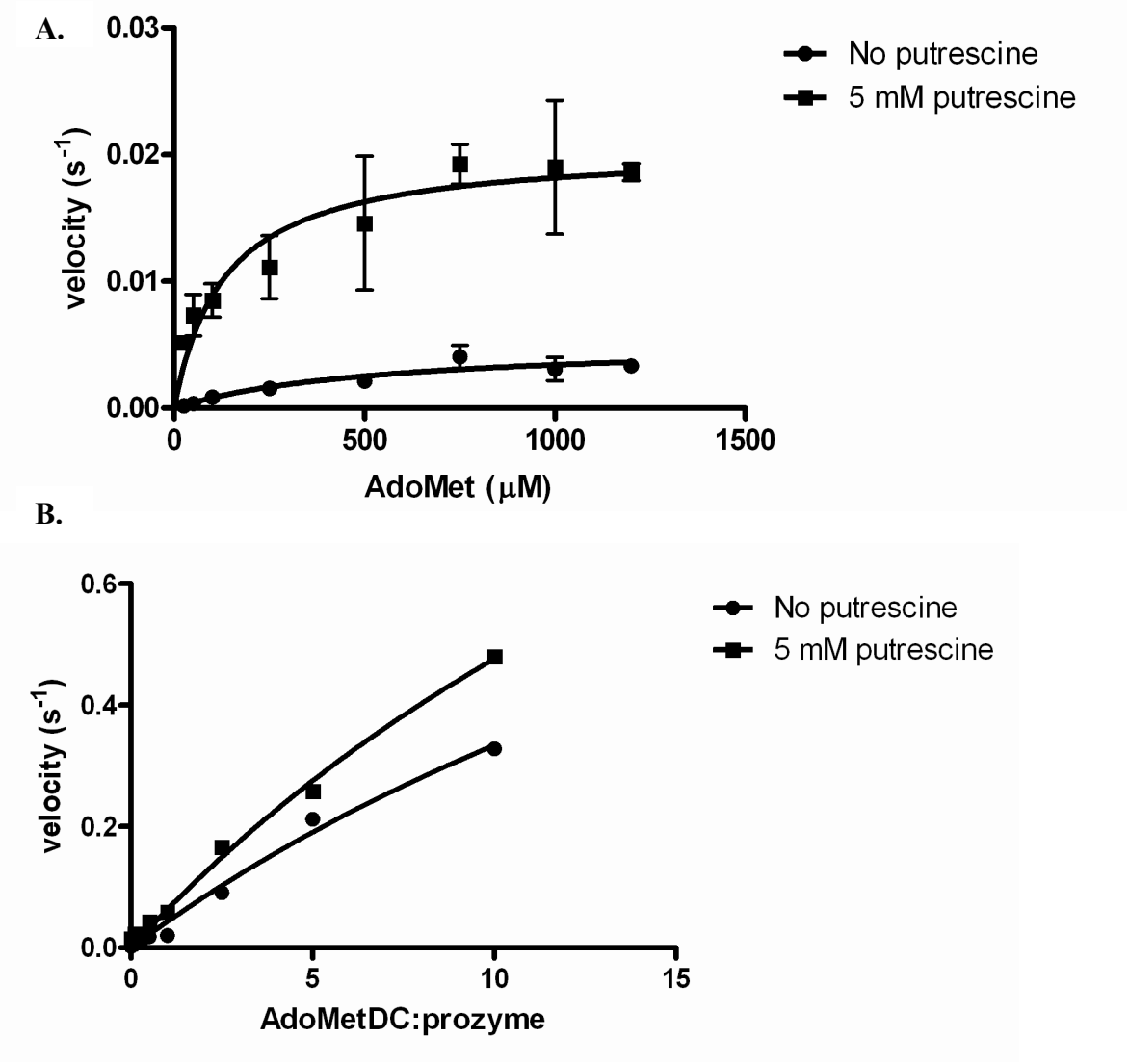


Figure 3.1 *T. cruzi* AdoMetDC is activated by prozyme. The kinetic parameterss of the recombinant *T. cruzi* AdoMetDC homodimer were determined by substrate titration (A), and the activation of AdoMetDC by prozyme is seen by the addition of increasing amount of *T. cruzi* prozyme to AdoMetDC (B).

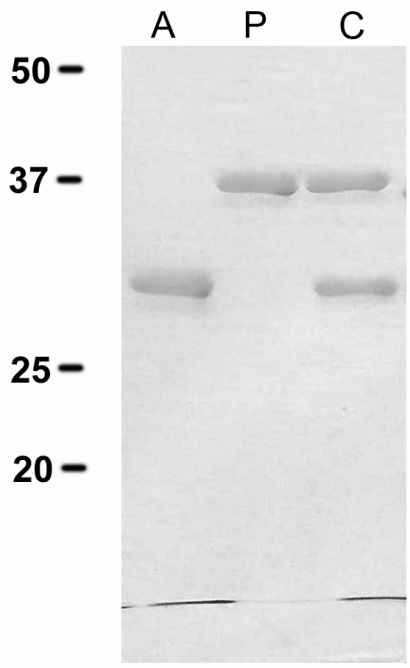


Figure 3.2 Co-purification of *T. cruzi* AdoMetDC and prozyme. The α and β subunits of AdoMetDC (A) are visualized by Commassie stain, while prozyme (P) is a single polypeptide. The co-purified complex (C) of AdoMetDC and prozyme remains together through three chromatography steps.

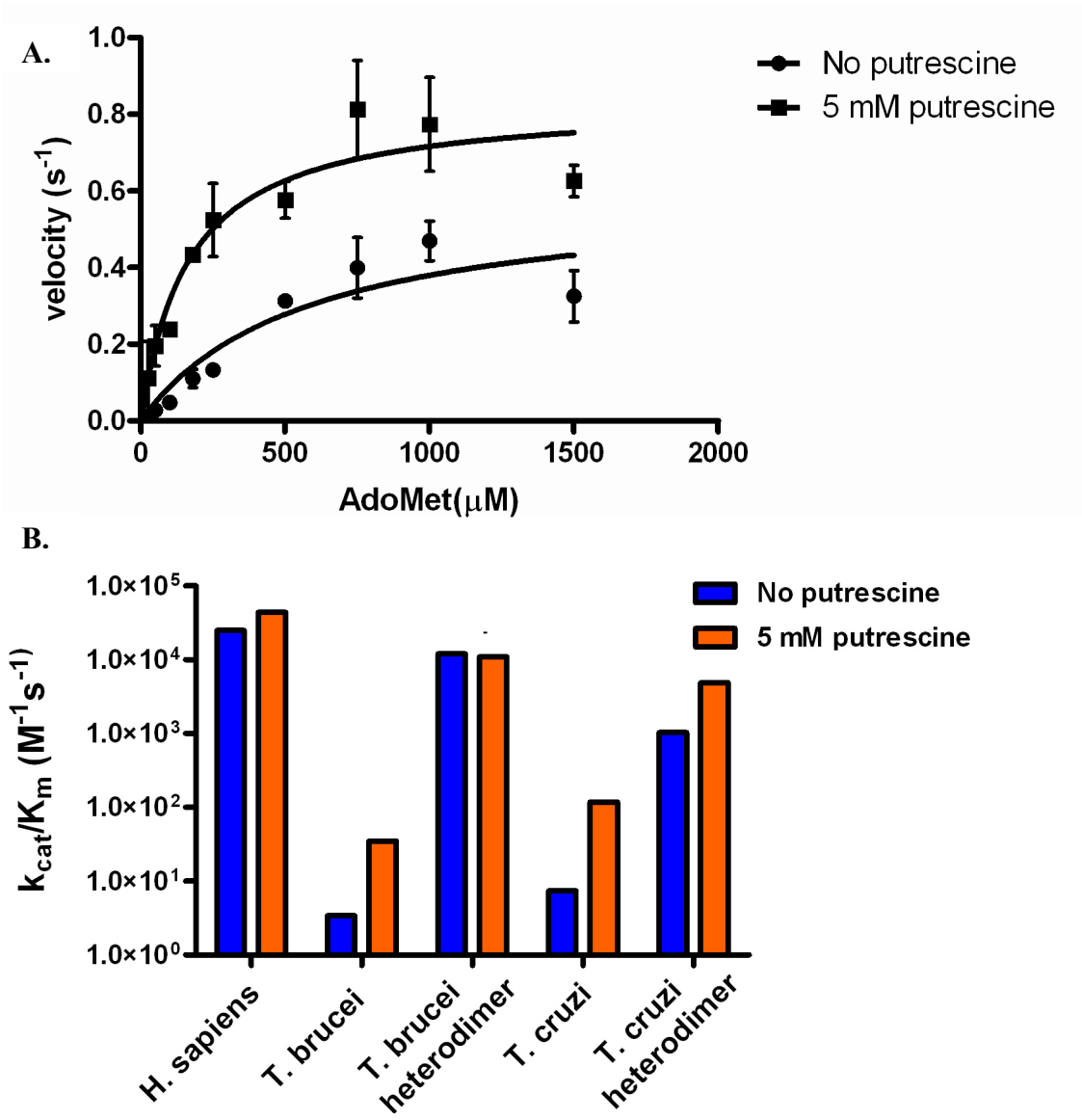


Figure 3.3 *T. cruzi* AdoMetDC/prozyme complex is stimulated by putrescine.

Kinetic analysis of the co-purified complex shows that *T. cruzi* AdoMetDC/prozyme is stimulated by putrescine (A), and a comparison of the catalytic efficiency of AdoMetDCs from human and the trypanosome species (B) demonstrates the variation of allosteric activation.

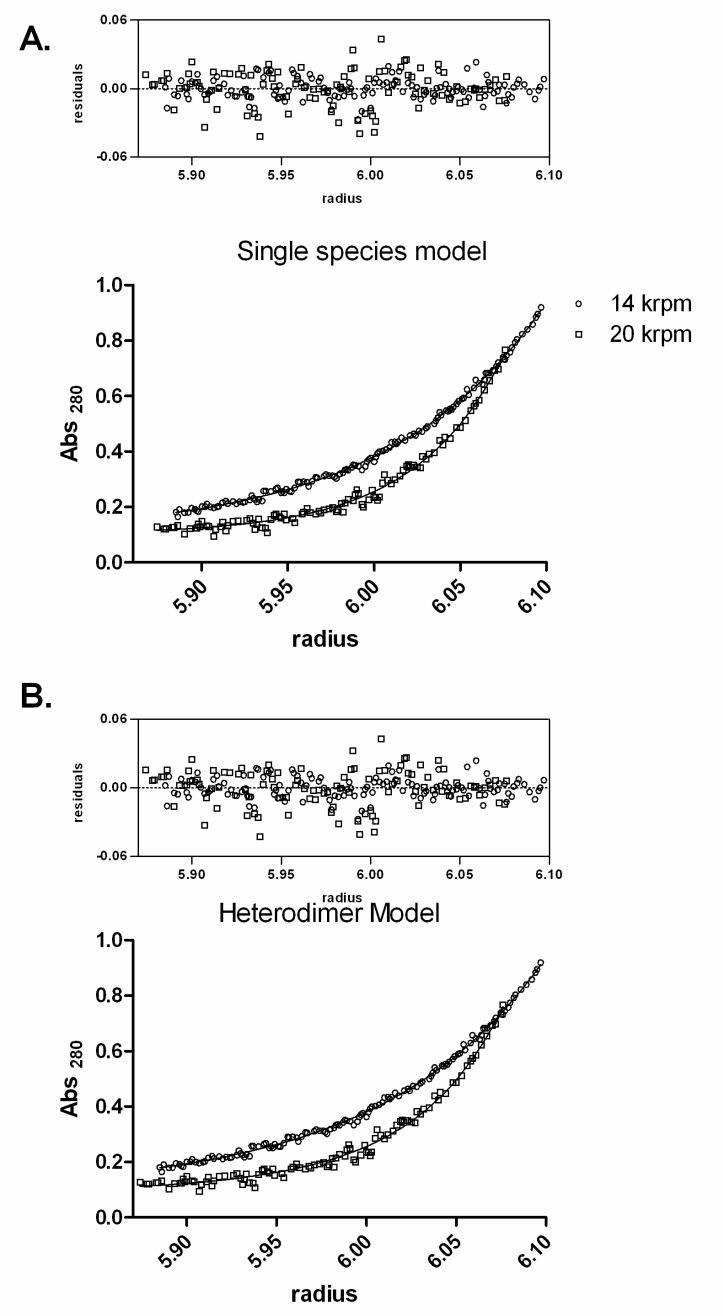


Figure 3.4 Analytical Ultracentrifugation of *T. cruzi* AdoMetDC/prozyme complex.

The molecular weight (single species model, A) and dimerization K_d (heterodimer model, B) of the co-purified complex (8 μM) were determined by analytical ultracentrifugation.

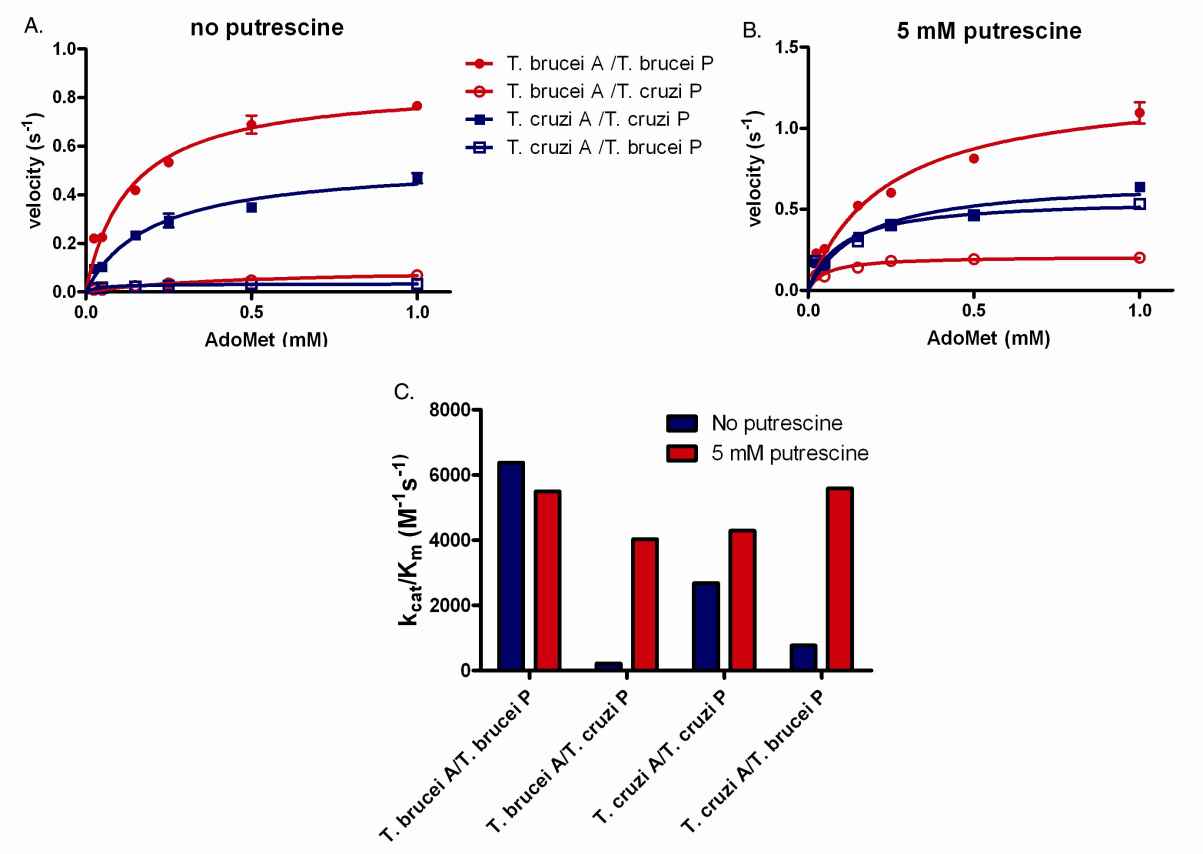


Figure 3.5 Activation of AdoMetDC by prozyme homologs from other species.

Substrate titration of AdoMetDC: prozyme at 1:10 was performed in the absence (A) or presence (B) of putrescine. A comparison of activation by homologous prozyme (C) indicates that both subunits are involved in the stimulation by putrescine seen in the heterodimer.

Gene	Vector	Site	Primer
Tc prozyme-Forward	pET 15b	NdeI	5′-CGCCATATGTTGGAGAGCACCTGGGCAGCCG-3′
Tc prozyme-Reverse	pET 15b	BamHI	5′-CGCGGATCCTTATTCGGCGCGAATATAGCTGG-3′
Tc DEV-Forward	pET 22b-DEV	SalI	5′-CGATAGTCGA**C**TCGAAATTAATACGACTCACTATAGG-3′
Tc DEV- Reverse	pET 22b-DEV	BamHI	5′-TATGCGGCCGCTTACTACTCTTCCACAGAATCTGTGG-3′

Table 3.1 Oligonucleotide primers Primers used to create the *T.cruzi* expression construct and the double expression vector.

	No putrescine			No putrescine		
	k_{cat} (s^{-1})	K_m (M)	k_{cat}/K_m ($M^{-1}s^{-1}$)	k_{cat} (s^{-1})	K_m (M)	k_{cat}/K_m ($M^{-1}s^{-1}$)
***T. cruzi* homodimer**	0.005	5.4×10^{-4}	9.3	0.021	1.3×10^{-4}	151.5
***T. cruzi* heterodimer**	0.6	5.8×10^{-4}	1033	0.84	1.7×10^{-4}	5027
***T. brucei* homodimer**	0.001	3.8×10^{-5}	3.4	0.008	2.4×10^{-5}	35
***T. brucei* heterodimer**	1.4	5.8×10^{-5}	12000	1.7	1.7×10^{-5}	11000
***T.brucei* A/*T.brucei* P**	0.86	1.3×10^{-4}	6379	1.3	2.3×10^{-4}	5496
***T.brucei* A/*T.cruzi* P**	0.098	4.5×10^{-4}	215	0.210	5.2×10^{-5}	4033
***T.cruzi* A/*T.cruzi* P**	0.53	1.9×10^{-4}	2684	0.69	1.6×10^{-4}	4292
***T.cruzi* A/*T.brucei* P**	0.034	4.4×10^{-5}	772	0.57	1.0×10^{-4}	5592

Table 3.2 Kinetic Analysis of AdoMetDC/prozyme complexes. Substrate titration of AdoMetDC homodimers, AdoMetDC/prozyme heterodimers, and prozyme homolog activated complexes were fit to the Michaelis-Menton equation to determine kinetic parameters.

CHAPTER FOUR

ADOMETDC KNOCKDOWN IN *T. BRUCEI* SHOWS ITS ROLE IN POLYAMINE/TRYPANOTHIONE REGULATION

A. INTRODUCTION

Though the polyamine pathway is tightly regulated in most cells, the control of this pathway in trypanosomatids is largely unknown. Trypanosomes use spermidine to generate trypanothione, a unique cofactor used in cellular redox reactions. We are interested in looking at the regulation of the polyamine and trypanothione pathways in *T. brucei.*

We have shown, in contrast to the AdoMetDC protein in humans, the active form of the enzyme in trypanosomes is an AdoMetDC/prozyme heterodimer (Willert, Fitzpatrick et al. 2007); the prozyme has the potential to act as a regulator of flux in this pathway. AdoMetDC has previously been implicated as a drug target by studies with chemical inhibition of the enzyme; here we complement these studies by genetically validating this enzyme as a drug target. The genetic validation and studies on the regulation of these pathways was accomplished by depleting the cells of AdoMetDC protein, then measuring the metabolites and protein levels of the other enzymes in the pathway.

AdoMetDC was depleted from the *T. brucei* cells by RNA interference (RNAi). RNAi is a process in which double stranded RNA triggers the destruction of homologous messenger RNA (mRNA). Long, double stranded RNA molecules are processed into

shorter fragments of 21-25 base pairs, called siRNAs (small, interfering RNAs). The siRNA is loaded into a RNA-induced silencing complex (RISC), which is activated when the siRNA is unwound. The RISC effector complex recognizes complementary mRNA and cleaves it, leading to the loss of mRNA and subsequently protein of the target (reviewed in (Ullu, Tschudi et al. 2004)). RNAi is functional in many organisms, including *T. brucei,* and though it is a natural process, it has become a very useful research tool.

To create an RNAi cell line, a construct that drives expression of double stranded AdoMetDC RNA was integrated into the *T. brucei* genome. This construct is tetracycline (tet) regulatable, and therefore is not expressed until tet is added to the culture. The stem-loop structure of double stranded RNA that is produced in response to tet is targeted to the AdoMetDC message. The message and protein levels are depleted, and there are several changes in the pathway resulting from this depletion. The response of the trypanosome to loss of AdoMetDC highlights the role of this protein in polyamine and trypanothione regulation and homeostasis.

B. MATERIALS AND METHODS

Trypanosome cell cultures

Bloodstream form trypanosomes were cultured in HMI-9 media supplemented with 10% serum in a at 37 °C, 5% CO_2 incubator (Hirumi and Hirumi 1989). When culturing trypanosomes, chicken serum (CS) replaced fetal bovine serum (FBS), which allowed for the addition of 100 μM spermidine without encountering polyamine oxidase-driven toxicity (Roberts, Scott et al. 2002). Cells were grown in media with the

appropriate antibiotics (G418, 2.5 μg/ml; hygromycin 5 μg/ml; phleomycin 2.5 μg/ml) and were split every 24 to 48 hours to maintain cultures in log phase (10^5 to 10^6 cells/ml). Cell densities of cultures were determined by counting on a hemocytometer (Brightline, Fisher). Growth curves are represented as total cell number (product of cell density and total dilution) and data was collected in triplicate.

The AdoMet RNAi trypanosomes were grown in the following four conditions: uninduced (no additional supplements); uninduced + spermidine (100μM spermidine (Sigma S4139) in the media); induced (1μg/ml tetracycline (Sigma) in the media); and induced + spermidine (100 μM spermidine plus 1 μg/ml tet in the media).

Generation of AdoMetDC RNAi construct and transgenic cell line

The pLEW100 and pJM326 vectors (generous gifts from Paul Englund) were used to generate the AdoMetDC RNAi plasmid. The stemloop RNAi vector was prepared as previously described (Wang, Morris et al. 2000). A 620 basepair portion of the *T. brucei* AdoMetDC gene (starting at the 78th coding nucleotide) was amplified by PCR from genomic DNA that was isolated from *T. brucei* 427 cells. Primer pairs used for PCR were:

A.: 5'-ACGAGCGAAGCTTCGAAGGGCCTGAGAAGC-3' (contains HindIII site) and 5'-GAAGGATCCATGAATCAGCTAGCAACTTCACTGAGC-3' (contains NheI site); B: 5'-AAAGCACGAGCGACGCGTCGAAGGGCCTGAGAAGC-3' (contains MluI site) and 5'-GAAGGATCCATGAATCATCTAGAACTTCACTGAGC-3' (contains XbaI site). The PCR product obtained with set A was inserted into the pJM326 vector, and the product obtained with set B was inserted into the pLew 100 vector. The AdoMetDC fragment fused to the stuffer region was excised from pJM326 with Hind III

and XbaI and inserted into the modified pLew100 vector. The resulting plasmid contains two copies of the AdoMetDC gene fragment in opposite orientation separated by the stuffer DNA sequence (Figure 4.1). The production of stemloop RNA (with double stranded RNA targeting the AdoMetDC message) is driven from the tetracycline inducible procyclin promoter.

Actively growing *T. brucei brucei* 90-13 bloodsteam form cells (a gift from George Cross) were transfected as previously described with a few exceptions (Huynh, Huynh et al. 2003). Cells were transfected with 80 µg AdoMet DC stemloop RNAi vector linearized with EcoRV, and a single pulse on the BioRad electroporation system was used. After transfection, cells were added to 24 ml HMI media containing G418 and hygromycin (antibiotics which sustain the host background) and incubated for 24 hours at 37 °C, 5% CO_2. Transformed cells, containing the construct integrated into the rDNA region of the genome, were selected for by the addition of phleomycin to the media. Synthesis of double stranded DNA targeting the AdoMetDC message in a stemloop structure was induced by the addition of tetracycline (tet: 1µg/ml) every 24 hours.

Preparation of protein samples and Western Blot Analysis

AdoMetDC RNAi trypanosomes (0.5 -1 x 10^8 cells) cultured in the four described conditions were collected 0, 2, 4 and 6 days after adding appropriate supplements to the media. Cells were pelleted at 3000 rpm and washed twice with 1 ml of cold phosphate-buffered saline (PBS, pH= 7.4). The pellets were resuspended in lysis buffer (50 mM HEPES pH 8, 100 mM NaCl, 5 mM 2-mercaptoethanol, 2 mM phenylmethylsulfonyl fluoride, 1 µg/ml leupeptin, 2 µg/ml antipain, 10 µg/ml benzamidine, 1 µg/ml pepstatin, 1 µg/ml chymostatin) and frozen in liquid nitrogen and

then thawed at 37 °C. The freeze/thaw cycle was repeated two more times, and then the lysate was centrifuged at 13,200 rpm for 5 minutes to remove debris. The protein content of the clarified lysate was determined by the Bradford method (Bradford 1976). The protein sample was boiled for 5 min in 1x SDS loading buffer (58 mM Tris-HCl 6.8, 2% (w/v) SDS, 5% glycerol, 155 mM 2-mercaptoethanol, 0.002% (w/v) bromophenol blue). Total protein (20 μg/lane) was separated by 12% SDS/PAGE (BioRad Mini Protean III system) and transferred to a polyvinylidene difluoride (PVDF) membrane (Hybond-P, Amersham). Membranes were blocked in 5% non-fat milk in Tris buffered saline (TBS: 20 mM Tris·HCl, 137 mM NaCl, pH 7.6). Primary and secondary antibody incubations were carried out in 5% milk TBS-T (TBS plus 0.1% (v/v) Tween-20).

Rabbit polyclonal antibodies (Babco, Richmond, CA) raised against recombinant *T. brucei* AdoMetDC, γ-GCS and ODC were diluted 2, 500- (AdoMetDC) or 10, 000- (γ-GCS and ODC) fold for immunoblotting (Huynh, Huynh et al. 2003; Willert, Fitzpatrick et al. 2007). Rabbit polyclonal antibody against *T. brucei* prozyme was raised (by Proteintech Group, Inc, Chicago, IL) to recombinant His_6-prozyme. The recombinant protein was purified as described (Willert, Fitzpatrick et al. 2007). Rabbit polyclonal antibody raised against *Leishmania donovani* spermidine synthetase (Cocalico Biologicals Inc), a gift from Dr. Buddy Ullman, which cross reacts with the *T. brucei* enzyme, was diluted 1000-fold for immunoblotting (Roberts, Jiang et al. 2001). Rat polyclonal antibodies raised against *T. brucei* trypanothione synthetase and *T. cruzi* trypanothione reductase (cross reactive with the *T. brucei* enzyme) were a gift from Dr. Alan Fairlamb, and were diluted 1000-fold for immunoblotting (Ariyanayagam, Oza et al. 2005). Horseradish peroxidase-(HRP) linked donkey anti-rabbit or anti-mouse IgG

secondary antibodies (Amersham Biosciences) were used at at 10,000-fold dilution. Antigen recognition was visualized using the ECL chemiluminescent HRP substrate reagents (Amersham) and exposure to film (Kodak Biomax XAR). All data were collected in triplicate.

Preparation of mRNA samples and Northern Blot Analysis

Messenger RNA (mRNA) was isolated from at least $1x10^8$ trypanosome cells using the micro polyA purist kit (Ambion) according to the manufacturer protocol. Northern blot analysis was facilitated by the NorthernMax kit (Ambion). RNA was quantified spectrophotometrically, and 1 µg/lane was separated by denaturing 1% agarose gel electrophoresis. The mRNA was transferred to a positively charged nylon membrane (BrightStar-Plus, Ambion) and crosslinked. Radiolabeled ($[^{32}P]$dATP) probes were prepared using the Strip-EZ PCR kit (Ambion). Probe templates were prepared from genomic or plasmid DNA. Probe hybridization was visualized by film exposure (Kodak biomax XAR).

Quantification of intracellular polyamine levels

Intracellular polyamine content was analyzed by reverse-phase HPLC. Samples of $1x10^7$ cells were pelleted and washed twice in PBS, then resuspended in 25 µL lysis buffer (described above, but lacking protease inhibitors) and subjected to three freeze/thaw cycles. Trichloroacetic acid was added to 10% (v/v), and the samples were incubated on ice for 10 minutes prior to pelleting debris and precipitated protein. Polyamines in 5 µL of the sample were labeled by conjugation to the fluorescent AccQ-tag reagent (6-aminoquinolyl-*n*-hydroxysuccinimidyl in acetonitrile, Waters) according to manufacturer protocol. HPLC buffers and separation have been previously described

(Osterman, Brooks et al. 1999). A Waters AccQtag (3.9 x 150 mm) was used for separation on Beckman System Gold HPLC with a Ranin Dynamax Fluorescence detector. Data were collected in triplicate.

Quantification of intracellular small molecule thiol levels

Reduced intracellular thiols were quantified as previously described with minor modifications (Fairlamb, Henderson et al. 1987; Huynh, Huynh et al. 2003). The pellets ($1x10^8$ trypanosome cells) were washed twice in PBS then resuspended in HEPPS buffer, pH= 8, (25 µl) and 8.5 mM monobromobimane solution (25µL), and subjected to three freeze/thaw cycles. The lysates were labeled for 3 minutes at 70°C, and cooled briefly before addition of methanesulphonic acid solution (50 µL). Lysates were incubated on ice for 10 minutes, then debris and protein precipitate were removed by centrifugation. The resulting supernatant was used for analysis on a Beckman System Gold HPLC and detected by a Ranin Dynamax Fluorescence detector. A Phenomonex Nucleosil C_{18} column (30 x 4.6 mm) was used for separation. Data was collected in triplicate.

C. RESULTS

Depletion of AdoMetDC by RNAi causes cell death in *T. brucei*

In order to genetically validate *T. brucei* AdoMetDC as drug target, we have depleted the cells of this protein by RNAi, and have shown that AdoMetDC is an essential enzyme for cell viability. The regulatable AdoMetDC RNAi construct (Figure 4.1) was stably integrated into the rDNA spacer region of the *T. brucei* genome in 90-13 BF cells, which express both the T7 polymerase and the tet repressor. After integration, the cells were subjected to limited dilution to generate a clonal cell line (refered to as

AdoMetDC RNAi cells). When integrated into the genome, the RNAi construct is silent until the addition of tet to the media; after induction, the dsRNA is produced as a stemloop structure. This dsRNA triggers the destruction of the AdoMetDC message through the RNAi process. To assess the effect of AdoMetDC RNAi, cells were cultured in media in the presence or absence of tet

The induction of RNAi against AdoMetDC by tet addition results in a decrease of AdoMetDC mRNA and protein, which remains throughout the experiment (Figure 4.2). The knockdown of AdoMetDC slows cell growth, and eventually all cells die (Figure 4.3A). This growth arrest response demonstrates that AdoMetDC is an essential parasite enzyme.

We next looked at the ability of spermidine, a downstream product of AdoMetDC, to rescue the cell death. In order to introduce high enough concentrations of spermidine to the media, we supplemented the BF culture media with chicken serum (CS) instead of fetal bovine serum (FBS) (Roberts, Scott et al. 2002). This substitution allowed us to add 100 μM spermidine to the media without incurring polyamine oxidase-induced toxicity. Spermidine restored the growth of induced AdoMetDC RNAi cells, and had no effect when added to uninduced cultures (Figure 4.3 B). The rescue of cell death by spermidine demonstrates that the RNAi effect is specific for AdoMetDC. Spermidine was able to rescue the cells when it was added at the start of induction (D0) or if it was added to the media up to four days post-induction (D2, D4). However, spermidine added to the media six days (D6) post induction did not rescue the cells, indicating that they are committed to death at this time.

AdoMetDC knockdown changes polyamine levels

We have shown by growth analysis of induced AdoMetDC RNAi cells that AdoMetDC is an essential enzyme, and therefore a validated drug target. To further characterize the cellular response to AdoMetDC knockdown, we looked for changes in the levels of polyamines in the uninduced and induced *T. brucei* cells cultured in the presence and absence of 100 μM spermidine. For these experiments, the spermidine was added at the same time as the tet.

Cells were cultured as described above, and samples were collected prior to induction and/or addition of spermidine and two, four and six days post-induction. The samples were lysed and processed to remove proteins and debris, then were derivatized with a fluorescent label, which attaches to amino groups. The samples were separated by reverse-phase HPLC, and the polyamines were quantified by fitting to a standard curve.

The induction of AdoMetDC RNAi leads to an increase in putrescine levels (5-9 fold) by two days of knockdown (Figure 4.4). Putrescine pools remain high throughout the experiment, in the presence and absence of exogenous spermidine. The spermidine levels are decreased in cells that have been induced for AdoMetDC RNAi. However, the reduction is not as great as we had expected; the values decrease by about 2 fold from initial conditions after six days of induction. The addition of spermidine to the induced culture partially restored the spermidine levels to about 80% of initial values. The putrescine and spermidine levels of uninduced samples were unchanged by the addition of spermidine to the media.

AdoMetDC knockdown changes polyamine levels

In *T. brucei,* spermidine is conjugated to glutathione to form a novel cofactor, trypanothione (TSH), and the precursor molecule, glutathionyl-spermidine (GSP). Since AdoMetDC RNAi has a small effect on spermidine pools, we wanted to see if there were changes in the levels of spermidine containing thiol molecules in the uninduced and induced *T. brucei* cells cultured in the presence and absence of 100 μM spermidine. For these experiments, the spermidine was added at the same time as the tet.

Cells were cultured as described above, and samples were collected prior to induction and/or addition of spermidine and two, four and six days post-induction. The samples were lysed and processed to remove proteins and debris, then derivatized to attach a fluorescent label to the thiol group. The samples were separated by reverse-phase HPLC, and the amounts of reduced small molecule thiols were quantified by fitting to a standard curve.

Under all conditions, the amount of glutathione remains fairly constant. There is no significant change in the amount of GSP or TSH between uninduced cells cultured in the presence or absence of exogenous spermidine. However, there is a drastic decrease in the amount of GSP and TSH found in the induced cells (Figure 4.5). These molecules are depleted to 4% (GSP) and 6 % (TSH) of initial values by six days post induction. The addition of exogenous spermidine to the media of AdoMetDC RNAi induced cells largely restores these pools of spermidine-containing small-thiol molecules.

Taken together, the analysis of metabolites in the polyamine and trypanothione biosynthesis pathway shows that knockdown of AdoMetDC perturbs the balance of these molecules in the *T. brucei* cell. The major result of the depletion of AdoMetDC is

observed in the levels of trypanothione and the precursor, glutathionyl-spermidine. Trypanothione has been shown to be an essential cofactor (Krieger, Schwarz et al. 2000; Ariyanayagam, Oza et al. 2005), and the loss of trypanothione may lead to the cell death of AdoMetDC RNAi trypanosomes, or the death may be due to a more complete depletion of the spermidine pools that may occur after all of the TSH and GSP is gone. The observation that *T. brucei* favors the maintenance of spermidine pools over trypanothione pools indicates that there is regulation of this pathway. Alternatively, there may be a large pool of spermidine that is not available for flux with trypanothione pools.

AdoMetDC knockdown induces protein expression of prozyme, ODC and TrypSyn

The metabolite analysis shows that depletion of AdoMetDC drastically reduces levels of trypanothione and the precursor, glutathionyl-spermidine, while maintaining about 50 % of the spermidine pools after six days of induction. The regulation of this pathway may be due to changes in the levels of biosynthetic proteins. We have used western blot analysis to look at the protein levels of AdoMetDC, prozyme, ODC (ornithine decarboxylase), SpdSyn (spermidine synthetase), TrypSyn (trypanothione synthetase) and TrypRed (trypanothione reductase) during AdoMetDC RNAi.

Cells were cultured as described above, and samples were collected prior to induction and/or addition of spermidine and two, four and six days post-induction. The samples were lysed in the presence of protease inhibitors, debris was removed, and the amount of total protein was measured. Total protein (20 μg) was loaded onto gels, transferred to a membrane, and probed for the proteins of interest, with tubulin as a loading control (Figure 4.6).

The induction of AdoMetDC depletes the cells of AdoMetDC protein at all time points sampled. The addition of exogenous spermidine had no effect on the knockdown. There was no change in the protein levels of SpdSyn or TrypRed during the induction of AdoMetDC RNAi. However, the induction of AdoMetDC RNAi stimulates proteins expression of prozyme, ODC and TrypSyn. The largest effect is seen in the induction of prozyme. The prozyme levels are increased by two days post-induction, and remain high in the presence and absence of spermidine. Though the expression is not stimulated as much as prozyme, ODC protein levels are also upregulated in the presence and absence of spermidine when the AdoMetDC RNAi is induced, which may lead to the increase in putrescine levels seen in these cells. TrypSyn is also induced, though to a lesser degree than prozyme or ODC, and may contribute to the maintenance of spermidine pools while trypanothione is depleted.

T. brucei does not often regulate proteins at the level of transcription; most transcripts are processed from a polycistronic transcript and trans-spliced to a 5' leader sequence and then polyadenylated. Regulation is found at the level of mRNA stability, protein stability or translation efficiency. We looked at the mRNA levels of AdoMetDC, prozyme, ODC and TrypSyn to see if there were changes in the amount of message present in cells undergoing AdoMetDC RNAi (Figure 4.7). As expected, the mRNA for AdoMetDC is decreased in these cells. There is no significant change in the mRNA levels of prozyme, ODC or TrypSyn. This indicates that the regulation of these proteins is not at the level of message stability, but must occur either at the level of translational initiation or protein stability.

D. CONCLUSIONS

Previously, chemical inhibition of AdoMetDC in *T. brucei* has implicated this enzyme as a drug target (Bacchi, Nathan et al. 1992; Yakubu, Majumder et al. 1993). Here, we have shown that knockdown of AdoMetDC leads to cell death, demonstrating that this is an essential enzyme and providing a genetic validation of AdoMetDC as a drug target to complement the chemical validation. We have previously demonstrated differences between the human and parasite AdoMetDC, which imply that species selective inhibitors to treat trypanosomiasis with minimal effect on the human enzyme may be discovered.

We used an inducible RNAi cell line to control the knockdown of AdoMetDC in *T. brucei.* We have shown that the cell death caused by induction of RNAi can be rescued by the addition of exogenous spermidine to the cell. The amount of spermidine added (100 μM) is much higher than that found in the human blood (0.3 μM, (Van Dobbenburgh, Houwen et al. 1983)), which is the environment where *T. brucei* live. Lower amounts were not able to rescue the cells (data not shown), indicating that trypanosomes *in vivo* would not be able to scavenge spermidine to overcome growth defects caused by AdoMetDC inhibition.

Polyamines are highly regulated in most cells; however, very little is known about polyamine regulation in *T. brucei*. The polyamine pathway is also involved in redox balance in *T. brucei*, as spermidine is used to form the essential trypanothione cofactor. We have previously shown that AdoMetDC is allosterically activated by prozyme, a protein that arose through gene duplication and mutational drift (Willert,

Fitzpatrick et al. 2007). Prozyme has the potential to act as a regulatory molecule in these cells.

Here we have demonstrated that AdoMetDC is a key protein involved in the regulation of polyamines and trypanothione pools. The knockdown of AdoMetDC leads to an almost complete depletion of trypanothione pools by six days after induction; at the same time point, the spermidine pools are only decreased by about half. The preference for maintaining spermidine pools at the expense of trypanothione establishes that *T. brucei* does indeed regulate this important pathway.

By western blot analysis, we have shown that the knockdown of AdoMetDC by RNAi results in the induction of three proteins involved in polyamine and trypanothione biosynthesis: prozyme, ODC and TrypSyn. TrypSyn is an interesting protein that has two domains with opposing functions. The C terminal synthetic domain produces TSH, while an N terminal CHAP domain is able to catalyze the conversion of trypanothione back to spermidine and glutathione (Oza, Ariyanayagam et al. 2003). Since TrypSyn can act in a catabolic manner to break down TSH to spermidine, the induction of these three enzymes may be the way that the cell attempts to maintain the spermidine pools in the absence of AdoMetDC activity. The increase of ODC helps to explain why the levels of putrescine are increased in these cells. The small increase in TrypSyn, or if the trypanosomes activate the catabolic nature of TrypSyn, may allow for the depletion of TSH prior to spermidine pools during AdoMetDC knockdown. The largest effect is seen in the upregulation of prozyme; the ability of this activating protein to be induced helps support the idea that prozyme is a regulatory protein. The prozyme has no intrinsic activity, and therefore cannot replace the AdoMetDC activity in the cell. The protein may be induced

as a mechanism to stimulate AdoMetDC activity, or prozyme may have another role in the cell. The induction of these proteins is seen during AdoMetDC RNAi in the presence and absence of spermidine. The trigger for this induction has not been determined, but a potential small molecule regulator may be AdoMet or dcAdoMet; these molecules would be effected when AdoMetDC is knocked down in the presence and absence of exogenous spermidine.

Taken together, these results demonstrate regulation of the polyamine and trypanothione biosynthetic pathway in *T. brucei.* AdoMetDC has a key role, as depletion of this protein has effects on the metabolism of the cell. Our data shows that the trypanosomes preserve their spermidine pools at the expense of trypanothione. Both polyamines and trypanothione are essential for growth of *T. brucei;* however, the organism appears to preferentially maintain spermidine pools. We have also demonstrated that some enzymes in the polyamine/trypanothione pathway can be modulated as a response to AdoMetDC knockdown. These changes fit with the idea of spermidine preference in *T. brucei*. The most highly induced protein in the pathway is prozyme, the allosteric activator of AdoMetDC. This protein has the potential as a key regulator of the pathway, and may have an additional role that has not yet been explored.

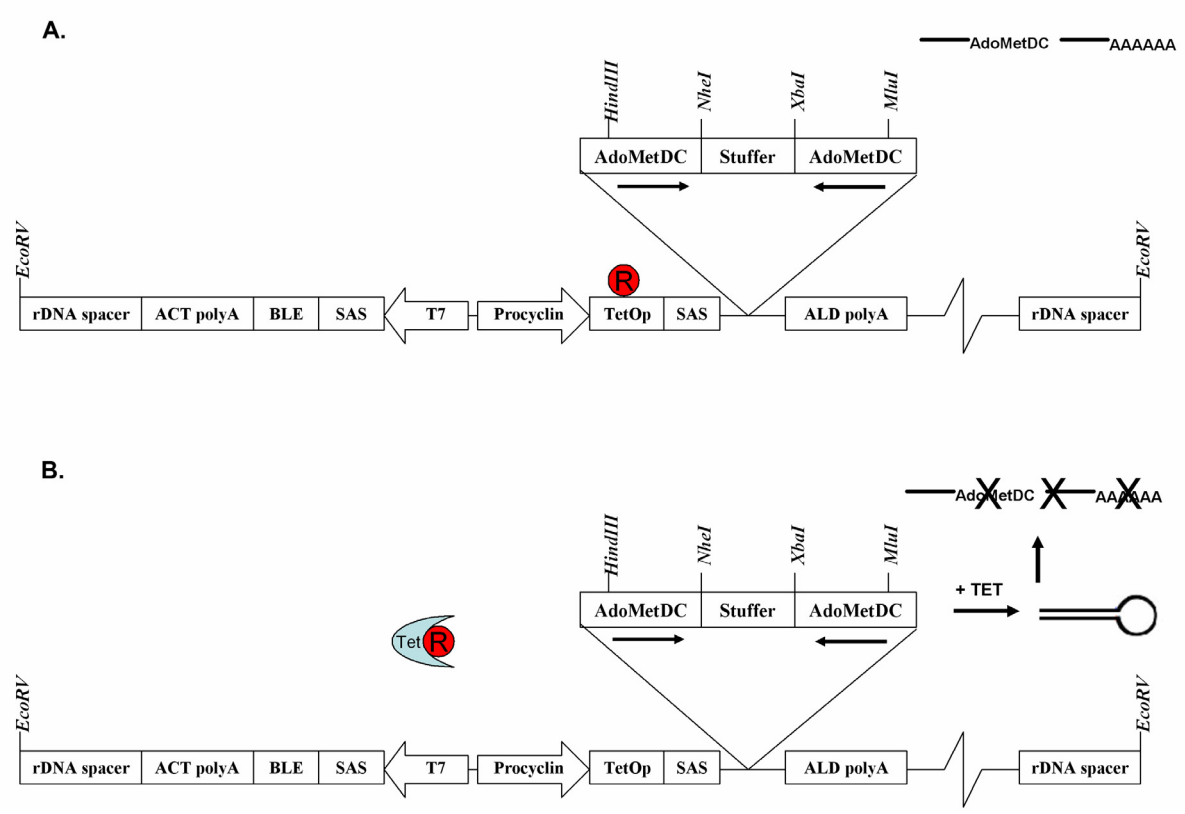


Figure 4.1 Regulatable AdoMetDC RNAi construct The inducible AdoMetDC construct was stably integrated into the *T. brucei* genome in the rDNA repeat region. In the absence of tet (A) there is no dsRNA produced from the construct, and the AdoMetDC message is intact. Once tet is added to the cell culture (B) the repression of the construct is relieved, and the stemloop dsRNA is produced, targeting the AdoMetDC mRNA for degradation.

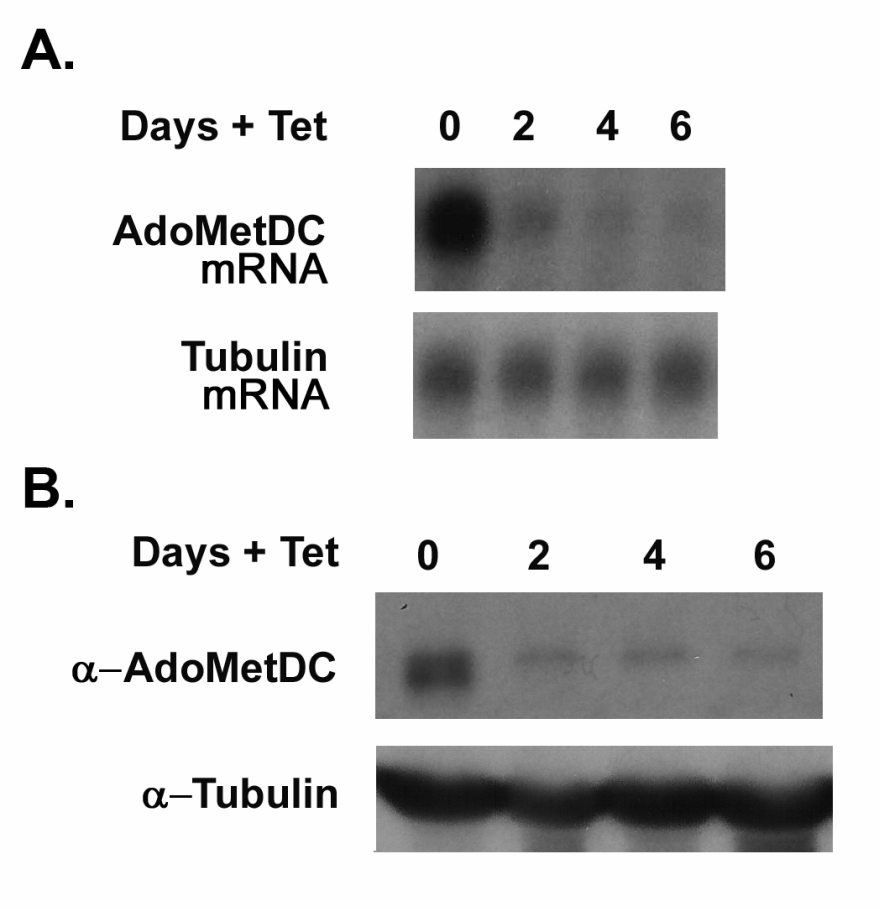


Figure 4.2 AdoMetDC RNAi knocks down mRNA and protein levels The induction of AdoMetDC RNAi by addition of tet to the culture media results in loss of AdoMetDC mRNA as shown by northern blot (A) and protein as shown by western blot (B) for the duration of the experiments.

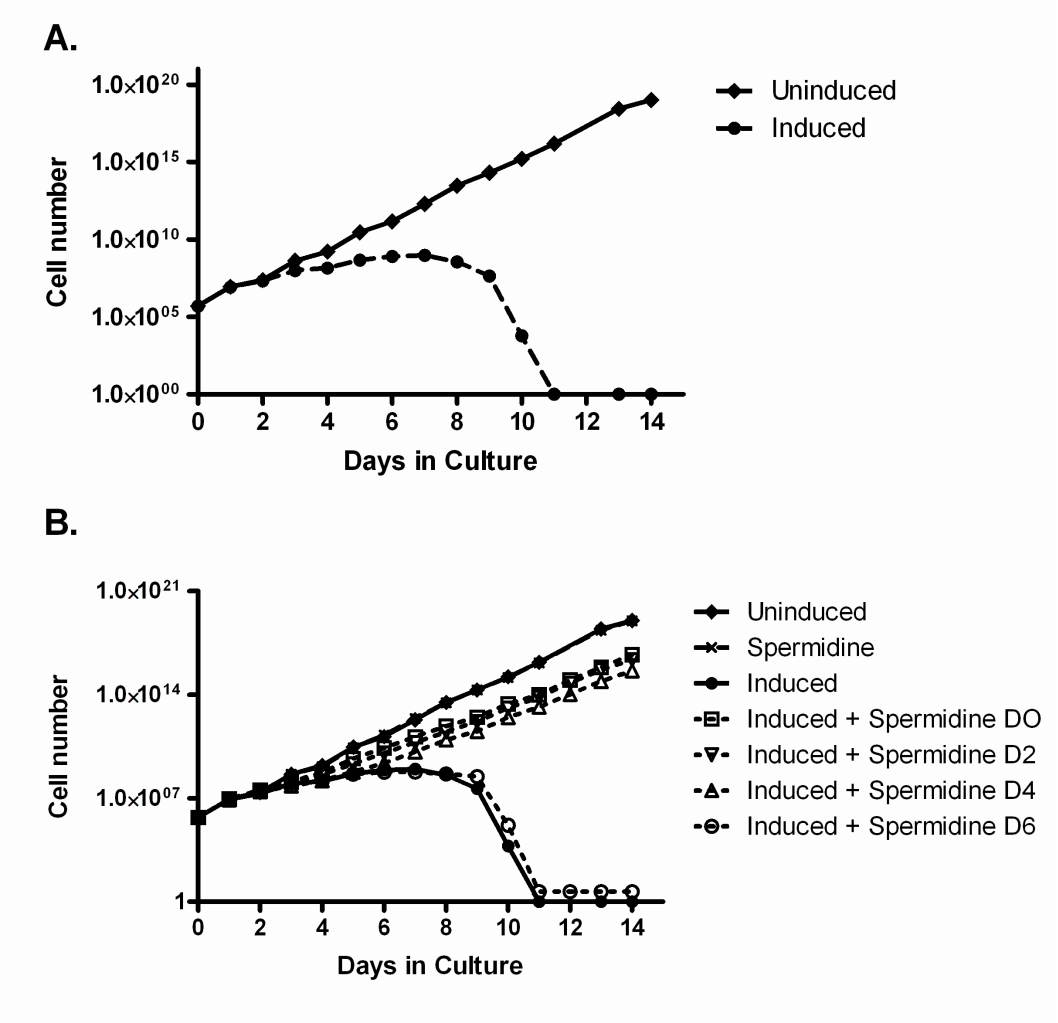


Figure 4.3 Growth of BF AdoMetDC RNAi cells The growth of AdoMetDC RNAi cells was monitored over two weeks. AdoMetDC is an essential enzyme, as depletion leads to cell death (A). This cell death can be rescued by the addition of spermidine (B); however, after 6 days of RNAi induction, the cells have committed to death and can no longer be rescued by spermidine.

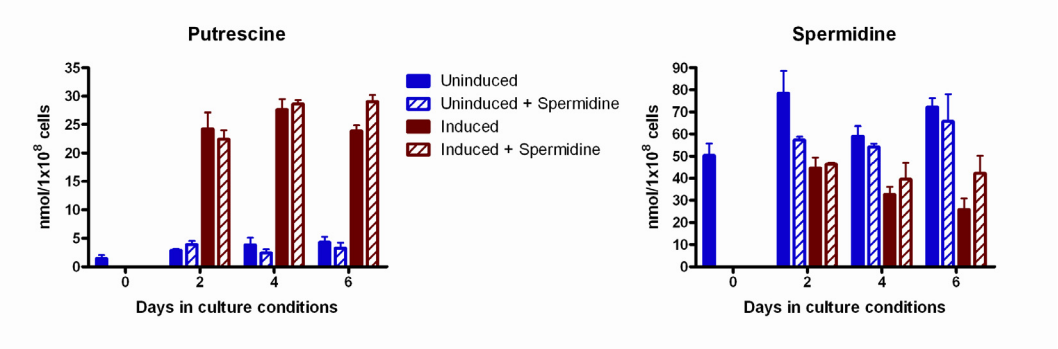


Figure 4.4 Quantification of polyamines Both uninduced and induced AdoMetDC RNAi cells were grown in the presence and absence of spermidine and the amount of putrescine and spermidine 2, 4 and 6 days post-induction were quantified.

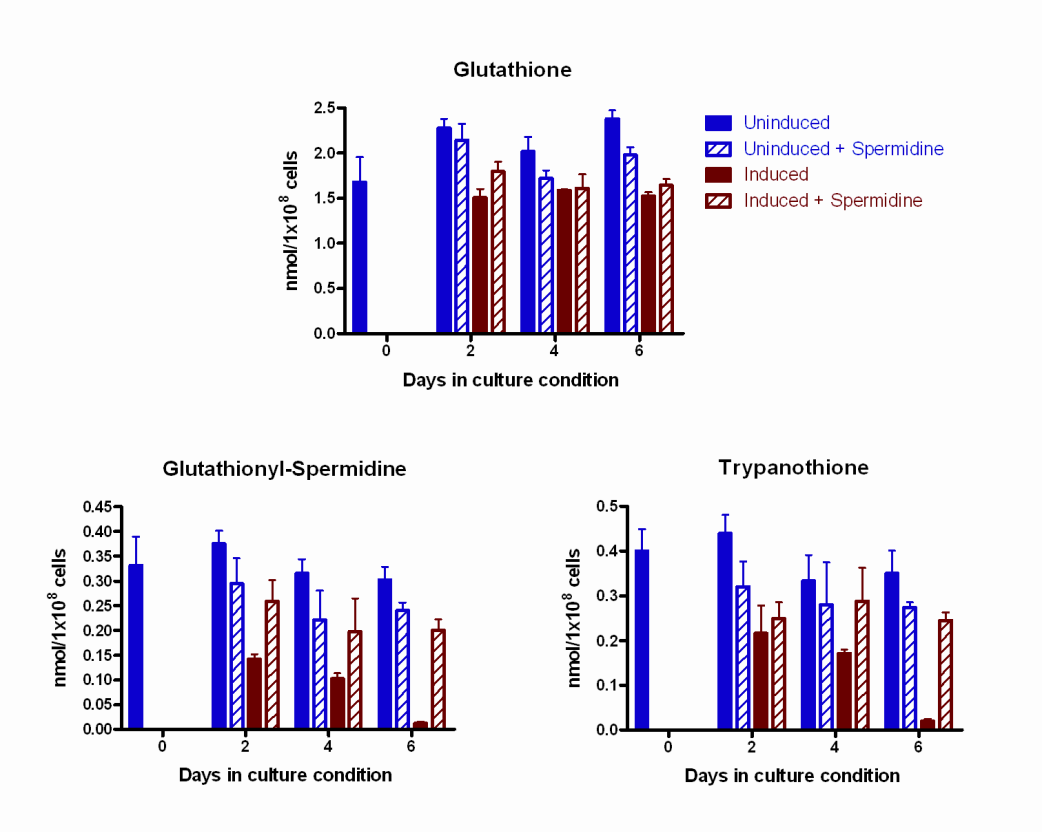


Figure 4.5 Quantification of small molecule thiols Both uninduced and induced AdoMetDC RNAi cells were grown in the presence and absence of spermidine and the amount of glutathione, glutathionyl-spermidine and trypanothione on days 2, 4 and 6 post-induction were quantified.

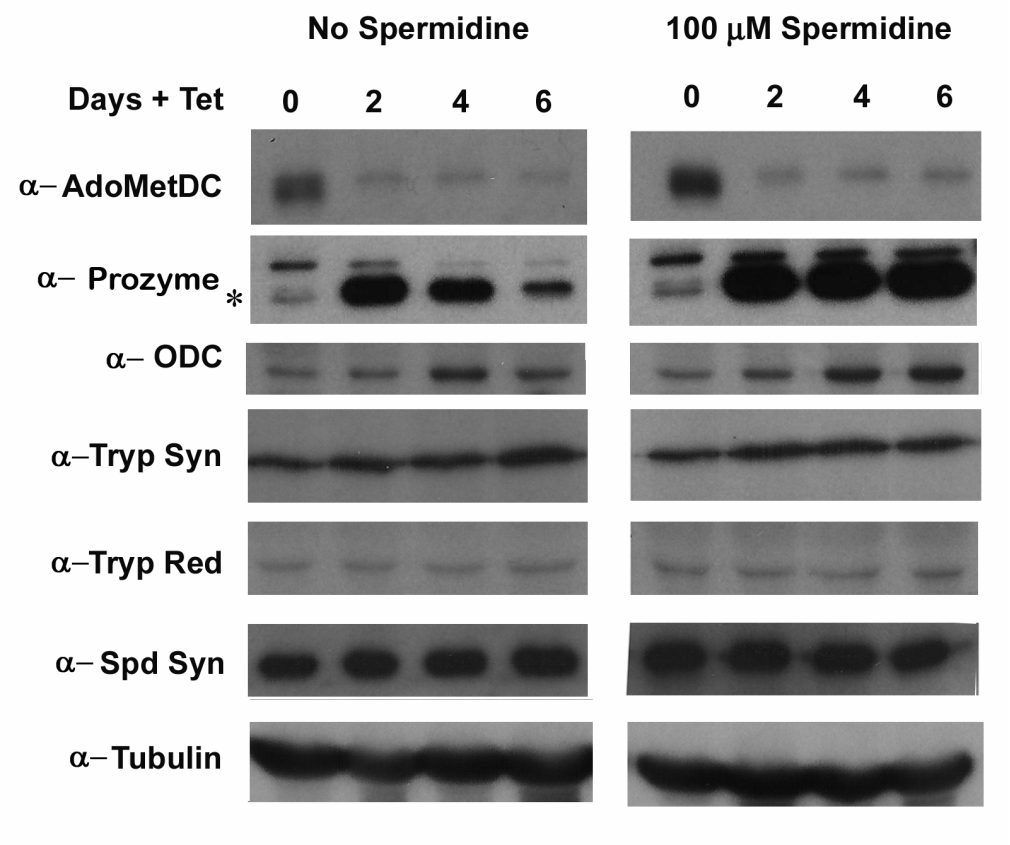


Figure 4.6 Western blot analysis of pathway enzymes The protein levels of AdoMetDC, prozyme, ODC, TrypSyn, TrypRed, SpdSyn and tubulin were measured in the absence (left) and presence (right) of exogenous spermidine. The asterisk marks the prozyme band. The day 0 sample on the 5 mM spermidine side (right) was cultured with 100 μM spermidine for six days. There are no changes in the protein levels of uninduced cells cultured in the presence or absence of exogenous spermidine (not shown)

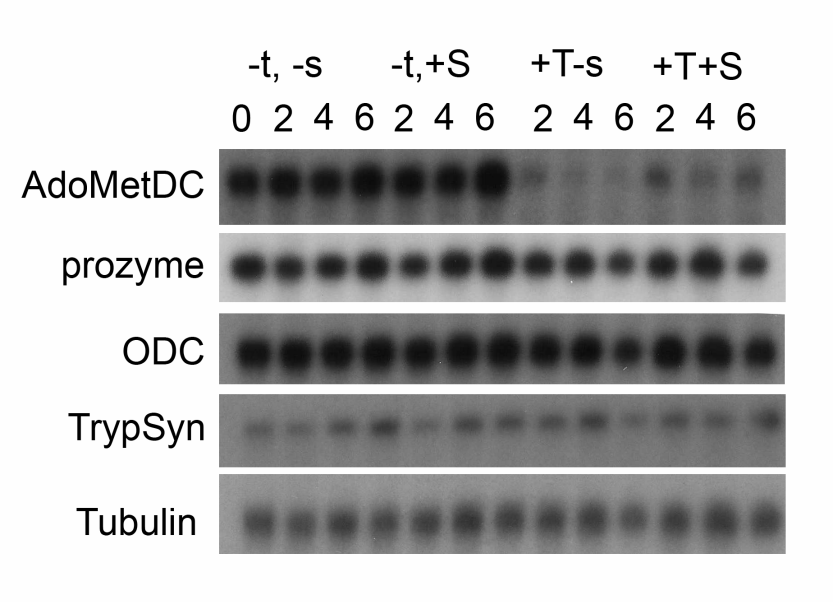


Figure 4.7 Northern blot analysis The levels of mRNA for AdoMetDC, prozyme, ODC and TrypSyn were assessed. Cells were cultured in the absence of tet and spermidine (-t,-s), presence of spermidine only (-t, +S), presence of tet only (+T-s) and presence of both tet and spermidine (+T+S).

Days with RNAi Induction	Spermidine Addition	**Polyamine Levels** (nmol/1x10^8 cells)		**Thiol Levels** (nmol/1x10^8 cells)		
		Putrescine	**Spermidine**	**GSH**	**GSP**	**T(SH)$_2$**
0	-	3.1 ± 0.6	64.9 ± 6.3	2.1 ± 0.2	0.33 ± 0.02	0.38 ± 0.02
2	-	24.2 ± 2.9	44.5 ± 4.8	1.5 ± 0.1	0.14 ± 0.01	0.22 ± 0.06
4	-	27.6 ± 1.8	32.6 ± 3.5	1.6 ± 0.01	0.10 ± 0 .01	0.17 ± 0.01
6	-	23.9 ± 1.0	25.8 ± 5.2	1.5 ± .04	0.01 ±0.003	0.02 ±.003
0	+	3.2 ± 0.4	58.9 ± 3.5	1.9 ± 0.1	0.25 ± 0.02	0.29 ± 0.01
2	+	22.4 ± 1.6	46.3 ± .74	1.8 ± 0.1	0.26 ± 0.04	0.25 ± 0.04
4	+	28.6 ± 0.6	39.5 ± 7.5	1.6 ± 0.2	0.20 ± 0.07	0.29 ± 0.07
6	+	29.0 ± 1.1	42.1 ± 8.0	1.6 ± 0.1	0.20 ± 0.02	0.25 ± 0.02

Table 4.1 Quantification of polyamine and thiol levels in AdoMetDC RNAi cells The amount of putrescine, spermidine, glutathione, glutathionyl-spermidine and trypanothione in induced and uninduced AdoMetDC RNAi cells cultures in the presence or absence of spermidine were quantified.

CHAPTER FIVE

ONGOING RESEARCH: THE ROLE OF PROZYME IN POLYAMINE/TRYPANOTHIONE REGULATION

A. INTRODUCTION

We have shown that a promising drug target against trypanosomiasis is *T. brucei* AdoMetDC, an important polyamine biosynthetic enzyme. The active form of the *T. brucei* enzyme is an AdoMetDC/prozyme heterodimer (Willert, Fitzpatrick et al. 2007); this and other differences between the homodimeric human AdoMetDC indicate that species-selective inhibitors can be developed. Studies of a genetic knockdown of AdoMetDC by RNAi have shown that AdoMetDC is an essential enzyme to growth of *T. brucei* and that it is a key protein involved in the regulation of polyamines and trypanothione (see chapter 4).

The response of the trypanosome to loss of AdoMetDC highlights the role of this protein in polyamine and trypanothione regulation and homeostasis. The parasites maintain pools of spermidine at the expense of trypanothione. When *T. brucei* is depleted of AdoMetDC by RNAi, there is a strong induction of prozyme protein levels. The observation that this protein can be induced by perturbances in the pathway help support the idea that prozyme is a regulatory protein.

In order to study the role of prozyme in polyamine and trypanothione regulation, we have created a prozyme conditional knock out (cKO) cell line. The conditional knockout has both endogenous copies of prozyme replaced by drug resistance cassettes,

and a regulatable copy of prozyme is expressed from a distant location in the genome only in the presence of tetracycline (tet).

We have used this cell line to assess the requirement for prozyme in *T. brucei* survival. We measured the growth of cells expressing and not expressing prozyme in the presence and absence of spermidine (at concentrations able to rescue the AdoMetDC RNAi cells). We have found that prozyme is an essential protein; the loss of prozyme expression leads to cell death. This cell death cannot be rescued by exogenous spermidine. The lack of spermidine rescue implies that prozyme may have another role in addition to its activation of AdoMetDC, or may be involved in spermidine uptake.

B. MATERIALS AND METHODS

Trypanosome cell cultures

Bloodstream form trypanosomes were cultured in HMI-9 medium supplemented with 10% serum in a at 37 °C, 5% CO_2 incubator (Hirumi and Hirumi 1989). When culturing trypanosomes, chicken serum (CS) replaced fetal bovine serum (FBS), which allowed for the addition of 100 μm spermidine without encountering polyamine-oxidase driven toxicity (Roberts, Scott et al. 2002). Cells were grown in media with the appropriate antibiotics (G418, 2.5 μg/ml; hygromycin 5 μg/ml**;** blasticidin 2.5 μg/ml) and were split every 24 to 48 hours to maintain cultures in log phase (10^5 to 10^6 cells/ml). Cell densities of cultures were determined by counting on a hemocytometer (Brightline, Fisher). Growth curves are represented as total cell number (product of cell density and total dilution), and data were collected in triplicate.

The prozyme cKO trypanosomes were grown in the following four conditions: not expressed (no additional supplements); not expressed + spermidine (100μM spermidine (Sigma S4139) in the medium); expressed (1μg/ml tetracycline (Sigma) in the medium); and expressed + spermidine (100 μM spermidine plus 1 μg/ml tet in the medium). Prozyme cKO cells are maintained in the presence of tet (1μg/ml) until the experiments are commenced. On day zero of the experiment, the cells are pelleted and washed in PBS. The washed cells are resuspended in tet-free medium, and then divided into media with the appropriate supplements for the four conditions described above.

Generation of prozyme knock out and regulatable expression constructs and transgenic cell line

The strategy for making conditional knockouts was similar to previously described schemes, with a few minor differences (Wirtz, Leal et al. 1999; Schnaufer, Panigrahi et al. 2001). We used the pLEW 13 and pLEW 90 vectors to create knock out constructs (provided by Ken Stuart, originally described by the George Cross lab). A 300 bp segment of prozyme 5' and 3' UTRs was introduced into these vectors. The pLEW100 vector (generous gift from Paul Englund) was altered to express blastocidin resistance instead of phleomycin resistance. This new cassette (pLEW 300) was used to generate the inducible expression plasmid. This plasmid expresses a Flag-tagged prozyme in the presence of tet. The primers used to generate the constructs are provided in Table 5.1.

Actively growing *T. brucei brucei* 427 bloodsteam form cells (provided by Ken Stuart) were transfected with the Amaxa nucleofector as described with a few exceptions. $3x10^7$ cells were resuspended in 100 μL Human T cell solution (Amaxa) and transfected with 20 μg of linearized vector by a single pulse (Burkard, Fragoso et al. 2007). After

transfection, cells were added to 24 ml medium containing antibiotics which sustain the host background and incubated for 24 hours at 37 °C, 5% CO_2. Transformed cells, containing the integrated construct were selected for by the addition of the appropriate antibiotic to the medium. The pLew13-prozyme SKO-N vector (which contains coding sequence for T7 polymerase and the G418 (N) resistance cassette inside the prozyme 5' and 3' UTRs) was integrated, selected for and cloned. This construct was used to for transfection, integration and selection of the pLew300-Flag prozyme construct (which contains coding sequence for a FLAG tagged prozyme and the blasticidin (B) resistance cassette targeted to the rDNA spacer region of the genome). In the presence of tet, the final pLew 90-prozyme SKO-H vector (which contains coding sequence for the tet repressor and the hygromycin (H) resistance cassette inside the prozyme 5' and 3' UTRs) was integrated, selected for and cloned out. Expression of Flag-tagged prozyme was induced by the addition of tet (1μg/ml) every 24 hours.

Preparation of genomic DNA

Genomic DNA was harvested from the 427 parental cell line, each single marker line (SKO-N and SKO-H), the single marker cell line with FLAG-prozyme (SKON-FP) and the cloned conditional knock outs (cKOs). Cells were grown under normal conditions, harvested, and resuspended in genomic lysis buffer (0.1 M NaCl, 0.025M EDTA, 0.5%SDS, 0.01M Tris·HCl pH = 8). RNAse A was added to a final concentration of 50 μg/ml, and the samples were incubated at 37 C for at least 2 hours. Then proteinase K was added to a final concentration of 0.2 μg/ml, and the samples were incubated overnight at 50 ºC, with shaking at 225 rpm. The next day, DNA was extracted by mixing with an equal volume of phenol: chloroform: isoamyl alcohol (25:24:1) and recovering

the aqueous layer. The DNA was then precipitated in the presence of ammonium acetate by ethanol. The precipitate was resuspended in TE buffer (10 mM Tris·HCl, pH = 7.5, 1 mM EDTA), and the concentration was determined spectroscopically.

Southern blot analysis

Southern blot analysis was used to confirm the genotypes of cKO cell lines. The probe was designed to recognize all of the constructs and the endogenous prozyme, and digestion with BanI (cuts the sequence GG/YRCC, where Y = C/T and R = A/G) will result in size differences for the hybridized fragment. The probe is a 600 bp region that starts 891 bp upstream of the prozyme ATG start site. Expected sizes of bands are: genomic prozyme 2026 bp; SKO-N 3733 bp; and SKO-H 1382 bp. Radiolabeled ([^{32}P]dATP) probes were prepared using the Strip-EZ PCR kit (Ambion), and the template was prepared from genomic DNA. The primers used for PCR of the probe template are provided in Table 5.1

We digested 10 μg genomic DNA with BanI overnight. The digested DNA was then precipitated and resuspended in TE and DNA loading dye. The samples were electroporated on a 1 % agarose gel, and the gel was rinsed in dH_2O, then depuriniated in 0.25 M HCl for 30 minutes at room temperature (RT) with agitation. The gel was rinsed again, then denatured by submerging in 1.5 M NaCl/ 0.5 M NaOH for 20 minutes at RT with agitation twice. The gel was rinsed again, then neutralized by submerging in 1.5 M NaCl/ 0.5 M Tris pH = 7 for 20 minutes at RT with agitation twice. The samples were transferred to a positively charged membrane (Ambion Bright Star) by vacuum in 10x SSC. The membrane was then crosslinked by UV, and prehybridized in Ambion UltraHyb solution. The membrane was hybridized with the probe overnight in the same

solution. After hybridization, the blot was washed in several steps with 2 to 0.1x SSC and 0.1% SDS. The washed blot was then placed in a phosphorimager cassette and visualized with the FLA 5000 phosphoimager.

Preparation of protein samples and western blot analysis

Prozyme cKO trypanosomes (0.5 -1 x 10^8 cells) were cultured in the four described conditions. Cells were pelleted at 3000 rpm (centrifuge) and washed twice with 1 ml of cold phosphate-buffered saline (PBS, pH= 7.4). The pellets were resuspended in lysis buffer (50 mM HEPES pH 8, 100 mM NaCl, 5 mM 2-mercaptoethanol, 2 mM phenylmethylsulfonyl fluoride, 1 µg/ml leupeptin, 2 µg/ml antipain, 10 µg/ml benzamidine, 1 µg/ml pepstatin, 1 µg/ml chymostatin) and frozen in liquid nitrogen and then thawed at 37 °C. The freeze/thaw cycle was repeated two more times, and then the lysate was centrifuged at 13,200 rpm for 5 minutes to remove debris. The protein content of the clarified lysate was determined by the Bradford method (Bradford 1976). The protein sample was boiled for 5 min in SDS loading buffer (58 mM Tris·HCl 6.8, 2% (w/v) SDS, 5% glycerol, 155 mM 2-mercaptoethanol, 0.002% (w/v) bromophenol blue).

Total protein (20 µg/lane) was separated by 12% SDS/PAGE (BioRad Mini Protean III system) and transferred to a polyvinylidene difluoride (PVDF) membrane (Hybond-P, Amersham). Membranes were blocked in 5% non-fat milk in Tris-buffered saline (TBS: 20 mM Tris·HCl, 137 mM NaCl, pH 7.6). Primary and secondary antibody incubations were carried out in 5% milk TBS-T (TBS plus 0.1% (v/v) Tween-20).

Rabbit polyclonal antibodies (Babco, Richmond, CA) raised against recombinant *T. brucei* AdoMetDC, γ-GCS and ODC were diluted 2, 500-(AdoMetDC) or 10, 000-(γ-

GCS and ODC) fold for immunoblotting (Huynh, Huynh et al. 2003; Willert, Fitzpatrick et al. 2007). Rabbit polyclonal antibody against *T. brucei* prozyme was raised (by Proteintech Group, Inc, Chicago, IL) to recombinant His_6-prozyme. The recombinant protein was purified as described (Willert, Fitzpatrick et al. 2007). Rabbit polyclonal antibody raised against *Leishmania donovani* spermidine synthetase (Cocalico Biologicals Inc), a gift from Dr. Buddy Ullman, which cross reacts with the *T. brucei* enzyme was diluted 1000 fold for immunoblotting (Roberts, Jiang et al. 2001). Rat polyclonal antibodies raised against *T. brucei* trypanothione synthetase and *T. cruzi* trypanothione reductase (cross reactive with the *T. brucei* enzyme) were a gift from Dr. Alan Fairlamb, and were diluted 1000-fold for immunoblotting (Ariyanayagam, Oza et al. 2005). Horseradish peroxidase-(HRP) linked donkey anti-rabbit or anti-mouse IgG secondary antibodies (Amersham Biosciences) were used at at 10,000 fold dilution. Antigen recognition was visualized using the ECL chemiluminescent HRP substrate reagents (Amersham) and exposure to film (Kodak Biomax XAR). All data were collected in triplicate.

C. RESULTS

Establishing a prozyme conditional knockout cell line

To study the effect of removing prozyme from *T. brucei* cells, we created a conditional knock out (cKO) cell line. In this line, the expression of a Flag-tagged prozyme is controlled by tet. After knock out of the first allele, the inducible construct is integrated into the genome in the rDNA spacer region; in the presence of tet it is expressed, but when tet is removed from the medium the expression is repressed. We

then knocked out the second allele, so that the conditional knockout has both endogenous copies of prozyme replaced by drug resistance cassettes, and the only available prozyme is expressed from a distant location in the genome. (A schematic of the constructs used is shown in Figure 5.1.) Attempts to knock out both prozyme alleles without the expression of prozyme from a heterologous site were unsuccessful. The fact that we were able to make this cKO line demonstrates that the knock out can be achieved at the endogenous location of prozyme in the genome. After the final transfection, two cell lines, cKO-8 and cKO-14, were subjected to limited dilution to generate clonal lines: cKO-8 A, B, C and cKO-14 A, B, C.

Southern blot confirmation of cKO cells

Genomic DNA was isolated from the parasites and the DNA was analyzed by southern blot analysis to confirm that the conditional knock out was achieved. The genomic DNA was digested so that the probe will hybridize to fragments of different sizes. The probe hybridizes to the genomic region 5' of the prozyme coding region, and is intact in all of the knocked in constructs. Genomic DNA from the parental cell line 427, each single cell line, SKO-N and SKO-H, the single knock out with Flag-prozyme SKO-N-FP, and the cKO cell lines cKO-8 A, B, C and cKO-14 A, B, C. The difference in the sizes of the bands on the southern blot confirms that the constructs have been integrated into the correct location in the genome (Figure 5.2).

The 427 parental line has only the endogenous prozyme, and the single knockouts as well as the single knock out with conditional Flag-prozyme have the endogenous gene and the appropriate integrated construct. The cKO-8 cloned lines and cKO-14A show both integrated vectors as well as the endogenous gene. This result may

be due to a mixed population of cells in the lines, or all three may be encoded in a single trypanosome genome by a mechanism used to retain an important gene, such as a chromosomal duplication. However, the clonal cell lines cKO-14B and cKO-14C show the desired arrangement. The genomic DNA from these cells shows that both constructs are integrated into the prozyme locus, and the endogenous gene is not present. These cells were used for all further experiments.

Tet regulation of Prozyme cKO cells

The cKO cells should only express prozyme when tet is present in the culture media. Since the cKO cells are maintained in the presence of tet, these cells were washed and resuspended in tet-free media. The cells were grown for two days in the presence of tet (Expressed), absence of tet (Not expressed) and in those conditions with exogenous spermidine (Expressed + Spd and Not expressed + Spd). Protein samples from these cells were probed for by antibodies against prozyme and the FLAG tag by western blot (Figure 5.3). The western blot clearly shows that FLAG-prozyme is only present in samples cultured with tet. The blot that was probed for prozyme has a faint line in the cells that do not express prozyme; this corresponds to a non-specific band.

Prozyme is an essential parasite enzyme

Since it was not possible to make a straight knock out of prozyme, we would expect that the deletion of this protein is lethal to the cells. The conditional knock out cell line was used to evaluate the effect of prozyme loss on the growth of *T. brucei.* The cells were grown for a week in the presence of tet (expressed) and the absence of tet (not expressed), and the growth was monitored (Figure 5.4).

The cells expressing FLAG-prozyme (+ tet) grow well, similar to the parental cell line. However, there is a severe growth deficit when the expression of prozyme is turned off (-tet). The growth of these cells is slowed by two days of culture without tet, at the point where no prozyme is expressed. The cell number begins to decline after five days without tet, and all cells are dead by seven days after the removal of tet.

Prozyme knockout cannot be rescued by spermidine

The cKO cells were grown in the presence and absence of spermidine. The growth of *T. brucei* cells cultured in the + tet (Expressed), -tet (Not expressed), + tet + spd (Expressed + spermidine) and –tet + spd (Not expressed + spermidine) was monitored. There was no difference in the growth of cells expressing prozyme in the presence or absence of spermidine. The addition of exogenous spermidine to the cells that are not expressing prozyme does not rescue the growth of these cells. There is a retardation of cell growth, followed by death of all cells in the culture by 6 days after the removal of tet (Figure 5.4).

Prozyme knockout induces changes in ODC and TrypSyn

The cKO cells grown in the presence (Flag-prozyme expressed) and absence (not expressed) of tet, with and without spermidine, were analyzed for the protein levels of other pathway enzymes by western blot. The protein levels of AdoMetDC, TrypRed, and SpdSyn are constant in the presence and absence of the prozyme protein. However, there is an induction of ODC and TrypSyn protein levels in the cells that do not express prozyme. This induction is seen in the presence and absence of spermidine. These proteins are also induced in the knockdown of AdoMetDC, indicating that this increase in protein levels may be a common response to loss of AdoMetDC activity.

D. CONCLUSIONS

In order to study the effect of prozyme on polyamine and trypanothione regulation, we attempted to create an inducible prozyme RNAi cell line, as we have done for AdoMetDC. Though the mRNA was decreased, the knockdown of the protein was not sufficient to cause an effect on cell growth. Since this is an activating protein, a moderate decrease in protein levels may be tolerated. Also, since the protein levels have been demonstrated to be inducible, the cells may up-regulate prozyme past the point where RNAi has an effect.

Since the RNAi approach was not feasible in this situation, we created a genetic knockout of prozyme. Trypanosomes are diploid, and therefore a complete knockout would have to replace both alleles. Since spermidine was able to rescue the AdoMetDC knockdown, we made several attempts to create a straight, double allele knockout in the presence of spermidine. The knockout of a single allele was very easy to achieve; however we were not able to make a double knockout. This was attempted in two cell lines with multiple constructs and drug resistance cassettes. Finally, we were successful in generating a conditional prozyme knock out cell line. Even though a straight knockout of prozyme was not achieved, the fact that we were able to make this cKO line demonstrates that the homologous recombination at this locus is not lethal to the cells. Since the straight knock out could not be made in the presence of spermidine, prozyme may have an additional role to the activation of AdoMetDC, or these cells may be deficient in spermidine uptake.

In the cKO cells lines, prozyme expression was demonstrated to be regulated by tet; after two days without tet there is no expression of the Flag-prozyme transgene. The

loss of this protein occurs in the presence and absence of spermidine. The trypanosomes that do not express prozyme stop growing, and all cells are dead by six days without tet. Unlike the situation for AdoMetDC knockdown cells, the prozyme knockout cannot be rescued by the addition of spermidine. This demonstrates that prozyme is an essential parasite enzyme. The loss of prozyme causes an increase in the protein levels of ODC and TrypSyn. Since these are the same proteins increased in the knockdown of AdoMetDC, this implies that the upregulation of these proteins is caused by loss of AdoMetDC activity.

Prozyme is a novel protein that we have recently characterized as an allosteric activator of *T. brucei* AdoMetDC (Willert, Fitzpatrick et al. 2007). However, the straight prozyme knockout cannot be created in the presence of spermidine, and the conditional prozyme knockout cannot be rescued by spermidine. This data implies that there may be another role for prozyme in these parasites. Another possibility is that these cells are unable to uptake spermidine. The parental cell used to generate the prozyme cKO cells (427) is different from the parental cell line used to generate the AdoMetDC RNAi cells (90-13), and these cell lines may have different spermidine uptake capacity. Experiments to measure the content of polyamines and thiols in the cKO cells are underway, and may shed some light on the uptake of spermidine in these cells.

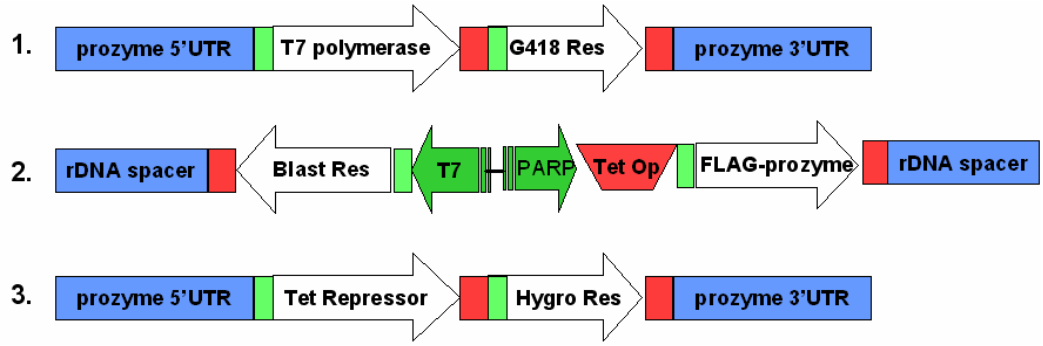


Figure 5.1 Constructs used to make prozyme cKO cells To make prozyme cKO cells, the first allele was knocked out by homologous recombination with construct 1. Next, the regulatable copy of prozyme, construct 2, was integrated into the rDNA spacer region of the genome. Finally, the second allele was knocked out with construct 3. This cell line was then cloned out by limited dilution.

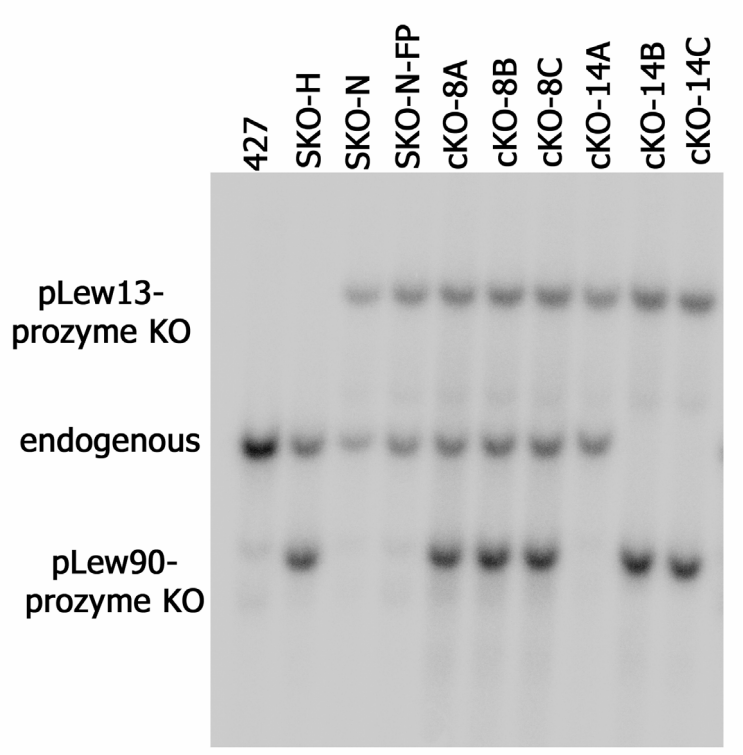


Figure 5.2 Southern blot of genomic DNA from cKO and precursor cell lines The genomic DNA was fragmented by restriction enzyme digestion and subjected to southern blot analysis. The probe hybridizes to a region of the genome 5' of the prozyme-coding region. Cell lines shown: 427 (parental), SKO-H (prozyme single knockout with hygromycin resistance), SKO-N (prozyme single knockout with G418 resistance), SKO-N-FP (SKO-N cells with FLAG-prozyme construct integrated into the rDNA region; blastacidin and G418 resistance), cKO-8 A-C and 14-A (SKO-N-FP cells transfected with the SKO-H construct that still display the endogenous prozyme gene) and cKO-14 B and C (SKO-N-FP cells transfected with the SKO-H construct that have been cloned out and show loss of the endogenous gene at both alleles) .

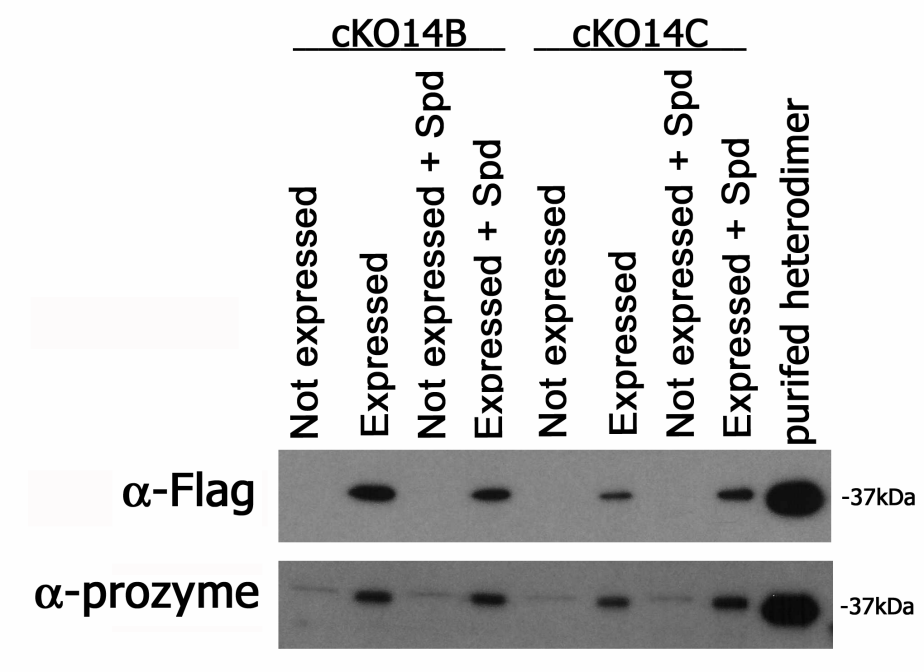


Figure 5.3 Removal of tet leads to loss of prozyme expression in cKO cells *T. brucei* cells were cultured in the presence (Flag-prozyme expressed) and absence (no prozyme expressed) of tet, with and without exogenous spermidine, for two days before the samples were harvested. Protein from the cell lysates was analyzed by western blot.

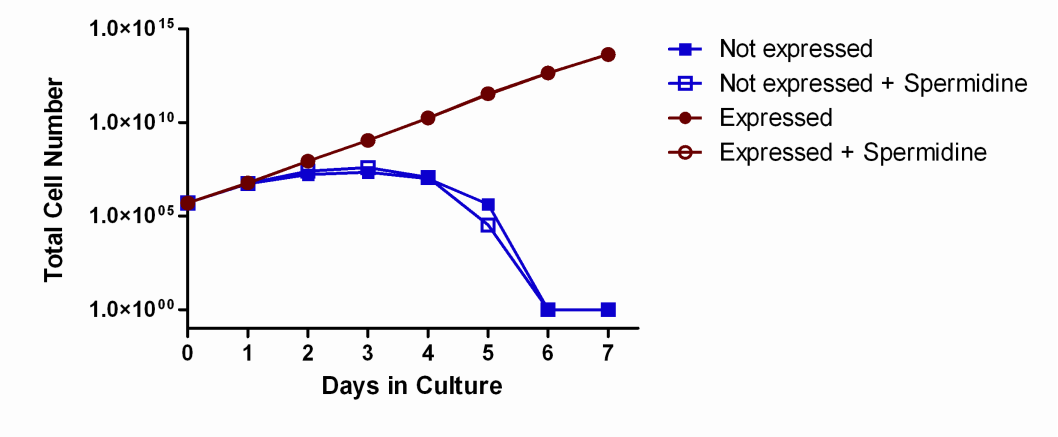


Figure 5.4 Prozyme knock out is lethal to *T. brucei* The loss of prozyme expression (by removal of tet, blue squares) leads to death of BF *T. brucei* cells after 6 days; the addition of spermidine (open symbols) to the media does not rescue the prozyme knock out cells.

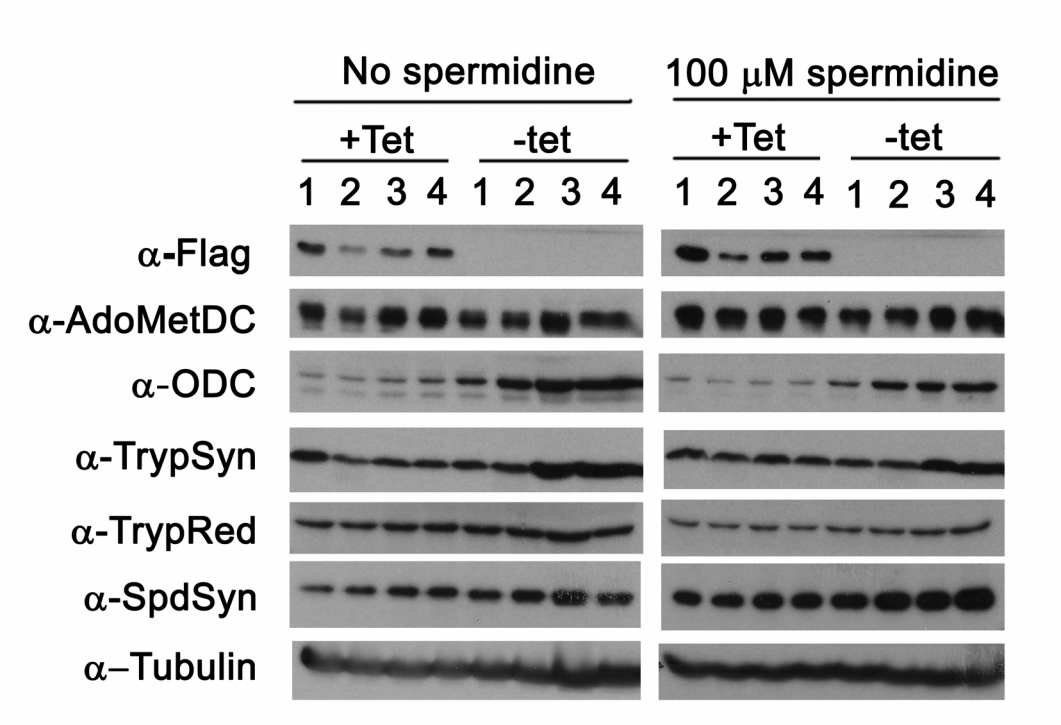


Figure 5.5. Prozyme knockout leads to changes in other pathway enzymes. *T. brucei* cells were cultured in the presence (Flag-prozyme expressed) and absence (no prozyme expressed) of tet, with and without exogenous spermidine. Protein from the cell lysates was analyzed by western blot.

Gene/locus	vector	sequence
prozyme 5'UTR forward	SKO (N or H)	5'-ATAGCGGCCGCGATGAGCGCCAATAAGAGTTTTACC-3'
prozyme 5'UTR reverse	SKO (N or H)	5'-ATAACGCGTCTCGAGTCCCTCTGGTGTAAAACCAG-3'
prozyme 3'UTR forward	SKO (N or H)	5-ATATCTAGATTTAAATGGTCACCCTGGTCAGCGCG-3'
prozyme 3'UTR reverse	SKO (N or H)	5'-ATAAGGCCTGCGGCCGCGGAGCAACTCAATAAAC-3'
FLAG-prozyme forward	pLEW300-FP	5'-CCCAAGCTTATGGACTACAAAGACGATGACG-3'
FLAG-prozyme reverse	pLEW300-FP	5'-CGCGGATCCTCGGCACTGCGTGCGTATGTGG-3'
891bp 5' forward	southern probe	5'-GCTTGGCTTCATTGATGTGCAGAAGGAGGTAGC-3'
291bp 5' reverse	southern probe	5'-CGGGTAAAACTCTTATTGGCGCTCATCGCAGG-3'

Table 5.1 Oligonucleotide primers Primers used to generate knock out and regulated expression constructs and to make the probe for southern blot analysis are given.

CHAPTER SIX

PERSPECTIVES

The neglected diseases caused by African (*T. brucei*) and South American (*T. cruzi*) trypanosomes are in need of new drugs for treatment. A unique area of trypanosome biology is the formation of trypanothione. This essential cofactor is generated by linking two molecules of glutathione with a molecule of spermidine, and is responsible for maintaining the redox balance of the cell, detoxification of free radicals, and participates in nucleic acid synthesis. By inhibiting enzymes involved in spermidine production, we will not only affect the polyamine homeostasis of the cell, but can also influence the amount of trypanothione available to the cell.

The polyamine pathway has been validated as a drug target, as DFMO, an inhibitor of the polyamine biosynthetic enzyme ODC, is a clinically utilized drug to treat African trypanosomiasis (Bacchi, Nathan et al. 1980). This drug is not effective against the Chagas disease, since the *T. cruzi* parasites do not have ODC (Ariyanayagam and Fairlamb 1997). However, inhibitors against another polyamine enzyme, AdoMetDC, have shown success in murine models of both diseases (Bacchi, Nathan et al. 1992; Yakubu, Majumder et al. 1993). The work presented in this dissertation has focused on trypanosomatid AdoMetDC.

We have genetically validated *T. brucei* AdoMetDC as a drug target by demonstrating that depletion of this protein leads to cell death in culture. I have also described the active form of the enzyme. In *T. brucei,* the activity of AdoMetDC is

stimulated by formation of a heterodimer with prozyme, a catalytically inactive paralog of AdoMetDC. I have also shown that the active form of the enzyme in *T cruzi* is the AdoMetDC/prozyme heterodimer. The related trypanosomatid, *L. major* also contains the prozyme gene, and is predicted to act in a similar manner. Using genetic manipulation of bloodstream form *T. brucei* cells, I have shown that both AdoMetDC and prozyme are essential for trypanosome survival. These manipulated cells have also been used to demonstrate the regulation of the polyamine and trypanothione biosynthesis pathways.

When looking for chemical inhibitors of AdoMetDC, it is important to test compounds in the presence of prozyme to recapitulate the situation found in *T. brucei.* Many AdoMetDC inhibitors have shown to be more effective on the human than the parasite enzyme; the species selectivity implies that there are differences that can be exploited in drug design. The trypanosome enzyme is a heterodimer, while the human enzyme is a monomer; this is another species selective difference that may facilitate the design of new inhibitors against these parasites. The heterodimeric form of an AdoMetDC protein is also a novel example of oligomeric state for this enzyme family.

The prozyme gene arose from a gene duplication event from AdoMetDC, and evolved by mutational drift. The two enzymes probably co-evolved, with AdoMetDC retaining activity but modified to require the prozyme to be made into the correct conformation for efficient catalysis, and the prozyme losing the catalytic ability and gaining an activator function. There are only a few other examples of the product of a gene duplication acting as regulators of active enzymes, and this is the first presentation of a gene duplicate acting as an allosteric activator. However, inactive homologs are very common and present in several diverse enzyme families. The discovery of the regulatory

role for a gene duplicate may be more common, and some potential "pseudogenes" may actually turn out to be "pseudo-enzymes" and regulate the function of an active enzyme.

The AdoMetDC from *T. cruzi* is also stimulated by prozyme. The main difference between the heterodimers from the two trypanosome species is that the activity of the *T. cruzi* complex is stimulated by putrescine, while the *T. brucei* catalysis is not. The mammalian AdoMetDC homodimer is also stimulated by putrescine. Since the *T. cruzi* parasites reside in an intracellular environment rich in polyamines and rely on uptake as their sole source of putrescine, this level of regulation may have been retained to monitor polyamine status. The *T. brucei* parasites live in the bloodstream where there is little polyamine content, and may have lost the regulation of AdoMetDC by putrescine. I have also shown that the prozyme from one trypanosome species can activate the AdoMetDC from the other to some extent. This result shows that they are functionally similar as well as homologous.

Polyamines are essential small molecules, and are found in millimolar concentrations in most cells. The levels of polyamines must be tightly controlled, as excess or insufficient polyamines can have deleterious results on the cell and/or organism. In mammalian cells, there are many layers of regulation; however most of these mechanisms are not present in trypanosomes. I depleted *T. brucei* cells of AdoMetDC by RNAi. This leads to cell death; however the cells can be rescued by the addition of spermidine, a downstream product of AdoMetDC. The knockdown of AdoMetDC leads to changes in the metabolites and protein levels of biosynthetic enzymes in the polyamine/trypanothione pathway. The results of this study show that the trypanosomes prefer to maintain the pools of spermidine at the expense of trypanothione.

When spermidine cannot be synthesized, the trypanothione pools may be used as a source to generate spermidine. The increase in enzyme levels of prozyme, ODC and TrypSyn all support the regulation of the pathway to favor spermidine.

Prozyme levels are highly induced in the AdoMetDC knockdown cells, supporting the idea that this is a regulatory protein. In order to further study the role of prozyme, I have generated a cell line for conditional knock out of this gene. These cells are currently being characterized. Preliminary results show that the prozyme is an essential parasite enzyme. The loss of prozyme leads to cell death, and this cannot be rescued by addition of the downstream product, spermidine. Similar to the AdoMetDC knockdown cells, the loss of prozyme leads to an increase in protein levels of ODC and TrypSyn. Currently, I am looking at the metabolite levels in the pathway to see what changes occur with the loss of prozyme. Spermidine may fail to rescue these cells because it is not taken up, or because prozyme has another role in the cell. An intriguing possibility is that prozyme plays a role in the uptake of spermidine, and therefore loss of this protein inhibits uptake, so the exogenous polyamine can no longer enter the cell.

Taken together, the data presented here demonstrates that the AdoMetDC/prozyme heterodimer is a promising drug target against *T. brucei* infections. Both subunits have been shown to be essential proteins for cell growth. AdoMetDC and prozyme appear to have an important role in the regulation of polyamine and trypanothione biosynthesis in *T. brucei*; here we have uncovered the first evidence for regulation of this pathway in these parasites. Future work on the role of prozyme may highlight other roles for this interesting protein.

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VITAE

Erin Kathleen Willert (née Hogan) was born in Endicott, New York, on March 7, 1981. She is the youngest daughter of Robert Leo and Patricia Ann Hogan and sister of Christy, Billy and Bridget. Erin married Timothy Andrew (Andy) Willert in April of 2006. Erin attended high school at Gilroy College in Castle Hill, NSW, Australia. After completion of her higher school certificate, she began her undergraduate studies at Beaver College (now Arcadia University) in Glenside, PA. She transferred to the University of Texas at Austin for her last two years, and graduated with honors, receiving her Bachelor of Science in Chemistry. Erin enrolled in the division of molecular and cellular biology graduate program at the University of Texas Southwestern Medical Center at Dallas in the fall of 2002, and joined the laboratory of Dr. Meg Phillips in the spring of 2003. Erin was appointed to the UTSW Pharmacological Sciences Predoctoral Training Grant (NIH NRSA) from 2004-2007. In the summer of 2007, she was a co-chair for the Gordon-Kenen Graduate Research Seminar on Polyamines. Also in the summer of 2007, she was a teaching assistant for the Biology of Parasitism summer course in Woods Hole, MA. After graduating with a Ph.D. from the Biochemistry program at UT Southwestern in January of 2008, she plans to pursue a postdoctoral position in the field of infectious disease.

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