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Cross-species activation of trypanosome S-adenosylmethionine decarboxylase by the regulatory subunit prozyme

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ABSTRACT

The protozoan parasite *Trypanosoma cruzi* is the causative agent of Chagas disease (American trypanosomiasis), a neglected disease of Central and South America. Polyamines are small organic cations that are required for cell growth and their biosynthesis has been the target of drug discovery efforts in both *T. cruzi* and the related *Trypanosoma brucei* parasites. Here we show that, as previously demonstrated for *T. brucei*, S-adenosylmethionine decarboxylase (AdoMetDC) from *T. cruzi* forms a heterodimer with prozyme, an inactive homolog that arose by gene duplication of the canonical enzyme uniquely in the trypanosomatids. The *T. cruzi* AdoMetDC/prozyme heterodimer is 110-fold more active than homodimeric AdoMetDC. Unlike for *T. brucei* AdoMetDC, the activity of the *T. cruzi* heterodimer is further stimulated by putrescine to generate an enzyme with similar catalytic efficiency to the fully activated *T. brucei* enzyme. The effects of prozyme on *T. cruzi* AdoMetDC are mediated by an increase in k_{cat}, while the predominant effect of putrescine is to lower the K_m. Finally we demonstrate that the cross-species heterodimers of *T. cruzi* and *T. brucei* AdoMetDC and prozyme pairs are functional, and that putrescine is required for prozyme to fully activate the mixed species heterodimers. These data demonstrate that prozyme mediated activation of AdoMetDC is a common mechanism required to regulate AdoMetDC activity in the trypanosomatids.

1. Introduction

The trypanosomatid parasites, *Trypanosoma cruzi* and *Trypanosoma brucei* cause significant human and veterinary disease worldwide [1–3]. In Africa 50 million people are at risk for contracting African sleeping sickness caused by *T. brucei*. The disease is fatal if untreated. In South America the causative agent of Chagas disease, *T. cruzi*, has an incidence of 200,000 cases each year and over 13 million people are chronically infected with the parasite. Chagas disease is a major cause of cardiomyopathy in the region. Current therapeutics for both Chagas disease and African sleeping sickness are limited, underscoring the need for the development of new and improved anti-trypanosomal drugs and drug targets.

Polyamines are small organic cations that are required for cell growth and proliferation [4]. Polyamines are synthesized from L-ornithine and decarboxylated S-adenosylmethionine (dcAdoMet) (Scheme 1), and the biosynthetic enzymes ornithine decarboxylase (ODC) and S-adenosylmethionine decarboxylases (AdoMetDC) have been studied as chemotherapeutic targets. α-Difluoromethylornithine (DFMO), a suicide inhibitor of ODC, is used clinically for the treatment of African sleeping sickness [1]. In contrast, *T. cruzi* does not encode an ODC gene, and therefore DFMO is not effective against this parasite (Scheme 1) [5,6]. Instead, the intracellular *T. cruzi* pathogen relies on putrescine uptake to obtain this polyamine precursor, which is facilitated by the abundance of putrescine found within host cells [7]. *T. cruzi* epimastigotes are also able to transport spermidine [8]. In contrast, *T. brucei*, an extracellular parasite, relies on *de novo* polyamine synthesis since there are limiting amounts of polyamines in the blood and lymph.

AdoMetDC catalyzes the decarboxylation of S-adenosylmethione to form dcAdoMet, which then serves as the amino propyl donor for the formation of spermidine from putrescine (Scheme 1). Both *T. cruzi* and *T. brucei* contain the gene for AdoMetDC, and inhibitors of this enzyme have been demonstrated to cure murine models of infection with either trypanosome species [9–11]. AdoMetDC is a pyruvoyl-dependent enzyme that derives the pyruvoyl cofactor from an auto-processing reaction that cleaves the polypeptide chain into a larger α-chain, containing the pyruvate at the N-terminus, and a smaller β-chain [12]. The human AdoMetDC enzyme is a homodimer ($\alpha_2\beta_2$) that

Abbreviations: AdoMetDC, S-adenosylmethionine decarboxylase; AdoMetDC/prozyme, the purified complex between AdoMetDC and prozyme; ODC, ornithine decarboxylase; DFMO, α-difluromethylornithine; AdoMet, S-adenosylmethionine; dcAdoMet, decarboxylated AdoMet.

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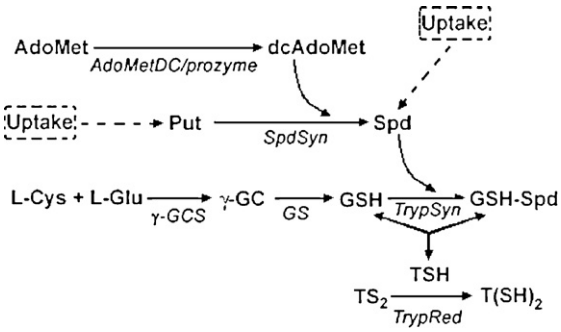

Scheme 1. The polyamine biosynthesis pathway in *T. cruzi*. AdoMet (S-adenosylmethionine); dcAdoMet (decarboxylated AdoMet); Put (putrescine); Spd (spermidine); SpdSyn (Spd synthetase); γ-GC (gamma-glutamylcysteine); γ-GCS (γ-GC synthetase); GSH (glutathione); GS (GSH synthetase); GSH-Spd (glutathionyl-spermidine); TSH (trypanothione); TrypSyn (TSH synthetase); TS_2 (oxidized TSH); $T(SH)_2$ (reduced TSH); TrypRed (TSH reductase).

is allosterically activated by putrescine [13,14]. Previous work has shown that the recombinant AdoMetDC enzyme from *T. cruzi* forms a homodimer that is stimulated by putrescine, however it has a much lower catalytic efficiency than human or plant homologs [15–17]. We recently demonstrated that the *T. brucei* AdoMetDC is allosterically activated by a regulatory protein, prozyme [18]. Prozyme is an inactive paralog of AdoMetDC that arose by gene duplication and is found only in the trypanosomatids. *T. brucei* prozyme and AdoMetDC form a high affinity heterodimer that is the functional species of the enzyme in the parasite. Furthermore prozyme protein expression levels are regulated in *T. brucei*, providing a mechanism to control pathway flux that is unique to these parasites [19].

The functional role for prozyme as an activator has so far only been demonstrated for *T. brucei* AdoMetDC, though phylogenic analysis suggests that both *T. cruzi* and *Leishmania* AdoMetDC will also be activated by prozyme [18]. Here we show that *T. cruzi* AdoMetDC is also activated by prozyme. The AdoMetDC/prozyme heterodimer from *T. cruzi* forms with high affinity stimulating the activity of the enzyme; however, in contrast to the *T. brucei* enzyme, the activity of the *T. cruzi* heterodimer is putrescine-dependent. Finally we demonstrate that prozyme and AdoMetDC from *T. brucei* and *T. cruzi* are able to form functional cross-species heterodimers, demonstrating that the dimer interface and mechanism of activation are conserved between the species.

2. Materials and methods

2.1. Cloning of T. cruzi prozyme and AdoMetDC

The expression construct (TcAdoMetDC) for *T. cruzi* AdoMetDC has been described previously [15]. The gene for *T. cruzi* prozyme was found by blast search of geneDB (http://www.genedb.org/, ID: Tc00.1047053509167.110) using *T. brucei* prozyme as the search sequence. *T. cruzi* prozyme was cloned and amplified by PCR from genomic DNA, and ligated into the pET 15b vector generating an N-terminal His_6-tagged construct. A double expression vector (DEV) that allows for co-expression of His-tagged AdoMetDC and untagged prozyme in *Escherichia coli* was also created. 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 DEV. Primers used for cloning were as follows: Tc prozyme-Forward for pET 15b (5′-CGCCATATGTTGGAGAGCACCTGGGCAGCCG-3′, includes an Nde I site); Tc prozyme-Reverse for pET 15b (5′-CGCGGATCCTTATTCGGCGCGAATATAGCTGG-3′, includes a BamHI site); Tc DEV-Forward for pET 22b-DEV (5′-CGATAGTCG-ACTCGAAATTAATACGACTCACTATAGG-3′, includes a SalI site); Tc DEV-Reverse for pET 22b-DEV (5′-TATGCGGCCGCTTACTACT-CTTCCACAGAATCTGTGG-3′, contains a NotI site).

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

T. cruzi AdoMetDC or prozyme were expressed individually in *E. 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 [15], 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. Cloning, expression and purification of His_6-tagged *T. brucei* AdoMetDC and prozyme has been described previously [18].

2.3. Sedimentation equilibrium by analytical ultracentrifugation

The molecular weight of the *T. cruzi* AdoMetDC/prozyme complex was 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 at 4, 6 and 8 μM (0.1–0.12 mL total volume) into a six-sector equilibrium centerpiece and equilibrated for data collection at 14,000 and 20,000 rpm. After equilibrium was reached (approximately 24 h), 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 (Eq. (1)), or a monomer-heterodimer model (Eq. (2)), as previously described [20], 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:

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

$$\begin{aligned} A = {} & c * \varepsilon_A * d * \exp[(M_A * (1 - \nu_{\text{bar }A} * \rho) * \omega^2 * (r^2 - r_o^2)/2 * R * T] \\ & + c * \varepsilon_B * d * \exp[(M_B * (1 - \nu_{\text{bar }B} * \rho) * \omega^2 * (r^2 - r_o^2)/2 * R * T] \\ & + c^2 * (\varepsilon_A + \varepsilon_B) * d * K_{a,AB} * \exp[((M_A * (1 - \nu_{\text{bar }A} * \rho) \\ & + (M_B * (1 - \nu_{\text{bar }B} * \rho)) * \omega^2 * (r^2 - r_o^2)/2 * R * T] + \delta \end{aligned} \quad (2)$$

where A = absorbance at 280 nm at radial position r; c = concentration at the meniscus in abs units; ε = extinction coefficient (TcADoMetDC monomer 61,310 $M^{-1}\,cm^{-1}$; Tcprozyme monomer 36,900 $M^{-1}\,cm^{-1}$; heterodimer 98,210 $M^{-1}\,cm^{-1}$; calculated by ExPASy ProtParam tool: http://www.expasy.ch/tools/protparam.html); d = pathlength (1.2 cm); M = molar mass (g/mol) (M_a = TcAdoMetDC monomer 44,000 Da; M_b = Tcprozyme monomer 37,000 Da); V_{bar} = partial specific volume (0.73 mL/g TcADoMetDC; 0.732 mL/g Tcprozyme; 0.731 mL/g Tc heterodimer); ρ = buffer density (1.005 g/mL); ω (angular