

Extremely rapid turnover of S-adenosylmethionine decarboxylase in *Crithidia fasciculata*

Sima Nasizadeh, Lo Persson*

Department of Physiological Sciences, Lund University, BMC F-13, S-221 84 Lund, Sweden

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Abstract The activity of S-adenosylmethionine decarboxylase (AdoMetDC) in *Crithidia fasciculata* was shown to be correlated to the growth of the parasite. An increase in activity was observed during exponential growth. Inhibition of protein synthesis induced an extremely rapid decay of AdoMetDC activity. The half-life of the enzyme was estimated to be about 3 min, which is the shortest half-life ever recorded for an eukaryotic AdoMetDC. The reduction in AdoMetDC activity was correlated with a decrease in AdoMetDC protein content, demonstrating a rapid turnover of the enzyme. No polyamine-mediated feedback regulation of AdoMetDC was observed in the parasite. © 2003 Federation of European Biochemical Societies. Published by Elsevier B.V. All rights reserved.

Key words: Polyamines; Enzyme turnover; S-adenosylmethionine decarboxylase; Trypanosomal; *Crithidia fasciculata*

1. Introduction

The polyamine biosynthesis pathway has been shown to be a potential target for drugs against various trypanosomal parasitic diseases [1,2]. The polyamines, putrescine, spermidine and spermine are aliphatic amines, which are essential for cell growth and differentiation [3,4]. In trypanosomatids, spermidine is also used for the synthesis of trypanothione, a unique spermidine–glutathione conjugate involved in the parasitic redox regulation [5]. Interestingly, the trypanosomatids do not contain spermine. Ornithine decarboxylase (ODC) catalyzes the first and what is often considered as the rate-limiting step in this pathway, namely the synthesis of putrescine from ornithine. Difluoromethylornithine (DFMO), which is an irreversible inhibitor of ODC, is an effective drug against the West African form of sleeping sickness, caused by the protozoan parasite *Trypanosoma brucei* [2]. The effectiveness of DFMO against sleeping sickness may be attributed, at least partly, to the differences in ODC turnover between the host and the trypanosomal parasite [6]. The parasitic ODC, which

is stable and thus has a slow turnover rate, is inactivated by DFMO, whereas the human host ODC, having a short half-life, is continuously regenerated.

S-Adenosylmethionine decarboxylase (AdoMetDC), catalyzes another important step in the polyamine biosynthetic pathway. Decarboxylated AdoMet provides the aminopropyl moiety for the synthesis of spermidine from putrescine (and of spermine from spermidine in organisms containing also this polyamine). In addition to providing the aminopropyl group for the synthesis of spermidine, AdoMet in the trypanosome parasites is utilized as the methyl donor for transmethylation reactions as well as for salvage of adenine and adenosine [7,8]. The decarboxylation of AdoMet has a pivotal role in the switching between cellular methylation and polyamine biosynthesis in the parasites [9].

AdoMetDC belongs to the unique group of enzymes having a covalently bound pyruvate as a prosthetic group [10,11]. The enzyme is synthesized as a proenzyme, which then is autocatalytically cleaved at a serine residue into two subunits. This cleavage generates the pyruvoyl group at the N-terminus of the larger subunit. In mammalian cells, the production of AdoMetDC is highly regulated at the levels of transcription and translation, whereas in the trypanosomatids it does not appear to be extensively regulated [10–12]. Nevertheless, AdoMetDC is an essential enzyme for the parasites and has been considered as an important chemotherapeutic target in the treatment of parasitic diseases [2]. The importance of AdoMetDC in *Leishmania donovani* was demonstrated by gene targeting [13]. Disruption of both copies of the AdoMetDC gene in the parasites made them polyamine auxotrophic. The parasites were unable to grow unless spermidine (no other polyamine was effective) was supplemented in their growth medium. In the absence of exogenous spermidine the parasites eventually died after 4 weeks. Furthermore, a variety of inhibitors against AdoMetDC have been shown to exert strong anti-parasitic effects [2]. Two of the most potent drugs against murine *T. brucei* infections were shown to be the AdoMetDC inhibitors, MDL 73811 [14] and CGP40251A [15], which, in contrast to DFMO, also cured mice infected with *T. brucei rhodesiense*, the causative agent of the East African form of sleeping sickness. Interestingly, the anti-protozoan drugs berenil and pentamine were both demonstrated to be effective inhibitors of trypanosomal AdoMetDC [16].

In the present study, we have investigated the properties of AdoMetDC in the trypanosomatid *Crithidia fasciculata*. It is demonstrated that, in contrast to other protozoan AdoMetDCs, *C. fasciculata* AdoMetDC has a very fast turnover rate, with a biological half-life as short as a few minutes.

*Corresponding author. Fax: (46)-46-2224546.
E-mail address: lo.persson@mphy.lu.se (L. Persson).

Abbreviations: AdoMet, S-adenosylmethionine; AdoMetDC, S-adenosylmethionine decarboxylase; ODC, ornithine decarboxylase; DFMO, difluoromethylornithine; MGBG, methylglyoxal-bis(guanyldrazone); IC₅₀, concentration that inhibits 50% of the enzyme activity

2. Materials and methods

2.1. Materials

S-Adenosyl-L-[carboxy- ^{14}C]methionine (58 mCi/mmol) and AdoMet were purchased from Amersham (Amersham, UK) and Sigma (St. Louis, MO, USA), respectively. SuperSignal West Dura and horseradish peroxidase conjugated goat anti-rabbit IgG were bought from Pierce (Rockford, IL, USA). Hyamine 10-X was obtained from Packard Instruments. Extract from AdoMetDC overproducing *L. donovani* and antibodies against *L. donovani* AdoMetDC were kind gifts of Drs. Sigrid Roberts and Buddy Ullman [13].

2.2. Cell culture

C. fasciculata of the strain ATCC 11745 was cultured in a completely defined medium, HOSMEM II [17]. The parasites were incubated at 28°C with mild shaking. Cells were seeded at a concentration of about 1×10^6 cells/ml and harvested for experiments (except for the growth curve) at mid-exponential growth ($10\text{--}15 \times 10^6$ cells/ml). The turnover of AdoMetDC was determined by measuring the decay of the enzyme activity after the addition of cycloheximide (50 $\mu\text{g}/\text{ml}$). The effects of polyamines on AdoMetDC activity was determined after growing the cells in the absence or presence of putrescine (0.5 mM), spermidine (10 μM) or spermine (10 μM) for 15 h. Aminoguanidine (1 mM) was supplemented to the medium when polyamines were added in order to inhibit oxidation by copper-containing amine oxidases.

2.3. AdoMetDC activity assay

The AdoMetDC activity was determined by measuring the release of $^{14}\text{CO}_2$ from S-adenosyl-L-[carboxy- ^{14}C]methionine. The parasites were sonicated in ice-cold AdoMetDC buffer containing 0.1 M Tris/HCl pH 7.5, 2.5 mM dithiothreitol, 0.1 mM EDTA and 2.5 mM putrescine. The sonicate was centrifuged at $30\,000 \times g$ at 4°C for 20 min. The reaction mixture usually contained 0.1–0.2 μCi of S-adenosyl-L-[carboxy- ^{14}C]methionine, 0.2 mM AdoMet, 2.5 mM putrescine and supernatant corresponding to $100\text{--}200 \times 10^6$ cells in a total volume of 250 μl . The samples were incubated at 28°C (which gave a higher enzyme activity than at 37°C) for 30 min in test tubes equipped with a rubber stopper. The $^{14}\text{CO}_2$ released was trapped in 0.1 ml of Hyamine 10-X in a polypropylene well attached to the rubber stopper. The enzymatic reaction was terminated by the addition of 0.3 ml 2 M HClO_4 followed by an additional incubation at 28°C for 45 min. The amount of radioactivity was measured using a liquid scintillation spectrometer. The enzyme assay was linear for the whole period of the assay. The K_m for AdoMet was determined by varying the substrate concentration in the reaction mixture between 0.025 and 0.4 mM.

2.4. Determination of substrate-mediated inactivation of

C. fasciculata AdoMetDC

The degree of substrate-mediated inactivation of *C. fasciculata* AdoMetDC was measured by preincubating cell extract at 28°C with or without 0.2 mM AdoMet for various time periods before analysis of AdoMetDC activity.

2.5. Western blot analysis

Aliquots from the cell extracts (corresponding to 5×10^6 cells) were analyzed by Western blot technique essentially as described in [18] using an antibody raised against *L. donovani* AdoMetDC [13]. The antibody was used in a dilution of 1:2000. The membrane was stained with the SuperSignal West Dura chemiluminescent substrate (Pierce). Prestained proteins (Bio-Rad, Hercules, CA, USA) and purified *L. donovani* AdoMetDC [13] were used as molecular mass markers.

3. Results and discussion

3.1. *C. fasciculata* AdoMetDC activity during cell growth

Although results from inhibitor studies [14,15] as well as gene targeting [13] have demonstrated that AdoMetDC fulfills important functions in trypanosomal growth, there are surprisingly few studies on the activity of this enzyme during growth of the parasites. Seltzer et al. [19] demonstrated a close relationship between AdoMetDC activity and cell growth of

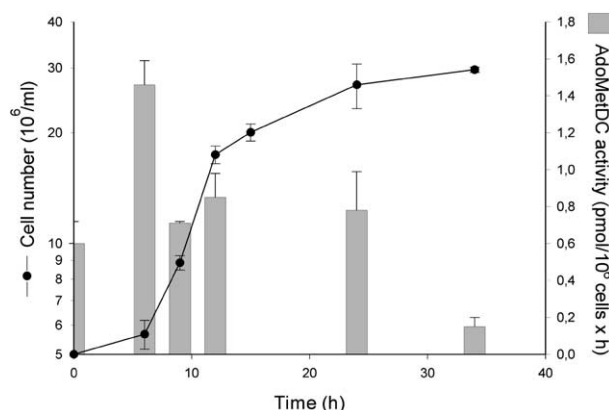


Fig. 1. AdoMetDC activity during growth of *C. fasciculata*. Cells from a high density (20×10^6 cells/ml) were reseeded at a density of 5×10^6 cells/ml. At various time points aliquots were taken for determination of cell number and AdoMetDC activity. Points and columns represent cell number and AdoMetDC activity, respectively. Mean $\pm$ S.D., $n = 3$.

T. brucei. Enzyme activity was high in parasites isolated during the exponential phase of growth, whereas enzyme activity in trypanosomes from the stationary phase was low. A similar observation was made in *L. donovani* by Mukhopadhyay and Madhubala [20]. Fig. 1 shows the activity of AdoMetDC during growth of *C. fasciculata*. As seen in this figure, the highest activity of AdoMetDC was noticed during late lag phase and early exponential phase. The AdoMetDC activity was markedly lower during the stationary phase.

The *C. fasciculata* AdoMetDC, like the enzymes from *T. brucei* [21] and *Trypanosoma cruzi* [22], was strongly activated by putrescine (results not shown). The apparent K_m for AdoMet was estimated to be about 0.2 mM (Fig. 2), which is comparable to that of *T. cruzi* AdoMetDC [22] but higher than that usually observed for mammalian AdoMetDC (50–100 μM) [23].

Methylglyoxal-bis(guanyldrazon) (MGBG) has been

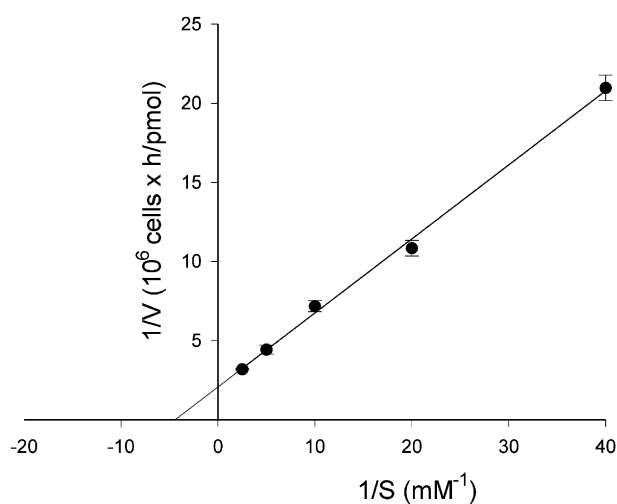


Fig. 2. Effect of substrate concentration on AdoMetDC activity. AdoMetDC activity was measured in the presence of 2.5 mM putrescine and various concentrations of AdoMet. The results is presented as a Lineweaver–Burk double reciprocal plot of reaction velocity ($1/V$) against substrate concentration ($1/S$). $K_m = 0.22$ mM. Mean $\pm$ S.D., $n = 3$.

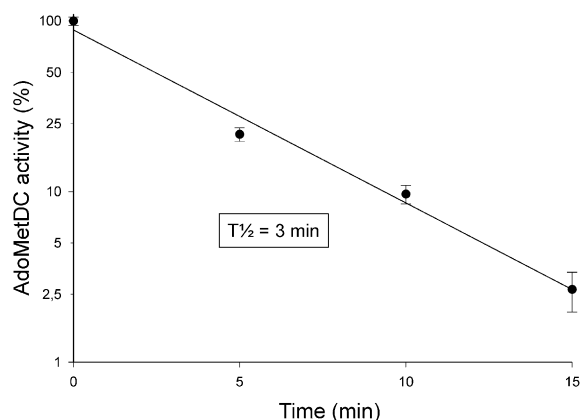


Fig. 3. Turnover of AdoMetDC in *C. fasciculata*. The half-life of AdoMetDC activity was determined by measuring the decay after inhibition of protein synthesis using cycloheximide. Mean $\pm$ S.D., $n=3$.

demonstrated to be a potent competitive inhibitor of mammalian AdoMetDC with an IC_{50} (concentration that inhibits 50% of the enzyme activity) of 1.5 μ M [24]. However, AdoMetDCs from *T. brucei* [21] and *T. cruzi* [22] were shown to be less sensitive to MGBG than the mammalian enzyme. The IC_{50} of MGBG for these enzymes were about 30 and 500 μ M, respectively. The *C. fasciculata* AdoMetDC was more sensitive to MGBG than AdoMetDC from *T. brucei* or *T. cruzi*. The concentration needed to give a 50% inhibition of enzyme activity in vitro was estimated to about 12 μ M (results not shown). In spite of this sensitivity observed in vitro, no effects were observed on *C. fasciculata* cell growth when MGBG was added to the growth medium (unpublished observation), which may indicate that MGBG is poorly taken up by the parasite.

3.2. Turnover of *C. fasciculata* AdoMetDC

Mammalian AdoMetDC, like ODC, has a very fast turnover with a half-life of usually less than 1 h [25,26]. In *T. brucei* [27] and *L. donovani* [13] AdoMetDC has been shown to be a stable enzyme. Also ODC has been demonstrated to be a stable enzyme in these parasites [6,28]. In contrast with *T. brucei* and *L. donovani*, *C. fasciculata* was shown to express an ODC with a fast turnover [29,30]. Thus, it was of great interest to determine the turnover of AdoMetDC in *C. fasciculata*. In order to measure the half-life of AdoMetDC in *C. fasciculata*, the parasites were treated with cycloheximide and aliquots were taken at different time intervals. Preliminary results indicated that the turnover of the enzyme was extremely fast, with a half-life in the minutes range. Hence, a time period of only 15 min was selected. As shown in Fig. 3, there was a rapid decay in AdoMetDC activity after inhibition of protein synthesis. The half-life was estimated to be as short as 3 min, which, to our knowledge, is the shortest half-life ever recorded for an eukaryotic AdoMetDC. Western blot analysis of the *C. fasciculata* extracts, using an antibody raised against *L. donovani* AdoMetDC, revealed a protein with a molecular mass similar to that of the larger subunit of *L. donovani* AdoMetDC (33 kDa), which disappeared after 6 min of cycloheximide treatment (Fig. 4). Thus, the decrease in enzyme activity was correlated with a decrease in AdoMetDC protein content.

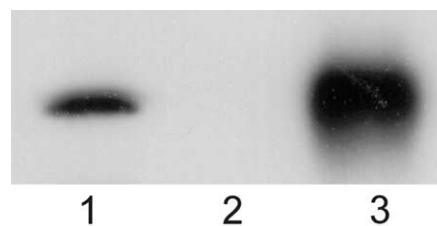


Fig. 4. Effect of cycloheximide on AdoMetDC protein content in *C. fasciculata*. Cells were grown in the absence or presence of cycloheximide (50 μ g/ml) for 6 min. The amount of AdoMetDC protein was determined by Western blot analysis as described in Section 2. Lane 1, control; lane 2, cycloheximide; lane 3, extract from AdoMetDC overproducing *L. donovani*.

As mentioned earlier AdoMetDC contains a covalently bound pyruvoyl group, which is important for the catalytic activity [10,11]. It has been shown that during the catalytic activity the pyruvoyl group can occasionally be transaminated converting the pyruvate into alanine, which irreversibly inactivates the enzyme [31,32]. The process can in some organisms be significant, inactivating a substantial percentage of the enzyme molecules. It is furthermore conceivable that the transaminated form may be degraded more rapidly, affecting the turnover of the enzyme. In order to determine whether substrate-mediated transamination of AdoMetDC may be a major phenomenon in *C. fasciculata*, we incubated cellular extracts in the presence or absence of 0.2 mM AdoMet for various time periods before analysis of AdoMetDC activity. As shown in Fig. 5, preincubation with AdoMet for up to 1 h did not affect the enzyme activity, indicating that substrate-mediated transamination of *C. fasciculata* AdoMetDC is a relatively rare event.

That the rapid decay in AdoMetDC activity seen in Fig. 3 was not related to a rapid inactivation of the enzyme during, or after, the sonication process was confirmed by the following findings: a) the enzyme assay was linear for at least 30 min, b) preincubating the enzyme for up to 1 h did not affect the enzyme activity, and c) treatment with cycloheximide for a short time induced a disappearance of AdoMetDC protein in the cells.

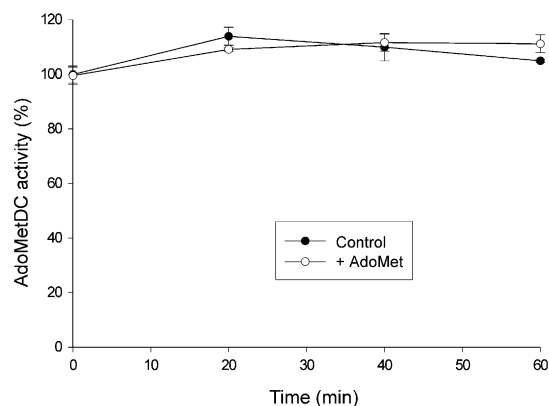


Fig. 5. Determination of substrate-mediated inactivation AdoMetDC. AdoMetDC was preincubated in the absence or presence of 0.2 mM AdoMet for various times before analyzed for enzyme activity. Mean $\pm$ S.D., $n=3$.

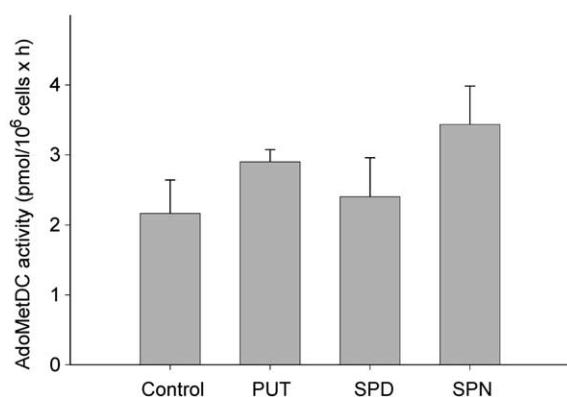


Fig. 6. Effect of polyamines on AdoMetDC activity. Cells were grown in the absence or presence of putrescine (PUT; 0.5 mM), spermidine (SPD; 10 μ M) or spermine (SPN; 10 μ M) for 15 h before analyzed for AdoMetDC activity. Mean $\pm$ S.D., $n=3$.

3.3. Effect of polyamines on *C. fasciculata*

AdoMetDC activity

Mammalian AdoMetDC is tightly regulated at the levels of transcription and translation [10–12]. Spermidine and spermine exert a negative feedback on the production of mammalian AdoMetDC at both these levels. In order to determine whether the polyamines have any regulatory effects on AdoMetDC production in *C. fasciculata* we incubated the parasites for 15 h in the absence or presence of putrescine, spermidine or spermine and then measured the AdoMetDC activity. As shown in Fig. 6, there was no decrease in AdoMetDC activity by the presence of polyamines in the medium, indicating that *C. fasciculata* AdoMetDC is not subject to a feedback-regulation by the polyamines. A similar observation has been made for *C. fasciculata* ODC, which also has a fast turnover ($T_{1/2}=30$ min) in the parasite [29].

3.4. Conclusions

In great contrast to the conditions in other protozoan parasites, both ODC [29] and AdoMetDC are turned over very quickly in *C. fasciculata*. The physiological function of this rapid turnover is not known. It may form the basis for an effective regulation of polyamine synthesis in the parasite. However, no evidence is yet obtained that there is a feedback control of polyamine synthesis in *C. fasciculata*, like that found in mammals [3]. Nevertheless, the production of metabolically unstable ODC and AdoMetDC may be still be related to a need to rapidly control the synthesis of polyamines or their metabolites in the parasite. Trypanothione for example is a polyamine metabolite which plays an essential role in protecting the parasite against various stressful conditions [5] and a close regulation of polyamine synthesis may be associated with the control of trypanothione production.

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