Volume 301, number 3, 261–264 FEBS 10959 April 1992

Ornithine decarboxylase from *Crithidia fasciculata* is metabolically unstable and resistant to polyamine down-regulation

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Received 3 March 1992

Ornithine decarboxylase (ODC) of *Crithidia fasciculata* extracts shows maximal activity during exponential growth of the parasite and decreases markedly in the stationary phase. The inhibition of protein synthesis by cycloheximide evoked a rapid loss of enzyme activity with a half-life of about 30 min. Upon removal of DFMO from *Crithidia* cultures treated with the drug for 24 h, the ODC activity increased at the same rate as total protein synthesis. The addition of putrescine at high concentrations to parasites cultivated in a synthetic medium showed that *Crithidia* ODC levels were not reduced by polyamines.

Ornithine decarboxylase; Enzyme turnover; Polyamine-dependent regulation; *Crithidia fasciculata*

1. INTRODUCTION

Ornithine decarboxylase (ODC) catalyzes the conversion of ornithine into putrescine, the first step of polyamine biosynthetic pathway in eukaryotic cells. Since polyamines play important roles in the synthesis of nucleic acids and proteins as well as in cell proliferation [1,2] it is not surprising that the ODC of mammalian cells is a precisely regulated enzyme; its activity can be modified by a wide variety of stimuli and physiological conditions [3,4].

Mammalian ODC has a very short half-life of about 20 min [5] and can be regulated inside the cell at different levels of gene expression, such as gene amplification [6–9], transcription [10], translation of the corresponding mRNA [11,12] and post-translational modifications which include oligomer assembly and protein degradation [10,13]. Some of these mechanisms are controlled by polyamine intracellular levels [14–16]. All the above mentioned properties and the fact that cell growth is usually related to polyamine endogenous concentrations can explain why ODC has been considered a likely appropriate target to block cell proliferation by using specific inhibitors of polyamine biosynthesis [17].

Studies of polyamine metabolism in several parasites have shown that *Trypanosoma brucei* and *Leishmania mexicana* contain rather stable ornithine decarboxylases [18,19]. This fact seems to explain the selective inhibitory effect of α-difluoromethylornithine (DFMO) on parasite proliferation [20], while the rapid turnover of the mammalian host ODC provides continuous synthesis of new active enzyme [20] and therefore a way to overcome the irreversible binding of DFMO. Recent reports have indicated that ODC activities from *T. brucei* and *L. mexicana* are not susceptible to polyamine regulation [18,19] in contrast to the well-known sensitivity of the mammalian cell enzyme to the same mechanism. On the other hand, *Plasmodium falciparum* ODC seems to be stable and can be regulated by polyamine endogenous levels [21].

The present work is the first report of a metabolically unstable ODC, the levels of which are not reduced by polyamine regulation. This enzyme has been obtained from the non-pathogenic trypanosomatid, *Crithidia fasciculata*, and is potentially useful for the investigation of the relationship between protein structure and regulation of ODC activity.

Abbreviations: ODC, ornithine decarboxylase; DFMO, α-di-fluoromethylornithine.

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2. MATERIALS AND METHODS

2.1. *Materials*

Brain heart infusion, tryptose and yeast extract were from Difco Laboratories, Detroit, MI. Minimal essential medium (SMEM) and amino acids were purchased from Gibco; vitamins, bases, haemin, polyamines, cycloheximide, pyridoxal 5'-phosphate, HEPES buffer and antibiotics were obtained from Sigma. L-[1-^{14}C]Ornithine (55.9 Ci/mol), L-[U-^{14}C]ornithine (266 Ci/mol) and L-[^{35}S]methionine (1,129 Ci/mol) were from New England Nuclear, Boston, MA. DFMO was a generous gift of Marion Merrel Dow Inc.

2.2. *Parasite cultures*

Crithidia fasciculata (ATCC 11745) was grown with shaking at 28°C in a rich liquid medium [22] supplemented with 0.2% of yeast extract, or in a completely defined medium (HOSMEM II) described

by Berens and Marr [23]. Parasite growth was followed by cell counting.

2.3. *Cell extract preparations and enzymatic assays*

Parasites harvested in the indicated growth phases were sedimented for 10 min at 3,000 × *g*, washed with 0.05 M HEPES buffer, pH 7.3, and resuspended at a concentration of 1 × 10^9 cells/ml in the same buffer containing 1 mM DTT, 0.1 mM EDTA and 1 mM pyridoxal 5'-phosphate. Nonidet P-40 was added (0.05% final concentration) and after 30 min samples were briefly sonicated. The parasite extracts were centrifuged for 20 min at 12,000 × *g* and supernatant fluids were used for enzymatic assays. All operations after cell harvesting were carried out at 0–4°C.

ODC activity was measured by the release of radioactive CO_2 from labeled ornithine, and the characterization of the reaction products as well as the calculation of the reaction stoichiometry were performed as previously described [19]. The standard reaction mixture contained 50 mM HEPES buffer, pH 7.3, 1 mM DTT, 0.1 mM EDTA, 0.5 mM pyridoxal 5'-phosphate and L-[1-^{14}C]ornithine (0.6 mM, 0.1–0.2 μCi), in a total volume of 0.05 ml. Radioactive CO_2 released during the reaction performed at 37°C was trapped on a piece of Whatman 3 MM filter paper soaked with 2 N KOH and then measured in a scintillation counter.

2.4. *In vivo studies of enzyme turnover and regulation*

In order to determine the in vivo turnover rate of ODC, and its susceptibility to polyamine regulation, *Crithidia* cultures were incubated with cycloheximide (50 μg/ml) or 10 mM putrescine, respectively. Aliquots were collected at the times indicated in each experiment and the enzymatic activities were measured.

Protein concentration was determined according to Lowry [24] after precipitation with deoxycholate and TCA [25].

3. RESULTS AND DISCUSSION

Decarboxylation of ornithine has been recently detected in *Crithidia fasciculata* extracts [26]. In order to study in detail the properties of this enzymatic activity we have used uniformly labeled ornithine as substrate. Under these conditions radioactive putrescine and CO_2 were characterized as the products formed, and the reaction stoichiometry has indicated that the decrease in the amount of ornithine was equivalent to putrescine formation (results not shown). In addition, while DFMO was able to inhibit the reaction almost completely (about 90%), aminooxyacetate only provoked a small effect. The latter compound at low concentrations is an inhibitor of ornithine 2-oxo acid amino-transferase and the subsequent oxidative decarboxylations [27]. All these results allowed us to discard other CO_2-releasing activities and conclude that the enzyme detected in *Crithidia* was a true ODC.

Table I

Catalytic properties of *Crithidia fasciculata* ODC

pH optimum	7.3
K_m ornithine	0.25 mM
K_m pyridoxal 5'-phosphate	0.09 mM
K_i DFMO	0.20 mM
K_m putrescine	>5 mM

The complete system and conditions of the reaction were as described in Materials and Methods. All values are the average of at least two independent determinations.

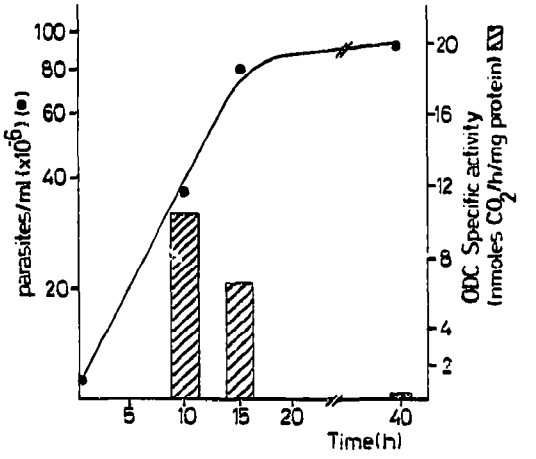

Fig. 1. ODC activities at different stages of *C. fasciculata* growth. 100 ml aliquots from a parasite culture in rich medium were harvested at the indicated times. Cell extract preparations and enzymatic assays were performed as indicated in Materials and Methods. ODC specific activities shown by bars are the average of two experiments.

Fig. 1 shows that the specific activity of the enzyme was maximal in the early stage of the parasite exponential growth, decreased in the late logarithmic phase and almost disappeared in the stationary phase.

Optimal enzymatic activity requires rather high concentrations of pyridoxal 5'-phosphate. When the cofactor was omitted both in the preparation of parasite extracts and in the reaction mixture, ODC activity was three-fold lower than at saturating levels of pyridoxal 5'-phosphate. On the other hand, in the absence of EDTA the enzymatic activity decreased by 35%.

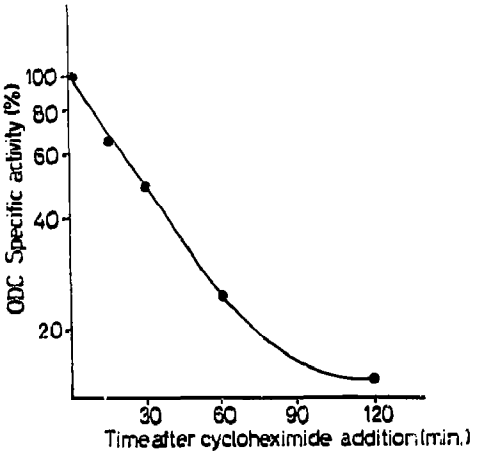

Fig. 2. In vivo stability of *C. fasciculata* ODC. Parasites were cultivated in HOSMEM II medium up to the log-phase and cycloheximide (50 μg/ml) was added. After different periods in the presence of the inhibitor cells were collected, lysed and ODC was assayed. Specific activities of ODC were expressed as percentages of the initial value obtained at the time of cycloheximide addition (6.7 nmol CO_2/h/mg of protein).

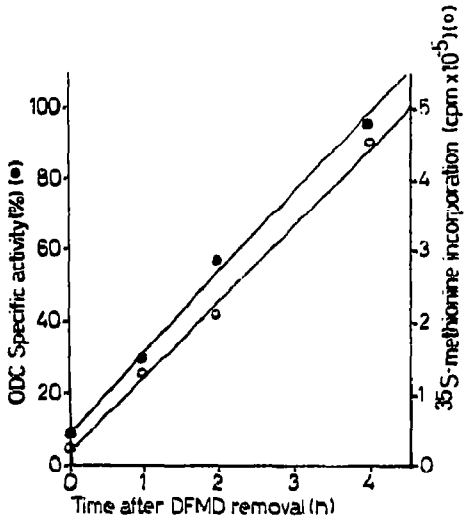

Fig. 3. Recovery of ODC activity after removal of DFMO from *C. fasciculata* cultures. Parasites were grown for 24 h in the HOSMEM II defined medium in the absence or presence of 10 mM DFMO. The culture containing the inhibitor was centrifuged, parasites were washed twice and resuspended in fresh medium without DFMO. After subsequent incubation, samples were collected at the indicated times and ODC was assayed. Enzymatic specific activities were expressed as percentages of the value corresponding to cells cultivated without DFMO (4.9 nmol CO_2/h/mg protein) (●). An aliquot of the culture after removal of DFMO was incubated with [^{35}S]methionine (5 μCi/ml, 0.2 μM) and the incorporation of radioactivity into TCA-insoluble material was measured at different times (○). All values are the average of duplicate experiments.

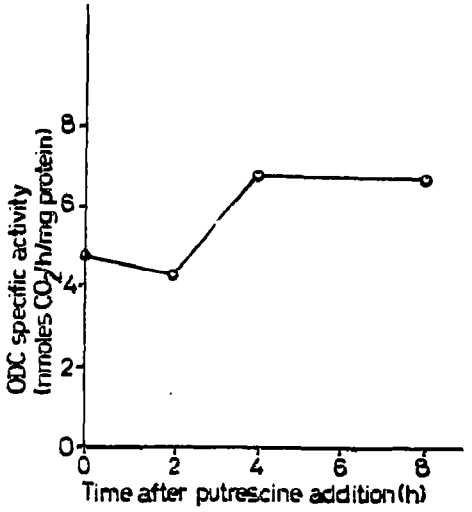

Fig. 4. Effect of putrescine addition to *C. fasciculata* cultures on ODC specific activity. Parasites were grown in the defined medium HOSMEM II with or without the addition of 10 mM putrescine. At the indicated times aliquots were collected and washed. Cell extracts were prepared and ODC was measured as indicated in Materials and Methods. Enzymatic specific activities corresponding to parasites cultivated in the absence of putrescine were 4.7 and 3.8 nmol CO_2/h/mg protein at 0 and 8 h, respectively.

Table I summarizes some properties of *Crithidia fasciculata* ODC. While the apparent K_m for ornithine is of the same order as the values reported for enzymes of other sources, the K_m for pyridoxal 5'-phosphate is markedly higher. DFMO is an irreversible inhibitor of *C. fasciculata* ODC, as it occurs with the enzyme from mammalian cells and several parasites [19,21,28,29]. Putrescine and spermidine are poor inhibitors of the reaction; their corresponding K_is are higher than 5 mM.

In order to study the in vivo turnover of *C. fasciculata* ODC, the specific activity of the enzyme was measured in cell extracts obtained from parasites cultivated for different times with cycloheximide (50 μg/ml). This treatment inhibited *Crithidia* protein synthesis almost completely (results not shown). Under these conditions ODC activity showed a rapid decay (Fig. 2). The enzyme half-life calculated from these data was about 30 min.

We have also investigated the recovery of enzyme activity upon removal of DFMO from cultures incubated for 24 h with the inhibitor. This drug provoked an almost complete inhibition of ODC (93%), and when parasites were washed and resuspended in the same volume of fresh culture medium, a rapid increase of ODC activity was detected (Fig. 3). After about 4 h in the absence of DFMO the enzyme reached normal levels; the rate of ODC activity revocery was similar to that of total protein synthesis. The increase in ODC activity should represent a balance between enzyme synthesis and degradation. Since the half-life of *Crithidia* ODC seems to be shorter than the average of the parasite proteins, the described results might indicate that the rate of synthesis of ODC molecules is also higher than the values corresponding to the marjority of the other parasite proteins.

Fig. 4 shows that the addition of 10 mM putrescine to *Crithidia* cultures for different periods of time caused a moderate increase of ODC levels. Similar results were obtained with spermidine. These data indicate that the *Crithidia* enzyme cannot be regulated by the polyamine-dependent feedback mechanism observed in other cells.

ODC has been intensively studied in many eukaryotic organisms. The enzymes from different sources share a common catalytic mechanism of putrescine formation using pyridoxal 5'-phosphate as cofactor, and they are irreversibly inactivated by DFMO. However, these enzymes differ widely in some other properties, such as their in vivo stability and their sensitivity to polyamine regulation.

Mammalian ODC has a very short half-life. The rapid enzyme degradation depends on the structure of this protein which contains two PEST regions (sequences rich in proline, glutamic acid, serine and threonine) [30], one of them near the carboxy-terminal end [18]. On the other hand, the feedback regulation of ODC activity by polyamines results from the reduction of enzyme synthesis and the increase in its degradation

rate. This regulation seems to depend on the amino acid sequence at the amino-terminus of the protein [18]. Recent work on *Trypanosoma brucei* ODC has indicated that this enzyme has a rather slow in vivo turnover and is not susceptible to regulation by polyamines, in contrast to the corresponding characteristics of the mouse ODC. However, the amino acid sequences of both enzymes show almost 70% of homology [18,31]. The distinctive properties of the *T. brucei* enzyme are related to different amino acid sequences at both carboxy- and amino-terminal ends of the molecule [31]. Earlier studies carried out in our laboratory have shown that *Leishmania mexicana* ODC is also metabolically stable and insensitive to polyamine-dependent regulation [19]. It is tempting to speculate that digenetic parasites such as *T. brucei*, *L. mexicana* and *P. falciparum* require a stable ODC for their biochemical adaptation to a complex life cycle which involves insects and mammals as alternating hosts. Conversely, a monogenetic trypanosomatid such as *Crithidia*, which is an insect parasite, might not need a special adaptation of polyamine metabolism and therefore retains an in vivo unstable ODC.

Acknowledgements: We are indebted to Dr. Sara H. Goldemberg for helpful discussion. This work was partially supported by grants from the Swedish Agency for Research Cooperation with Developing Countries (SAREC) and the Consejo Nacional de Investigaciones Cientificas y Técnicas (Argentina). N.S.G. and I.D.A. are career investigators of the latter institution. C.C. is a fellow of the Instituto de Investigaciones Bioquímicas 'Fundación Campomar'.

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