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Trypanosoma cruzi epimastigotes lack ornithine decarboxylase but can express a foreign gene encoding this enzyme

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Abstract ***Trypanosoma cruzi*, a pathogenic protozoan causing Chagas disease, lacks ornithine decarboxylase (ODC), the enzyme catalyzing the first step of polyamine biosynthetic pathway in eukaryotic cells. Our results indicate that the auxotrophy for diamines of *T. cruzi* epimastigotes is due to the absence of an active ODC gene in these parasites and not to the inability for the expression of this gene. The introduction of an exogenous complete coding region from *Crithidia fasciculata* ODC gene inserted in an expression vector specific for trypanosomatids induces the normal expression of the foreign genetic information allowing the transformed *T. cruzi* to overcome the exogenous polyamine requirement for growth. The enzyme expressed in the transformed parasites has shown a considerably extended metabolic stability. The loss of ODC activity in *T. cruzi* might be related to the parasite adaptation to the intracellular stages of its life cycle.**

Key words: Gene expression; Ornithine decarboxylase; Enzyme turnover; *Trypanosoma cruzi* transformation; *Crithidia fasciculata*

1. Introduction

The first step of polyamine biosynthesis in eukaryotic organisms (except plants) occurs exclusively by the conversion of ornithine into putrescine, and this reaction is catalyzed by ornithine decarboxylase (ODC). This enzyme plays a key role in the polyamine pathway, and since these polycationic substances are essential for macromolecular synthesis [1,2], it is not surprising that ODC activity can regulate cell growth and differentiation [3–5].

Several studies have been carried out on various aspects of polyamine metabolism and transport in pathogenic protozoa with the aim of investigating whether specific inhibitors of these processes can be used to alter the proliferation, infectivity or differentiation of these organisms in a way appropriate for the design of new strategies against parasitic diseases [6].

Results from our and other laboratories have shown that ODC activity could not be detected in *Trypanosoma cruzi* epimastigotes [7–10]. This situation is unique among all other trypanosomatids studied so far where ODC is present and plays essential roles in metabolic regulation and proliferation [11–13]. In this regard it has recently been shown that several wild-type species of *Leishmania* sp. promastigotes growing in synthetic media stopped their proliferation after a treatment with α-difluoromethylornithine (DFMO), a specific and irreversible inhibitor of ODC, which causes the depletion of the intracellular polyamine pool [11,14]. It has also been reported that the construction of *Trypanosoma brucei* and *Leishmania donovani* cell lines deficient in the ODC gene by disruption of its two alleles gave rise to parasites which were unable to grow in putrescine-free media unless either the ODC gene was introduced back by transfection or the culture medium was supplemented with polyamines [13,15].

The absence of ODC activity in *T. cruzi* epimastigotes explains the inability of DFMO to inhibit this parasite growth [9,10].

The lack of de novo putrescine synthesis in *T. cruzi* seems to be compensated by the presence of a constitutive and active transport system specific for putrescine which cannot be stimulated by DFMO and allows the uptake of putrescine from the external medium [8,16,17]. In contrast, other trypanosomatid protozoa such as *Crithidia fasciculata* and *Leishmania mexicana* have shown an inducible putrescine transport system. In the latter cases the uptake of putrescine is markedly enhanced after the depletion of intracellular polyamines [8,16,18].

In the present work we have investigated the underlying causes of ODC activity absence in wild-type *T. cruzi* epimastigotes. In addition we were able to obtain transformed parasites showing a high level of expression of exogenous ODC genes.

2. Materials and methods

2.1. Materials

Brain heart infusion, tryptose and yeast extract were obtained from Difco Laboratories (Detroit, MI, USA). Minimal essential medium (SMEM) and amino acids were from GIBCO BRL (Gaithersburg, MD, USA); vitamins, bases, haemin, polyamines, pyridoxal 5′-phosphate, HEPES buffer, antibiotics and cycloheximide were purchased from Sigma (St. Louis, MO, USA). L-[1-^{14}C]ornithine (58 Ci/mol and L-[^{35}S]methionine (1.129 Ci/mol) were from DUPONT/NEN (Boston, MA, USA) and L-[U-^{14}C]ornithine (248 Ci/mol) from Amersham Life Science (England). DFMO was a gift from the Merrell Dow Research Institute (Cincinnati, OH, USA).

2.2. Parasite cultures

T. cruzi strains, Tulahuen 2 [19], CL Brenner clone [20] and RA [21] were cultivated with shaking at 28°C in a rich medium called BHT [22]. In some experiments a semi-defined medium SDM-79 [23] with very low content of polyamines has been used. Haemin (20 mg/l), 10% heat-inactivated fetal calf serum and antibiotics (100 μg/ml streptomycin and 100 U/ml penicillin) were added to all cultures. Parasite growth was usually initiated at 4–8×10^6 cells/ml and followed by cell counting.

2.3. In vivo labeling with radioactive amino acids

Parasites were collected by centrifugation (3000×*g*, 10 min) at the

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Abbreviations: ODC, ornithine decarboxylase; DFMO, α-difluoromethylornithine

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exponential phase of growth, washed once with PBS and resuspended in the same buffer at a concentration of 10^9 cells per ml. L-[U-^{14}C]ornithine (6 μCi/ml) was added and after incubation for 2 h at 37°C parasites were sedimented, washed with PBS and resuspended in 50–100 μl of 0.2 M perchloric acid. Cell extracts were neutralized with KOH and precipitates removed by centrifugation. The supernatant fluids were analyzed by paper electrophoresis as previously described [24] and the radioactive compounds were detected with a radiochromatogram scanner.

In order to follow protein biosynthesis, parasites were labeled with ^{35}S-methionine and the incorporation into 10% trichloroacetic acid-insoluble material was measured [25].

2.4. In vivo studies of ODC metabolic stability

To determine the in vivo turnover rate of ODC, cycloheximide (50 μg/ml) was added to the corresponding cultures to stop protein synthesis and the remaining enzymatic activity was measured on aliquots collected after different times [26].

2.5. Preparation of cell extracts and enzymatic assays

Cell extracts were obtained essentially as described previously [27] and the enzymatic activity was measured by the release of $^{14}CO_2$ from L-[1-^{14}C]ornithine [25]. The identity of the putrescine formed during the reaction was confirmed using L-[U-^{14}C]ornithine followed by electrophoretic analysis of the reaction products. Protein concentration was measured according to Bradford [28] using BSA as standard.

2.6. Kinetic parameters of ODC

The determination of apparent K_m values for ornithine of the enzyme expressed in *T. cruzi* as well as the studies of time-dependent inactivation by DFMO and the subsequent calculation of the corresponding K_i values were carried out as previously described [25,27].

2.7. Polyamine analysis

Parasites or samples from culture media were resuspended in 0.2 M perchloric acid. After centrifugation, NaOH was added to the supernatant liquids and different aliquots treated with benzoyl chloride. The benzoyl derivatives were separated by HPLC on a Beckman system equipped with a C_{18} reverse-phase column and analyzed spectrophotometrically [29].

2.8. Construction of recombinant plasmids bearing the ODC coding region from C. fasciculata and transfection of T. cruzi

A DNA fragment containing the whole coding region of the ODC gene from *C. fasciculata* [30] was amplified by polymerase chain reaction (PCR) using as template a 4.5 kb *Sac*I DNA fragment obtained from *Crithidia* genomic DNA which had been subcloned into pBluescript KS$^+$ and characterized previously [30]. The 4.5 kb fragment contains the open reading frame of *C. fasciculata* ODC and long segments of the 5′ and 3′ untranslated regions. For the amplification we have used the sense primer 5′-CGCATGGATCCATGAGTAACCATGACGTCT-3′ and the antisense primer 5′-CGCATGGATCCTTACAACGCCACGGAAGAA-3′ which correspond to the 5′ and 3′ terminal sequences of *Crithidia* ODC coding region, respectively, both extended by an oligonucleotide (11 b long) containing a *Bam*HI site. The reaction was carried out in a total volume of 100 μl which contains 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.25 mM of each dNTP, 25 pmoles of each primer and 2.5 units of Taq DNA polymerase. PCR cycles were: 94°C, 30 s; 55°C, 60 s; 72°C, 4 min for 30 cycles followed by an extension step of 7 min at 72°C. The product of the reaction (a DNA fragment 2188 bp long) was digested with *Bam*HI, purified from agarose gel and inserted in the *Bam*HI site of the expression vector pRIBOTEX [31], kindly provided by Dr. R. Hernández (Universidad Nacional Autónoma de México), which contains the transcription start point of *T. cruzi* rRNA genes and the neomycin phosphotransferase gene able to induce resistance to Geneticin (G 418). We have obtained two recombinant plasmids designated pODC-7 and pODC-8, bearing the ODC coding region inserted in the sense and antisense orientations, respectively (Fig. 1). The control of both constructions was performed by restriction mapping and nucleotide sequencing. Electroporation of various wild-type strains of *T. cruzi* epimastigotes with the recombinant plasmids described above was carried out by using a slight modification of procedures already described [32,33]. After transfection, parasites were diluted with rich medium and incubated during 48 h at 28°C to allow their recovery before the addition of Geneticin (G 418) at a final concentration of 500 μg/ml as the antibiotic for selection. Subsequent growth was followed by cell counting. Samples of *T. cruzi* cultures before and after transfection were taken at different times to look for DNA and RNA sequences with homology to ODC gene as well as for ODC activity determinations.

2.9. Southern and Northern blot analyses

DNA from wild-type and transfected *T. cruzi* was prepared according to Medina-Acosta and Cross [34]. After digestion with restriction enzymes, the resulting DNA fragments were separated by electrophoresis on agarose gels, transferred to nylon membranes (Hybond N$^+$ from Amersham), and hybridized to a ^{32}P-labeled probe (810 bp) specific for ODC gene. This probe was obtained by PCR of *C. fasciculata* DNA using a pair of primers complementary to both strands of a segment of the ODC coding region and ^{32}P-dCTP as the radioactive nucleotide.

Total RNA from parasites before and after transfection was obtained using an 'Ultraspec' RNA isolation system [35]. For identification of ODC transcripts, samples containing 20 μg of total RNA were separated by electrophoresis on a 1% agarose gel containing 2.2 M formaldehyde and transferred to nylon membranes. Northern hybridization was carried out with the same radioactive probe used for DNA analysis.

3. Results and discussion

3.1. Lack of ODC activity in wild-type T. cruzi epimastigotes and polyamine requirement for the parasite proliferation

Previous studies have shown that *T. cruzi* epimastigotes do not contain a significant activity of ODC [7,10]. We have confirmed these results working with different strains of *T. cruzi* (Tulahuen 2, RA and CL) where the specific activity of the enzyme found in parasite extracts was lower than 0.1 nmole CO_2/h/mg of protein. Moreover, experiments performed with intact parasites failed to detect the in vivo conversion of ornithine into putrescine as shown in Fig. 2. Instead, the only radioactive compound formed during the reaction was presumably 4-aminobutanamide derived from ornithine by oxidative decarboxylation ([36], unpublished results).

In agreement with the above mentioned results *T. cruzi* epimastigotes grew normally in a rich medium (Fig. 3A) but their proliferation was brought to a halt after a few passages in a semi-defined medium (SDM-79) (Fig. 3B) which contains

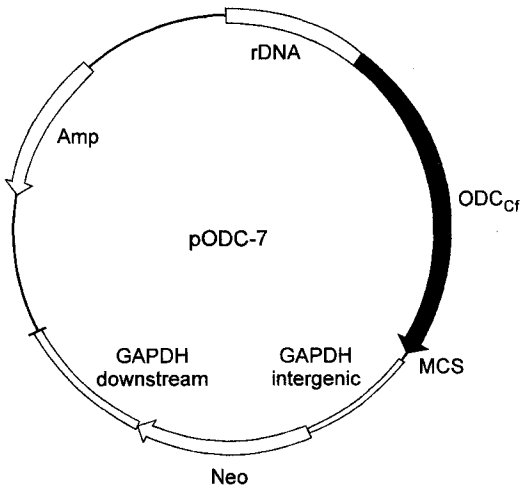

Fig. 1. The transformation plasmid pODC-7 containing the *C. fasciculata* ODC coding region in the sense orientation.

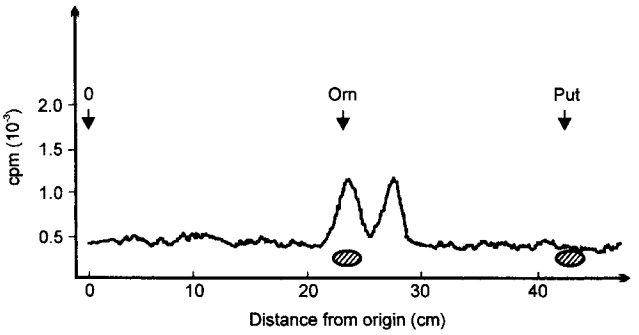

Fig. 2. Analysis of wild-type *T. cruzi* extracts obtained from parasites incubated in vivo with radioactive ornithine. Washed protozoa were resuspended in PBS and incubated with L-[U-^{14}C]ornithine as indicated in Section 2. After treatment with perchloric acid and neutralization, the cell extracts were analyzed by paper electrophoresis. The corresponding scans are shown. Shaded spots indicate the position of standard ornithine (Orn) and putrescine (Put).

only traces of polyamines (undetectable putrescine and cadaverine, and 0.27 μM spermidine). The non-proliferating parasites were able to resume growth upon addition of exogenous polyamines to the semi-defined medium, indicating that these substances are essential requirements for the normal growth of *T. cruzi* epimastigotes. When the protozoa are cultivated in rich medium which contains rather high levels of polyamines, these compounds can be taken up from the external medium. The fact that exogenous polyamines have to be added for the normal growth of wild-type *T. cruzi* epimastigotes in a semi-defined medium confirms the absence of ODC activity in these parasites and at the same time argues against the possibility of an active arginine decarboxylase in *T. cruzi*, as it has been previously claimed by several authors [37,38].

3.2. Search of the ODC gene and its mRNA in T. cruzi epimastigotes before and after transfection

The lack of ODC activity in *T. cruzi* might be due to the absence of the corresponding active gene, to the inability of its expression inside the parasite or to the direct inhibition of the enzymatic activity. In order to investigate these possibilities we have looked for DNA sequences and RNA transcripts from *T. cruzi* with some degree of homology to other trypanosomatid ODC genes which have been already cloned and sequenced [30,39]. For these experiments we have used DNA and total RNA preparations obtained from several strains of *T. cruzi* epimastigotes before and after transfection with recombinant plasmids bearing the *C. fasciculata* ODC coding region. These plasmids have been prepared by insertion of the *C. fasciculata* ODC complete ORF in the multicloning site (MCS) of the expression vector pRIBOTEX [31] as described in Section 2.

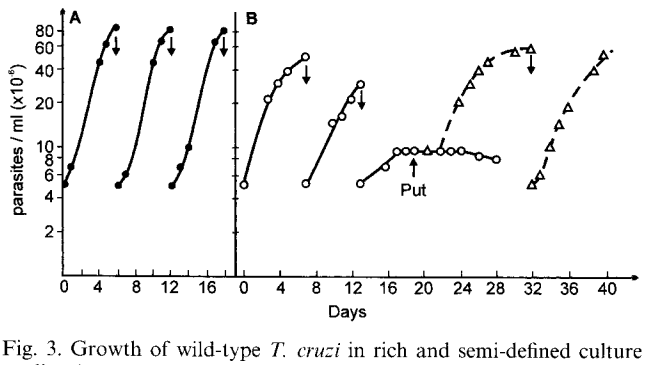

Fig. 3. Growth of wild-type *T. cruzi* in rich and semi-defined culture media. A: *T. cruzi* epimastigotes were continuously grown by serial passages in BHT medium (●). B: Aliquots taken from cultures shown in A were diluted about 10-fold with semi-defined medium and cultures were followed for several passages in the latter medium (○) until growth was arrested. At this point (upward arrow) 0.1 mM putrescine was added to a portion of the culture and incubation was resumed for several weeks (△). At the times indicated by downward arrows cultures were diluted with fresh medium in the absence or presence of putrescine.

Fig. 4 shows the results of Southern and Northern analyses using a radioactive ODC specific probe which hybridizes with genomic DNA from both *C. fasciculata* and *L. mexicana*. Both transfected trypanosoma DNA preparations gave hybridization bands while the DNA from wild-type *T. cruzi* Tulahuen 2, RA or CL strain did not show any signal unless the membrane was exposed for a very long time to X-ray films (Fig. 4A). These results strongly suggest that wild-type *T. cruzi* does not contain DNA sequences with a significant homology to ODC genes from other trypanosomatids. Furthermore, the untransfected *T. cruzi* could not survive after several weeks of culture in the presence of Geneticin (G 418) whereas the parasites electroporated with the plasmids pODC-7 and pODC-8 were resistant to the antibiotic, indicat-

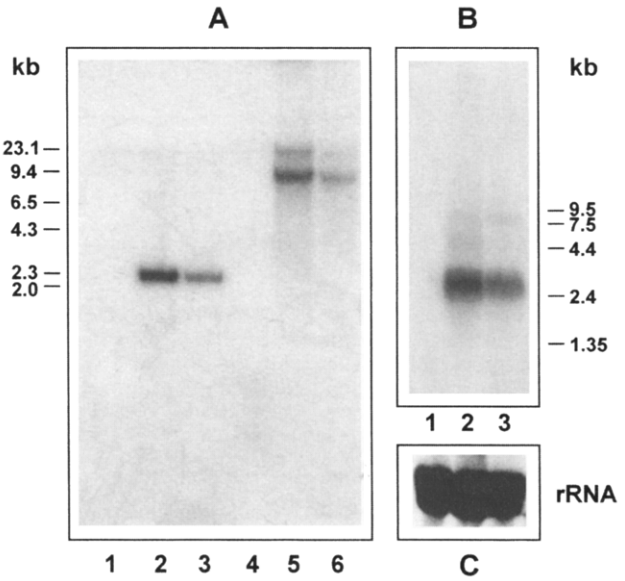

Fig. 4. Southern and Northern blot analyses of DNA and RNA from wild-type and transformed *T. cruzi*. A: 15 μg of DNA from wild-type or transformed *T. cruzi* Tulahuen 2 grown in BHT medium were digested with *Bam*HI (lanes 1, 2 and 3) or *Eco*RI (lanes 4, 5 and 6) and then subjected to electrophoresis on a 1.2% agarose gel. After transfer to a nylon membrane the hybridization was carried out with a radioactive probe specific for *C. fasciculata* ODC. Lanes 1 and 4 correspond to DNA from wild-type *T. cruzi*; lanes 2 and 5 to parasites transformed with plasmid pODC-7; and lanes 3 and 6 to trypanosomes transformed with plasmid pODC-8. B: Total RNA (20 μg) obtained from wild-type *T. cruzi* Tulahuen 2 (lane 1), pODC-7 transformed parasites (lane 2) or pODC-8 transformed trypanosomes (lane 3) cultivated in BHT medium were subjected to electrophoresis on a 1% agarose/2.2 M formaldehyde gel, transferred to a nylon membrane and hybridized with the radioactive ODC specific probe used in Southern analysis. C: The ethidium bromide-stained rRNA bands are shown as the loading control.

Table 1
ODC activities in wild-type and transformed *T. cruzi*

Vector	ODC specific activity
None (wild-type Tulahuen 2 strain)	< 0.1
pODC-7 (48 h after electroporation)	568
pODC-7 (60 days after electroporation)	1725
pODC-8 (60 days after electroporation)	< 0.1

Cell extract preparations and enzyme assays were performed as indicated in Section 2. All values are the averages of at least two independent determinations.

ing that both transfections had been successful. The hybridization bands corresponding to parasites transformed with the plasmid pODC-7 showed higher intensity than those obtained from trypanosomes transfected with the plasmid containing the ODC coding region in the counter-clockwise orientation, suggesting a lower level of plasmid replication in the latter case.

The Northern blot analysis depicted in Fig. 4B clearly shows that the protozoa transfected with the recombinant plasmids bearing the ODC coding region in both orientations were able to express the ODC gene giving rise to specific transcripts. Samples from wild-type trypanosomes failed to produce any hybridization signal.

3.3. ODC activity in transformed T. cruzi

In accordance with the results of Southern and Northern hybridization analyses we have found that cells electroporated with pODC-7 were able to express a high level of ODC activity when measured under conditions of either transient or stable transformation. These results seem to indicate that the intracellular medium of *T. cruzi* does not contain inhibitors of ODC activity. In fact we have found that cell extracts from wild-type *T. cruzi* did not decrease the in vitro activity of ODC. The lysates corresponding to untransfected parasites or *T. cruzi* transformed with pODC-8 (containing the ODC coding region in the counter-clockwise orientation with respect to the transcription start point) showed only traces of the enzyme activity (Table 1).

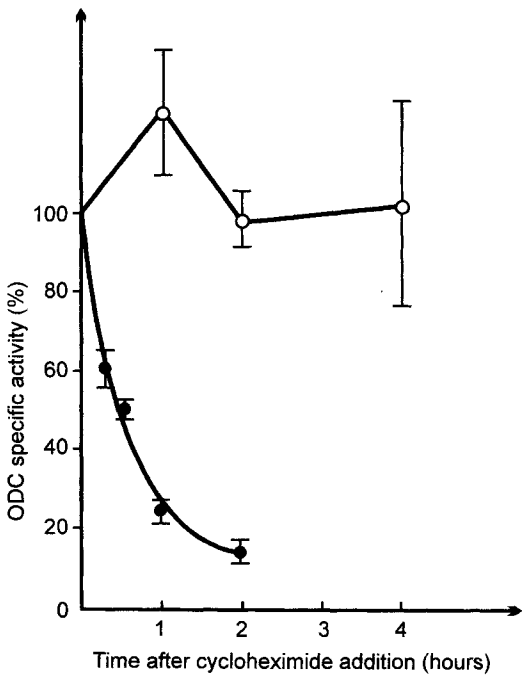

Fig. 5. In vivo stability of ODC activity expressed in *C. fasciculata* or in transformed *T. cruzi*. Cycloheximide (50 μg/ml) was added to log-phase parasite cultures and different samples were collected at the indicated times for ODC assays. Specific activities of ODC in *C. fasciculata* (●) and transformed *T. cruzi* (○) were expressed as percentages of the corresponding initial values obtained at the time of cycloheximide addition.

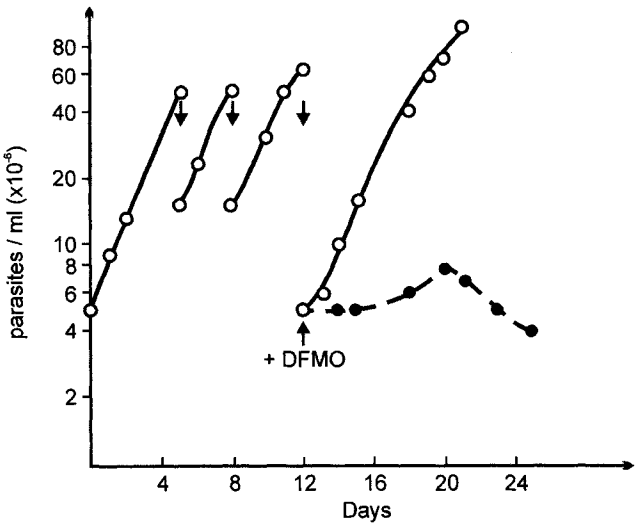

Fig. 6. Growth of transformed *T. cruzi* in semi-defined medium. After transformation with the exogenous ODC gene *T. cruzi* epimastigotes were able to grow continuously in SDM-79 medium (○). At the time indicated by the upward arrow 5 mM DFMO was added to a fraction of the culture and subsequent growth was followed by cell counting (●).

Preliminary studies to assess the stability of ODC expression in pODC-7 transfected parasites have indicated variable but very significant levels of the enzyme activity even 60 days after transfection and incubation with G 418 (Table 1). The elucidation of more details about the stable transformation will require the isolation of individual clones from our heterogeneous *T. cruzi* populations. The presence of ODC activity in intact parasites grown in semi-defined medium after transfection with the plasmid pODC-7 has been confirmed by the detection of the in vivo partial conversion of radioactive ornithine into putrescine in addition to the oxidative decarboxylation product 4-aminobutanamide which is also present in wild-type trypanosomes (Fig. 2 and unpublished results).

3.4. Properties of ODC in transformed T. cruzi

We have determined the kinetic parameters of the enzyme expressed in transformed trypanosomes. The K_m for ornithine (0.26 ± 0.02 mM) as well as the apparent K_i for DFMO (0.33 ± 0.04 mM) gave values very similar to those obtained for the ODC extracted from *C. fasciculata* [26]. However, both activities showed very different metabolic stabilities. While the enzyme has a half-life of only 30 min in *C. fasciculata* [26], when expressed in *T. cruzi* it showed a remarkably extended half-life longer than 4 h (Fig. 5). The unexpected change in ODC metabolic stability of the crithidia enzyme when expressed inside *T. cruzi* might be due to the different properties of the proteolytic machineries present in both parasitic protozoa. A similar variation of ODC turnover has been reported for the mouse ODC expressed in *T. brucei* [40].

3.5. Growth of transformed T. cruzi in semi-defined medium

Since *T. cruzi* transfected with the plasmid pODC-7 showed a high level of enzymatic activity, we could expect that these parasites should have developed the ability to grow normally in the medium SDM-79 (containing only traces of polyamines) where the wild-type *T. cruzi* epimastigotes as well as the parasites transfected with the plasmid pODC-8 failed to proliferate. This was in fact the case as indicated in Fig. 6. The parasites expressing the heterologous ODC gene could grow continuously in semi-defined medium; furthermore this proliferation could be arrested and subsequently trypanosomes died when cultures were treated with DFMO, the specific and irreversible inhibitor of ODC.

It is interesting to mention that both wild-type and pODC-8 transfected *T. cruzi* grew equally well in BHT medium.

The auxotrophy for diamines of wild-type *T. cruzi* epimastigotes is now well established [7,10]. Our results strongly support the idea that this deficiency is due to the absence of an active ODC gene in these parasites, and not to the incapacity for the expression of this gene, since the introduction of an exogenous complete coding region from crithidia ODC gene in a context appropriate for transcription induces the normal expression of this new genetic information. Concomitantly, the transformed *T. cruzi* epimastigotes are able to overcome the diamine requirement observed for the proliferation of wild-type trypanosomes. We can speculate that the loss of ODC activity in *T. cruzi* could be related to the adaptation of the parasite to the intracellular stages of its life cycle.

The absence of ODC in *T. cruzi* epimastigotes as well as the remarkable different properties between the proteolytic systems of this parasite and *C. fasciculata* might give important clues to understand the evolution of mono- and digenetic trypanosomatids.

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References

[1] Geiger, L.E. and Morris, D.R. (1980) J. Bacteriol. 141, 1192–1198.
[2] Algranati, I.D. and Goldemberg, S.H. (1997) Trends Biochem. Sci. 2, 272–274.
[3] Pegg, A.E. (1988) Cancer Res. 48, 759–774.
[4] Marton, L.J. and Pegg, A.E. (1995) Annu. Rev. Pharmacol. Toxicol. 35, 55–91.
[5] Heby, O. and Emanuelsson, H. (1981) Med. Biol. 59, 417–422.
[6] Bacchi, C.J. and McCann, P.P. (1987) in: P.P. Mc Cann, A.E. Pegg and A. Sjoerdsma (Eds.), Inhibition of Polyamine Metabolism: Biological Significance and Bases for New Therapies, Academic Press, Inc., Orlando, pp. 317–344.
[7] Algranati, I.D., Sánchez, C.P. and González, N.S. (1990) in: S.H. Goldemberg and I.D. Algranati (Eds.), The Biology and Chemistry of Polyamines, IRL Press, Oxford, pp. 137–146.
[8] González, N.S., Ceriani, C. and Algranati, I.D. (1992) Biochem. Biophys. Res. Commun. 188, 120–128.
[9] Hunter, K.J., Le Quesne, S.A. and Fairlamb, A.H. (1994) Eur. J. Biochem. 226, 1019–1027.
[10] Ariyanayagam, M.R. and Fairlamb, A.H. (1997) Mol. Biochem. Parasitol. 84, 111–121.
[11] González, N.S., Sánchez, C.P., Sferco, L. and Algranati, I.D. (1991) Biochem. Biophys. Res. Commun. 180, 797–804.
[12] Coons, T., Hanson, S., Bitonti, A.J., Mc Cann, P.P. and Ullman, B. (1990) Mol. Biochem. Parasitol. 39, 77–90.
[13] Li, F., Hua, S., Wang, C.C. and Gottesdiener, K.M. (1996) Mol. Biochem. Parasitol. 78, 227–236.
[14] Hunter, K.J., Strobos, C.A.M. and Fairlamb, A.H. (1991) Mol. Biochem. Parasitol. 46, 35–44.
[15] Jiang, Y., Roberts, S.C., Jardim, A., Carter, N.S., Shih, S., Ariyanayagam, M., Fairlamb, A.H. and Ullman, B. (1999) J. Biol. Chem. 274, 3781–3788.
[16] González, N.S., Ceriani, C. and Algranati, I.D. (1993) An. Asoc. Quim. Argent. 81, 117–125.
[17] Le Quesne, S.A. and Fairlamb, A.H. (1996) Biochem. J. 316, 481–486.
[18] González, N.S. and Algranati, I.D. (1994) Cell. Mol. Biol. 40, 907–914.
[19] Segura, E.L., Subias, E., Esteva, M., Cabeza Meckert, P., Brozina, A. and Laguens, R.P. (1980) Medicina (Buenos Aires) 40, 97–102.
[20] Cano, M.I., Gruber, A., Vázquez, M., Cortés, A., Levin, M.J., González, A., Degrave, W., Rondinelli, E., Zingales, B., Ramírez, J.L., Alonso, C., Requena, J.M. and da Silveira, J.F. (1995) Mol. Biochem. Parasitol. 71, 273–278.
[21] González Cappa, S.M., Katzin, A.M., Anasco, N. and Lajmanovich, S. (1981) Medicina (Buenos Aires) 41, 549–555.
[22] Cazzulo, J.J., Franke de Cazzulo, B.M., Engel, J.C. and Cannata, J.J.B. (1985) Mol. Biochem. Parasitol. 16, 329–343.
[23] Brun, R. and Schonenberger, M. (1979) Acta Trop. 36, 289–292.
[24] Cataldi, A.A. and Algranati, I.D. (1989) J. Bacteriol. 171, 1998–2002.
[25] Sánchez, C.P., González, N.S. and Algranati, I.D. (1989) Biochem. Biophys. Res. Commun. 161, 754–761.
[26] Ceriani, C., González, N.S. and Algranati, I.D. (1992) FEBS Lett. 301, 261–264.
[27] Sánchez, C.P., Mucci, J., González, N.S., Ochoa, A., Zakin, M.M. and Algranati, I.D. (1997) Biochem. J. 324, 847–853.
[28] Bradford, M.M. (1976) Anal. Biochem. 72, 248–254.
[29] Kotzabasis, K., Christakis-Hampsas, M.D. and Roubelakis-Angelakis, K.A. (1993) Anal. Biochem. 214, 484–489.
[30] Svensson, F., Ceriani, C., Wallström, E.L., Kockum, I., Algranati, I.D., Heby, O. and Persson, L. (1997) Proc. Natl. Acad. Sci. USA 94, 397–402.
[31] Martínez-Calvillo, S., López, I. and Hernández, R. (1997) Gene 199, 71–76.
[32] Hariharan, S., Ajioka, J. and Swindle, J. (1993) Mol. Biochem. Parasitol. 57, 15–30.
[33] Lu, H.Y. and Buck, G.A. (1991) Mol. Biochem. Parasitol. 44, 109–114.
[34] Medina-Acosta, E. (1993) Mol. Biochem. Parasitol. 59, 327–330.
[35] 'Chaosolv', Biotecx Bulletin # 21 (1992) Biotecx Laboratories, Inc.
[36] Smith, T.A. and Marshall, J.H.A. (1988) Phytochemistry 27, 703–710.
[37] Majumder, S., Wirth, J.J., Bitonti, A.J., Mc Cann, P.P. and Kierszenbaum, F. (1992) J. Parasitol. 78, 371–374.
[38] Schwarcz de Tarlovsky, M.N., Hernández, S.M., Bedoya, A.M., Lemmel, E.M. and Isola, E.L. (1993) Biochem. Mol. Biol. Int. 30, 547–558.
[39] Hanson, S., Adelman, J. and Ullman, B. (1992) J. Biol. Chem. 267, 2350–2359.
[40] Bass, K.E., Sommer, J.M., Cheng, Q.L. and Wang, C.C. (1992) J. Biol. Chem. 267, 11034–11037.