Amino Acids (2010) 38:645–651
DOI 10.1007/s00726-009-0425-6

REVIEW ARTICLE

Polyamine metabolism in *Trypanosoma cruzi*: studies on the expression and regulation of heterologous genes involved in polyamine biosynthesis

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Received: 7 July 2009 / Accepted: 6 September 2009 / Published online: 3 December 2009

Abstract Biochemical studies have shown that *Trypanosoma cruzi* and *Toxoplasma gondii* are the only eukaryotic organisms so far described which are auxotrophic for polyamines. Both parasites are unable to carry out the de novo biosynthesis of putrescine, and therefore they need the addition of exogenous polyamines to the culture medium for their normal proliferation. Further investigations at the molecular level have demonstrated that the wild-type *T. cruzi* genome does not contain ornithine or arginine decarboxylase-like nucleic acid sequences, and that the corresponding genes have been presumably lost during evolution. Since *T. cruzi* behaves as a deletion mutant for ornithine decarboxylase (ODC) and arginine decarboxylase (ADC) genes, this parasite has been selected to study the regulation of the expression of heterologous genes involved in polyamine biosynthesis in other organisms. The resulting transgenic parasites have been useful tools to analyze the different stages of gene expression after transformation, as well as the mechanisms of drug resistance induction and the post-translational processing of enzyme precursors.

Keywords Polyamine auxotrophy · *Trypanosoma cruzi* · Transgenic parasites · Gene expression · DFMO resistance · Post-translational processing

Abbreviations

ODC	Ornithine decarboxylase
ADC	Arginine decarboxylase
DFMO	α-Difluoromethylornithine
DFMA	α-Difluoromethylarginine
PCR	Polymerase chain reaction
PLP	Pyridoxal 5′-phosphate

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Introduction

Trypanosoma cruzi is a parasitic protozoan causing the Chagas' disease. This organism has a digenetic life cycle involving an insect vector and a mammalian host. The parasite undergoes major morphological, physiological and biochemical changes during the different stages of its life cycle. The epimastigote form is non-infective and proliferates extracellularly in the insect gut. After differentiation to metacyclic trypomastigotes they can infect the mammalian host through the insect contaminated faeces. The animal or human host trypanosomes replicate inside as intracellular parasites after transforming into amastigotes (Vickerman 1985; Tyler and Engman 2001; Kohl et al. 2003). The protozoan life cycle is completed when the triatomine bugs become infected by sucking the blood of an already contaminated mammalian host (Tyler and Engman 2001).

Chagas' disease affects more than fifteen million people mainly in Latin America (Heby et al. 2007; Carrillo et al. 2006) causing cardiac and intestinal damage which leads eventually to premature mortality. There is no effective vaccine against this disease and the existing drugs used in its chemotherapy are usually rather toxic or can induce the emergence of resistant parasites (Borst and Ouellette 1995). For these reasons, many studies carried out in different laboratories are investigating the metabolic pathways of *T. cruzi*. The aim of this research is the

identification of differences between the protozoan metabolism and those of its mammalian host; this knowledge might be useful to detect appropriate targets for new specific therapeutic drugs against Chagas'disease.

T. cruzi metabolism is largely controlled by post-transcriptional and post-translational mechanisms, including pre-mRNA processing, mRNA stability, modification and degradation of proteins, compartmentalization, protein folding, oligomerization and metabolic regulation (Clayton 2002; Carrillo et al. 2007; Kahana 2007).

Since parasites live inside other organisms, a significant number of the essential metabolites for their survival and proliferation can be obtained directly from the host cells through different transport systems without the need to synthesize them. Therefore, parasites have adapted to these conditions and retained only a limited capacity of metabolism in comparison with a mammalian cell (Fairlamb 1989, 2002). In some cases, a number of metabolic pathways can be inhibited in certain life cycle stages and activated in others according to the parasite requirements and the environmental conditions. In other cases, protozoan complete pathways can be absent because the genes coding for the enzymes which catalyze some or all of the biochemical reactions involved have been lost during evolution.

Metabolic analysis of *T. cruzi* has indicated that glucose as well as other sugars and amino acids can be used to obtain energy. Glycolysis takes place in glycosomes and the electron transport chain occurs in the parasite mitochondrial apparatus (Opperdoes and Michels 2001; Opperdoes and Coombs 2007). The pentose phosphate pathway is also present (Cronin et al. 1989), while a whole tricarboxylic acid cycle is not active (van Hellemond and Tielens 1997). Glycolysis produces glycero-3-phosphate which can be oxidized by an enzyme requiring Mg^{2+}. In trypanosomes the metal requirement can be fulfilled by the polyamines spermidine or spermine (Bacchi et al. 1974).

Some enzymes involved in haem biosynthesis have not been detected in *T. cruzi* which therefore requires exogenous haem for its normal growth. Trypanosomes cannot synthesize folates; hence these substances must be obtained from external sources (Ouellette et al. 2002; Klaus et al. 2005).

De novo purine synthesis does not occur in *T. cruzi* (Marr and Berens 1983) and the ability of this parasite to synthesize amino acids varies in different cases. While some enzymes involved in the formation of the branched-chain amino acids phenylalanine, tyrosine and methionine have not been detected in *T. cruzi*, other amino acids such as glycine, serine, cysteine and proline can be synthesized in this parasite. On the other side, the conversions of asparagine to aspartate and that of glutamic acid into histidine were observed only in *T. cruzi* and not in other trypanosomatids (Opperdoes and Coombs 2007).

Since argininosuccinate synthase and arginase have not been found in *T. cruzi*, the urea cycle is not functional (Yoshida and Camargo 1978; Opperdoes and Coombs 2007). In addition, this protozoan is one among the few eukaryotic organisms that are unable to synthesize putrescine from ornithine (Algranati et al. 1990; Ariyanayagam and Fairlamb 1997; Carrillo et al. 1999, 2003; Cook et al. 2007). These characteristics and other aspects of polyamine metabolism in *T. cruzi* as well as the expression in this parasite of exogenous genes related to the biosynthesis of polyamines will be discussed in detail in the present review. Although there are significant stage specific differences in the metabolic state of the protozoan, we will refer mainly to cultured epimastigote stages of the strains Tulahuen or RA grown in vitro in rich or semi-synthetic media.

Polyamine transport and intracellular pools in trypanosomatids

It is well known that polyamines play essential roles in cell proliferation and differentiation (Pegg 1988; Wallace et al. 2003; Heby and Emanuelsson 1981). Many studies on polyamine intracellular pools and their involvement in protozoan growth have shown that in addition to the ubiquitous putrescine and spermidine, the derivative N^1,N^8-bis-glutathionyl-spermidine (trypanothione) is also present in trypanosomatids (Fairlamb et al. 1985; Fairlamb and Cerami 1985, 1992). This compound is essential to maintain the redox equilibrium in the parasites and plays the metabolic and antioxidant functions of glutathione (Fairlamb and Cerami 1992).

When *T. cruzi* epimastigotes or *Leishmania mexicana* promastigotes were cultivated in axenic media almost free of polyamines and collected at different stages of growth, free polyamine analyses of the corresponding extracts have detected putrescine and spermidine. The levels of both substances decreased markedly during growth, being maximal in early logarithmic cultures and minimal in stationary phase. In *T. cruzi* at exponential growth, the ratio of spermidine to putrescine concentrations was about 4–1 (8 and 2 nmoles/10^8 parasites, respectively), while in *Leishmania* the opposite ratio was found (80 and 20 nmoles/10^8 cells for putrescine and spermidine). These results are consistent with a clearly higher conversion of putrescine into spermidine detected in *T. cruzi* as compared to *Leishmania* (Algranati et al. 1990). Although putrescine and spermidine uptake in *T. cruzi* markedly exceeds that in *Leishmania*, the opposite situation was observed for the uptake of basic amino acids which were rather low in *T. cruzi* (Algranati et al. 1990).

Polyamine transporters were recently identified and functionally characterized in *T. cruzi* (Carrillo et al. 2006). In this parasite, the permease is able to carry out the uptake of spermidine and less efficiently that of putrescine and arginine.

The treatment of *L. mexicana* or *Crithidia fasciculata* cultures with α-difluoromethylornithine (DFMO) for different times elicited a marked increase (five to tenfold) of putrescine uptake. On the contrary, the transport of this polyamine remained essentially unchanged in *T. cruzi* incubated in the absence or presence of the drug, indicating that this parasite is not sensitive to DFMO (González et al. 1992, 1993).

Polyamine metabolism in *T. cruzi* and other trypanosomatids

In order to study the biosynthetic pathways of polyamines in *T. cruzi, Trypanosoma brucei, C. fasciculata* and several species of *Leishmania*, various groups have used physiological, biochemical and molecular genetics approaches.

Physiological studies

T. cruzi epimastigotes can be grown in vitro in axenic media either rich or semi-synthetic. Working with different wild-type strains of *T. cruzi*, it has been observed that the parasites were able to proliferate continuously in a rich medium, but growth stopped after a few passages in a semi-defined medium containing only traces of polyamines (Carrillo et al. 1999). The non-proliferating parasites remained viable for several weeks in the synthetic medium and were able to resume normal growth upon addition of exogenous polyamines to the cultures. These results confirmed previous reports from various laboratories indicating that *T. cruzi* epimastigotes are unable to synthesize putrescine de novo (Carrillo et al. 1999). The possible role of ornithine or arginine as polyamine precursors in *T. cruzi* has been ruled out because none of these amino acids were able to support the parasite continuous multiplication in the defined medium, and growth in the presence of ornithine or arginine ceased at the same time as in unsupplemented cultures (Carrillo et al. 2003). In all cases, the proliferation could be resumed by addition of putrescine or spermidine. The parasite growth under different conditions showed a good correlation with the corresponding intracellular levels of polyamines. In fact, it has been demonstrated that spermidine was the essential polyamine for *T. cruzi* proliferation, since putrescine addition to a non-growing culture together with cyclohexylamine which inhibits its conversion to spermidine did not reinitiate the parasite growth even though putrescine levels increased (González et al. 2001).

Biochemical studies

All the attempts to detect ornithine decarboxylase (ODC) enzymatic activity in different strains of wild-type *T. cruzi* were unsuccessful (Ariyanayagam and Fairlamb 1997; Carrillo et al. 1999; Müller et al. 2001). The incubation of intact parasites or cell extracts with radioactive ornithine only gave negligible amounts of putrescine and the release of CO_2 in parasite extracts was less than 0.1 nmoles/h/mg of protein (Carrillo et al. 1999). In agreement with this finding, the growth of wild-type *T. cruzi* could not be reduced by high concentrations of DFMO, supporting the conclusion that ODC enzymatic activity is absent in this parasite (Carrillo et al. 2007).

It has been previously reported that the compound difluoromethylarginine (DFMA), a specific inhibitor of the enzyme arginine decarboxylase (ADC) was able to block the infection of macrophages by *T. cruzi* trypomastigotes and was able to inhibit the proliferation of intracellular amastigotes (Kierszenbaum et al. 1987; Yakubu et al. 1992). These effects could be reversed by addition of agmatine or putrescine to the culture medium (Majunder et al. 1992). These results were interpreted as indicating that *T.cruzi* trypomastigotes were able to synthesize putrescine by a two-step reaction involving ADC and the subsequent conversion of agmatine into putrescine (Cohen 1998). Other authors have found very low ADC activities measured by CO_2 release in extracts of *T.cruzi* epimastigotes incubated with radioactive arginine (Hernández and Schwarcs de Tarlovsky 1999). However, in the latter case, the enzyme presence had not been confirmed by the identification of its reaction product. Due to the controversial results about the putative presence of ADC in *T. cruzi*, this possibility was reinvestigated in various laboratories. The addition of labeled arginine to extracts of different strains of *T. cruzi* under the conditions of ADC assay previously described (Hernández and Schwarcs de Tarlovsky 1999) did not show the formation of significant amounts of agmatine or putrescine. The concomitant release of CO_2 could indicate only traces of ADC activity or a non-enzymatic decarboxylation (Smith and Marshall 1988). These results are in agreement with Fairlamb's studies on polyamine and trypanothione syntheses in *T. cruzi* from arginine or agmatine. The described experiments support the conclusion that *T. cruzi* epimastigotes are auxotrophic for polyamines as the result of its inability to synthesize putrescine (Ariyanayagam and Fairlamb 1997; Carrillo et al. 2007). Therefore, this polyamine (or spermidine) should be supplied by the external medium in order to maintain the parasite normal growth (Carrillo et al. 2006).

Analysis of polyamine metabolism in different stages of *T. cruzi* life cycle indicated that trypomastigotes as well as amastigotes were able to partially convert arginine into

citrulline. On the other hand, the enzymatic activities of ODC and ADC had not been detected in any stage of the parasite cycle (Bacchi et al. 2001).

Early studies have shown that *T. cruzi* and other trypanosomatids, as well as animal cells, can convert putrescine into spermidine by addition of an aminopropyl group coming from decarboxylated S-adenosylmethionine. This reaction is catalyzed by the enzyme spermidine synthase (Algranati et al. 1990; Cohen 1998). The biosynthesis and reduction of trypanothione in protozoa have been described in detail by Fairlamb's group (Fairlamb et al. 1985; Fairlamb and Cerami 1992). The retro-conversion of spermidine into putrescine has been reported by Bacchi's group in *T. cruzi* epimastigotes as well as in the other stages of the parasite life cycle (Bacchi et al. 2001).

The absence of ODC and ADC enzymatic activities in intact parasites might be the result of a deficient uptake of ornithine and arginine, but transport measurements have indicated that both amino acids are normally taken up and utilized by *T. cruzi*. In addition, the search for inhibitors of ODC and ADC activities in the parasite extracts gave essentially negative results (Carrillo et al. 2007).

Molecular genetics studies

The failure to detect the enzymes ODC and ADC in wild-type *T. cruzi* epimastigotes might be due to the possible absence of the corresponding genes in the parasite genome. In fact, hybridization analysis with radioactive specific probes homologous to conserved regions of *ODC* or *ADC* genes from many organisms has indicated the absence of ODC- and ADC-like nucleotide sequences in the genome of wild-type *T. cruzi* (Carrillo et al. 1999, 2004).

Bioinformatic analyses based on available data from the *T. cruzi* genome project were used to design degenerate primers for low-stringency PCR amplification reactions with genomic DNA from various wild-type strains of *T. cruzi* as templates. The amplified products were cloned and sequenced. None of the resulting sequences showed a partial identity with any reported *ODC* or *ADC* genes (Carrillo et al. 2004, 2007). In accordance with these results, phylogenetic analyses carried out recently concluded that an ancestor of *T. cruzi* has lost the *ODC* gene during evolution. *T. brucei* and other related trypanosomatids were then able to recover a new copy of a homologous gene by horizontal transfer from a vertebrate organism. In contrast, *T. cruzi* has permanently lost its *ODC* gene (Steglich and Schaeffer 2006).

All the described evidence has shown that *T. cruzi* behaves as a natural deletion mutant of *ODC* and *ADC* genes. Thus, it could be expected that the auxotrophy for polyamines might be reversed after the parasite transformation with plasmids bearing *ODC* or *ADC* gene coding regions obtained from exogenous sources. This kind of experiment has been carried out by transfection of wild-type *T. cruzi* with recombinant plasmids constructed with an appropriate expression vector specific for this parasite transformation (Kelly et al. 1992; Martínez Calvillo et al. 1997) and the coding regions of *C. fasciculata ODC* gene (Svensson et al. 1997) or oat ADC cDNA (Bell and Malmberg 1990). In both cases, the transgenic parasites were able to show high levels of ODC or ADC enzymatic activities in a transient expression which afterwards became stable (Carrillo et al. 2007; Serra et al. 2006). Parasites containing the foreign *ODC* gene became autotrophic for polyamines since they could grow continuously in synthetic media after acquiring the ability to synthesize putrescine (Carrillo et al. 1999). On the contrary, transformed *T. cruzi* expressing oat ADC was not able to overcome polyamine auxotrophy because agmatine could neither perform polyamine functions nor be converted into putrescine due to the parasite lack of agmatinase enzymatic activity (Carrillo et al. 2004).

The normal expression of heterologous *ODC* or *ADC* genes in transgenic *T. cruzi* clearly indicated that the intracellular medium of this protozoan does not contain any repressor of *ODC* or *ADC* genes expression (Carrillo et al. 2007). The above-mentioned results confirmed that the wild-type *T. cruzi* lacks the *ODC* and *ADC* genes which encode the enzymes catalysing the first steps of the two known polyamine pathways (Ariyanayagam and Fairlamb 1997; Carrillo et al. 1999). These findings opened the possibility of using this parasite as a model system to study the expression of foreign genes coding for polyamine biosynthesis.

DFMO resistance in transformed *T. cruzi* containing an exogenous *ODC* gene

After transformation of wild-type parasites with plasmids containing a heterologous *ODC* gene, *T. cruzi* became autotrophic for polyamines and at the same time sensitive to DFMO. This drug inhibited the acquired ODC activity and was able to arrest the transgenic parasite proliferation in a semi-defined culture medium (Carrillo et al. 1999). Following a period of growth inhibition lasting several weeks, the continuous presence of DFMO at a rather high concentration provoked the spontaneous re-initiation of proliferation. The induced resistance to DFMO of ODC-transformed parasites was neither caused by a reduced uptake of the drug nor by a mutation of the enzyme target which could have decreased its affinity for the inhibitor; it was rather due to the selection of transgenic parasites highly resistant to the drug (at least 100-fold higher than the sensitive protozoa). After the complete removal of DFMO, the resistant parasites showed to contain ODC

enzyme with a specific activity about five times higher than in DFMO-sensitive transformed *T. cruzi*. It is interesting to emphasize that the enzyme from drug-resistant parasites is still completely inhibited by DFMO added to in vitro assays. The reduction of the parasite susceptibility to the drug can be accounted by the increased activity of the enzyme in DFMO-resistant *T. cruzi.* The hybridization analysis with labeled probes specific for *ODC* gene has shown that the *ODC* gene copy number has been duplicated in DFMO-resistant transgenic *T. cruzi* and that these genes appeared integrated into the parasite genome. The determination of *ODC* transcription and translation products has indicated that multiple molecular mechanisms as transcript processing, translation and protein assembly and folding might contribute to the development of DFMO resistance in ODC-transformed parasites (Carrillo et al. 2007).

Regulation of oat *ADC* gene expression in *T. cruzi* and post-translational processing of the enzyme

The expression of a plant *ADC* gene in *T. cruzi* epimastigotes has also been studied after parasites transformation with a recombinant plasmid containing the coding region of oat *ADC* gene (Carrillo et al. 2004). The new enzymatic activity characterized as arginine decarboxylase appeared soon after transfection during a transient period, and then decreased markedly for several weeks reaching very low levels. However, when cultures of ADC-transfected *T. cruzi* were incubated for long times in the presence of geneticin, the antibiotic used for selection of plasmid-containing parasites, the ADC enzymatic activity reappeared about 4 weeks after transfection and then increased and became permanently expressed at rather high levels (Serra et al. 2006).

The ADC-transgenic parasites have been an useful tool to investigate the fate of the transforming plasmid and the organization of *T. cruzi* genome at different times of post-transfection. Experiments of PCR amplification and DNA hybridization have indicated that during the first day, the transforming plasmid remained as a free episome which was able to express transiently the heterologous *ADC* gene. Later, the free plasmid was almost completely degraded by endogenous nucleases. However, the addition of geneticin 48 h after transformation and the continuous presence of the drug for several weeks induced the selection of parasites containing two or more units of the transfected plasmid integrated in tandem into the parasite genome. At this time, the expression of the exogenous *ADC* gene became stable (Serra et al. 2006).

Experiments performed with several arginine decarboxylases from different plants have shown that their enzymatic activities appeared after the proteolytic processing of a precursor polypeptide synthesized by direct translation of the corresponding messenger RNA (Malmberg and Cellino 1994; Hanfrey et al. 2001). A partially purified proteolytic activity from oat extracts was able to cleave an inactive 66 kDa polypeptide precursor producing an active ADC formed by two peptide chains of 42 and 24 kDa held together as heterodimer molecules (Malmberg and Cellino 1994). Since ADC enzymatic activity appeared in *T. cruzi* after the described transformation, the precursor polypeptide primarily synthesized by the parasite translational machinery should be a good substrate for a correct proteolysis carried out by enzymes that are normally found in the parasite internal medium. These enzymes exhibit probably non-specific proteolytic activities. This assumption, supported by the lack of *ADC* gene in wild-type *T. cruzi* genome (Carrillo et al. 2004), has been confirmed by experiments showing that a transcription/translation product obtained in vitro with a recombinant plasmid containing the *ADC* coding region was correctly processed by purified cruzipain, one of *T. cruzi* main proteases (Cazzulo et al. 1990; Serra et al. 2009). The described results have shown that the post-translational processing of ADC can also occur inside the wild-type parasite. Therefore, the precursor polypeptide modification seems to depend on the presence of a protease-sensitive loop in the structure of the protein rather than on the availability of some specific proteolytic enzymes inside the parasites. The amino acid sequence alignment of oat ADC with a group of PLP-dependent decarboxylases allowed us to predict that the ADC protein structure contains an exposed interdomain between the N- and C-terminal moieties. This segment appears to be the protease-sensitive region of ADC protein (Serra et al. 2009).

Conclusions

T. cruzi epimastigotes lack both ODC and ADC enzymatic activities and therefore are unable to synthesize putrescine de novo. Molecular genetics studies have shown that the parasite genome contains neither *ODC* nor *ADC*-related nucleotide sequences. These findings have suggested that *T. cruzi*, which behaves as a natural deletion mutant for both *ODC* and *ADC* genes, might be a convenient system to investigate the expression of heterologous genes involved in polyamine biosynthesis. *T. cruzi* epimastigotes transformed with recombinant plasmids bearing the coding region of *C. fasciculata ODC* or oat *ADC* genes have been used for this purpose. Both types of transgenic parasites were able to show a transient as well as a stable expression of the foreign genes. These results indicate that the parasite intracellular medium contains neither any repressor of *ODC* or *ADC* genes expression nor any inhibitor of the new enzymatic activities.

Both heterologous genes integrate into the parasite genome at the onset of the stable period of expression. The presence of DFMO for several weeks in cultures of *ODC*-transformed protozoa elicited the amplification of the foreign gene with the simultaneous induction of drug resistance.

ADC-transgenic *T. cruzi* was able to perform the correct post-translational processing necessary to convert the inactive polypeptide precursor into an active enzyme. This processing is presumably carried out by non-specific proteolytic activities of the parasite.

Acknowledgment I thank Dr. Sara H. Goldemberg for helpful discussions. This work was supported by the University of Buenos Aires and the National Research Council (CONICET, Argentina).

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