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Aspartate transport and metabolism in the protozoan parasite *Trypanosoma cruzi*

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Abstract

Aspartate is one of the compounds that induce the differentiation process of the non-infective epimastigote stage to the infective trypomastigote stage of the protozoan parasite *Trypanosoma cruzi*. L-aspartate is transported by both epimastigote and trypomastigote cells at the same rate, about 3.4 pmol min^{-1} per 10^7 cells. Aspartate transport is only competed by glutamate suggesting that this transport system is specific for anionic amino acids. Aspartate uptake rates increase along the parasite growth curve, by amino acids starvation or pH decrease. The metabolic fate of the transported aspartate was predicted in silico by identification of seven putative genes coding for enzymes involved in aspartate metabolism that could be related to the differentiation process.
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Keywords: *Trypanosoma cruzi*; Metabolite transport; Aspartate; Amino acid transporter; Glutamate; Chagas disease

1. Introduction

Chagas' disease is a major health and economic problem in South and Central America. The causative agent is the hemoflagellate *Trypanosoma cruzi*, which is transmitted to humans by blood-sucking triatomine vectors. At present, an infection prevalence of 18 million people is estimated, with about 5 million symptomatic cases [1]. No efficient and well-tolerated therapy is available as yet, especially against the chronic form of the disease. Therefore, basic and applied research on the mechanism of pathogenesis is of great importance. Metacyclogenesis is one of the steps of the *T. cruzi* life cycle, by which the epimastigote, a replicative and non-infective stage in the insect vector, differentiates into trypomastigote, the non-replicative and infective stage, which is capable to infect mammalian hosts. One of the most common methods used for parasite in vitro metacyclogenesis mimics conditions found in the hindgut of the triatomine insect vector. Accordingly, epimastigote cells are exposed to transient nutritional stress in Triatomine Artificial Urine (TAU) medium and further treated in TAU supplemented with aspartate, glutamate, proline and glucose [2–4]. These amino acids were selected by their ability to promote metacyclogenesis but the

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transport and metabolic processes involved have not been completely elucidated.

Metabolites transport processes constitute the first step of their metabolic pathways determining the availability of substrates in the intracellular medium. In spite of the importance to characterize parasite transporters, only two amino acid transport systems, one for proline and the other for arginine, were described in *T. cruzi* [5–8]. Both transport systems have two components, one with high-affinity and the other with low-affinity for their substrates. That feature has been proposed as an evolutionary adaptation to environments in which the amino acid concentration is significantly different, i.e., the mammalian blood and the insect hindgut. Moreover, arginine transport shows an adaptative regulation in response to different extracellular stimuli and parasite replication capability [5,7,8].

On the other hand, amino acids such as aspartate are relevant in *T. cruzi* energetic metabolism [9]. Only one enzyme that metabolizes aspartate has been cloned and characterized in *T. cruzi*, the aspartate carbamoyltransferase (EC 2.1.3.2), which produces carbamoyl–aspartate from carbamoyl–phosphate and L-aspartate constituting the second step in the de novo pyrimidine biosynthesis [10]. Two other enzyme activities were reported in *T. cruzi*, an aspartate aminotransferase (EC 2.6.1.1) that represents the final step of methionine recycling in a wide variety of parasitic organisms [11,12] and an adenylosuccinate synthase (EC 6.3.4.4) that catalyzes the condensation of aspartate with IMP [13].

The biochemical characterization of aspartate transport in the parasite *T. cruzi* is herein reported. The possible fate of the transported aspartate was predicted using a bioinformatic approach.

2. Materials and methods

2.1. Cell cultures

Epimastigotes of the CL Brener strain were cultured at 28 °C in plastic flasks (25 cm^2), containing 5 ml of LIT medium (started with 10^6 cells per milliliter) supplemented with 10% fetal calf serum, 100 U/ml penicillin, and 100 μg/ml streptomycin [14]. The parasites were subcultured with passages each 7 days, unless otherwise indicated. At the indicated times, cells were counted using a hemocytometric chamber. Trypomastigotes, (CL Brener strain) were obtained as previously described [15]. LLC-MK2 monolayers were grown in DME medium supplemented with 5% fetal calf serum at 37 °C. During infection, fetal calf serum concentration was reduced to 2%. The trypomastigotes, released from the cultured cells around the 5th–6th day after inoculation, were washed three times in DME before used for further experiments.

2.2. Aspartate transport assays

Aliquots of epimastigote or trypomastigote cultures (10^7 parasites) were grown for the indicated periods. The parasites were centrifuged at 8000*g* for 30 s, and washed once with phosphate-buffered saline (PBS) pH 7.0. Cells were then resuspended in 1 ml of PBS supplemented with 2% glucose, preincubated 2 h at 28 °C to decrease endogenous L-aspartate pool, and then centrifuged at 8000*g* for 30 s. Cells were resuspended in 0.1 ml PBS and then 0.1 ml of the transport mixture containing 200 μM L-[2,3-^{3}H] aspartic acid (Amersham Biosciences; 1.2 μCi) was added, unless otherwise indicated. Following incubation for 3 min at 28 °C, cells were centrifuged as indicated above, and washed twice with 1 ml of ice-cold PBS. Pellets were then resuspended in 0.2 ml of water and counted for radioactivity in UltimaGold XR liquid scintillation cocktail (Packard Instrument Co., Meridien CT, USA). Non-specific transport and carry over were measured in transport mixtures containing 20 mM L-aspartate [5]. The transport assay was previously validated by comparison with short-time assays performed by using a rapid technique based on separation of cells from the incubation medium by centrifugation through a dibutylphtalate/dinonylphtalate oil mixture (2:1; v:v) as described by Pereira et al. [5] and Le Quesne and Fairlamb [16]. Assays were run at least in triplicates. Cell viability was assessed by direct microscopic examination. Enzyme kinetic constants were calculated following the procedures of Hanes or Lineweaver and Burk as described by Dixon and Webb [17].

2.3. RT-PCR technique

Total RNA samples from 10^8 epimastigotes were isolated using the Trizol® (GIBCO, BRL) method according to the manufacturer instructions, and used for reverse transcription with ThermoScript® reverse transcriptase (Invitrogen) and Oligo (dT)20 following the manufacturer instructions. PCR reactions were performed by standard protocols using 1 μl of the reverse transcription mixture and the following set of primers (forward and reverse): Adenylosuccinate synthetase: 5′ GAG GCG AAT GTT CCG TAT 3′ and 5′ CGG GCA TTA CTC AAC CA 3′ ; aspartate–ammonia ligase: 5′ GTT GTG GCG ACG GAA CAC 3′ and 5′ TAA GCT GGC GTC GAA GTG 3′ ; aspartate aminotransferase: 5′ GGA CTG AGC GGC ACA GG 3′ and 5′ CAA AGC TTG GCG CAT TTC 3′ ; aspartate carbamoyltransferase: 5′ CGC ATC ATG ACG CCA CT 3′ and 5′ TCT GCA GAC GCG TTG TGT 3′ ; asparaginase: 5′ GCA GAC CCG TCG TTG GAG 3′ and 5′ GGT GGC CTC AAT GGT CAT GT 3′. Control reactions for PCR were performed using total RNA preparations instead of cDNA [18]. All the obtained

products were sequenced using an ABI PRISM® 3100 Genetic Analyzer with Computer Workstation (Applied Biosystems) and submitted to the GenBank under the following accession numbers: AY762326 (asparaginase), AY762327 (aspartate aminotransferase), AY762328 (aspartate–ammonia ligase), and AY762329 (adenylosuccinate synthetase).

2.4. Bioinformatics

Reference protein sequences for each enzyme analyzed were obtained from the Swiss-Prot database (http://us.expasy.org/sprot/) and blasted against preliminary *T. cruzi* genome sequence data at "The Institute for Genomic Research" (http://www.tigr.org). The obtained individual nucleotide sequences were then assembled into contigs using phrap ("Phragment Assembly Program") (http://www.phrap.org/) [19] and the conceptual translation of the deduced open reading frames were analyzed as previously described [20].

3. Results

3.1. Biochemical characterization of aspartate transport

The range in which L-aspartate uptake is proportional to the incubation time was determined first. During the first 3 min of incubation the uptake velocity was constant, and calculated as 3.4 pmol min^{-1} per 10^7 cells (±0.3) (Fig. 1, inset). After that, a decrease of 52% in the 15th min and 77% in the 30th min was observed. The calculated transport rate was dependent on L-aspartate concentration, however, a low non-saturable component was also detected at concentrations above 0.3 mM. In Lineweaver–Burk plots, maximum velocity (V_{max}) and the apparent Michaelis-Menten constant (K_m) values, were 3.4 pmol min^{-1} per 10^7 cells, and 32 μM, respectively (Fig. 1). The specificity of the transport system was evaluated by competition analysis using 67-fold molar excess (1 mM) of amino acids from different groups, and 15 M L-[^{3}H] aspartate (0.47-fold the apparent K_m value). Aspartate transport was only inhibited by glutamate (55.4% inhibition), but not by lysine, serine, methionine, glycine, tyrosine, arginine or glutamine. Similar results were obtained using the same concentration of the competitors and 200 μM L-[^{3}H] aspartate, when the inhibition by glutamate was 88.9% (Table 1). It was observed that the transport activity was strongly dependent on pH, increasing with the diminution of

Table 1
Specificity of aspartate transport in *T. cruzi* epimastigotes

Treatment	Aspartate 15 μM Activity ± SD (%)	Aspartate 200 μM Activity ± SD (%)
No treatment	100.0 ± 17.9[a]	100.0 ± 7.1[b]
Lysine	92.1 ± 15.9	52.7 ± 1.3
Serine	92.2 ± 10.6	82.9 ± 14.7
Methionine	108.9 ± 10.9	80.7 ± 12.2
Glutamate	*44.6 ± 16.5*	*11.1 ± 4.8*
Aspartate	*26.7 ± 13.4*	*9.2 ± 10.1*
Glycine	72.3 ± 10.2	60.6 ± 8.0
Tyrosine	68.1 ± 12.2	74.7 ± 16.0
Arginine	76.2 ± 12.8	50.9 ± 12.6
Glutamine	63.2 ± 17.7	51.6 ± 7.4

T. cruzi epimastigotes were washed (see Section 2) and resuspended in phosphate-buffered saline at 28 °C, containing 1 mM of the indicated amino acid and a concentration of 15 or 200 μM [^{3}H] L-aspartate. Control transport activities (100%) correspond to 1.0 pmol min^{-1} per 10^7 cells at a 15 μM L-aspartate concentration[a] or 3.4 pmol min^{-1} per 10^7 cells at a 200 μM L-aspartate concentration[b]. Values represent the means and the standard deviation of three assays.

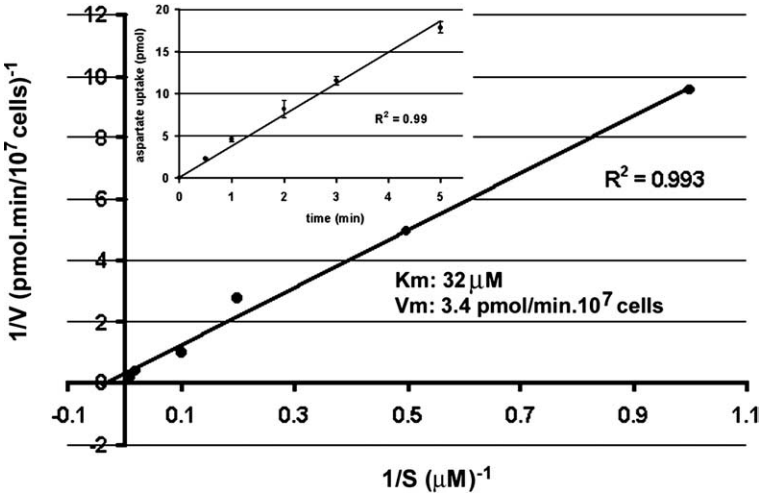

Fig. 1. Kinetics of aspartate transport. Initial rates of aspartate transport (V_0) were measured as a function of aspartate concentration in the range 1–100 μM, as described under Section 2. Maximum velocity (V_{max}) and an estimate for the apparent Michaelis-Menten constant (K_m) value, were 32 pmol min^{-1} per 10^7 cells, and 3.4 μM, respectively, calculated using a Lineweaver–Burk plot. Inset: initial velocity (V_0) determination. Aspartate transport was measured during a 5 min interval.

pH from 8 to 4. The transport activity reached maximum values at pH 4, about 3.4-fold higher than the activity measured at pH 7, or 4-fold when compared with pH 8 (Fig. 2).

3.2. Regulation of aspartate transport

In order to test if aspartate transport shows an adaptative regulation mechanism, parasites were assayed in different conditions. First, epimastigote cells were starved in PBS-glucose up to 3 h to decrease the endogenous L-aspartate pool before the aspartate transport assay. An increase in the aspartate transport proportional to the starvation period was detected reaching maximum values after 3 h, with a 6.7-fold increase when compared to the control without starvation. Epimastigote cells were also assayed for aspartate transport during the exponential and stationary phases of culture growth. During the interval between the 4th and 14th day of culture aspartate transport increased about 5.3-fold, from 0.7 pmol min^{-1} per 10^7 cells (±0.08) on the 4th day to 3.7 pmol min^{-1} per 10^7 cells (±0.25) on the 14th day. At last, aspartate uptake was measured in the infective stage trypomastigote at the same rate than mid-log epimastigote forms. It is worth mentioning that LIT cultured cells were at least 95% epimastigote forms.

3.3. Bioinformatic prediction of aspartate metabolism in T. cruzi

Thirty-three different aspartate metabolizing enzyme sequences were used as baits for tblastn searches in the unassembled *T. cruzi* genome at "The Institute for Genomic Research" (TIGR) website. Five contigs containing open reading frames coding for putative enzymes that metabolize free-aspartate were then assembled, including: aspartate carbamoyltransferase, aspartate aminotransferase, aspartate–ammonia ligase, adenylosuccinate synthetase and asparaginase. The previously described aspartate carbamoyltransferase gene (GenBank accession number: AB074139) from *T. cruzi* [10] was used as a control of the searching procedure. In addition, two open reading frames coding for putative enzymes that metabolize polypetide-associated aspartate were also found: Protein–L-isoaspartate (D-aspartate) *O*-methyltransferase (PCMT) and aspartyl aminopeptidase (Table 2). RT-PCR amplification assays were performed using specific primers for the five predicted enzyme genes and total RNA from *T. cruzi* epimastigotes and trypomastigotes. As Fig. 3 (upper panel, cDNA) shows, the expression of all tested genes was demonstrated in epimastigote cells. All the predicted genes were also amplified from *T. cruzi* genomic DNA as positive controls (Fig. 3, upper panel, DNAg) and the results confirmed by sequencing all the obtained products.

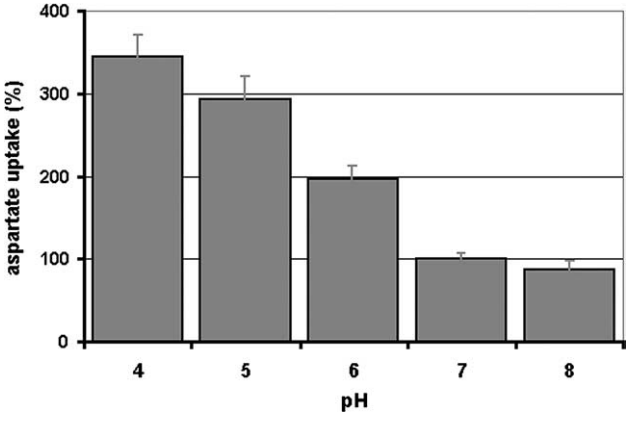

Fig. 2. pH dependency of aspartate transport in *T. cruzi* epimastigotes. *T. cruzi* epimastigotes were washed (see Section 2) and resuspended in buffer acetate containing 145 mM Na^+ and 4.5 mM K^+, for pHs 4 and 5, or PBS for pHs 6, 7 and 8 at 28 °C, in the presence of 200 μM [^{3}H] L-aspartate. Control transport activities (100%) correspond to 3.4 pmol min^{-1} per 10^7 cells in PBS pH 7.0. Bars represent the means and the standard deviation of three assays.

4. Discussion

A high-affinity L-aspartate transport system in the protozoan parasite *T. cruzi* was herein characterized. The estimated K_m value for aspartate transport in *T. cruzi* is similar to those reported for other eukaryotic organisms, i.e., glutamate/aspartate transporters from the "Excitatory Amino Acid Transporters" family (EAAT) [21]. Several amino acids were used to estimate substrate specificity by measuring the percent of inhibition of L-aspartate uptake. Aspartate transport was only competed by L-glutamate suggesting that a glutamate/aspartate transporter mediates the transport activity herein described. Our group recently characterized in silico 60 different *T. cruzi* putative amino acid transporters, clustered in 12 groups, from the "Amino Acid/Auxin Permeases" (AAAP) family [20]. However, the substrate specificity of these transporters remains unknown. It is worth mentioning that in other organisms, such as yeast and plants, members of the AAAP family mediate the aspartate uptake together with other amino acids [22,23].

Some adaptive changes in the aspartate transport process were also studied. When parasites from different days of culture were compared, an increase in aspartate uptake was observed accompanying the reduction in parasite replication rates, the increase in cell density, and the nutrient depletion of the medium. The uptake of aspartate in trypomastigotes was measured at the same rate of mid-log epimastigotes indicating that aspartate is transported by the parasite not only in the insect vector, but also in the mammalian host. These regulation mechanisms are quite different to those

Table 2
Bioinformatic prediction of aspartate metabolism

No.	Enzyme name	EC	Reaction catalized	Best match
1	Aspartate carbamoyltransferase[a]	2.1.3.2	Carbamoyl phosphate + L-*aspartate* ↔ Phosphate + *N*-carbamoyl–L-*aspartate*	AB074139 *Typanosoma cruzi*
2	Aspartate aminotransferase	2.6.1.1	L-*Aspartate* + 2-oxoglutarate ↔ oxaloacetate + L-glutamate	AAK73815 *Typanosoma brucei*
3	Aspartate–ammonia ligase	6.3.1.1	ATP + L-*aspartate* + NH_3 ↔ AMP + diphosphate + L-asparagine	NP991407 *Yersinia pestis*
4	Adenylosuccinate synthetase	6.3.4.4	GTP + IMP + L-*aspartate* ↔ GDP + phosphate + adenylosuccinate	CAD19432 *Leishmania major*
5	Asparaginase	3.5.1.1	L-asparagine + H_2O ↔ L-*aspartate* + NH_3	AAO52247 *Caenorhabditis elegans*
6	Protein–L-isoaspartate (D–aspartate) *O*-methyltransferase (PCMT)	2.1.1.77	*S*-adenosyl–L-methionine + protein L-beta-*aspartate* ↔ *S*-adenosyl–L-homocysteine + protein L-beta-*aspartate* methyl ester	AAH56106 *Xenopus laevis*
7	Aspartyl aminopeptidase	3.4.11.21	Release of an N-terminal *aspartate* from a peptide	NP036232 *Homo sapiens*

Genes coding for putative aspartate metabolism enzymes in *T. cruzi* identified using reference protein sequences from other organisms as baits for blast searches at the *T. cruzi* genome project (TIGR).
[a] Control of the searching procedure (GenBank Accession number: AB074139).

previously reported for cationic amino acid transport in *T. cruzi* [5,7,8]. In addition, aspartate transport seems to be a substrate-regulated process, since an amino acid starvation induces a 6.7-fold increase in the transport rate. This might indicate the presence of a rapid mechanism to restore the intracellular aspartate concentration.

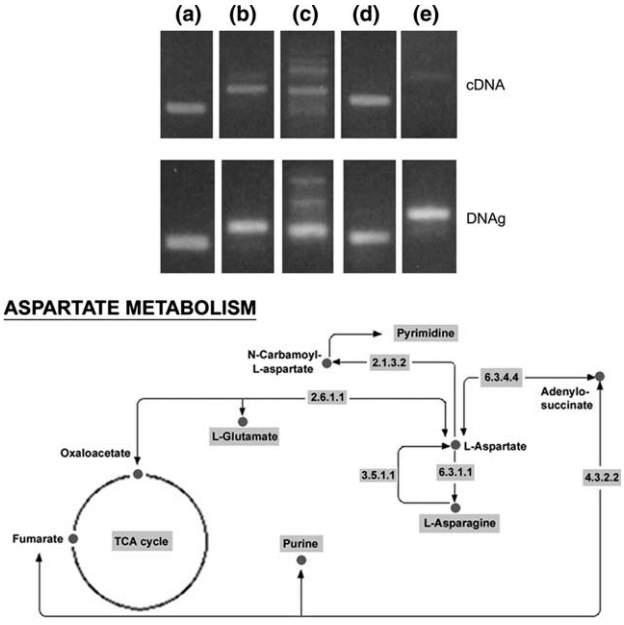

Fig. 3. mRNA expression analysis of deduced enzymes and a model of the aspartate metabolism in *T. cruzi*. Upper panel: agarose gels displaying the RT-PCR products obtained by amplification of the five genes corresponding to each enzyme (lanes (a) to (e)) from epimastigote total RNA (cDNA). Control reactions were performed using for PCR genomic DNA instead of cDNA (DNAg). Lanes: (a) adenylosuccinate synthetase (509 bp), (b) aspartate–ammonia ligase (726 bp), (c) aspartate aminotransferase (687 bp), (d) aspartate carbamoyltransferase (579 bp), and (e) asparaginase (945 bp). Lower panel: schematic representation of the aspartate metabolism in *T. cruzi*. EC numbers correspond to: aspartate carbamoyltransferase (2.1.3.2), aspartate aminotransferase (2.6.1.1), asparaginase (3.5.1.1), adenylosuccinate lyase (4.3.2.2), aspartate–ammonia ligase (6.3.1.1) and adenylosuccinate synthetase (6.3.4.4).

The activity of the aspartate transport system is sensitive to the extracellular pH since it was observed that the transport activity was increased with the decrease of the pH from 8 to 4. Similar results were previously obtained for other *T. cruzi* amino acid transporters, such as arginine and proline, where the maximum transport activities were determined in the 4 to 5 pH range [6,8]. Parasites are exposed to variations in the extracellular pH during its life cycle, for example within the digestive tract of the insect vector (pH 5–9) [2,24], mammalian blood (neutral pH), mammalian intracellular parasitophorous vacules (pH 4.5–5.5) [24] and cytoplasm (neutral pH). Therefore, an increase in the transport of arginine, proline and aspartate should be expected in a more acidic environment present in the insect and in the mammalian hosts. In this context it is important to note that at pH as low as 4.0, amino acid residues in the transporter would be protonated, a condition that usually hinders activity. However, the existence of independent genes encoding similar transporter isoforms to function at different extracellular pH has been previously suggested [25].

Using the available data from the Swiss-Prot database and the *T. cruzi* genome project, a total of seven enzymes involved in aspartate metabolism were identified. Five of the identified genes codify for putative enzymes that use free aspartate as substrate. Three of them belong to a group of enzymes that mediate the conversion of aspartate to other amino acids: asparaginase, aspartate–ammonia ligase and aspartate aminotransferase belong to this group. The second group, including adenylosuccinate synthetase and aspartate carbamoyl transferase, participates in the nucleoside biosynthetic

and salvage pathways. The aspartate aminotransferase is well known as part of the malate–aspartate shuttle for the transport of reducing equivalents between cytosol and mitochondria. At least one of these enzymes, adenylosuccinate synthetase, has a potential importance as a target for chemotherapeutical agents. Inhibition of this enzyme in *Plasmodium falciparum* leads to death of the parasite, because the parasite lacks a de novo purine biosynthetic pathway and depends exclusively on the purine salvage pathway for its purine nucleotides. [26]. In addition, adenylosuccinate synthetase together with adenylosuccinate lyase, seems to be responsible for the selective amination of allopurinol ribonucleotide in *T. cruzi*. The metabolically produced AMP analog may be the agent, or an agent precursor that accounts for the anti-growth activity of the allopurinol in these organisms [13]. Despite the searching procedure, which was focused on free aspartate metabolizing enzymes, two sequences of enzymes that use polypeptide-associated aspartate as substrate were also found. Protein–L-isoaspartate (D-aspartate) *O*-methyltransferase (PCMT) is an enzyme that catalyzes the transfer of a methyl group from *S*-adenosylmethionine to the free carboxyl groups of D-aspartyl or L-isoaspartyl residues in a variety of peptides and proteins and plays a role in the repair and degradation of these damaged proteins. The second enzyme was an aspartyl aminopeptidase that releases an N-terminal aspartate from a peptide.

The model showed in the Fig. 3 (lower panel) summarizes the three main fates of aspartate in *T. cruzi*, nucleoside (purine and pyrimidine) salvage and biosynthesis, amino acids (asparagine and glutamate) biosynthesis and as an alternative energy source via TCA cycle. These data led us to hypothesize that aspartate acts on metacyclogenesis not as a signaling molecule but as a precursor of primary metabolites necessary to the differentiation processes. The results herein presented provide an insight on the role played by aspartate in trypanosomes.

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