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FEMS Microbiology Letters 247 (2005) 65–71

FEMS MICROBIOLOGY Letters

www.fems-microbiology.org

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

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Received 13 October 2004; received in revised form 23 March 2005; accepted 19 April 2005

First published online 30 April 2005

Edited by D.P. Wakelin

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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doi:10.1016/j.femsle.2005.04.029

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

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