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Biochemical characterization of stage-specific isoforms of aspartate aminotransferases from *Trypanosoma cruzi* and *Trypanosoma brucei*

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ABSTRACT

Three genes encoding putative aspartate aminotransferases (ASATs) were identified in the *Trypanosoma cruzi* genome. Two of these *ASAT* genes, presumably corresponding to a cytosolic and mitochondrial isoform, were cloned and expressed as soluble His-tagged proteins in *Escherichia coli*. The specific activities determined for both *T. cruzi* isozymes were notably higher than the values previously reported for *Trypanosoma brucei* orthologues. To confirm these differences, *T. brucei* mASAT and cASAT were also expressed as His-tagged enzymes. The kinetic analysis showed that the catalytic parameters of the new recombinant *T. brucei* ASATs were very similar to those determined for *T. cruzi* orthologues. The cASATs from both parasites displayed equally broad substrate specificities, while mASATs were highly specific towards aspartate/2-oxoglutarate. The subcellular localization of the mASAT was confirmed by digitonin extraction of intact epimastigotes. At the protein level, cASAT is constitutively expressed in *T. brucei*, whereas mASAT is down-regulated in the bloodstream forms. By contrast in *T. cruzi*, mASAT is expressed along the whole life cycle, whereas cASAT is specifically induced in the mammalian stages. Similarly, the expression of malate dehydrogenases (MDHs) is developmentally regulated in *T. cruzi*: while glycosomal MDH is only expressed in epimastigotes and mitochondrial MDH is present in the insect and mammalian stages. Taken together, these findings provide evidence for a metabolically active mitochondrion in the mammalian stages of *T. cruzi*, and suggest that the succinate excreted by amastigotes more likely represents a side product of an at least partially operative Krebs cycle, than an end product of glycosomal catabolism.

1. Introduction

Trypanosoma brucei and *Trypanosoma cruzi* are the causative agents of African and American trypanosomiases, respectively. In tropical and subtropical regions of Latin America and Africa, these parasitic infections represent some of the most consequential diseases in terms of human suffering and economic hardship. Current clinical treatments are highly inefficient due to the low effectiveness and high toxicity of the available drugs. Moreover, these parasites develop resistance to the available chemotherapy, and at least in the case of African trypanosomes, production of suitable vaccines is unlikely due to the antigenic variation of these parasites [1].

Trypanosomatids have a digenetic life cycle and are transmitted to humans and animal reservoirs by the bite of a specific insect vector. Consequently, these parasites spend part of their life cycles in the gut of a hematophagous insect, where glucose does not represent the main energy source. By contrast, nutrients such as amino acids, easily obtained from the blood meal, are actively degraded and the derived 2-oxoacids are believed to feed the Krebs cycle [2,3]. Aspartate aminotransferases (ASATs) from parasitic protozoa have been reported to transaminate aromatic and dicarboxylic amino acids in addition to other amino donors such as glutamine, alanine, leucine, etc. Hence, these isozymes might provide the 2-oxoacids required for Krebs cycle functionality. Moreover, based on the capability of these broad specificity enzymes to convert 2-oxo-4-methylthiobutyrate (OMTB) into methionine, it has also been postulated that ASATs in parasitic protozoa might be also involved in methionine recycling [4].

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Early studies also showed that *T. cruzi* and *T. brucei* were able to reduce, in different degrees, the 2-oxoacids derivatives resulting from aromatic amino acid transamination to the corresponding aromatic 2-hydroxyacids [5,6]. In *T. cruzi* epimastigotes, two cytosolic enzymes are responsible for this catabolic process: a very abundant tyrosine aminotransferase (TAT) and an aromatic L-2-hydroxyacid dehydrogenase (AHADH). The former exhibits high sequence similarity (≅70%) to the mammalian liver enzyme, but has notably broader substrate specificity. *T. cruzi* TAT displays the highest activities towards amino donors such as alanine, the three aromatic amino acids and methionine, while pyruvate is the best amino acceptor [7,8]. The second enzyme of this pathway, AHADH, probably derived from a cytosolic malate dehydrogenase (MDH) no longer present in the parasite, efficiently reduces the aromatic 2-oxoacid derivatives to the corresponding 2-hydroxyacids [9,10]. By contrast, in *T. brucei* the aminotransferases involved in this process have not been identified yet, and AHADH appears to be absent [11,12].

Trypanosomes, when introduced into the human host, undergo dramatic morphological and metabolic changes, which result in the parasite adaptation to proliferate in specific mammalian niches. Thus, *T. brucei* bloodstream forms, which solely depend on glucose for energy production, easily multiply in the host's blood by taking advantage of this nutrient abundance. Since in this stage the metabolic activity in the mitochondrion is almost completely down-regulated, the end product of glycolysis, pyruvate, is not further oxidized but excreted [3,13]. By contrast, *T. cruzi* presents two mammalian stages, the non-replicative trypomastigotes and the replicative amastigotes. The former dwell in the blood whereas the latter are intracellular parasites that easily proliferate inside muscle cells of heart and digestive systems. In amastigotes, glucose transporters are believed to be virtually absent [14], whereas the Krebs cycle is expected to be essential, and amino acid oxidation thought to represent the main source of metabolic energy. Also, gluconeogenesis has been postulated to be vital to maintain the synthesis of glycoproteins and glycoinositolphospholipids. The substrates required for the *de novo* synthesis of glucose might be provided by the coupled action of cASAT and mASAT isozymes, phosphoenolpyruvate carboxykinase and mitochondrial MDH [14,15]. However, very little is known about the metabolic processes functional in the mammalian stages of *T. cruzi*.

In the present study we have demonstrated that *T. cruzi* and *T. brucei* cASATs are able to transaminate aromatic and dicarboxylic amino acids equally well, whereas mASATs are highly specific towards the substrate pair L-aspartate/2-oxoglutarate. Moreover, *T. brucei* and *T. cruzi* ASAT isozymes exhibit different expression patterns: *T. brucei* mASAT is only present in procyclics while cASAT is expressed during the whole life cycle of this parasite. Unlike *T. brucei*, *T. cruzi* cASAT is specifically expressed in the mammalian stages, whereas mASAT is constitutively expressed. Also in *T. cruzi*, the expression of MDHs is developmentally regulated, mMDH being expressed along the whole life cycle, whereas gMDH is only present in epimastigotes.

2. Materials and methods

2.1. Parasite and culture

Epimastigotes of *T. cruzi* CL Brener clone were grown in axenic medium, harvested and washed as previously described [16]. Amastigotes and trypomastigotes were obtained from infected Vero cell monolayers [17].

Procyclic forms of *T. brucei* stock 427 were grown at 28 °C on SDM-79 culture medium (JRH Biosciences, Lenexa, KS, USA), supplemented with hemin (7.5 mg/l) and 10% fetal calf serum, and harvested by centrifugation at 6000 × *g* for 10 min at 4 °C. The bloodstream forms of *T. brucei* stock 427 were grown in male Wistar rats. Highly infected rats were bled by cardiac puncture under diethyl ether anesthesia, using heparin as an anticoagulant. The blood was diluted with phosphate-buffered saline pH 8.0 containing 1% (w/v) glucose. Trypanosomes were separated from blood constituents by DEAE-cellulose chromatography [18].

2.2. DNA purification

Total DNA was isolated from *T. cruzi* epimastigotes and *T. brucei* procyclics as previously described [19].

2.3. Cloning and expression of T. brucei and T. cruzi cytosolic and mitochondrial ASATs isozymes

The nucleotide sequences encoding *T. cruzi* putative cytosolic and mitochondrial ASATs (Tc00.1047053503841.70, XP_807788; Tc00.1047053510945.70, EAN86462) and *T. brucei* ASATs (AAK73815; AAK73816) were amplified by PCR. In each case, two sets of primers were designed on the bases of the nucleotide sequences assigned to each of the four *ASAT* genes in genome project database (http://www.genedb.org).

TbASATc fw (NdeI)	5′CATATGTCCAGGCCCTTTAAG3′
TbASATc rev (SalI)	5′GTCGACTCACTTGTTACGCACGTGTCGG3′
TbASATm fw (NdeI)	5′CATATGGGCAAACCGGATCCGATCCTCG3′
TbASATm rev (XhoI)	5′GTCGACTCATTTAGTAACGTTG3′
TcASATc fw (NdeI)	5′CATATGGCGATCCGATGCCTCTG 3′
TcASATc rev (EcoRI)	5′ GAATTCTCATTCCGTGACGGTTC 3′
TcASATm fw (NdeI)	5′ AATCATATGCGCGCGCCTCCAGA3′
TcASATm rev (HindIII)	5′ AAGCTTATTTCGAAACATCGTGAAAGGC3′

The forward primers corresponding to *T. brucei* and *T. cruzi* ASATs contain NheI, NdeI, restriction enzyme sites (underlined), whereas the reverse primers contain SalI, EcoRI and HindIII restriction enzyme sites (underlined). The PCR reactions were carried out using 100 ng of *T. cruzi* or *T. brucei* total DNA and Pfu-Turbo polymerase (Stratagene). The PCR settings were 5 min at 95 °C and 25 cycles under the following conditions: 95 °C for 45 s, 56 °C for 45 s and 72 °C for 1 min. Then, a final extension step was performed at 72 °C for 10 min. A single fragment (≅1.2 Kb) was amplified in each of the four reactions and upon agarose gel purification, each of the DNA fragments was cloned into pGEM-Teasy vector and fully sequenced. In order to obtain the recombinant enzymes, each of the amplified DNA fragments corresponding to *T. cruzi* and *T. brucei* ASATs were subcloned into pET28a+ expression vector (Novagen, Darmstadt, Germany). The 5′ends of the four genes were similarly extended with a nucleotide sequence encoding a 6xHis-Tag and a thrombin cleavage site. All these constructs were fully sequenced and the resulting plasmids containing each of the genes encoding the four ASAT isozymes were used to transform *Escherichia coli* BL21-CodonPlus (DE3)RIL. Bacteria were grown in LB medium containing 12.5 μg/ml tetracycline and 30 μg/ml kanamycin, at 37 °C, until an $OD_{600\,nm}$ of 0.6 was reached. Then, isopropyl-α-D-thiogalactopyranoside (IPTG) was added to a final concentration of 0.05 mM, to induce the expression of *T. brucei* and *T. cruzi* ASAT isozymes. The cultures were further grown overnight at 27 °C. For the purification of the His-tagged enzymes, the bacterial cells were harvested by centrifugation and the pellets suspended in 20 mM HCl–Tris buffer, pH 7.9, containing 5 mM imidazole and 500 mM NaCl. The suspended bacterial cells were disrupted by three cycles of sonic disintegration at 4 °C (Branson 450 Sonifier), at 80% maximum power. Samples were supplemented with pyridoxal phosphate (PLP) at a final concentration of 4 μM, incu-