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FEMS Microbiology Letters 236 (2004) 79–84

FEMS MICROBIOLOGY Letters

www.fems-microbiology.org

Biochemical characterization of a low-affinity arginine permease from the parasite *Trypanosoma cruzi*

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Received 29 January 2004; received in revised form 24 March 2004; accepted 17 May 2004

First published online 26 May 2004

Abstract

Trypanosoma cruzi, the etiological agent of Chagas disease, uses arginine for several metabolic processes, including energy reserves management. In the present work, a novel low-affinity arginine transport system has been studied. Maximum velocity (97 pmol min^{-1} per 10^7 cells), and an estimate for the apparent K_m value (350 μM) of this arginine transporter, were 6-fold and 80-fold higher respectively, when compared with the previously described high-affinity arginine transport system. This transport activity seems to be H^+-mediated, presents a broad specificity by other amino acids such as methionine, and is regulated along the parasite growth curve and life cycle.

Keywords: *Trypanosoma cruzi*; Metabolite transport; Arginine; Amino acid transporter; Chagas disease

1. Introduction

Trypanosoma cruzi is the causative agent of Chagas' disease, a zoonosis affecting approximately 18 million people in the Americas [1]. Amino acids are relevant in several cellular processes in trypanosomes beyond the protein synthesis. In the particular case of *T. cruzi*, it was demonstrated that amino acids such as proline, glutamate and aspartate are relevant in the parasite energetic metabolism [2]. These amino acids also have a role in the differentiation from the insect replicative, non-infective form to the non-replicative infective form [3], and it was recently shown that proline participates in the differentiation between the mammalian intracellular epimastigote stage and the infective form trypomastigote [4]. On the other hand, arginine participates in the cell energy management through an arginine kinase activity and constitutes a key substrate for several metabolic pathways [5,6]. Our group has described an arginine kinase in *T. cruzi* and *T. brucei* that converts arginine into phosphoarginine, a phosphagen with a role as energy reservoir [7–9]. Moreover, in *T. cruzi*, this amino acid is essential for the in vitro cultivation of epimastigote cells [10]. Arginine kinase specific activity and enzyme amounts increase continuously during exponential growth, reaching maximum values at the stationary phase of growth, suggesting a regulatory function under starvation stress conditions [11]. This hypothesis was confirmed using transgenic *T. cruzi* cells that overexpress the arginine kinase gene [12].

The transport of amino acids in parasites may be regarded as the first step of their metabolic pathways. The kinetic, thermodynamic and mechanistic properties of the transport processes determine the availability of the substrate in the intracellular medium. This fact makes the characterization of transporters a major goal aiming to metabolic research and drug design. However,

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0378-1097/$22.00
doi:10.1016/j.femsle.2004.05.021

only two amino acid transport systems, one for proline and the other for arginine, were described in *T. cruzi* [7,13,14].

Our group has characterized a high-affinity L-arginine transporter with a calculated K_m value in the micromolar range (4.2 μM). This arginine transport activity also showed high substrate specificity, among several amino acids and derivatives, only L-homoarginine, D-arginine, L-canavanine, L-ornithine and L-citrulline elicited a weak inhibition on the arginine transport. After uptake, the major product detected was characterized as phosphoarginine [7]. Several ways to regulate arginine transport such as medium composition, cell density, replication rate and arginine starvation were described. In addition, a relationship between arginine kinase specific activity and substrate transport with *T. cruzi* stages was established [14].

The evolutionary selection of a high-affinity arginine permease in the insect stage of the parasite life cycle could be explained by the low arginine concentrations in the vector midgut, but the functionality of this permease in the mammalian blood stream stage trypomastigote is uncertain. Herein, we demonstrated the presence of a second arginine transport system in *T. cruzi*, with low-affinity for the substrate, and kinetic properties compatibles with the arginine concentrations of human plasma [15].

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^{-1} penicillin, and 100 μg ml^{-1} streptomycin [17]. The parasites were subcultured with passages each 5 days, unless otherwise was indicated. At the indicated times, cells were counted using a hemocytometric chamber. Trypomastigotes, (CL Brener strain) were obtained as previously described [18]. 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 fifth to sixth day after inoculation, were washed three times in DME before used for further experiments.

2.2. Arginine transport assays

Aliquots of epimastigote or trypomastigote cultures (0.6×10^7 parasites) were grown for 5 days, unless otherwise was indicated. The parasites were centrifuged at 8000*g* for 30 s, and washed once with phosphate-buffered saline (PBS). Cells were then resuspended in 1 ml of PBS, preincubated 2 h at 28 °C to diminish endogenous L-arginine pool, and then centrifuged at 8000*g* for 30 s. Cells were then again resuspended in 0.1 ml PBS and then added 0.1 ml of the transport mixture containing 2 mM L-[2,3-^{3}H] arginine (NEN/DuPont, Boston, MA, USA; 0.4 μCi) or the indicated concentrations. Following incubation for 10 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 100 mM L-arginine [7]. Assays were run at least by triplicate. 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 [19].

2.3. Competition and inhibition assays

Competition assays were performed by incubation of the parasites with the standard transport mixture and 5-fold excess of competing metabolites (10 mM) as described [13]. The effects of cations or pH were evaluated by washing and resuspending the cells in the corresponding solutions prior to the assay. For experiments involving ionophores, 0.5 mM FCCP (Carbonyl Cyanide *p*-Trifluoromethoxyphenylhydrazone) in dimethylsulfoxide (Sigma, St. Louis, MO, USA), in the presence or absence of 5 mg ml^{-1} oligomycin (Sigma, St. Louis, MO, USA), the parasites were resuspended in the corresponding solution and used immediately for transport assays, as described above. For experiments involving respiratory chain inhibitors 200 mM rotenone and 0.5 mM antimycin A in PBS (Sigma, St. Louis, MO, USA), or the ATPases inhibitor oligomycin 5 mg ml^{-1} in PBS, parasites were preincubated for 30 min with the inhibitors prior to the transport assay [13]. All assays were performed at least in triplicates and data are representative of at least three independent experiments. The presence of a proton-dependant plasma membrane potential was evaluated by microscopical observation using fluorescence with Rhodamine 123, according to the manufacturer's instructions (Molecular Probes, Eugene, OR, USA). All assays were performed at least in triplicates and data are representative of at least three independent experiments. Viability of the parasites under the assay conditions was evaluated by microscopic observation. Viability of the parasites under the assay conditions was evaluated by microscopic observation.

3. Results

3.1. Kinetics of arginine transport

Transport of L-arginine by *T. cruzi* epimastigotes was found to be roughly proportional to incubation time up to 10 min (Fig. 1(a)). Thereafter, the transport rate declined. The transport rate was dependent on L-arginine concentration and was partially saturable, a smaller non-saturable component was also measured. In Lineweaver–Burk plots, maximum velocity (V_{max}) and an estimate for the apparent Michaelis–Menten constant (K_m) value, were 97 pmol min^{-1} per 10^7 cells, and 350 μM, respectively, without subtraction of the V_{max} of the previously reported high-affinity arginine transporter (Fig. 1(b)). In this way, these values represent the kinetic parameters of the global physiological arginine transport under condition of high-arginine concentrations, independently of the possibility that both transport systems were active or not. However, if the V_{max} value of the previously reported high-affinity arginine transporter was subtracted to each data point the corrected V_{max} and an estimate apparent K_m values became 145 pmol min^{-1} per 10^7 cells and 945 μM, respectively [7].

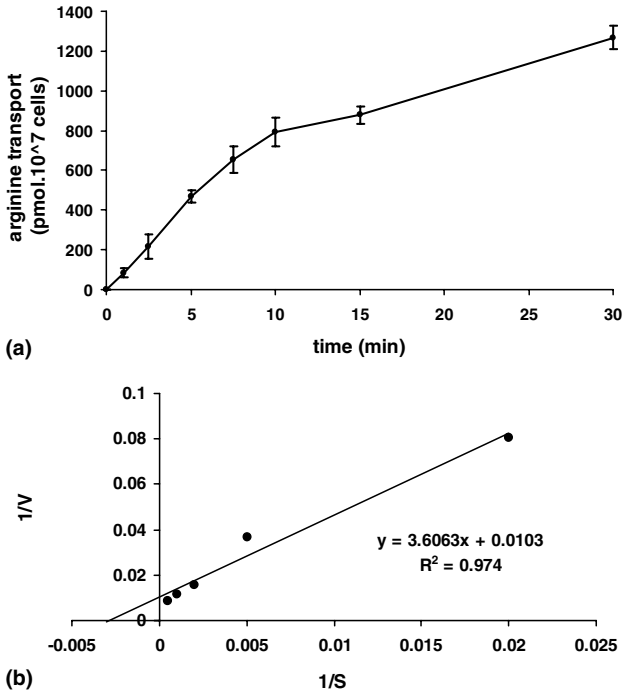

Fig. 1. (a) Initial velocity (V_0) determination. Arginine transport was measured during a 30 min interval. (b) Kinetics of arginine transport. Initial rates of arginine transport (V_0) were measured as a function of arginine concentration in the range 50–2000 μM, as described under Section 2. Maximum velocity and an estimate for the apparent Michaelis–Menten constant (K_m) value, were 97 pmol min^{-1} per 10^7 cells, and 350 μM, respectively, calculated using a Lineweaver–Burk plot.

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

Amino acid (10 mM)	Arginine 0.2 mM Activity ± SD (%)
None	100 ± 7.52
Aspartate	84.70 ± 12.42
Glutamine	81.58 ± 5.91
Serine	66.17 ± 13.14
D-Arginine	63.44 ± 5.55
Glycine	61.24 ± 5.77
Tyrosine	59.62 ± 9.41
Lysine	54.79 ± 6.62
Methionine	46.50 ± 3.53
Arginine	20.39 ± 5.02

Trypanosoma cruzi epimastigotes were washed (see Section 2) and resuspended in phosphate-buffered saline at 28 °C, containing the corresponding amino acid in a concentration 50-fold higher than L-arginine. Radioactive L-arginine (0.2 mM, 0.4 mCi) was immediately added.

3.2. Specificity of arginine transport

The specificity of the transport system was evaluated by competition analysis using 50-fold molar excess of amino acids from different groups, and 0.2 mM arginine (0.57-fold the estimated apparent K_m value). The highest inhibition was observed with methionine (hydrophobic), followed in decreasing order by lysine (basic), tyrosine (aromatic), glycine (small), serine (nucleophilic), glutamine (amide) and aspartate (acidic). The fact that D-arginine was not a competitor of L-arginine transport indicates the stereospecificity of the transport system (Table 1). The pattern of amino acid specificity for this permease resembles previously reported broad transport activities from African trypanosomes [20] but it was completely different to the observed for the high-affinity arginine permease.

3.3. Effects of temperature and activation energy

The low-affinity arginine transporter presented a basal, nearly constant activity at temperatures between 15 and 25 °C. About at 25 °C, a breakpoint in the uptake curve was observed, increasing the slope between that temperature and 40 °C. Finally, decay in V_0 was observed in assays performed at temperatures between 40 and 50 °C (Fig. 2). In order to determine activation energy (Ea) an Arrhenius plot was performed using data from Fig. 2. The calculated value was 324 kJ mol^{-1} with a correlation coefficient (r^2) of 0.93. The optimal temperature for L-arginine transport was about 40 °C. Above this temperature transport declined sharply (Fig. 2). The Q_{10} value was also calculated, being 5.12 for the range of temperature between 25 and 40 °C.

3.4. Driving force determination

L-Arginine uptake was insensitive to monovalent cations Na^+ and K^+ present in the extracellular medium

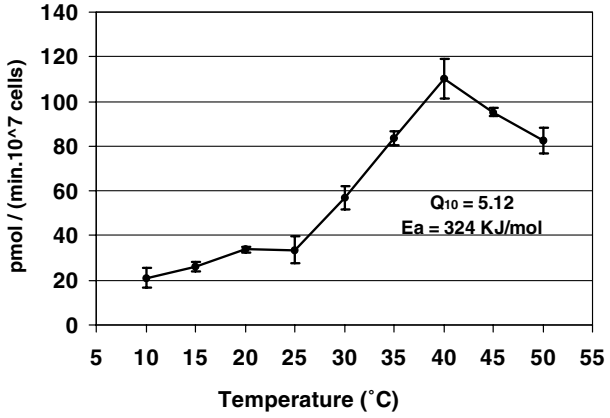

Fig. 2. Temperature dependence of L-arginine uptake in epimastigotes of *Trypanosoma cruzi*. L-Arginine transport velocity was measured as a function of temperature in the range 10–50 °C. Activation energy and Q_{10} values are also indicated.

(Inset, Fig. 3). The effect of the pH on the uptake of arginine was also evaluated. As can be seen (Fig. 3) the activity of the low affinity arginine transport system is sensitive to the extracellular pH. It was observed that the transport activity was increased in an approximately linear way with the diminution of the pH from 8 to 5.5. The activity reached maximum values at a pH range between 4.5 and 5, decaying at pH 4. When the activity was assayed in the range of pH corresponding to the maximum activity, it was increased by approximately two times with respect to control conditions (PBS at a pH 7.4). The arginine transport activity was also evaluated in the presence of the F_0F_1 inhibitor oligomycin, the H^+ gradient uncoupling FCCP, and a mixture of rotenone and antimicin A as respiratory chain inhibitors. The activity was not affected when the parasites were treated with oligomycin or the mixture of rotenone/antimicin A, immediately or preincubated by 30 min previous to the assay. The H^+ ionophore FCCP decreased the arginine transport by approximately 50% (significantly different in t test at $p < 0.05$) when used alone or after a 30 min incubation in the presence of oligomycin (Table 2). The H^+ gradient disruption at the cytoplasmic membrane level was determined by fluorescence with Rhodamine 123 (not shown), and was attained independently of the preincubation of the parasites for 30 min with oligomycin.

3.5. Arginine transport along the parasite growth curve and life cycle

Trypanosoma cruzi epimastigote cells were assayed for arginine transport during the exponential and stationary phases of culture growth. During the interval between the fourth and 14th day of culture arginine transport decreased about 4-fold, from 124 pmol min^{-1} per 10^7 cells (±15) on the fourth day to 33 pmol min^{-1} per 10^7 cells (±2.8) on the 14th day. Arginine transport was about 2,7-fold lower in trypomastigote than epimastigote forms from the 7th day of culture (Table 2). It is worth to mention that LIT cultured cells were at least 95% epimastigote form. These results are quite different to those obtained for the high-affinity arginine permease, which is completely inactive on the stationary phase of parasite growth as well as in trypomastigote cells [14].

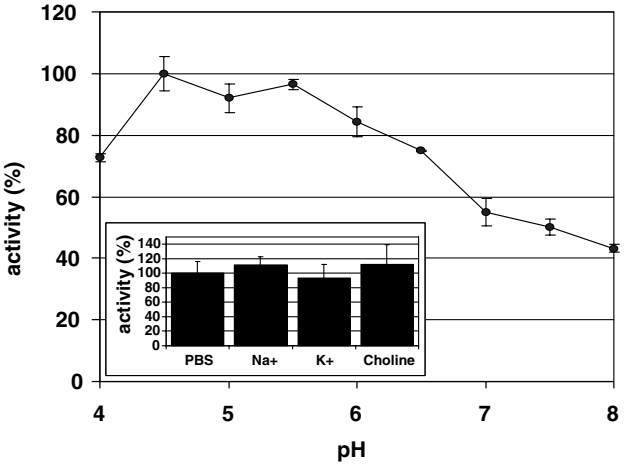

Fig. 3. Effects of pH and monovalent cations on arginine transport. Initial transport velocities (V_0) were measured at pH ranging from 4 to 8. The inset showed the effect of added cations on L-arginine uptake. Arginine transport was measured in phosphate buffer saline (145 mM Na^+ and 4.5 mM K^+) or in phosphate buffers containing only Na^+ (149.5 mM) or K^+ (149.5 mM) as cations. Choline (139.7 mM) was used as control. The 100% of L-arginine transport correspond to 106.9 pmol min^{-1} per 10^7 cells.

Table 2
Effects of inhibitors and parasite stage regulation of arginine transport

Treatment	Activity ± SD (%)
Control	100 ± 8.1
+ Oligomycin (5 μg ml^{-1})	97.2 ± 12.4
+ Oligomycin (5 μg ml^{-1})[a]	84.2 ± 9.5
+ Rotenone (200 μM) + antimycin A (0.5 μM)	93.0 6.3
+ Rotenone (200 μM) + antimycin A (0.5 μM)[a]	95.1 ± 5.6
+ FCCP (0.01 μM)	91.62 ± 12.3
+ FCCP (0.05 μM)	52.95 ± 11.8
+ FCCP (0.5 μM)	45.08 ± 5.3
+ FCCP (0.5 μM) + oligomycin (5 μg ml^{-1})	52.5 ± 10.1
Epimastigotes (day 7)	100 ± 11.0
Trypomastigotes	36.8 ± 3.4

Trypanosoma cruzi epimastigotes or trypomastigotes were washed (see Section 2) and resuspended in phosphate-buffered saline at 28 °C, containing the desired inhibitor. Radioactive L-arginine (2 mM, 0.4 mCi) was immediately added. For long-term inhibitions cells were resuspended in buffer containing the inhibitors and preincubated for 30 min before radioactive arginine was added. Incorporations were calculated as percentage of the controls± SD. All assays were performed at least in triplicates and data are representative of at least three independent experiments.

[a] Preincubated for 30 min at 28 °C.

4. Discussion

Trypanosoma cruzi has a complex life cycle which occurs in a wide variety of environments with very different compositions, mainly the insect vector gut, mammalian blood and mammalian host cell cytoplasm, imposing variable nutritional conditions to the parasite stages. In this sense, we reinforce the importance to understand the *T. cruzi* adaptive changes in nutrient transport processes and energy metabolism [21]. In the present work, a novel arginine transport activity was identified and characterized in *T. cruzi*. The component of the arginine transport system herein described, has low-affinity for its substrate L-arginine (estimated apparent K_m: 350 μM) and a moderate transport capacity with a maximum velocity of 97 pmol min^{-1} per 10^7 cells. This system presents low substrate specificity, since its activity was competed by methionine, and it resulted partially inhibited by lysine, tyrosine and glycine. The driving force was also determined. This activity was not dependent on the Na^+ or K^+ concentration. However, it showed strong pH dependence, suggesting that the extracellular pH is a relevant factor in the regulation of this transport activity. The hypothesis of a H^+/arginine symporter activity was suggested by the fact that inhibitors of the ATP synthesis or the respiratory chain affected poorly the activity, but the H^+ ionophore FCCP diminished it by more that 50%.

The amino acid transporters of *T. cruzi* studied up to now consisted of two L-proline transport systems [13], and a high-affinity arginine transporter [7]. The high-affinity arginine permease presents a K_m and V_{max} values of 4.2 μM and 12 pmol min^{-1} per 10^7 cells indicating that both systems function in strongly different conditions in terms of substrate concentration. As arginine is an amino acid involved in energy management, and it can function as energy reserve, its availability in the intracellular medium is critical for parasite survival. The fact that *T. cruzi* posses different arginine permeases warrant the arginine supplies in low and high arginine concentration environments. For example, the arginine concentration in mice plasma varies in the range of 87–290 μM [15]. The arginine concentration inside the cytoplasm of several human cells is in the range of 0.1 and 1 mM [15], and the free arginine concentrations in the triatomine gut are in the range 0.3–0.8 mM [16]. However, the concentration of free L-arginine must reach maximum values during the blood ingestion, near the concentration in the mammalian host blood, and it presumably decays to lower values along the insect vector gut due to the intestinal absorption. In this context, it is not surprising to find more than one transport system presenting different K_m, and so, having the ability to function in such different contexts. In the case of proline transport, a high-affinity system was defined, with a K_m of 0.31 mM and V_{max} of 6 pmol min^{-1} per 10^7 cells, and a low-affinity system presenting values of K_m and V_{max} of 1.36 mM and 32.5 pmol min^{-1} per 10^7 cells [13]. The results obtained for the arginine transporter herein described have some characteristics that resemble the high affinity proline transport system such as their K_m values and the driving force. However, their capacities, in terms of V_{max}, are quite different, probably due to the differences in cell requirements for their substrates. In a first view, these facts may suggest the existence of a single transporter with broad specificity that may function as arginine and proline permease. However, the high-affinity L-proline transport activity was not competed by 10-fold excess of arginine, suggesting that they are different transport systems [13].

The calculated activation energy for the low-affinity arginine transporter is 324 kJ mol^{-1}, about 10-fold higher than the value corresponding to the high-affinity system (31.1 kJ mol^{-1}) [7]. Interestingly, as occurs with the L-proline low-affinity system, the low-affinity arginine transporter presented a strong variation in terms of activity between 25 and 40 °C. This characteristic could be related with an adaptive advantage as *T. cruzi* is subjected to a wide variation in environmental temperatures around 28 °C in the midgut of the insect vector and 37 °C in the mammalian host. Thus, the increase in arginine uptake capacity may have physiological significance when temperature is raised from 28 °C, the optimal growth temperature of insect stage forms, to 37 °C, the temperature at which the infection in the mammalian host is carried out. The existence in *T. cruzi* of two arginine transport systems showing a wide variation of activation energies confers on the parasite the ability of having functionally stable arginine transport systems in a broad range of thermodynamic conditions. The arginine transport through the low-affinity system is developmentally regulated since it is about 2.7 times higher in epimastigotes than in trypomastigotes. However, it is worth to stress that arginine is actively transported in trypomastigotes by this system instead of the previously reported high-affinity one [14]. These data, taken together with the expression pattern of arginine kinase [14], a key enzyme involved in the arginine metabolism in this parasite, strongly suggest that arginine is also essential in the infective mammalian host stage.

The presence of amino acid transporters with characteristics of H^+ amino acid symporters and low specificity, is compatible with the recent observation that there exists a particularly large family of amino acid transporter genes corresponding to the amino acid/auxin permease family (AAAP, TC 2.A.18) in *T. cruzi* ([22] and Bouvier et al. unpublished). One of the characteristics of some members of this family is the fact that their activity is H^+ dependent. Our group has recently identified at least 60 genes corresponding to this group of permeases in the *T. cruzi* genome, but the confirmation of this hypothesis requires further investigation.

Considering that the transport of amino acids is in fact the first step of their metabolic pathways the characterization of the corresponding transport systems in *T. cruzi* is potentially important for metabolic research and trypanocidal drug design.

Acknowledgements

We are deeply grateful to Prof. M. Júlia M. Alves for helpful advice and comprehension. This study was supported in part by Consejo Nacional de Investigaciones Científicas y Técnicas (Argentina), University of Buenos Aires (Argentina), Agencia Nacional de Promoción Científica y Tecnológica (Argentina), Fundación Antorchas (Argentina), and Fundação de Amparo à Pesquisa do Estado de São Paulo (Brazil). C.A.P. is career member of CONICET and L.A.B. is fellow of the latter institution A.M.S. is fellow of the Fundação de Amparo à Pesquisa do Estado de São Paulo (Brazil). We would also like to extend special thanks to Ursula C. Vermelha Urias and Prof. Gina P. R. de Madeira for critically reading the manuscript.

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