Arginine metabolism in *Trypanosoma cruzi* is coupled to parasite stage and replication

Claudio A. Pereira[a,1], Guillermo D. Alonso[a,1], Soledad Ivaldi[a], Ariel Silber[b], Maria Júlia M. Alves[b], León A. Bouvier[a], Mirtha M. Flawiá[a], Héctor N. Torres[a,*]

[a] *Instituto de Investigaciones en Ingeniería Genética y Biología Molecular, Consejo Nacional de Investigaciones Científicas y Técnicas, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, INGEBI, Vuelta de Obligado 2490, 1428 Buenos Aires, Argentina*
[b] *Laboratório de Bioquímica de Parasitas, Instituto de Química, Universidade de São Paulo, São Paulo, Brazil*

Received 7 May 2002; revised 26 July 2002; accepted 28 July 2002

First published online 7 August 2002

Edited by Judit Ovádi

Abstract **L-Arginine plays an essential role in the energetic metabolism of *Trypanosoma cruzi*. In this work we propose a relationship between L-arginine uptake, arginine kinase activity and the parasite replication ability. In epimastigote cultures L-arginine uptake decreases continuously accompanying a cell replication rate reduction. The use of conditioned or fresh medium mimics uptake variations. Interestingly, in non-replicative trypomastigote cells, L-arginine uptake was undetectable. The association between L-arginine uptake and cell replication was demonstrated using the antimitotic agent hydroxyurea. Arginine kinase, the enzyme responsible for phosphoarginine and ATP synthesis, also shows a differential activity in epimastigote and trypomastigote parasite stages.** **© 2002 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.**

Key words: Arginine transport; Arginine uptake; Arginine kinase; Phosphoarginine; *Trypanosoma cruzi*

1. Introduction

Knowledge of the intake and metabolism of medium components by parasites is essential for the design and improvement of anti-parasitic drugs. Intake mechanisms include passive diffusion, endocytosis, receptor-mediated uptake or transporter-mediated uptake [1].

Arginine is a key substrate for several metabolic pathways. Moreover, in *Trypanosoma cruzi*, the flagellated protozoan causing Chagas' disease, this amino acid is essential for in vitro cultivation of epimastigote cells [2].

A high-affinity, very specific L-arginine transport has been characterized in *T. cruzi*. In epimastigotes, this process undergoes saturation and is dependent on L-arginine concentration. Among several amino acids and their derivatives, only L-homoarginine, D-arginine, L-canavanine, L-ornithine and L-citrulline elicited a weak inhibition of the L-arginine transport. After uptake, the major product detected was characterized as phosphoarginine [3]. Phosphoarginine plays a critical role as an energy reserve, since the high-energy phosphate is easily transferred to ADP when ATP is needed. It has been proposed that phosphoarginine supports bursts of cellular activity before metabolic events such as glycogenolysis, glycolysis and oxidative phosphorylation are switched on [4–6]. Arginine kinase is a key enzyme of the energetic metabolism, described for the first time in *T. cruzi* by our laboratory [7]. Epimastigote growth rate and arginine kinase specific activity have been correlated. Enzyme amounts and specific activity increased continuously during exponential growth, reaching maximum values at the stationary phase of growth. Therefore, arginine kinase was proposed as a regulator of energetic reserves under starvation stress conditions [8]. Herein, we describe several ways to regulate L-arginine uptake such as medium composition, cell density, replication rate and L-arginine starvation. In addition, a relationship between arginine kinase specific activity, substrate transport and *T. cruzi* stages is proposed.

2. Materials and methods

2.1. Reagents

Reagents were from Sigma Chemical Co. (St. Louis, MO, USA).

2.2. Parasite cultures and cell extracts

Epimastigote cells 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/ml) supplemented with 10% heat-inactivated fetal calf serum (FCS), 100 U/ml penicillin, and 100 μg/ml streptomycin [9]. At the indicated times, cells from three independent culture flasks were counted using a hemocytometric chamber.

Trypomastigote cells (CL Brener strain) were obtained as previously described [10]. LLC-MK_2 cell monolayers were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% FCS at 37°C. During infection, the FCS concentration was reduced to 2%. The trypomastigotes, released from the LLC-MK_2 cultures around day 5–6 after inoculation, were washed three times in DMEM before being used for further experiments. Each LLC-MK_2 culture flask was used for a single trypomastigote infection. Aliquots (0.1–2 ml) were harvested by centrifugation at $1500\times g$ for 10 min and washed three times with phosphate-buffered saline (PBS). Trypomastigotes were counted using a hemocytometric chamber and viability was assessed by direct microscopic examination.

Epimastigote or trypomastigote cell pellets were then resuspended in 50 mM HEPES buffer, pH 7.3, containing 0.01 mg/ml leupeptin, 25 U/ml aprotinin and 0.5 mM phenylmethylsulfonyl fluoride, and lysed by six cycles of freezing in liquid N_2 and thawing at 4°C. The extracts were then centrifuged at $10\,000\times g$ for 10 min.

2.3. Uptake assays

Aliquots of epimastigote or trypomastigote cell cultures (3×10^7 parasites), grown for the mentioned periods of time, were centrifuged at $8000\times g$ for 30 s, and washed once with PBS. Cells were then resuspended in 1 ml of PBS, preincubated 2 h at 30°C to deplete the endogenous L-arginine pool [11,12], and then centrifuged at

*Corresponding author. Fax: (54)-11-4786 8578.
E-mail address: torres@dna.uba.ar (H.N. Torres).

[1] C.A.P. and G.D.A. are joint first authors.

0014-5793/02/$22.00
PII: S0014-5793(02)03157-5

8000×*g* for 30 s. Cells were then again resuspended in 1 ml of PBS containing 25 μM L-[2,3-^{3}H]arginine (NEN/DuPont, Boston, MA, USA; 0.5 μCi). Following incubation for 10 min at 30°C, cells were centrifuged as indicated above, and washed with 1 ml of ice-cold PBS. Pellets were then resuspended in 1 ml of water and counted for radioactivity in UltimaGold XR liquid scintillation cocktail (Packard Instrument Co., Meriden, CT, USA). Non-specific uptake and carry-over were measured in uptake mixtures containing 100 mM L-arginine [3]. Assays were run at least in triplicate. Cell viability was assessed by direct microscopic examination.

2.4. Arginine kinase assay

The incubation mixture contained 25 mM HEPES buffer, pH 7.3, 2 mM ATP, 5 mM $MgCl_2$, 10 mM 2-mercaptoethanol, 1 mM L-[2,3-^{3}H]arginine (NEN Life Science Products, Boston, MA, USA; 0.5 μCi per assay), and the enzyme source (8 μg of protein), in a total volume of 0.2 ml. Incubations were carried out 10 min at 30°C. Reactions were stopped by the addition of 1 ml of 25 mM HEPES buffer, pH 7.3, containing 10 mM L-arginine and 5 mM EDTA (stop buffer). The mixtures were then resolved by passage through a strong anion exchange resin, Dowex AG 1-X4, 200–400 mesh, chloride form (1 ml, Bio-Rad Laboratories, Hercules, CA, USA) mounted into tulip columns and equilibrated with stop buffer. After loading the samples, the columns were washed with 3 ml of 25 mM HEPES buffer, pH 7.3, and eluted with 2 ml of 1 M NaCl, 0.1 N HCl [7]. Results are the average of triplicate assays. The authenticity of phosphoarginine as reaction product was validated by procedures described elsewhere [7].

2.5. Western blot analysis

SDS–PAGE of protein samples was carried out as described by Laemmli [13]. Polypeptides were electrotransferred from polyacrylamide gels to Hybond-C membranes (Amersham Pharmacia Biotech, Piscataway, NJ, USA). For antibody reaction, the transferred membranes were blocked with a 5% (w/v) non-fat milk suspension for 2 h, after incubation for 2 h with a 1:1000 dilution of the mouse polyclonal anti-arginine kinase antiserum obtained previously in our laboratory [7]. Detection was carried out by incubating with a 1:2000 dilution of sheep anti-mouse IgG conjugated with horseradish peroxidase (Amersham Pharmacia Biotech). The latter was developed with the Renaissance Western blot Chemiluminescence Reagent Plus (NEN Life Science Products).

2.6. Hydroxyurea treatment

Hydroxyurea acts as an inhibitor of ribonucleotide reductase. Consequently, DNA replication is inhibited and parasite replication is arrested. Hydroxyurea has been used to arrest the cell cycle of a wide variety of cells from the lower eukaryote order Kinetoplastida, including *T. cruzi* [14]. *T. cruzi* epimastigote cells (10^6 cells/ml) were grown in LIT medium for 2 days. A filtered hydroxyurea solution was added to the cell cultures to a final concentration of 20 mM. After 24 h of treatment cells were washed twice in PBS and resuspended in conditioned medium from control cells (without treatment) corresponding to the same day of culture for 24 h [14]. Cell viability was assessed by direct microscopic examination.

2.7. Analytical methods

Protein was determined according to Bradford [15].

3. Results

3.1. Arginine uptake during epimastigote growth

During the interval between the 3rd (d3) and 15th day (d15) of epimastigote cell cultures, L-arginine uptake decreased continuously from 30.85 pmol/min/10^7 cells (±0.62) on d3 to 1.47 pmol/min/10^7 cells (±0.13) on d15 (approximately 21-fold). Fig. 1A shows the inverse correlation between L-arginine uptake and cell density, the latter increasing approximately 12.5-fold in the same period of time while the replication rate decreased from 1.93 to 0.43 cell duplications per day (Fig. 1B).

In order to check whether medium components might influence L-arginine uptake, epimastigote cell cultures from d3 were incubated for 16 h in conditioned medium obtained from 15-day grown parasites (d15 medium) or cells from d15 were incubated in fresh medium. A 9.7-fold increase in the L-arginine uptake was observed when d15 parasites were treated with fresh medium, and a 5.8-fold decrease was observed when d3 parasites were treated with conditioned medium from d15 parasites (Table 1).

To test the kinetics of the increase in L-arginine uptake, d15 parasites were assayed after a fresh medium treatment for 2, 4, 8, and 16 h. L-Arginine uptake increased by a rate of

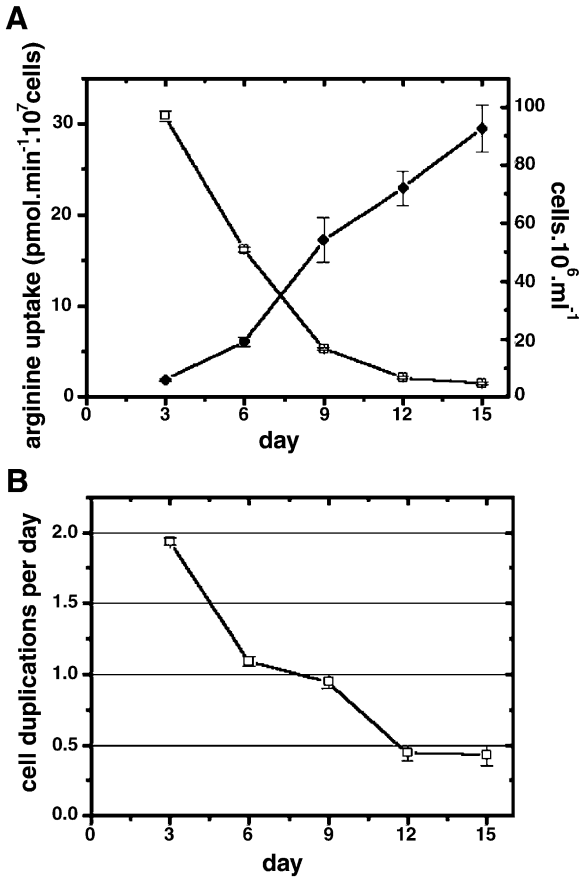

Fig. 1. *T. cruzi* L-arginine uptake as a function of cell growth. A: Cell densities (●) and L-arginine uptake (□) in epimastigotes growing between d3 and d15. B: Cell replication rate (number of cell divisions per day), calculated as the average at 3-day intervals.

Table 1
L-Arginine uptake under different culture conditions

Condition	V_{max} (pmol/min/10^{-7} cells)	S.D. (±)
(a) d3	30.85	0.62
(b) d3+CM	5.32*	0.14
(c) d3 w/o starv	9.07*	0.21
(d) d15	1.47	0.13
(e) d15+FM	14.26*	0.22
(f) d15 w/o starv	0.86*	0.06

D3 (and d15 epimastigote cells were assayed for arginine. Control cells without treatment (a and d); d3 cells treated with conditioned medium (CM, b); d3 and d15 cells without starvation (w/o starv, c and f) and d15 cells treated with fresh medium (FM, e).
*$P < 0.05$, significantly different in *t*-test. Treated cultures (d3+CM, d3 w/o starv, d15+FM and d15 w/o starv) were compared with the untreated controls (d3 and d15 respectively).

1.33 pmol/min/10^7 cells per hour for the first 8 h and 0.27 pmol/min/10^7 cells per hour in the subsequent 8 h.

3.2. Influence of the culture medium conditions and L-arginine starvation on the L-arginine uptake

Modified LIT media were prepared omitting one or more components and then parasites from d15 were treated with the different fresh media for 16 h. All the treatments significantly increased the L-arginine uptake similarly to complete LIT medium, except the media without a carbon source, including tryptose, liver extract, glucose or serum in an additive manner. The number of parasites was normalized in all treatments (data not shown).

In *T. cruzi*, the absence of L-arginine in the pre-incubation medium (PBS or PBS plus 11 mM glucose) increased the uptake rate of d3 parasites 3.4-fold with 3 h of pre-incubation time. When cells were pre-incubated with 10 mM unlabeled L-arginine, washed to eliminate the amino acid, and then subjected to a standard uptake assay, the increase in the uptake rate was abolished. No effects were observed when L-arginine was replaced by other amino acids (data not shown). When parasites from d15 were subjected to starvation for 3 h the increment was 1.7-fold (Table 1).

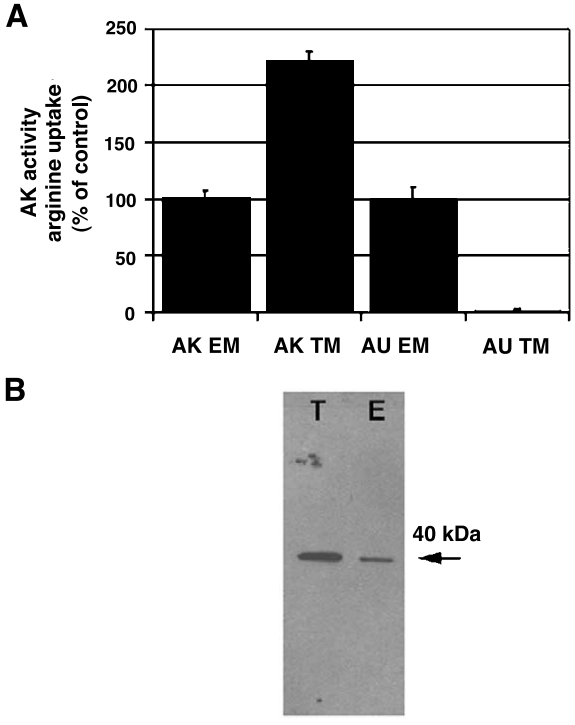

Fig. 2. Arginine kinase activity and expression, and L-arginine uptake in epimastigote and trypomastigote cells. A: Epimastigote (EM) and trypomastigote (TM) cells were assayed for arginine kinase activity (AK) (10 μg of soluble extracts) and L-arginine uptake (AU). The results were expressed as percentage of the epimastigote corresponding values. B: Western blot analysis. SDS–PAGE of protein samples (30 μg) from trypomastigote (T) and d7 epimastigote (E) cells were transferred to a Hybond C membrane and incubated with a mouse polyclonal arginine kinase antiserum. Arrow indicates the band corresponding to arginine kinase (40 kDa).

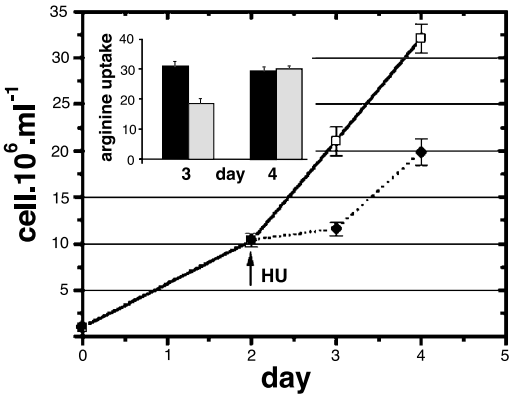

Fig. 3. Effect of hydroxyurea treatment on L-arginine uptake. Epimastigote cells were grown for 2 days in LIT medium. One culture was treated with the antimitotic hydroxyurea (arrow). The graph represents the parasite density of the hydroxyurea-treated culture (●) or the control culture (□). The inset shows the L-arginine uptake by the control (black columns) or by the hydroxyurea-treated culture (gray columns).

3.3. Relationship between the L-arginine uptake and arginine kinase activity along the parasite growth curve and life cycle

Previous reports from our group describe the increase in arginine kinase expression and activity along the parasite growth phases [8]. These data suggest a relationship between arginine kinase activity and L-arginine uptake with the cell replication capacity. In order to test the latter hypothesis, these processes were investigated in trypomastigotes, the non-replicative stage of *T. cruzi*. Arginine kinase activity from cell culture-derived trypomastigotes was 2.5-fold higher (0.3 nmol/min/mg protein) than that from d7 epimastigotes (0.12 nmol/min/mg protein). These results were confirmed by Western blot analysis of the expressed enzyme (Fig. 2A,B). L-Arginine uptake was at least 30-fold lower in trypomastigotes than in d7 epimastigotes (Fig. 2A). It is worth mentioning that LIT cultured cells were at least 95% epimastigote stage.

3.4. Effect of cell division arrest on L-arginine uptake

Epimastigote cells from d2 were treated with the antimitotic hydroxyurea (20 mM) in LIT medium. After 24 h of treatment, the cell replication rate decreased from 1.02 (control) to 0.11 (hydroxyurea) duplications per day and the L-arginine uptake decreased by 40% with respect to the control values (Fig. 3). When cells were washed and reinoculated into a conditioned medium from control cells (d3) for 24 h, the hydroxyurea-treated group recovered 100% of the L-arginine uptake and the replication rate reached 0.8 cell duplications per day (Fig. 3). No hydroxyurea-mediated toxicity was observed during the 24-h exposure.

4. Discussion

Nutritional conditions during the *T. cruzi* life cycle impose a restriction to energy availability. The parasites are exposed to a wide range of conditions during their complex life cycle. In the mammalian host the trypomastigote and amastigote cellular stages benefit from a rather constant environment, including a steady supply of glucose and amino acids. In

contrast, feeding status determines variations in the availability of nutrients for the epimastigote stage in the insect vector [16]. These data reinforce the importance of understanding the *T. cruzi* adaptive changes in nutrient transport processes and energetic metabolism.

In this work we report a continuous decrease in the L-arginine uptake accompanying the reduction in parasite replication rates, together with an increase in cell density and consequent nutrient depletion of the medium. Furthermore, L-arginine uptake shows a negative correlation with the previously described increase in the arginine kinase expression and activity [8]. Treating the cells with conditioned medium or fresh medium mimics L-arginine uptake variations. In addition, omission of any carbon source in LIT medium increases the L-arginine uptake at a lower rate when compared to the complete medium, and decreases parasite replication. It is postulated that changes in medium conditions and/or the appearance of a density-derived factor that affects cell replication ability, modulate L-arginine uptake.

Previous works report substrate-regulated arginine transporters, such as mammalian CAT-1 and CAT-2 [17–19], and this effect is termed adaptive regulation. Accordingly, L-arginine uptake was herein reported to increase with L-arginine starvation.

L-Arginine uptake and arginine kinase activity were also investigated in the non-replicative form, trypomastigote. The absence of L-arginine uptake and the higher rate of arginine kinase activity observed in trypomastigotes, when compared to the replicative cellular stage epimastigote, led us to postulate a correlation between cell replication and arginine metabolism. In addition, the results obtained with the cells treated with the antimitotic hydroxyurea support this hypothesis.

In trypanosomatids, amino acids can be used as a carbon source, in particular, insect stages grow even better on amino acids than glucose. In addition, 98–99% of the total carbon consumption is used in the replication process [20]. In vivo, the epimastigote cell replication rate is dramatically altered depending on the nutritional state of the insect vector [16]. Taking these data into account, it can be postulated that under nutritional stress conditions, the cell replication rate decreases and the parasites can use phosphoarginine reserves in order to synthesize ATP whereas the produced free arginine can be used as a carbon source. The replication-associated decrease in the arginine flux facilitates the arginine kinase reaction in the ATP synthesis direction.

Acknowledgements: This study was supported in part by the Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET, Argentina), the University of Buenos Aires (UBA), the Agencia Nacional de Promoción Científica y Tecnológica (Argentina), and the Third World Academy of Sciences (TWAS) (Trieste, Italy), and the World Health Organization-Tropical Disease Research (WHO-TDR). H.N.T., M.M.F. and C.A.P. are Career Members of CONICET; G.D.A. and S.I. are fellows of the latter institution. We are deeply grateful to Professor Walter Colli for critically reading the manuscript.

References

[1] de Koning, H. (2001) Int. J. Parasitol. 31, 512–522.
[2] Dusanic, D.G. (1992) in: Parasitic Protozoa, Vol. 1 (Kreier, J.P. and Baker, J.R., Eds.), pp. 137–194, Academic Press, London.
[3] Pereira, C.A., Alonso, G.D., Paveto, M.C., Flawiá, M.M. and Torres, H.N. (1999) J. Eukaryot. Microbiol. 46, 566–570.
[4] Hird, F. (1986) Comp. Biochem. Physiol. 85B, 285–288.
[5] Huennekens, F.M. and Whiteley, H.R. (1960) in: Comparative Biochemistry, Vol. 1 (Florkin, M. and Mason, H.S., Eds.), pp. 107–180, Academic Press, New York.
[6] Morrison, J.F. (1973) In: The Enzymes, Vol. 8 (Boyer, P.D., Ed.), pp. 457–486, Academic Press, New York.
[7] Pereira, C.A., Alonso, G.D., Paveto, C., Iribarren, A., Cabanas, M.L., Torres, H.N. and Flawiá, M.M. (2000) J. Biol. Chem. 275, 1495–1501.
[8] Alonso, G.D., Pereira, C.A., Remedi, S., Paveto, C., Cochella, L., Ivaldi, S., Gerez de Burgos, M., Torres, H.N. and Flawiá, M.M. (2001) FEBS Lett. 498, 22–25.
[9] Castellani, O., Ribeiro, L.V. and Fernandes, J.F. (1967) J. Protozool. 14, 447–451.
[10] Andrews, N. and Colli, W. (1982) J. Protozool. 29, 264–269.
[11] Low, B.S. and Grigor, M.R. (1995) J. Biol. Chem. 270, 27577–27583.
[12] White, M.F. and Christensen, H.N. (1982) J. Biol. Chem. 257, 4450–4457.
[13] Laemmli, U.K. (1970) Nature 227, 105–132.
[14] Galanti, N., Dvorak, J., Grenet, J. and McDaniel, J. (1994) Exp. Cell Res. 214, 225–230.
[15] Bradford, M.M. (1976) Anal. Biochem. 72, 248–254.
[16] Kollien, A.H. and Schaub, G.A. (2000) Parasitol. Today 16, 381–387.
[17] Hyatt, S.L., Aulak, K.S., Malandro, M., Kilberg, M.S. and Hatzoglou, M. (1997) J. Biol. Chem. 272, 19951–19957.
[18] Kakuda, D.K., Finley, K.D., Maruyama, M. and MacLeod, C.L. (1998) Biochim. Biophys. Acta 1414, 75–84.
[19] Aulak, K.S., Mishra, R., Zhou, L., Hyatt, S.L., de Jonge, W., Lamers, W., Snider, M. and Hatzoglou, M. (1999) J. Biol. Chem. 274, 30424–30432.
[20] ter Kuile, B.H. (1997) J. Bacteriol. 179, 4699–4705.