FEBS Letters 498 (2001) 22–25 FEBS 24898

Arginine kinase of the flagellate protozoa *Trypanosoma cruzi*

Regulation of its expression and catalytic activity

Guillermo D. Alonso[a], Claudio A. Pereira[a], María S. Remedi[b], M. Cristina Paveto[a], Luisa Cochella[a], M. Soledad Ivaldi[a], Nelia M. Gerez de Burgos[b], Héctor N. Torres[a], Mirtha M. Flawiá[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, Vuelta de Obligado 2490, 1428 Buenos Aires, Argentina*
[b] *Cátedra de Química Biológica, Facultad de Medicina. Universidad Nacional de Córdoba, Córdoba, Argentina*

Received 30 January 2001; revised 19 March 2001; accepted 3 May 2001

First published online 15 May 2001

Edited by Judit Ovádi

Abstract In epimastigotes of *Trypanosoma cruzi*, the etiological agent of Chagas' disease, arginine kinase activity increased continuously during the exponential phase of growth. A correlation between growth rate, enzyme-specific activity and enzyme protein was observed. Arginine kinase-specific activity, expressed as a function of enzyme protein, remains roughly constant up to 18 days of culture. In the whole range of the culture time mRNA levels showed minor changes indicating that the enzyme activity is post-transcriptionally regulated. Arginine kinase could be proposed as a modulator of energetic reserves under starvation stress condition.

Key words: Phosphagen; Arginine kinase; Guanidino kinase; Phosphoarginine; *Trypanosoma cruzi*

1. Introduction

Arginine kinase (EC 2.7.3.3) belongs to a family of conserved proteins with phosphotransferase activity, the guanidino kinases, being creatine kinase the best-known member. These kinases catalyze the synthesis of *N*-phosphorylated guanidino compounds by the reversible transfer of a phosphoryl group from ATP to an enzyme-specific guanidino acceptor [1]:

$$\mathrm{MgATP} + \text{guanidino acceptor} \leftrightarrow \text{P-guanidino acceptor} + \mathrm{MgADP} + \mathrm{H^+}$$

Phosphoarginine plays a crucial role as an energy reserve because the high-energy phosphate can be transferred when a renewal of ATP is needed. It has been proposed that phosphoarginine supports bursts of cellular activity until catabolic events such as glycogenolysis, glycolysis and oxidative phosphorylation are switched on [1,2]. Guanidino kinases have been reported widely distributed among metazoan organisms [3], but only recently, we have presented the first description of a guanidino kinase in a flagellar unicellular parasite: the arginine kinase from *Trypanosoma cruzi* [4], the etiological agent of Chagas' disease in Latin America. *T. cruzi* arginine kinase gene encodes a 357-amino acids polypeptide with a deduced molecular weight of 40 201. The amino acid sequence shows all the characteristic consensus blocks of the guanidino kinase family, and a putative 'actinine-type' actin-binding domain [4].

In *T. cruzi* the relationship between arginine kinase activity and energy requirements is a relevant and unresolved issue. The most energy-demanding processes in trypanosomatids are cell division, motility, stage differentiation and host-cells invasion [5]. Nutritional conditions during the *T. cruzi* life cycle also impose restriction to energy availability. The parasites support a wide range of conditions during its complex life cycle. For example, in the mammalian host the trypomastigote and amastigote stages benefit from a rather constant environment, including a steady supply of glucose and amino acids; on the contrary in the insect vector, the feeding status determines variations in the availability of nutrients for the epimastigote forms [6].

Taking into account these facts and the relevance of *N*-phosphorylated guanidino compounds in energy-transfer reactions, the present studies were thought as the first step in the study of the arginine kinase regulation in relation to changes in the composition of the culture medium during the in vitro parasite growth phases.

2. Materials and methods

2.1. Reagents

Enzymes and molecular biology reagents were provided by Promega Corporation (Madison, WI, USA), other reagents were from Sigma Chemical Co. (St. Louis, MO, USA).

2.2. Parasite cultures and cell extracts

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 ml) supplemented with 10% fetal calf serum, 100 U/ml penicillin, and 100 μg/ml streptomycin [7]. At the indicated times, cells were counted using a hemocytometric chamber. Aliquots (0.1–1.6 ml) were harvested by centrifugation at $1500 \times g$ for 10 min and washed

*Corresponding author. Fax: (54)-11-4786 8578.
E-mail: mflawia@proteus.dna.uba.ar

PII: S0014-5793(01)02473-5

three times with phosphate-buffered saline. Cell pellets were then resuspended in 50 mM HEPES buffer, pH 7.3, containing 0.01 mg/ml leupeptin, 25 U/ml aprotinin, 0.5 mM phenylmethylsulfonyl fluoride and 14 mM 2-mercaptoethanol, 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. Arginine kinase assay

The incubation mixture contained 25 mM HEPES buffer, pH 7.3, 2 mM ATP, 5 mM magnesium acetate, 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 enzyme source (8 μg of protein), in a total volume of 0.2 ml. Incubations were carried out 10 min at 30°C, and 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, AG 1-X4, 200–400-mesh chloride form (Bio-Rad Laboratories, Hercules, CA, USA) mounted into 1-ml tulip columns 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 [4].

Under the assay conditions enzyme activities were proportional to incubation time and enzyme protein. No change in the kinetic parameters of arginine kinase has been observed in extracts from cell cultures performed at different periods.

Experiments were carried out in three independent flask cultures from which three samples were taken at each indicated time for cell counting and enzyme assay. Enzyme activities were expressed as μmol of [^{3}H]arginine converted to [^{3}H]phosphoarginine per min of incubation per mg of protein in the extract. Enzyme-specific activities were given as μmol of [^{3}H]arginine converted to [^{3}H]phosphoarginine per min of incubation per mg of enzyme protein. The latter was calculated from data obtained from Western blots of each extract revealed with a specific anti-arginine kinase antibody (see below) referred to those performed with homogeneous recombinant *T. cruzi* arginine kinase. In studies of enzyme-specific activities, as a function of growth time, the amount of protein representing the same enzyme activity (expressed as μmol per min of incubation) was loaded in each gel lane.

2.4. Western and Northern blot analysis

Procedures for SDS–PAGE of protein samples were performed as described by Laemmli [8]. Polypeptides were electrotransferred from polyacrylamide gels to Hybond-C membranes (Amersham Pharmacia Biotech). Arginine kinase antiserum was obtained from female mice, BALB/c strain, immunized by an intraperitoneal injection of 50 mg of recombinant arginine kinase [4] plus 0.1 ml of incomplete Freund's adjuvant followed, 15 days later, by a booster intraperitoneal injection of 50 mg of recombinant protein. After 15 days, mice were bled by exposing the ocular cavity. For reaction with the antibody, 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, detection was carried out by incubating with a 1:2000 dilution of sheep anti-mouse Ig conjugated with horseradish peroxidase (Amersham Pharmacia Biotech). The latter was developed with the Renaissance Western blot Chemiluminescence reagent plus (NEN Life Science Products).

Total RNA, for Northern blot analysis, was isolated from 10^8 cells, using the RNAgents/Total RNA isolation system (Promega, Madison, WI, USA). Samples were electrophoresed on a 1.5% agarose under denaturing conditions, transferred to a Hybond N+ nylon membrane (Amersham Pharmacia Biotech) and hybridized with the radiolabeled full-length arginine kinase gene as a probe [4].

Enzyme and mRNA levels were determined in three independent experiments.

2.5. Analytical methods

Glucose and ammonia concentration of the media were estimated using the reducing sugar [9,10] and indophenol colorimetric reactions respectively (for details on this latter reaction, see: http://ecosystems.mbl.edu/ARC/data_doc/ammonia.html). Protein was determined according to Bradford [11]. Densitometric analysis of electrophoretic bands was performed with the Scion Image software (http://www.scioncorp.com).

3. Results

3.1. Arginine kinase activity along the parasites growth curve

Arginine kinase activity in *T. cruzi* epimastigote cells increased continuously during the exponential phase of growth. However, when the culture reached the late exponential phase, the activity remained unchanged (Fig. 1A). Differences in activities between early and late exponential phases were higher than seven-fold. The inset of this figure shows the existence of a high degree of correlation ($y = 0.0036x$, $R^2 = 0.95$) between growth rate and enzyme-specific activity. In addition, maximum increase was observed between days 3 and 6 (three-fold). In order to study whether some component of the media influences the activity of arginine kinase, epimastigote cultures from day 3 were incubated for 24 h in medium obtained from parasites from day 9. An increase in the arginine kinase activity up to day-9 levels was observed (data not shown).

3.2. Arginine kinase expression analysis

The level of enzyme protein was evaluated by Western blotting in three independent cultures. A unique 40-kDa polypeptide band corresponding to the size of *T. cruzi* arginine kinase [4] was observed along the growth curve. The increase in

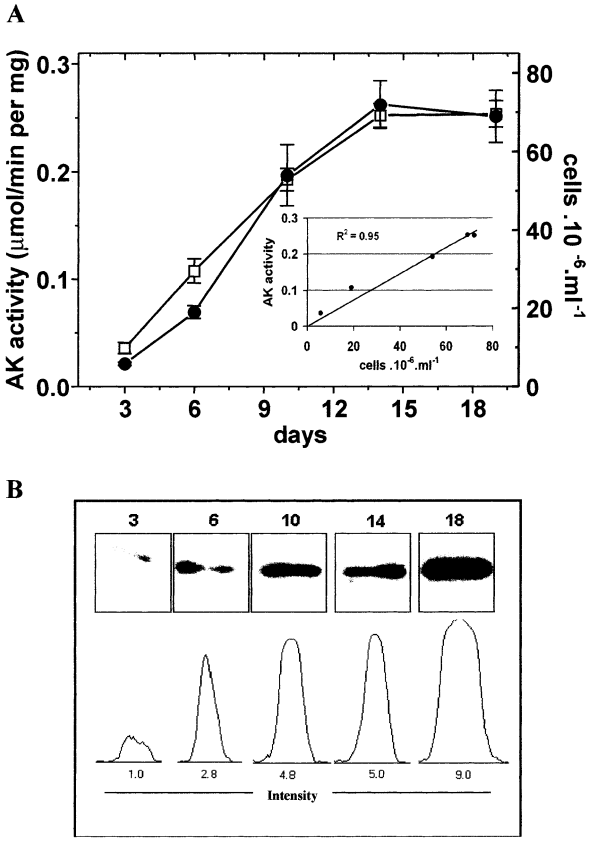

Fig. 1. *T. cruzi* arginine kinase-specific activity as a function of cell growth. A: Soluble extracts from days 3 to 19 were assayed for arginine kinase activity as described under Section 2 (empty squares). Solid circles indicate cell number. The inset shows the correlation between arginine kinase activity and cell number ($y = 0.0036x$, $R^2 = 0.95$). B: Western blot analysis. SDS–PAGE of protein samples (30 μg) from days 3 to 18 were transferred and incubated with 1:1000 dilution of mouse polyclonal anti-arginine kinase antiserum. Band quantification was performed by densitometry using the Scion Image program (Scion Corp. USA).

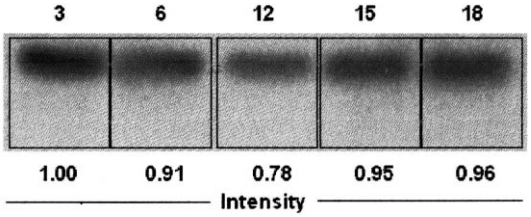

Fig. 2. Northern blot analysis of *T. cruzi* arginine kinase. Total RNA (20 μg) from days 3 to 18 was subjected to agarose–formaldehyde electrophoresis then, the transferred filter was hybridized with full-length arginine kinase gene as a probe. Ribosomal RNAs were used as size markers and loading control. Band quantification was performed by densitometry using the Scion Image program (Scion Corp. USA).

enzyme protein roughly resembled the increase in enzyme-specific activity (Fig. 1B).

It is important to emphasize that arginine kinase-specific activity, expressed, as a function of enzyme protein, remains roughly constant up to 18 days of culture. Values were 11.3, 11.0, 11.8, 13.4, and 10.1 μmol of [^{3}H]arginine converted to [^{3}H]phosphoarginine per min of incubation per mg of enzyme protein for the 3th, 6th, 10th, 14th and 18th day of culture respectively.

In order to establish if any transcription regulatory step was involved in the determination of arginine kinase activity levels, Northern blot analysis of total RNA population was performed in triplicate cultures. mRNA levels in one of the cultures showed minor variations during the whole range of culture time (Fig. 2), suggesting that the arginine kinase gene is not regulated at the transcriptional level.

3.3. Cell growth and culture medium conditions

In order to gather information on those cell culture parameters that may be involved in the regulation of energetic metabolism and the possible influence on the arginine kinase activity during *T. cruzi* growth, glucose and ammonia levels as well pH variations were monitored. As it is shown in Fig. 3A, the exhaustion of the carbon source glucose, widely preceded the beginning of the stationary phase and the arrest of the increase of enzyme-specific activity. In addition, the excretion of ammonia started coincidently with the diminution of glucose concentration. This suggests that a shift from hydrocarbon to nitrogen-containing carbon sources is operating after the 6th day of culture. As expected, the pH of the medium did not vary significantly during the first 3 days of culture, but strongly decreased about 1.15 units, between days 3 and 6 (Fig. 3B). This phenomenon could be attributed to the excretion of succinate, lactate and acetate, characteristic of the aerobic fermentation of trypanosomatids [12–14]. Over the 6th day, the pH value increases linearly at about 0.1 pH units per day, indicating the start-point of the oxidative metabolism of amino acids (Fig. 3B).

4. Discussion

The present study reports the characterization of arginine kinase levels from the protozoan parasite *T. cruzi* as a function of growth time. Enzyme activity and enzymatic protein levels increased continuously during the exponential phase of growth, the enzyme-specific activity remained constant.

mRNA levels showed minor changes indicating a post-transcriptional regulation. In addition, a clear correlation between growth rate and enzyme activity was observed, as well as a conditioned medium-dependent increase in the activity, followed by a complete reset of the enzyme to basal levels after each culture passage. These findings let us speculate that a high cell density-derived nutritional condition such as energetic stress or a yet to identify factor may up-regulate the level of protein expression and the enzyme activity.

The discrepancies observed from days 14 to 18 between the respective arginine kinase activities, that remain constant, and the increasing enzyme protein levels, might indicate a post-translational modification of the protein that reduce the enzyme activity.

In a time span of about 2 weeks of epimastigote cell growth in liquid medium, two different nutritional conditions were operative: during the first one glucose is consumed and the medium pH decreased continuously. The second one took place after the 6th day of culture and is characterized by

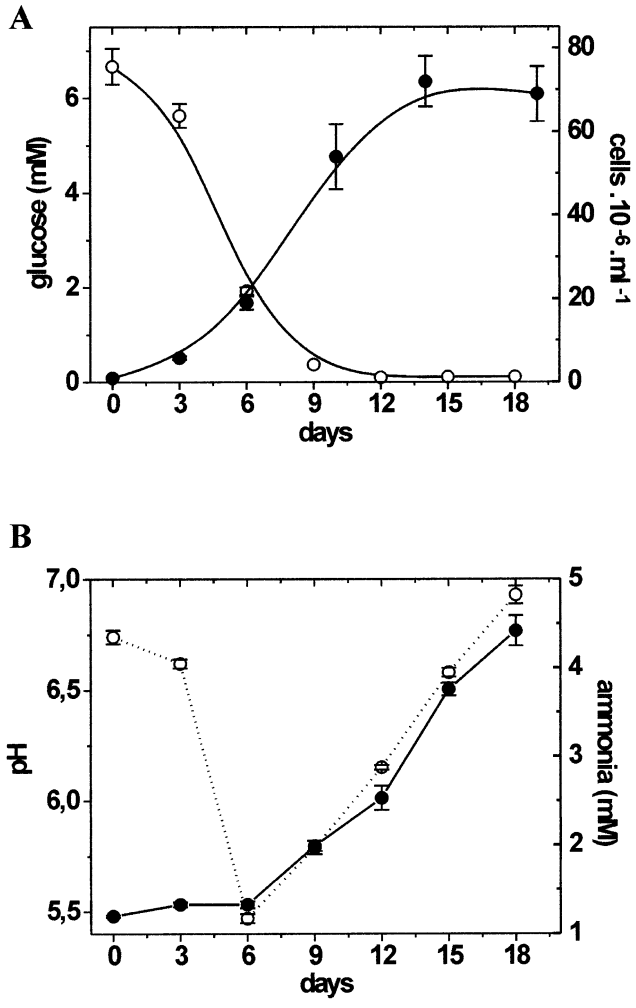

Fig. 3. Analysis of culture parameters as a function of cell growth. A: Glucose determination along the parasite growth curve. Glucose concentration of the media from days 0 to 18 was estimated using the Somogyi–Nelson method (empty circles). Solid circles indicated cell number. B: Ammonia determination along the parasite growth curve. Ammonia concentration of the media from days 0 to 18 was estimated using the indophenol colorimetric reaction (solid circles). Empty circles indicate the pH of the media.

ammonia excretion and the increase of the pH in the medium. This latter phenomenon is explained by the stimulation of the oxidative metabolism of amino acids [13], which is parallel with a significant increase of protease activities in late growth phases [15].

Arginine kinase in the parasite *T. cruzi* is responsible for the production of a high-energy phosphate reserve compound, the phosphagen phosphoarginine [4]. Since synthesis of phosphagens is reversible, the same enzyme can degrade the compound when ATP and/or arginine are needed. Moreover, the requirement of arginine and other nitrogen-rich compounds as alternative carbon sources during the late phase of growth, explains the necessity to up-regulate arginine kinase through its continuous synthesis [1,16].

In Trypanosomatidae the genome is organized in polycistronic units that usually contain tandem repeats of the same, or very similar, open reading frame, which are separated by short intergenic regions [17]. Although transcription of these units is constitutive, genes within a unit can show different expression patterns [18]. The regulation of gene expression in these organisms is mostly exerted at the post-transcriptional level, including mRNA maturation, stability and translation [19]. Consistently with these observations, transcriptional regulation of the arginine kinase gene was not detected in this work; therefore, the up-regulation on the arginine kinase levels in *T. cruzi* could be attributed to post-transcriptional regulatory mechanisms.

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), the International Center for Genetic Engineering and Biotechnology (Trieste, Italy) and the Argentine–Brazilian Center for Biotechnology. M.C.P., H.N.T., M.M.F., and N.M.G.B are Career Members of CONICET; G.D.A and C.A.P. are fellows of the later institution.

References

[1] Hird, F. (1986) Comp. Biochem. Physiol. 85B, 285–288.
[2] Huennekens, F.M. and Whiteley, H.R. (1960) in: Phosphoric acid anhydrides and other energy-rich compounds (Florkin, M. and Mason, H.S. Eds.), Comparative Biochemistry, 1, pp. 107–180, Academic Press, New York.
[3] Morrison, J.F. (1973) in: Arginine kinase and Other Guanidino Kinases (Boyer, P.D., Ed.), The Enzymes, 8, pp. 457–486, Academic Press, New York.
[4] Pereira, C.A., Alonso, G.D., Paveto, C., Iribarren, A., Cabanas, M.L., Torres, H.N. and Flawia, M.M. (2000) J. Biol. Chem. 275, 1495–1501.
[5] Brener, Z. (1973) Annu. Rev. Microbiol. 27, 347–382.
[6] Kollien, A.H. and Schaub, G.A. (2000) Parasitol. Today 16, 381–387.
[7] Castellani, O., Ribeiro, L.V. and Fernandes, J.F. (1967) J. Protozool. 14, 447–451.
[8] Laemmli, U.K. (1970) Nature 227, 105–132.
[9] Somogyi, M. (1945) J. Biol. Chem. 160, 61–74.
[10] Nelson, N. (1944) J. Biol. Chem. 153, 375–386.
[11] Bradford, M.M. (1976) Anal. Biochem. 72, 248–254.
[12] Cazzulo, J.J. (1992) FASEB J. 6, 3153–3161.
[13] Adroher, F.J., Osuna, A. and Lupiañez, J.A. (1990) Mol. Cell. Biochem. 94, 71–82.
[14] ter Kuile, B.H. (1997) J. Bacteriol. 179, 4699–4705.
[15] Remedi, M.S., Scaraffia, P., Rodriguez, M., Bronia, D.H. and Gerez de Burgos, N.M. (1997) Acta Physiol. Pharmacol. Therap. Latinoam. 47, 64–68.
[16] Sanchez-Moreno, M., Fernandez-Becerra, M.C., Castilla-Calvente, J.J. and Osuna, A. (1995) FEMS Microbiol. Lett. 133, 119–125.
[17] Vanhamme, L. and Pays, E. (1995) Microbiol. Rev. 59, 223–240.
[18] Myler, P.J., Audleman, L., deVos, T., Hixson, G., Kiser, P., Lemley, C., Magness, C., Rickel, E., Sisk, E., Sunkin, S., Swartzell, S., Westlake, T., Bastien, P., Fu, G., Ivens, A. and Stuart, K. (1999) Proc. Natl. Acad. Sci. USA 96, 2902–2906.
[19] Teixeira, S.M., Kirchhoff, L.V. and Donelson, J.E. (1995) J. Biol. Chem. 270, 22586–22594.