Trypanosoma cruzi Arginine Kinase Characterization and Cloning

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This work contains the first description of a guanido kinase in a flagellar unicellular parasite. The enzyme phosphorylates L-arginine and was characterized in preparations from Trypanosoma cruzi, the etiological agent of Chagas’ disease. The activity requires ATP and a divalent cation. Under standard assay conditions (1 mM L-arginine), the presence of 5-fold higher concentrations of canavanine or histidine produced a greater than 50% enzyme inhibition. The base sequence of this enzyme revealed an open reading frame of 357 amino acids and a molecular weight of 40,201. The amino acid sequence shows all of the characteristic consensus blocks of the ATP:guanidino phosphotransferase family and a putative “actin-type” actin-binding domain. The highest amino acid identities of the sequence, about 70%, were with arginine kinases from Arthropoda. Ten and chromosome blots revealed that the kinase is encoded by a single-copy gene. Moreover, Northern blot analysis showed an mRNA subpopulation of about 2 kilobases, and Western blotting of T. cruzi-soluble polypeptides revealed a 40-kDa band. The finding in the parasite of a phosphagen and its biosynthetic pathway, which are totally different from those in mammalian host tissues, points out this arginine kinase as a possible chemotherapeutic target for Chagas’ disease.

**N-Phosphorylated guanido compounds, commonly referred to as phosphagens, play a critical role as an energy reserve because of high energy phosphate that can be transferred when the renewal of ATP is needed.** It has also been proposed that these compounds function in spatial buffering of cellular energy production sites. So, phosphagens act as reserves not only of ATP but also of inorganic phosphate, which is mostly returned to the medium by metabolic consumption of ATP. Phosphoarginine is the main reserve of high energy phosphate compounds in a wide variety of invertebrates. In addition phosphocreatine, phosphoglycoryamine, phosphoaurocryamine, phosphohypotaurine, and phospholymbricine are also found, whereas in vertebrates the only present is phosphocreatine (1, 2).

Arginine kinase (EC 2.7.3.3) is a member of a conserved family of phosphotransferases which also includes creatine kinase. These enzymes catalyze the reversible transfer of a phosphoryl group from ATP to a guanidino acceptor, which can be either an amino acid (e.g., lombricine or arginine) or a carbonyl (e.g., creatine or glycocyamine; Reaction 1).

\[
\text{MgATP} + \text{guanido acceptor} \rightleftharpoons \text{P-guanido acceptor} + \text{MgADP} + \text{H}^+ \\
\text{Reaction 1}
\]

Arginine kinase is present in Annelida, Celerentara, Platyzelmintes, Nemertea, Mollusca, Phoroniida, Arthropoda, Echinodermata, Hemichordata, and Chordata, where, like other phosphagen kinases, it maintains ATP homeostasis during muscle contraction (1, 2).

Common in most of these phosphotransferases is the presence in the binding sites of substrates of five arginine residues interacting with ATP, two carbonyl amino acids, and one cysteine residue interacting with the guanido acceptor group (3). In addition, some arginine kinases contain a domain for interaction with actin (4).

Most of the enzymatic reactions studied in trypanosomatids, which involves L-arginine, are related to the ornithine-arginine pathway. Distinct genus of trypanosomatids utilizes different enzymes in arginine catabolism. Members of the Trypanosoma genus include T. cruzi, the causative agent of Chagas’ disease, devoid of ornithine decarboxylase, arginine decarboxylase, and arginase (5, 6). In addition, the existence of the nitric oxide pathway in T. cruzi was demonstrated recently by this laboratory. This signaling pathway involves a putative L-glutamate/N-methyl-D-aspartate receptor, a nitric oxide synthase, and a guanylyl cyclase and seems to be a control step in epimastigote flagellar motility (7, 8). The existence of a high affinity and very specific L-arginine transporter was also demonstrated in T. cruzi (9).

The present article provides wide information on the enzymatic and genetic characterization of T. cruzi arginine kinase.

**EXPERIMENTAL PROCEDURES**

Reagents—Enzymes and PCR 1 reagents were provided by Promega Corporation (Madison, WI). Oligonucleotides were from Biosynthesis, Inc. (Lewisville, TX), and other reagents were from Sigma Chemical Co. (St. Louis, MO).

Parasite Culture, Cell Extract, and Enzyme Purification—Unless otherwise indicated, proteins were taken from 2490 parasites and cell extracts were prepared as described (10). Cell extracts were obtained from parasites propagated in insect cell cultures (11). Extracts were used as described (11). Protein concentrations were determined by the method of Bradford (12).

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AF070451, AI035659, AF028619, P48610, F51545, Q27355, AE001338, P00732, P12277, F51546, and Q15991.

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**The abbreviations used are:** PCR, polymerase chain reaction; bp, base pair(s); kb, kilobases; kbp, kilobase pair(s).
Trypanosoma cruzi Arginine Kinase

Effect of divalent cations and nucleotide triphosphates on T. cruzi arginine kinase activity

| Additions/omissions | Arginine kinase | Recombinant AK |
|---------------------|----------------|---------------|
|  | µmol · min⁻¹ · mg⁻¹ | µmol · min⁻¹ · mg⁻¹ |
| None (control)      | 0.81 ± 0.04 | 10.65 ± 0.25 |
| Minus ATP           | 0.00 ± 0.02 | 0.0 ± 0.05 |
| Minus Mg²⁺, plus 10 mM EDTA | 0.11 ± 0.09 | 0.28 ± 0.02 |
| Minus Mg²⁺, plus 5 mM Mn²⁺ | 0.74 ± 0.04 | 0.27 ± 0.02 |
| Minus Mg²⁺, plus 5 mM Ca²⁺ | 0.47 ± 0.03 | 0.73 ± 0.14 |
| Plus 5 mM Zn²⁺      | 0.05 ± 0.01 | 0.56 ± 0.052 |
| Minus Mg²⁺, plus 5 mM Zn²⁺ | 0.00 ± 0.05 | 0.19 ± 0.017 |
| Plus 5 mM Cu²⁺      | 0.02 ± 0.07 | 0.50 ± 0.262 |
| Minus Mg²⁺, plus 5 mM Cu²⁺ | 0.00 ± 0.10 | 0.15 ± 0.097 |
| Minus ATP, plus 2 mM dATP | 0.49 ± 0.10 | 0.76 ± 0.059 |
| Minus ATP, plus 2 mM GTP | 0.16 ± 0.122 | 0.03 ± 0.003 |

putative arginine kinase sequence of 287 bp was amplified from T. cruzi DNA as template using two degenerated primers with the sequences GA/CT/GA/CT/CACCTT/C/TG/C/T/C/T/AA/A/G/G (forward) and GTCGCT/AG/CC/G/C/A/G/A/G/T/CT/G/AA/G/G/C/G/G/C (reverse). Primer structures were deduced from arginine kinase amino acid sequences from Apis mellifera, Drosophila melanogaster, Penaeus japonicus, and Caenorhabditis elegans (see “Results” and Fig. 2A). Incubation mixtures contained 400–700 ng of T. cruzi DNA, 200–500 ng of each primer and 4 µm MgCl₂. Amplification conditions were as follows: 5 min at 95 °C, 35 cycles of 2 min at 95 °C, 1 min at 55 °C, and 1.5 min at 72 °C, followed by an extension step at 72 °C for 10 min. A 287-bp product was obtained by PCR amplification, subcloned, and sequenced. This product showed an 83% identity with the Trypanosoma brucei rhodesiense expressed sequence tag (Accession Number W00186) and a high identity with others related guanidine kinases.

Labeling of DNA Probes—Probes were labeled with the random primer DNA labeling kit from Bio-Rad using [α-32P]dCTP (NEN Life Science Products) as the labeled nucleotide.

Screening of a T. cruzi Genomic Library—A T. cruzi genomic library from the CL Brener clone was obtained from Dr. M. Levin. This library was prepared by Dr. Edison Rondinelli (Instituto de Biofisica, Universidade de Río de Janeiro, Brazil) in the λ phage replacement vector Lambda FIX II (Stratagene, La Jolla, CA), containing 12–15-kbp inserts and a unique XhoI site, and is available through the European Collection of Animal Cell Cultures (ECACC, Weybridge, UK). Approximately 100,000 independent recombinant phages were screened with the 287-bp PCR fragment as a probe and, after three to five cycles of selection, three phage clones giving positive hybridization signals were selected.

Sequencing of Phage DNA—The positive phage clones were subjected to sequencing using the PRISM Ready Reaction DyeDeoxy Terminator Cycle Sequencing Kit (Perkin-Elmer, Foster City, CA). This procedure requires the Applied Biosystems model 373A DNA automatic sequencer. Mixtures were supplemented with double-stranded phage DNA and the corresponding oligonucleotide. Reactions were performed in the model 480 DNA Thermal Cycler (Perkin-Elmer) following the manufacturer’s instructions.

Northern and Southern Blot Analysis—For Northern blot analysis, 10–20 µg of total RNA samples from epimastigote cells were electrophoresed on a 1.5% agarose gel, transferred to a Hybond N nylon membrane (Amersham Pharmacia Biotech), and hybridized with the full-length arginine kinase gene as a probe. The Southern blot analysis was performed using aliquots of 5 µg of DNA previously digested with the following endonucleases: AccI, BamHI, BglI, and EcoRI. The products were resolved by electrophoresis in a 0.8% agarose gel, transferred, and hybridized as described for Northern blots.

Fractionation of Chromosomal Bands by Pulsed-field Gel Electrophoresis—Pulsed-field gel electrophoresis was performed in a CHEF electrophoresis cell (Bio-Rad) with a hexagonal electrode array. Agarose blocks containing about 10⁷ epimastigote forms were prepared as described (14). The separation of the chromosomal bands was carried out in 1.0% Gel Seakem agarose (FMC BioProducts, Rockland, ME) in 0.5 x TBE (45 mM Tris, 45 mM boric acid, 1 mM EDTA, pH 8.3), and run in the same buffer. The electrophoretic conditions were as follows: 16 h, 6...
The effects of different compounds were tested on the native and recombinant arginine kinase (AK) standard assay, as described under “Experimental Procedures,” in the presence of 1 mM arginine and a 5-fold molar excess (5 mM) of the metabolite screened. Data are the means ± S.D. of triplicates.

| Addition          | Arginine kinase inhibition | Recombinant AK inhibition |
|-------------------|---------------------------|--------------------------|
|                   | μmol · min⁻¹ · mg⁻¹ | %                       | μmol · min⁻¹ · mg⁻¹ | %         |
| None (control)    | 1.20 ± 0.27              | 0                       | 15.99 ± 0.541     | 0         |
| l-Histidine       | 0.52 ± 0.22              | 56                      | 11.74 ± 0.085     | 27        |
| l-Lysine          | 0.90 ± 0.055             | 25                      | 13.58 ± 0.045     | 15        |
| Glycine           | 1.13 ± 0.145             | 15                      | 15.34 ± 0.235     | 4         |
| l-Isoleucine      | 1.02 ± 0.064             | 15                      | 14.55 ± 0.453     | 9         |
| l-Aspartate       | 0.90 ± 0.084             | 25                      | 13.43 ± 0.260     | 16        |
| l-Glutamate       | 0.89 ± 0.007             | 31                      | 13.81 ± 0.580     | 14        |
| N-Methyl-l-arginine| 0.86 ± 0.055            | 28                      | 13.1 ± 1.127      | 17        |
| L-Nitroarginine   | 0.85 ± 0.037             | 29                      | 11.95 ± 0.412     | 25        |
| d-Arginine        | 1.27 ± 0.020             | 0                       | 16.59 ± 0.170     | 0         |
| l-Homoarginine    | 0.80 ± 0.016             | 33                      | 13.83 ± 0.256     | 13        |
| l-Ornithine       | 1.05 ± 0.085             | 12                      | 14.53 ± 0.376     | 9         |
| l-Citrulline      | 1.17 ± 0.032             | 12                      | 13.75 ± 0.096     | 14        |
| Creatine          | 1.05 ± 0.011             | 13                      | 13.39 ± 0.305     | 16        |
| Canavanine        | 0.27 ± 0.044             | 77                      | 9.30 ± 0.357      | 42        |
| Agmatine          | 0.96 ± 0.182             | 20                      | 13.47 ± 1.283     | 16        |
| Putrescine        | 1.29 ± 0.045             | 0                       | 17.77 ± 0.544     | 0         |
| Ethylguanidine    | 0.94 ± 0.051             | 22                      | 14.27 ± 0.739     | 11        |
| Guanidine propionic acid | 0.97 ± 0.097  | 18                      | 13.57 ± 0.831     | 15        |
| Amino guanidine   | 1.01 ± 0.004             | 16                      | 14.68 ± 0.344     | 8         |
| Guanidine         | 1.11 ± 0.067             | 7                       | 14.07 ± 0.321     | 12        |
| Methylguanidine   | 1.19 ± 0.042             | 1                       | 13.81 ± 0.083     | 14        |
| Urea              | 1.15 ± 0.079             | 4                       | 14.69 ± 0.067     | 8         |
| γ-Guanidine butyramide | 1.16 ± 0.100  | 3                       | 15.74 ± 0.078     | 2         |

The effects of several amino acids, arginine/guanidine derivatives, and polyamines on *T. cruzi* arginine kinase were tested. Under standard assay conditions (1 mM L-arginine), the presence of 5-fold higher concentrations of canavanine and histidine produced a greater than 50% inhibition. Other compounds such as L-homoarginine, L-glutamate, N-methyl-L-arginine, L-nitroarginine, L-lysine, L-aspartate, agmatine, and ethylguanidine elicited inhibitions of between 20 and 35% (Table II). Michaelis-Menten constant ($K_i$) values for both ATP and L-arginine were about 0.3 mM, and the optimum pH was 8.2 (results not shown). Identical results were obtained with the two kinase assay procedures (see “Experimental Procedures”).

The reaction product was characterized by mass spectrometry. Standard phospho-L-arginine and arginine kinase reaction products were obtained with the same FAB" signals: m/z 113 and 207; FAB" signals were also identical: m/z 255 and 283.

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and C. elegans belong to the lowest evolved metazoa and posses arginine kinases with reported genetic sequences. All of these sequences share amino acid identities of about 60–70% (see Fig. 2). Taking into account this information, a set of primers was designed and used to amplify a putative arginine kinase from T. cruzi DNA. The only product resulting from this amplification was a fragment of 287 bp. After sequencing, the fragment revealed a 79% amino acid identity with an equivalent domain of D. melanogaster arginine kinase. The 287-bp fragment was used to probe a T. cruzi genomic library in \( \lambda \) FIX. Following a tertiary screening, three clones were isolated with 12–15-kbp inserts. One of these, when subjected to sequencing, revealed an open reading frame corresponding to a polypeptide of 357 amino acids (Fig. 1) with a molecular weight of 40,201 and a theoretical isoelectric point of 7.04.

This protein sequence shows all of the characteristic consen-
sus blocks of the ATP:guanidino phosphotransferase family (see “Experimental Procedures”): BL001112A (positions 52–95), BL001112B (positions 116–149), BL001112C (positions 161–191), BL001112D (positions 259–288), and BL001112F (positions 306–354). BL001112E houses the consensus CPTNLGT (positions 271–277), which partially corresponds to the active site of the kinase. In addition, a putative “actin-in-type” actin binding domain (18), DAK-TFLVWVNE, was found (positions 214–224).

5'- and 3'-noncoding sequences were also characterized. A proposed “pyrimidine-rich” region and a splice leader acceptor site were located at the 5'-noncoding region, whereas downstream of the coding region, a 309-bp noncoding sequence was found, apparently followed by an open reading frame. This sequence is identical to a T. cruzi expressed sequence tag (GenBank AI035059).
coli—The expressed enzyme was purified to near homogeneity as described under “Experimental Procedures” (Fig. 3) and assayed for biochemical properties. It was recovered with a specific activity of about $10^{-16}$ moles $\cdot$ min $^{-1}$ $\cdot$ mg of protein $^{-1}$. This value was 10 times lower than those reported for some arthropod and mollusk muscle arginine kinases (19–21). On the other hand, some kinetic properties of the recombinant and the native \textit{T. cruzi} enzymes were similar but not identical; for example, the $K_m$ value for ATP and L-arginine was about 0.3 mM for both preparations (results not shown). On the other hand, the recombinant enzyme was less active with Mn$^{2+}$ than the native one (Table 1).

\textbf{Southern, Northern, Western, and Chromosome Blot Analysis—}Southern blots were carried out on DNA from the \textit{T. cruzi} Tulahuen 2 strain digested with four restriction endonucleases. Hybridization analysis using as probe the fragment carrying the entire \textit{T. cruzi} arginine kinase sequence revealed that \textit{T. cruzi} arginine kinase is encoded by a single-copy gene (Fig. 4). These results are consistent with those obtained by pulsed-field gel electrophoresis analysis of \textit{T. cruzi} chromosomes (chromosome blot), in which only two chromosomal bands of 880 and 847 kbp were detected (see Fig. 4).

**Fig. 2.** Amino acid sequence comparison (panel A) and phenogram (panel B). Panel A, alignment of arginine kinase (AK) sequences: \textit{T. cruzi} (GenBank AF070451), \textit{A. mellifera} (AF023619), \textit{D. melanogaster} (P48610), \textit{P. japonicus} (P51545), \textit{C. elegans} (Q27355), \textit{C. trachomatis} (AE001338), \textit{Homo sapiens} mitochondrial creatine kinase (CKM) (P00732), \textit{Homo sapiens} brain creatine kinase (CKB) (P12277), \textit{N. diversicolor} glycoccyamine kinase (GK) (P51546), and \textit{E. fetida} lombricine kinase (LK) (O15991). Identical residues are indicated by points, and dashes indicate gaps. Panel B, phenogram as well as sequence comparison were performed using the Clustal method from LaserGene software (22). Numbers represent the percentage of divergence.
On the other hand, Northern blot analysis of T. cruzi RNA using the fragment carrying the entire T. cruzi arginine kinase sequence revealed a mRNA subpopulation of about 2.0 kb (Fig. 4B).

Western blot analysis of polypeptides in an epimastigote soluble extract, using the antiserum against T. cruzi recombinant arginine kinase, revealed a 40-kDa polypeptide band (Fig. 4D).

**Distribution of the Arginine Kinase in Trypanosomatids—**

Cytosolic fractions of some lower eukaryotic organisms were assayed for arginine kinase activity. The activity was present in the Tulahuen 0, Tulahuen 2 and G T. cruzi strains, as well as in: *Trypanosoma rangeli*, *Herpetomonas muscarum*, and *Leptomonas samueli*. No activity was detected in *Crithidia fasciculata*, *Leishmania chagasi*, *Plasmodium falciparum*, *Toxoplasma gondii*, *Euglena gracilis*, and *Acanthamoeba polyphaga* (results not shown).

**DISCUSSION**

The present study reports the biochemical characterization and the analysis of the genetic sequence of arginine kinase from the flagellate protozoan *T. cruzi*. The enzyme was partially purified from epimastigote cells and from an *E. coli* expression system. Except for the behavior to divalent cations and some inhibitors both preparations showed similar kinetic properties. This may be attributable to an inappropriate folding of the recombinant enzyme or the presence of a histidine tag.

Because this enzyme presents an extraordinary amino acid homology compared with those from crustaceans and insects, the evolutionary origin of its sequence is under discussion. Indeed, it is accepted that arthropods were the first hosts for trypanosomatids. Later, some of them diverged to parasitize plants and warm blooded animals. Thus, it may be speculated that some kind of horizontal genetic transfer between arthropods and trypanosomatids might have occurred during evolution.

An interesting fact is related to the role of arginine kinase in trypanosomatids and, in general terms, in protozoa organisms from which it was believed that phosphoguanidino phosphagens were excluded. In this regard, the presence of a putative actin-like actin binding domain in the sequence of this enzyme clearly suggests a relationship with cytoskeletal structures related to cell movement, particularly flagellar movement and other processes requiring a high energy consumption. From the experience in *T. cruzi*, it is evident that the role of phosphagens is not restricted to multicellular organisms bearing movements driven by muscle tissue.

A last point is related to therapeutic implications of arginine kinase. Because this kinase is not present in mammalian tissues, it could be a possible target for the future development of chemotherapeutic agents against Chagas’ disease and other parasitic diseases caused by related organisms. For this purpose, a rational approach would involve three steps: 1) identification of the parasite of a unique biochemical compound different from those in the host cell (in our case phosphoarginine); 2) characterization of the target enzyme responsible for the production of such compound (the arginine kinase described here); and 3) search for a specific inhibitor of this enzyme.

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