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# *Trypanosoma cruzi* Arginine Kinase Characterization and Cloning

## A NOVEL ENERGETIC PATHWAY IN PROTOZOAN PARASITES*

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**Claudio A. Pereira, Guillermo D. Alonso, M. Cristina Paveto, Adolfo Iribarren, M. Laura Cabanas, Héctor N. Torres, and Mirtha M. Flawiá‡**

*From the Instituto de Investigaciones en Ingeniería Genética y Biología Molecular, Consejo Nacional de Investigaciones Científicas y Técnicas, and Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Buenos Aires, Argentina*

**This work contains the first description of a guanidino kinase in a flagellar unicellular parasite. The enzyme phosphorylates L-arginine and was characterized in preparations from *Trypanosoma cruzi*, the ethiological 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 "actinin-type" actin-binding domain. The highest amino acid identities of the *T. cruzi* sequence, about 70%, were with arginine kinases from Arthropoda. Southern and chromosome blots revealed that the kinase is encoded by a single-copy gene. Moreover, Northern blot analysis showed an mRNA subpopulation of about 2.0 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 chemotherapy target for Chagas' disease.**

*N*-Phosphorylated guanidino compounds, commonly referred to as phosphagens, play a critical role as an energy reserve because of the 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, phosphoglycocyamine, phosphotaurocyamine, phosphohypotaurocyamine, phosphoopheline, and phospholombricine are also found, whereas in vertebrates the only one 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 carboxylate (*e.g.* creatine or glycocyamine; Reaction 1).

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

REACTION 1

Arginine kinase is present in Annelida, Celenterata, Platyhelmintes, Nemertea, Mollusca, Phoronida, Arthropoda, Equinodermata, 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 carboxylate amino acids, and one cysteine residue interacting with the guanidino 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* epimastigotes. One of the major products of L-arginine uptake was characterized as phosphoarginine (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

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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, AI035059, AF023619, P48610, P51545, Q27535, AE001338, P00732, P12277, P51546, and O15991.*

‡ To whom correspondence should be addressed: INGEBI, Vuelta de Obligado 2490, 1428 Buenos Aires, Argentina. Tel.: 54-11-4783-2871; Fax: 54-11-4786-8578; E-mail: mflawia@proteus.dna.uba.ar.

[1] The abbreviations used are: PCR, polymerase chain reaction; bp, base pair(s); kb, kilobases; kbp, kilobase pair(s).

otherwise indicated, *T. cruzi* epimastigote forms of the Tulahuen 2 strain were cultured at 28 °C in a medium containing 5 g/liter liver infusion, 5 g/liter Bacto-tryptose (Difco Laboratories, Detroit, MI), 68 mM NaCl, 5.3 mM KCl, 22 mM $Na_2HPO_4$, 0.2% glucose, 0.002% hemin supplemented with 10% fetal calf serum, 10 units/ml penicillin, and 10 mg/liter streptomycin. Cell viability was assessed by direct microscopic examination.

For arginine kinase purification, epimastigotes from 7-day cultures (late logarithmic phase) were collected by centrifugation at 1,000 × *g*, washed three times with 25 mM Hepes adjusted to pH 7.3 with Tris and containing 1.8 mM $CaCl_2$, 0.8 mM $MgSO_4$, 2.7 mM KCl, 1.2 mM $KH_2PO_4$, 154 mM NaCl, and 5.5 mM D-glucose (buffer A). The pellet was then resuspended (1 ml/each 30 ml of culture) in 25 mM Hepes buffer, pH 7.3, containing 0.01 mg/ml leupeptin, 2 mg/ml soybean trypsin inhibitor, 1 mM benzamidine, 25 units/ml Trasylol, 0.1 mM phenylmethylsulfonyl fluoride, and 0.5 mM tosyl-lysine chloromethyl ketone, and lysed by six cycles of freezing in liquid $N_2$ and thawing at 4 °C. The extract was then centrifuged 5 min at 8,000 × *g*. The supernatant thus obtained was used as a source of epimastigote proteins for the arginine kinase purification.

The supernatant fluid (5.0 ml) was applied to a 5 × 1-cm Whatman DE-52 column equilibrated with 25 mM Hepes buffer, pH 7.6, and washed with 25 ml of the same buffer solution. The enzyme activity was recovered in the percolate. After a concentration step using Amicon ultracentrifugation cells, the sample (0.5 ml) was applied to a Superose-6 HR10/30 column (Amersham Pharmacia Biotech) equilibrated as above. The effluent, obtained at a rate of 0.3 ml/min, was collected in 0.5-ml fractions. The peak activity fractions were pooled and used as a source of kinase activity. These fractions were enriched 80-fold in enzyme activity with a recovery of about 55% (specific activity 0.8–1.2 $\mu$mol·min$^{-1}$·mg of protein$^{-1}$; see Fig. 3*B*).

Purification of recombinant arginine kinase was performed on a Ni-NTA agarose column (QIAGEN, Valencia, CA) according to the manufacturer's instructions for denatured polypeptides. The eluate was then dialyzed successively for 24 h against decreasing urea concentrations (from 7.0 M to none) in 0.1 M phosphate buffer, pH 7.2, containing 7 mM 2-mercaptoethanol, 10% glycerol, and 0.15 M NaCl, to obtain recombinant arginine kinase with a specific activity of 10–16 $\mu$mol·min$^{-1}$·mg of protein$^{-1}$ and a yield of 10–20 mg of protein/liter of culture (see Fig. 3*A*).

*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; 0.5 $\mu$Ci/assay), and an enzyme source (0.1–8 $\mu$g of protein) in a total volume of 0.2 ml. Incubations were carried out for 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) 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. The eluates were counted for radioactivity.

The assay method for arginine kinase activity is validated for the ulterior analysis of the anion exchange eluate products by mass spectrometry (see below). Under the conditions described above, phosphoarginine but not L-arginine was retained by the anion exchange resin. In addition, the eluate fraction contained less than 2% of free L-arginine and more than 95% of the phosphoarginine produced.

Arginine kinase activity was assayed alternatively using a modification of the procedure described by France *et al.* (10). The reaction mixture contained 25 mM Hepes, pH 7.3, 10 mM L-arginine, 2 mM [$\gamma$-$^{32}$P]ATP (NEN Life Science Products), 5 mM magnesium acetate, 10 mM 2-mercaptoethanol, and an enzyme source in a final volume of 0.2 ml. The reaction was carried out for 10 min at 30 °C and stopped by the addition of trichloroacetic acid to a final concentration of 1.4% and heating for 1 min at 100 °C. Mixtures were cooled 1 min on ice and then extracted with isobutyl alcohol/$(NH)_4MoO_4$ as described by Richards *et al.* (11). Aliquots of 0.1 ml of the organic phase were counted for radioactivity. For both procedures, the enzyme activity was proportional to the incubation time (2–20 min) and enzyme protein (0.1–1.0 $\mu$g protein of Superose fraction).

*Preparation of T. cruzi DNA and RNA*—DNA was obtained from an epimastigote lysate, treated with proteinase K, by phenol-chloroform extraction and centrifugation through a CsCl gradient. Total RNA was prepared from approximately 3.7 × 10$^{10}$ cells (99% epimastigotes), according to Chomszynski and Sacchi (12).

*Amplification of a Putative T. cruzi Arginine Kinase Sequence*—A putative arginine kinase sequence of 287 bp was amplified from *T. cruzi* DNA as template using two degenerated primers with the sequences GA(C/T)GA(C/T)CACTT(C/T)CT(G/T/C)TT(C/T)AA(A/G)G (forward) and GTCGT(A/G)CC(G/A/C)AG(A/G)TT(C/T)G(A/T)(G/T)GG(A/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. 2*A*). Incubation mixtures contained 400–700 ng of *T. cruzi* DNA, 200–500 ng of each primer and 4 mM $MgCl_2$. 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 guanidino kinases.

TABLE I

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

0.8 $\mu$g of partially purified *T. cruzi* soluble extract and 0.2 $\mu$g of the recombinant protein were assayed for arginine kinase (AK) activity with the standard method as described under "Experimental Procedures," with the modifications listed in the table. A control reaction was performed in the presence of 2 mM ATP and 5 mM MgAcO. Data are the means ± S.D. of triplicates.

| Additions/omissions | Arginine kinase | Recombinant AK |
|---|---|---|
| | $\mu mol \cdot min^{-1} \cdot mg^{-1}$ | $\mu mol \cdot min^{-1} \cdot mg^{-1}$ |
| None (control) | 0.81 ± 0.004 | 10.65 ± 0.257 |
| Minus ATP | 0.00 ± 0.002 | 0 ± 0.005 |
| Minus $Mg^{2+}$, plus 10 mM EDTA | 0.11 ± 0.010 | 0.28 ± 0.027 |
| Minus $Mg^{2+}$, plus 5 mM $Mn^{2+}$ | 0.74 ± 0.044 | 0.27 ± 0.022 |
| Minus $Mg^{2+}$, plus 5 mM $Ca^{2+}$ | 0.47 ± 0.032 | 7.03 ± 0.147 |
| Plus 5 mM $Zn^{2+}$ | 0.05 ± 0.019 | 0.56 ± 0.052 |
| Minus $Mg^{2+}$, plus 5 mM $Zn^{2+}$ | 0.00 ± 0.005 | 0.19 ± 0.017 |
| Plus 5 mM $Cu^{2+}$ | 0.02 ± 0.007 | 0.50 ± 0.262 |
| Minus $Mg^{2+}$, plus 5 mM $Cu^{2+}$ | 0.00 ± 0.010 | 0.15 ± 0.097 |
| Minus ATP, plus 2 mM dATP | 0.49 ± 0.101 | 7.69 ± 0.059 |
| Minus ATP, plus 2 mM GTP | 0.16 ± 0.122 | 0.03 ± 0.003 |

*Labeling of DNA Probes*—Probes were labeled with the random primer DNA labeling kit from Bio-Rad using [$\alpha$-$^{32}$P]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. Edson Rondinelli (Instituto de Biofisica, Universidade de Rio de Janeiro, Brazil) in the $\lambda$ phage replacement vector Lambda FIX II (Stratagene, La Jolla, CA), containing 12–15-kbp inserts into the *Xho*I site, and is available through the *T. cruzi* genome initiative (13). Approximately 100,000 independent recombinant phages were screened with the 287-bp PCR fragment as a probe and, after three rounds 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 $\mu$g 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 $\mu$g of DNA previously digested with the following endonucleases: *Acc*I, *Bam*HI, *Bgl*I, and *Eco*RI. 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 10$^7$ 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 × 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

TABLE II

*Effect on T. cruzi arginine kinase of several amino acids, arginine/guanidine derivatives and polyamines*

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 |
|---|---|---|---|---|
| | $\mu mol \cdot min^{-1} \cdot mg^{-1}$ | % | $\mu mol \cdot min^{-1} \cdot mg^{-1}$ | % |
| None (control) | 1.20 ± 0.027 | 0 | 15.99 ± 0.541 | 0 |
| L-Histidine | 0.52 ± 0.022 | 56 | 11.74 ± 0.085 | 27 |
| L-Lysine | 0.90 ± 0.055 | 25 | 13.58 ± 0.045 | 15 |
| Glycine | 1.19 ± 0.145 | 1 | 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 | 0.82 ± 0.007 | 31 | 13.81 ± 0.580 | 14 |
| *N*-Methyl-L-arginine | 0.86 ± 0.055 | 28 | 13.25 ± 1.127 | 17 |
| L-Nitroarginine | 0.86 ± 0.037 | 28 | 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 | 1.06 ± 0.085 | 12 | 14.53 ± 0.376 | 9 |
| L-Citrulline | 1.17 ± 0.032 | 2 | 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 | 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 |

V/cm pulsed each 60–90 s; 20 h, 3 V/cm pulsed each 200–500 s; and 24 h, 2.7 V/cm pulsed each 500–1,200 s, maintained at constant temperature (14 °C). After electrophoresis, the gel was stained with ethidium bromide (0.1 μg/ml) and photographed. Chromosomes from *Saccharomyces cerevisiae* (Life Technologies, Gaithersburg, MD) were used as molecular mass standards. The gel was treated for 10 min with 0.25 M HCl and washed twice with bidistilled water. DNA was denatured in a solution containing 0.4 M NaOH and 1 M NaCl (two incubations, 20 min each). The transfer of nucleic acids and hybridization conditions were the same as those described for Northern blots.

*Expression of T. cruzi Arginine Kinase in Escherichia coli*—A fragment carrying the entire *T. cruzi* arginine kinase sequence was obtained by PCR using as primers the following oligonucleotides: CGCGGATCCAACTCAGTCACGATGGCC (5′-end carrying a *Bam*HI site) and CGGAATTCGGCTCACCTCGCAGATTC (3′-end carrying an *Eco*RI site). Conditions were similar to those described above. After double digestion with *Bam*HI and *Eco*RI, the fragment thus generated was purified with the Wizard PCR Preps Kit (Promega) and ligated to the pRSET A vector (Invitrogen, Carlsbad, CA) treated previously with *Eco*RI and *Bam*HI. This plasmid transcribes under the control of the T7 promoter and includes a polyhistidine tag. Expression was performed in the BL21(DE3)pLysS host (*E. coli* B, $F^-$, dcm, ompT, hsdS, ($rb^-$, $m_B^-$), gal λ(DE3),[pLysS, $Cam^r$ ]). An overnight culture was carried out in 700 ml of LB medium containing ampicillin and chloramphenicol. Induction was performed with 100 mM isopropyl-1-thio-β-D-galactopyranoside for 60 min at 37 °C.

*Western Blot Analysis*—Arginine kinase antiserum was obtained using female mice from the BALB/c strain immunized by an intraperitoneal injection of 50 μg of recombinant arginine kinase plus 0.1 ml of incomplete Freund's adjuvant followed, 15 days later, by a booster intraperitoneal injection of 50 μg of recombinant protein. After 15 days, mice were bled by exposing the ocular cavity. Procedures for SDS-polyacrylamide gel electrophoresis of protein samples were carried out as described by Laemmli (15). Polypeptides were electrotransferred from polyacrylamide gels to Hybond-C membranes (Amersham Pharmacia Biotech). For reaction with the antibody, the transferred membranes were blocked with a 5% (w/v) non-fat milk suspension for 30 min. After incubation for 2 h with a 1:100 dilution of the mouse anti-arginine kinase antiserum, detection was carried out by incubating with a 1:2,000 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).

*Analytical Methods*—L-Arginine and phosphoarginine characterization was performed by mass spectrometry using a ZAB-SE Q4F (Micromass, UK) mass spectrometer (high mass, 800; low mass, 50; resolution, 1,000; time (s/dec), 4; ionization mode, FAB− and FAB+; accelerating voltage, 8,000 V). Protein was assayed as described by Bradford (16).

Sequences were analyzed, compared, and aligned using Advanced BLAST version 2.0, Clustal W, and LaserGene software (DNASTAR, Inc.). Consensus sequences in *T. cruzi* arginine kinase were located with BLOCKS (17).

## RESULTS

*General Characterization of T. cruzi Arginine Kinase Activity*—An arginine kinase activity was detected in partially purified preparations from *T. cruzi* epimastigote soluble extracts. The activity requires ATP and a divalent cation such as $Mg^{2+}$, $Mn^{2+}$, or $Ca^{2+}$; dATP or GTP is less efficient than ATP in phosphorylating L-arginine (Table I). Michaelis-Menten constant ($K_m$) 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 gave the same $FAB^+$ signals: *m/z* 113 and 207; $FAB^-$ signals were also identical: *m/z* 255 and 283.

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). Inhibition constant ($K_i$) values for canavanine and homoarginine were calculated because both metabolites produced inhibition of arginine kinase activity as well as arginine transport in epimastigote cells (9). $K_i$ values for canavanine and homoarginine were 6.0 mM, and 7.0 mM respectively (results not shown).

*Cloning of T. cruzi Arginine Kinase*—Available arginine kinase sequences were selected as a source of information to amplify a putative homologue from *T. cruzi* DNA. Arthropods and helminths such as *A. mellifera, D. melanogaster, P. japoni-*

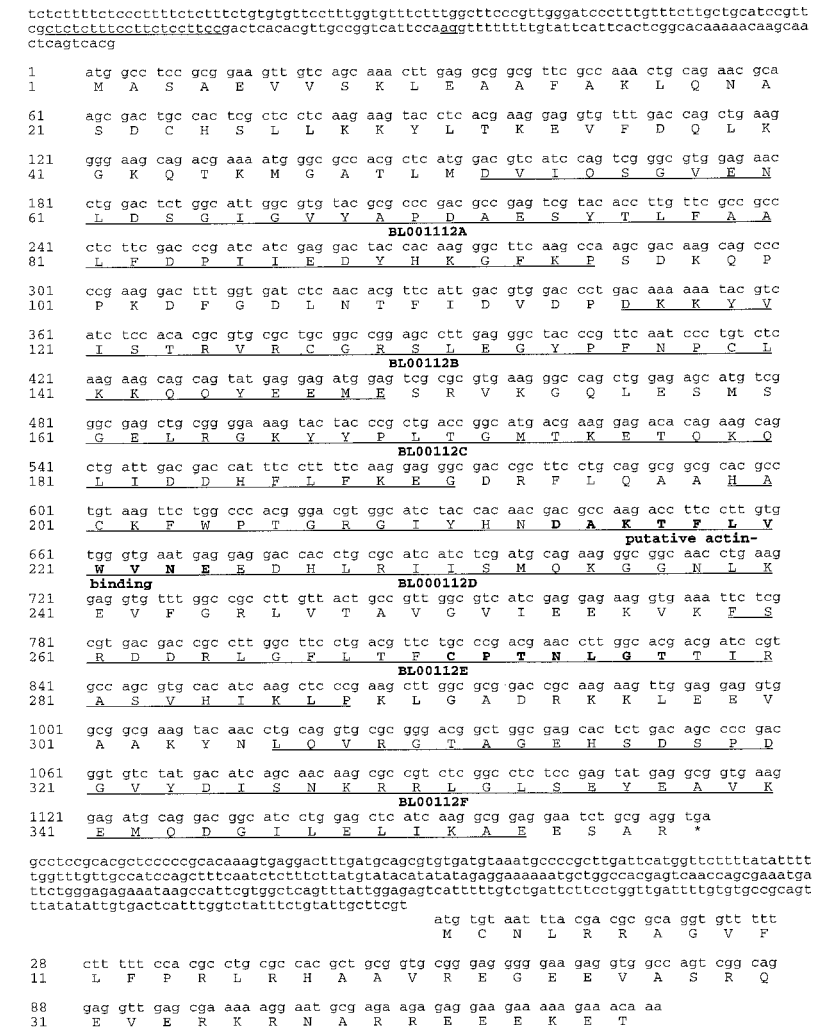


FIG. 1. **Sequence analysis of *T. cruzi* arginine kinase gene and the 5′-/3′-noncoding neighbor sequences found in a *T. cruzi* λ FIX genomic clone.** Characteristic consensus sequences according to the BLOCKS data base (17) are indicated. *Underlined letters*, base sequences of the 5′-terminal polypyrimidine tract and presumed *trans*-splicing acceptor site (ag) and consensus sequences of guanidine kinases. Bases in *bold type* indicate the putative actin binding and the signature sequence pattern of ATP:guanidino kinases: CP(S/T)N(I/L)GT, including the reactive cysteine at the residue 271. An open reading frame immediately following a 309-bp noncoding sequence was found to be identical to a *T. cruzi* expressed sequence tag (GenBank AI035059).

*cus,* 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 λ FIX II. 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 consensus blocks of the ATP:guanidino phosphotransferase family (see "Experimental Procedures"): BL001112A (positions 52–95), BL00112B (positions 116–149), BL00112C (positions 161–191), BL00112D (positions 199–245), BL00112E (positions 259–288), and BL00112F (positions 306–354). BL00112E houses the consensus CPTNLGT (positions 271–277), which partially corresponds to the active site of the kinase. In addition, a putative "actinin-type" actin binding domain (18), DAKTFLVWVNE, 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) reported by the *T. cruzi* genome initiative. The protein coded by this open reading frame of 82 amino acids is quite unusual: it contains 15 arginines, 5 lysines, and has an isoelectric point of 10.8.

*Amino Acid Identities of T. cruzi Arginine Kinase and Guanidino Kinases from Other Species*—The highest amino acid identities of the *T. cruzi* sequence were with arginine kinases of *A. mellifera* (insect), *D. melanogaster* (insect), *P. japonicus* (crustacean), and *C. elegans* (helminth): 69.6, 66.9, 71.8, and 58.5%, respectively. Identity with a putative bacterial arginine kinase, from *Chlamydia trachomatis*, was rather poor: 11.2%. When the comparison was made with other guanidino kinases, the degree of homology was 39% with the human creatine kinase, 38% with the lombricine kinase from *Eisenia fetida* (annelid), and 36% with the guanidino acetate kinase of *Neanthes diversicolor* (annelid) (Fig. 2*A*).

A phylogenetic tree was constructed from the 10 ATP:guanidino phosphotransferase sequences analyzed (Fig. 2*B*). The phenogram indicates the existence of three major groups: one corresponding to arthropod and helminth arginine kinases; the second to a variety of creatine, lombricine, and glycocyamine kinases; and a third group that comprises a bacterial arginine kinase. *T. cruzi* arginine kinase clearly belongs to the first group.

*Expression of Recombinant T. cruzi Arginine Kinase in E.*

**A**

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Majority              MVDAAVLNK------LEEGFPKLSAASDCKSLLKKYLTKEVFDKLKGK-KTPLGATLDDVIQSGVEN-G---LDSGVGIYAPDAESYTLFADLFDPIIED
                                10        20        30        40        50        60        70        80        90       100
AK  T.cruzi           .AS.E.VS.------..AA.A..QN....H.............Q....-Q.KM....M.........-----....I.V.............A........
AK  A.mellifera       ...Q...D.------..T..S..-SS..S........S.D...Q..T.-..SFDS..L.C....I..-----.............A.............
AK  D.melanogaster    .......A.------....YA..-....S..............N..N.GHAH.QVDPA......L..-----H..ASA.......A..V.......F...
AK  P.japonicus       -......E.------.QA..K..E..T..........S.DI......Q-..S.....L.........-----.............A.....P........
AK  C.elegans         .TATPEVQ.-----SI..VYT..QG....S.....H...D.VA.N.S.-..R.....L......G..-----.......................N.V..E
AK  C.trachomatis     .LPNHI.TAIA---TIKHSLRTETPRPI.TLS.SRN.SVSK.VPCLS.ENKRD--V.ETIAKQFSAIE.EEFFVLPLKDLPIWQRECL.EHY..PYHLGS
CKM H.sapiens         .PFGNTH..FKLNYKP..EY.D..KHNN-H--MA.V..L.LYK..RD.-E..S.F.V.....T..D.P.HPFI-MT..CV.G.E...EV.KE......S.
CKB H.sapiens         .PFSNSH.ALKLRFPA.DE..D...HNN-H--MA.V..P.LYAE.RA.-S..S.F.......T..D.P.HPYI-MT..CV.G.E...EV.K.........
GK  N.diversicolor    .-FKDYS---REKF-AK.N..D..KHNN---VMASH..Y.LYE.YWD.-V..N.V...KC..T..D.P.NKFYGKKT.CVFG.EH..ET.K.F..RV..EI
LK  E.fetida          .----------PKFTARQN..DYKSNGH-.CMVGMH..EDMYER.YEL-R..N.VSI.KC..PS.D.T.-----RII.LV.G.P...EV.KE...AV.NEK

Majority_____         YHGGFKPTDKHPATDLGD-NLVGGGDLDP--KYVISTRVRCGRSLEGYPFNPCLSEAEYKEMEKKVSSALSSLEG-ELKGKYY---PLTGMTKETQQQLI
                             110       120       130       140       150       160       170       180       190       200
AK  T.cruzi           ..K....S..Q.PK.F..--.NTFI.V..DK.....................KKQQ.E...SR.KGQ.E.MS.-..R....---.........K...
AK  A.mellifera       ......K.....PK.F..--VDSL.N...ANEPIV.................T..Q.....E....T..G...-....TF.---.....S.....K..
AK  D.melanogaster    ......K......SNF..--VSTF.NV..TNE.............MQ........T..Q.....S....T..G...-.....F.---.....E.AV.....
AK  P.japonicus       ..V...Q.....NK.F..--VSSFVNV..EGQ.............M.........T..Q....QQ....T......-....T.F---.....S..V..K..
AK  C.elegans         ..N...A..TQ..M...EK.VGELA....EG.FIV...I......Q..........TN..M..TRMKEIFN.ITDP....T..---.....DE..KKK..
AK  C.trachomatis     .LE.EALIVNQAG.L.AGI..RDHLVIHGVD----------------FVWQ.EVLLQKLIDLDIRLQQS..FAFSSDFG--FLTAD..RCG.ALIARAFVH
CKM H.sapiens         R...Y......K-...NHE..K..D....--N..L.S...T...IK..TLP.HC.RG.RRAV..LSVE..N..T.-.F.....---..KS..EKE.....
CKB H.sapiens         R...Y..S.E.K-...NPD..Q..D....--N..L.S...T...IR.FCLP.HC.RG.RRAI..LAVE.....D.-D.A.R..---A.KS..EAE.....
GK  N.diversicolor    I.H-...E.V......DETK....V-F.E--...K.C.I.....VR.VCLP.AM.R..RRLV..V..N..GG.KE-D.A...F---...T.NDKDMEA..
LK  E.fetida          K....G......PP..NANA....Q-F..--...K.A.I.T...VK.FCLP.SI.R..RR.V.RIIVD..AG...-D.A.V..---..KK..P.QEK...

Majority _____        DDHFL-F-KEGDRLLQAAGACRDWPDGRGIYHND-AKTFLVWVNEEDHLRIISMQKGGDLGEVFRRLVTGLNEIEKLVK-----SFRNDRLGFLTFCPTN
                             210       220       230       240       250       260       270       280       290       300
AK  T.cruzi           .....-.-.....F....H..KF..T........-.....................N.K...G....AVGV..EK..-----...D............
AK  A.mellifera       .....-.-.....F....N...F..T........-D......C............M.....Q.Y....HAV.....RLL------..H............
AK  D.melanogaster    .....-.-.....F....N...F..S........-.......C............Q.....QIYK....AV.....R.P------..HD............
AK  P.japonicus       .....-.-.....F....N...Y..A........-N..................M.....Q.....TSAV.....RIP------..HH............
AK  C.elegans         A....-.-.....F.K..N.N.Y..N....F..E-K...................I..-.DD.KKICSDMK..I..IH...S.SKE.I......Q....T
AK  C.trachomatis     HVPA.KYGDALSE..VP-------------.QREF.SSS.IKNRILR..GMLTHSCCL.LQEALDATSWIQ.GMSMQWI.DS.----------.HP.WNP
CKM H.sapiens         .....-.D.PVSP..L.S.MA.....A...W...-N.S.....GL.GG..V..AH..KHP-....ILTR.R..K.....VD.A.V.S.F.V..AD...S.
CKB H.sapiens         .....-.D.PVSP..L.S.MA.....A...W...-N..............V.......NMK...T.FC...TQ..T.F.SKDYE.MW.PH..YILT..S.
GK  N.diversicolor    E....-.E.PTGA..TTS.CA.........W..N-G.N....I.....I..........MRA..S.FGR..T.V.R.M.EKGYELM..E...YICT....
LK  E.fetida          A....-.Q.PTGH.MVNS..V.....A...W..K-D....I.I....QV...A..H...VKA..E.FSR..TQ..G.M.KHGHE.AWSE...YICT..S.

Majority _____        LGTTVRASVHIKLPKLSADK-KFEEVAAKLNLQVRGTGGEHTEAEGGVYDISNKRRLGLSEFEAVQEMVDGVLELIELEKSLE----I-------------------------
                             310       320       330       340       350       360       370       380       390       400       410
AK  T.cruzi           ....I..........G..RK.L......Y.......A...SDSPD..............Y...K..Q..I....KA.E.AR
AK  A.mellifera       ...............A.NRA.L..I.G.F.......R.........I...........T.YQ..K..H..IA...K...E.
AK  D.melanogaster    ....IL......V...PSN.A.L.....Y....ANPR-........S........M..T.....K..Y..IT...K....
AK  P.japonicus       ...............A.NRD.L....G.Y.......R.........I........M..T..Q..K..Q..I.Q..KM..EM
AK  C.elegans         ...............I..-.DD.KKICSDMK..I..IH...S.SKE.I......Q....T.YQ..RQ.Y..LKK.....AAA
AK  C.trachomatis     IKNRILR..GMLTHSCCL.LQEALDATSWIQ.GMSMQWI.DS.----------.HP.WNPL.---WDLRR.H.A.YNQDT---ANRS.EKEVIAQIRAKATK-PQAERLIIRI
CKM H.sapiens         ...GL.GG..V..AH..KHP-....ILTR.R..K.....VD.A.V.S.F.V..AD...S..V.Q..LV....KLMV.M..K..KGQS.DDMIPAQK
CKB H.sapiens         ...GL..G......N.GKHE-..S..LKR.R..K.....VD.A.V...F.V..AD...F..V.L..MV....KL...M.QR..KGQA.DDLMPAQK
GK  N.diversicolor    ...V......LR.AN.EK..-R.DDFL...R.GK......SSL..DST.....LA...K..R.L..VL....NV...AD.R..AGKP.DDLTPRLNSSTGTSISATASRHMTL
LK  E.fetida          ...GL.....LQ.H...KHP-....IILAFH..K.........VDD......RA..KK..R.F..LLI...GK...Y..L..AGKS.DDVLPA----------------SLKG
```

**B**

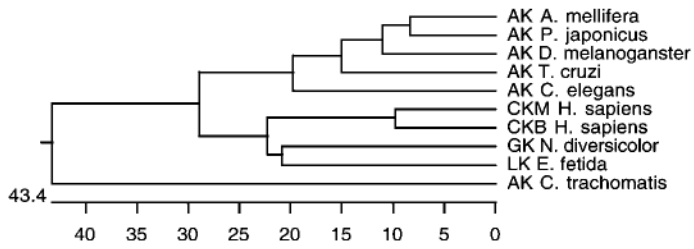


FIG. 2. **Amino acid sequence comparison (*panel A*) and phenogram (*panel B*).** *Panel A*, alignment of arginine kinase (AK) sequences: *T. cruzi* (GenBank AF070451), *A. mellifera* (AF023619), *D. melanogaster* (P48610), *P. japonicus* (P51545), *C. elegans* (Q27535), *C. trachomatis* (AE001338), *Homo sapiens* mitochondrial creatine kinase (CKM) (P00732), *Homo sapiens* brain creatine kinase (CKB) (P12277), *N. diversicolor* glycocyamine kinase (GK) (P51546), and *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.

*coli*—The expressed enzyme was purified to near homogeneity as described under "Experimental Procedures" (Fig. 3*A*) and assayed for biochemical properties. It was recovered with a specific activity of about 10–16 $\mu$moles·min$^{-1}$·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 *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 I).

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

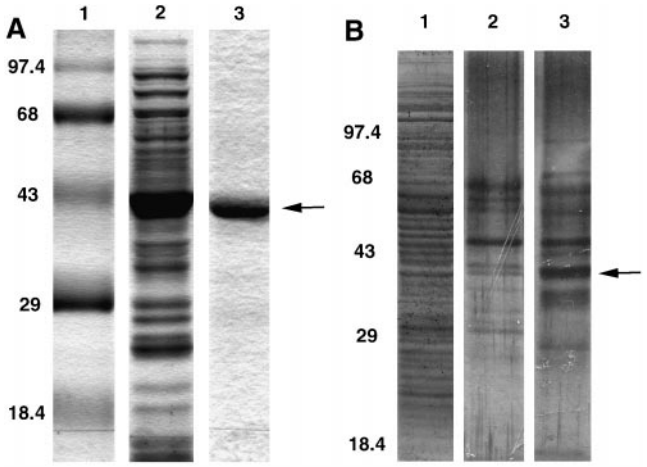


FIG. 3. **SDS-polyacrylamide gel electrophoresis of the recombinant and native *T. cruzi* arginine kinase.** *Panel A*: *lane 1*, molecular weight standards; *lane 2*, 100 $\mu$g of total protein extracted from an *E. coli* culture induced with 100 mM isopropyl-1-thio-$\beta$-D-galactopyranoside; *lane 3*, 10 $\mu$g of the Ni-agarose column purified recombinant protein. *Panel B*: *lane 1*, 100 $\mu$g of cytosolic proteins; *lane 2*, 10 $\mu$g of DE-52 percolate (pH 7.6) proteins; *lane 3*, 10 $\mu$g of Superose 6-purified fraction. The arginine kinase position is indicated by the *arrow*.

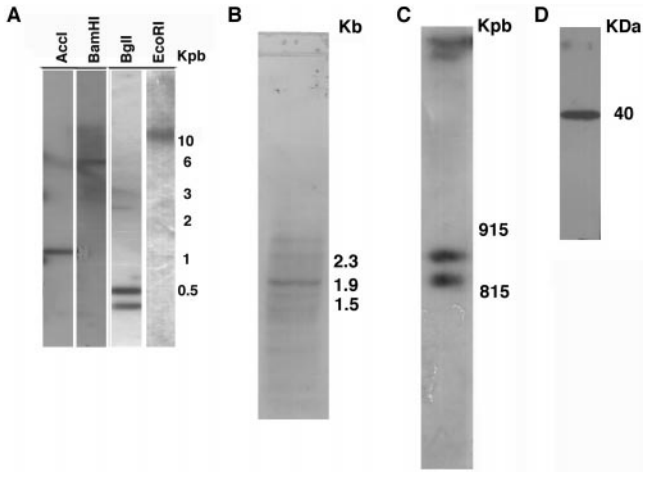


FIG. 4. **Southern (*panel A*), Northern (*panel B*), chromosome (*panel C*), and Western (*panel D*) blot analysis of *T. cruzi* arginine kinase sequences and polypeptides.** *Panel A*, 5 $\mu$g of genomic DNA was digested with the restriction enzyme indicated, transferred, and hybridized using the arginine kinase gene as a probe. Molecular mass marker positions are indicated. *Panel B*, total RNA (15 $\mu$g) 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. *Panel C*, chromosome blot. The fractionation of chromosomal bands were performed by pulsed field gel electrophoresis, with about $10^7$ epimastigote form (see "Experimental Procedures") using *S. cerevisiae* chromosomes as molecular mass standards (Life Technologies, Inc.). The probe was the same described above. *Panel D*, S100 subcellular fraction was subjected to Western blot analysis in SDS-polyacrylamide gel electrophoresis. The band position of 40 kDa was obtained by comparison with molecular weight standard proteins (New England BioLabs Inc.).

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. 4*B*).

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. 4*D*).

*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 actinin-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 in 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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