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Screening of Substrate Analogs as Potential Enzyme Inhibitors for the Arginine Kinase of *Trypanosoma cruzi*

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ABSTRACT. Arginine kinase catalyzes the transphosphorylation between phosphoarginine and ADP. Phosphoarginine is involved in temporal ATP buffering and inorganic phosphate regulation. *Trypanosoma cruzi* arginine kinase phosphorylates only L-arginine (specific activity 398.9 · mUE·min^{-1} · mg^{-1}), and is inhibited by the arginine analogs, agmatine, canavanine, nitroarginine, and homoarginine. Canavanine and homoarginine also produce a significant inhibition of the epimastigote culture growth (79.7% and 55.8%, respectively). Inhibition constants were calculated for canavanine and homoarginine (7.55 and 6.02 mM, respectively). In addition, two novel guanidino kinase activities were detected in the epimastigote soluble extract. The development of the arginine kinase inhibitors of *T. cruzi* could be an important feature because the phosphagens biosynthetic pathway in trypanosomatids is different from the one in their mammalian hosts.

Key Words. Canavanine, guanidino kinase, homoarginine, phosphagen kinase, phosphoarginine.

ARGININE kinase (ATP:arginine phosphotransferase; EC 2.7.3.3) catalyzes the reversible transphosphorylation between *N*-phospho-L-arginine and ADP (Huennekens and Whiteley 1960). Phosphoarginine plays a critical role as an energy reserve because the high-energy phosphate can be transferred to ADP when a renewal of ATP is needed. It has been proposed that phosphoarginine supports bursts of cellular activity until metabolic events, such as glycogenolysis, glycolysis, and oxidative phosphorylation, are switched on (Hird 1986; Morrison 1973). Arginine kinase is present in Annelida, Coelenterata, Platyhelminthes, Nemertea, Mollusca, Phoronida, Arthropoda, Echinodermata, Hemichordata, and Chordata (Morrison 1973). Another seven guanidino kinases have been identified. Creatine kinase, the best known member of this super-family, is found in vertebrates and is widely distributed throughout the lower chordates and invertebrates. Thalassemine kinase, glycocyamine kinase, taurocyamine kinase, hypotaurocyamine kinase, lombricine kinase, and opheline kinase were discovered later, exclusively in the tissues of worm-like marine groups. Their substrates possess the characteristic guanidino group but differ in terms of other chemical features (Ellington 2001).

We have reported the molecular and biochemical characterization of arginine kinases in *Trypanosoma cruzi*, the etiological agent of Chagas' disease, a major health and socioeconomic problem in Latin America (Pereira et al. 1999; Pereira et al. 2000) and *Trypanosoma brucei*, the etiological agent of African human sleeping sickness and Nagana in livestock (Pereira et al. 2002). The arginine kinase expression during growth of *T. cruzi* suggests a correlation between the enzyme activity, nutrient availability, and cell density or replication rate. In this way, arginine kinase could be proposed as a regulator of energy reserves under starvation stress conditions (Alonso et al. 2001).

The finding in trypanosomatid parasites of a phosphagen and its biosynthetic pathway, which are totally different from those in mammalian host tissues, points to arginine kinase as a possible chemotherapeutic target. This work presents a first step in the development of inhibitors of the arginine kinase of *T. cruzi* based on arginine analogs. In addition, we describe two novel guanidino kinase activities present in the epimastigote soluble fraction.

MATERIALS AND METHODS

Reagents. Reagents were provided by Sigma Chemical Co. (St. Louis, MO).

Parasite culture, lysis and preparation of a semi-purified enzyme fraction. Epimastigotes of the CL Brener strain were cultured at 28 °C in plastic flasks (25 cm^2), containing 5 ml of liver infusion-tryptose (LIT) medium (inoculated with 10^6 cells per milliliter) supplemented with 10% fetal calf serum, 100 U/ml penicillin, and 100 μg/ml streptomycin (Castellani et al. 1967). Parasites were harvested by centrifugation at 1,500 *g* for 10 min and washed three times with phosphate-buffered saline (PBS). Cell pellets were resuspended in 50 mM HEPES buffer pH 7.3, containing 0.01 mg/ml leupeptin, 25 U/ml aprotinine, and 0.5 mM phenyl-methyl-sulphonyl fluoride. The cells were lysed by six cycles of freezing in liquid N_2 and thawing at 4 °C. The extracts were then centrifuged at 10,000 *g* for 10 min. The supernatant fluid (S10) was applied to a 5 × 1 cm Whatman DE-52 column that had been pre-equilibrated with 25 mM HEPES buffer, pH 7.6. The column was washed with 25 ml of the same buffer and the enzyme activity was recovered in the effluent with a specific activity 10-fold higher than the original extract.

Arginine kinase assays. Two assays were employed, one indirect (enzyme-coupled) and the other direct (isotopic) assay. For the enzyme-coupled assay, an incubation mixture containing 100 mM Tris-HCl buffer pH 8.2, 1.5 mM ATP, 1.5 mM $MgCl_2$, 0.5 mM DTT, 1.5 mM phospho(enol)pyruvic acid, 0.3 mM NADH, 5 units of lactic dehydrogenase (Sigma Chemical Company, L-2518), 5 units of pyruvate kinase (Sigma Chemical Company, P-7768), and the enzyme source (about 40 μg/ml) of partially purified enzyme (DE-52 effluent) or 240 μg/ml of the soluble (S10) fraction was used. After a 5-min preincubation at 35 °C, reactions were started by adding arginine to a final concentration of 1 mM for the competition assays or 10 mM for the phosphorylation assays, in a spectrophotometer cuvette to a final vol. of 1 ml. Arginine kinase activity was calculated from the consumption of NADH, measured as a decrease in absorbance at 340 nm, during 10-min reactions (Hird and McLean 1983). To determine whether the compounds tested for inhibition of arginine kinase activity in the indirect competition assay inhibited the coupling enzymes, all the drugs were tested in the presence of 1.5 mM ADP to bypass the arginine kinase reaction. Negative controls were performed using reaction mixtures without ATP, enzyme source or arginine.

The direct (isotopic) assay was performed using an incubation mixture containing 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, USA; 0.5 μCi per assay), and the enzyme source (40–400 μg/ml of protein), in a total vol. of 0.2 ml. Incubations were carried out for 10 min at 30 °C. Reactions were stopped by the addition of 1

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Table 1. Phosphorylation of guanidino compounds inferred from NADH consumption in an enzyme-coupled assay using the partially purified arginine kinase of *Trypanosoma cruzi* or a soluble fraction from epimastigote cells.[a]

Compound	Partially purified arginine kinase		Epimastigote soluble fraction	
	Specific activity (mUE·min^{-1}·mg^{-1})	SD (±)	Specific activity (mUE·min^{-1}·mg^{-1})	SD (±)
L-arginine	398.9[b]	40.3	34.6[b]	9.0
Nitroarginine	0	—	0	—
D-arginine	0	—	0	—
L-histidine	0	—	6.3	1.2
Homoarginine	2.9	4.7	0	—
Canavanine	0	—	0	—
Glycocyamine	0	—	23.1[b]	0.7
Agmatine	0	—	0	—
Creatine	0	—	13.7[b]	2.7
Guanidine	0	—	4.8	3.7
Aminoguanidine	0	—	0	—
Guanidine butyrate	0	—	3.2	1.1

[a] For phosphorylation each test compound was assayed indirectly by depletion of ATP in the enzyme-coupled assay. The values represent means ± one standard deviation calculated from three independent assays.
[b] Significantly different from zero in *t*-test at $p < 0.05$.

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 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 (Pereira et al. 2000). Results are the averages of triplicate assays. The authenticity of phosphoarginine as a reaction product was validated by the procedures described elsewhere (Pereira et al. 2000).

Michaelis-Menten constant (Km) and inhibition constants (Ki) for L-arginine, canavanine, and homoarginine were calculated using the standard isotopic arginine kinase assay with partially purified arginine kinase (40 μg/ml of protein). Eight different concentrations of L-arginine and four of canavanine or homoarginine were used.

Table 2. Competition analysis of substrates for the arginine kinase of *Trypanosoma cruzi*.[a]

Compound (10 mM)	Inhibition (%)	SD (±)
L-arginine	0	6.3
Nitroarginine	52.6[b]	15.9
D-arginine	0	16.9
L-histidine	2.4	16.6
Homoarginine	38.2[b]	11.9
Canavanine	54.6[b]	8.7
Glycocyamine	0	14.5
Agmatine	79.3[b]	4.9
Creatine	12.7	14.7
Guanidine	0	13.2
Aminoguanidine	2.6	14.5
Guanidine butyrate	4.3	14.6

[a] Arginine kinase substrate competition analysis was performed using the indirect enzyme-coupled kinase assay, containing 1 mM L-arginine as substrate and about 40 μg/ml of partially-purified enzyme. Reactions were started after a 5-min preincubation at 35 °C by adding arginine (to 1 mM) and the indicated compound (to 10 mM). The values represent means ± one standard deviation calculated from three independent assays.
[b] Significantly different from the control assay (L-arginine) in *t*-test at $p < 0.05$.

Cell growth inhibition assays. Epimastigotes were cultured at 28 °C in plastic 96-wells plates, containing 0.1 ml of LIT medium (inoculated with 10^6 cells/ml). The treatments were added the first day at a 10 mM concentration, and the plate included control cells without treatment. At the indicated times, cells were counted using a hemacytometric chamber. Cultures were performed in triplicate.

Analytical methods. Protein was determined according to Bradford (Bradford 1976).

RESULTS AND DISCUSSION

Phosphorylation assay using partially purified arginine kinase. Phosphorylation of compounds containing a guanidino group was evaluated using an indirect assay that detects the conversion of ATP to ADP by an enzyme-coupled system (Hird and McLean 1983). Different compounds, including L-arginine (Km 0.33 mM, determined using the direct isotopic assay), were tested at 10 mM concentration. Partially purified arginine kinase from *T. cruzi* (purified 10-fold with respect to the initial supernatant fraction) phosphorylated L-arginine with a specific activity of 398.9 · mUE·min^{-1} · mg^{-1}. The enzyme is stereospecific and phosphorylation of D-arginine was not detected. Phosphorylation by the arginine kinase of *T. cruzi* was not observed for the following: the phosphagens creatine and glycocyamine (guanidinoacetate); the natural amino acid L-histidine; the arginine analogs homoarginine, canavanine and agmatine; guanidine and their derivatives amino guanidine and guanidine butyrate; and the negative control (the guanidino blocked compound, nitroarginine) (Table 1). These results reveal a difference between the arginine kinases of *T. cruzi* and insects: arginine analogs are phosphorylated by insect (*Manduca sexta*) arginine kinases (Rosenthal et al. 1977), but the arginine kinase of *T. cruzi* seems to be highly specific.

Phosphorylation assay using *T. cruzi* soluble extract. To determine if compounds other than arginine could be phosphorylated by *T. cruzi* extracts, an indirect enzyme-coupled phosphorylation assay was performed using a crude soluble fraction (240 μg protein per ml). The same compounds used with the partially purified arginine kinase were each tested at a 10 mM concentration. Strikingly, L-arginine and only the other

Table 3. Growth inhibition of epimastigotes of *Trypanosoma cruzi* cell cultures by arginine analogs.[a]

Treatment	Cell number ($\times 10^6$)	SD (±)	Inhibition (%)
Control	21.7	1.0	—
Nitroarginine	23.85	0.9	—
Agmatine	26.3	1.8	—
Homoarginine	9.6[b]	0.6	55.8
Canavanine	4.4[b]	0.8	79.7

[a] 10^6 epimastigote cells were grown on 96-well plates containing 0.1 ml of liver infusion-tryptose (LIT) medium and 10 mM of the indicated compounds. The values represent means ± one standard deviation calculated from three independent assays.

[b] Significantly different from control assay in *t*-test at $p < 0.05$.

two phosphagens assayed were phosphorylated by the epimastigote soluble fraction. L-arginine was phosphorylated at a velocity of 34.6 mUE · min^{-1} · mg^{-1}, glycocyamine at 23.1 mUE · min^{-1} · mg^{-1}, and creatine at 13.7 mUE · min^{-1} · mg^{-1} (Table 1). These results suggest the presence of other guanidino kinases in *T. cruzi* that will require further characterization. Previous studies from our group reported that anti-*T. cruzi* arginine kinase antibody cross-reacts in soluble extract of *T. brucei* with the arginine kinase of *T. brucei* and with another polypeptide that is similar in size to a creatine kinase or glycocyamine kinase (Pereira et al. 2002). It is noteworthy that both substrates (creatine and glycocyamine) are present in the *T. cruzi* mammalian hosts, glycocyamine being an intermediate in the creatine biosynthesis from arginine (Ellington 2001).

Arginine kinase substrates competition analysis. Although no guanidino compounds were tested as substrates with the partially purified arginine kinase, we tested them for their capacity to act as arginine kinase inhibitors binding to the guanidino specificity region of the enzyme (Zhou et al. 1998). An indirect enzyme-coupled phosphorylation assay was performed using 10-fold molar excess of the competitor (1 mM L-arginine plus 10 mM of the competitor) and partially purified enzyme (40 μg/ml). Agmatine significantly inhibited the enzyme activity to 79.3%, canavanine to 54.6%, nitroarginine to 52.6%, and homoarginine to 38.2% (Table 2). All other compounds, included D-arginine, produced no effects on the arginine kinase activity.

In order to confirm the inhibition results obtained using the enzyme-coupled assay, a direct isotopic assay using L-[^{3}H] arginine was performed in the presence of the same compounds and partially purified arginine kinase (40 μg/ml) or a epimastigote soluble fraction (400 μg/ml). In concordance with the previous enzyme-coupled assay, only canavanine, nitroarginine, and homoarginine produced higher and statistically significant inhibition compared with the control without treatment. In contrast, agmatine (decarboxylated arginine) caused no inhibition of arginine kinase activity.

Inhibition constants (Ki) values were calculated for canavanine and homoarginine using the isotopic assay because both metabolites were able to inhibit arginine kinase activity as well as epimastigote growth. Ki values for canavanine and homoarginine were 7.55 mM and 6.02 mM, respectively, and the inhibition kinetics were competitive in both cases.

Inhibition of epimastigote growth by arginine analogs. We analyzed the in vivo effect of the arginine kinase competitive inhibitors, adding 10 mM of each one in independent epimastigote cell cultures (Table 3). The results obtained after a 5-day treatment revealed that canavanine inhibited cell growth by 79.7% with respect to the control without treatment, and homoarginine inhibited cell growth by 55.8%. These results suggest that the observed effect could be mediated by an inhibition of the parasite's arginine kinase, in addition to possible toxic effects produced in other metabolic pathways, such as protein synthesis.

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