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Research brief

Trypanosoma cruzi: Multiple nucleoside diphosphate kinase isoforms in a single cell

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ABSTRACT

Nucleoside diphosphate kinases (NDPKs) are multifunctional enzymes involved mainly in the conservation of nucleotides and deoxynucleotides at intracellular levels. Here we report the characterization of two NDPKs from the protozoan parasite *Trypanosoma cruzi*, the etiological agent of Chagas disease. TcNDPK1 and TcNDPK2 were biochemically characterized presenting different kinetic parameters and regulation mechanisms. NDPK activity was mainly detected in soluble fractions according to the digitonin extraction technique; however 20% of the activity remains insoluble at digitonin concentrations up to 5 mg ml^{-1}. TcNDPK1 is a short enzyme isoform, whereas TcNDPK2 is a long one containing a DM10 motif. In addition, two other putative NDPK genes (TcNPDK3 and TcNDPK4) were detected by data mining at the *T. cruzi* genome database. The large number and diversity of NDPK isoforms are in agreement with those previously observed for other *T. cruzi* phosphotransferases, such as adenylate kinases.

1. Introduction

Nucleoside diphosphate kinases (NDPK, EC 2.7.4.6) are ubiquitous and conserved enzymes essential for cellular homeostasis. They play a key role in the maintenance of intracellular ratios of NTPs and dNTPs catalyzing the reversible phosphorylation of nucleoside diphosphates to nucleoside triphosphates (Parks and Agarwal, 1973) as follows:

$$N_1TP + N_2DP \leftrightarrow N_1DP + N_2TP$$

Although the high-energy phosphate is mainly supplied by ATP these enzymes have broad substrate specificity and use the ribose and deoxyribose forms of both, purine and pyrimidine nucleotides. It was postulated a "ping-pong" mechanism with a phosphoenzyme (phosphohistidine) intermediary involved in the transfer reaction (Lascu and Gonin, 2000).

The crystal structures and biochemical studies have shown that prokaryotic enzymes have a tetrameric structure, whereas the eukaryotic ones are hexamers. Each NDPK monomer have a molecular mass of 15–18 kDa and are conserved during evolution, presenting about 43% identity between *Escherichia coli* and humans enzymes (Lascu and Gonin, 2000).

Far more than house-keeping enzymes, a lot of accumulated evidence involves NDPKs in many biological processes other than the biosynthetic one. NDPKs appear to be associated in signal transduction by providing GTP to G proteins activation (Bominaar et al., 1993; Randazzo et al., 1992). Regarding to the high-energy phosphohistidine formation it was also postulated a cell signaling role because the phosphate can be transferred to different acceptors such as other histidine residue as part of a phosphorylation cascade (Lu et al., 1996) or to serine/threonine or aspartate residues in other proteins (MacDonald et al., 1993; Wagner et al., 1997; Wagner and Vu, 2000). In this sense some functions in cell development, proliferation, differentiation, tumor metastasis, apoptosis and motility were attributed (MacDonald et al., 1993; Biggs et al., 1990; Kantor et al., 1993; Rosengard et al., 1989; Lombardi et al., 2000; Lacombe et al., 2000). It was also suggested that NDPK activity could be involved in bacterial two-component signal transduction systems (Stock et al., 2000). During the last years, some roles in DNA processing have also been assigned. In humans NM23-H2/NDPK B appears to be a transcription factor because of its sequence-specific DNA binding activity. Also the recognition, cleavage and structural alterations of the DNA targets suggest a possible participation in DNA repair (Postel et al., 1993,2000; Postel, 1999). In addition, *E. coli* NDPK is capable of bind and cleave DNA molecules (Levit et al., 2002).

An interesting model to study the role of NDPKs is constituted by protozoan parasites, including *Trypanosoma cruzi*, the causative agent of Chagas disease. In contrast to mammals, these organisms are not able to synthesize purines de novo and therefore rely upon the salvage pathway in which free purines are converted subsequently to nucleotides involving NDPK activities (Hammond and Gutteridge, 1984; Marr, 1991). These parasites constitute a serious health problem that affect millions of Americas, so this metabolic pathway could be a possible target for future drug development. In *T. cruzi*, a soluble NDPK activity was previously purified and biochemically characterized (Ulloa et al., 1995). Interestingly, in

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Trypanosoma brucei a nuclear isoform of NDPK has been identified (Hunger-Glaser et al., 2000). The presence of NDPK isoforms within different cellular compartments may be critical for their biological functions and also for their specific role in the phosphotransfer network involved in the cellular energy transport and homeostasis.

In this work we report the molecular and biochemical characterization of different NDPK isoforms from *T. cruzi*.

2. Materials and methods

2.1. Parasite cultures and cell extracts

Epimastigotes of the CL Brener strain, were cultured at 28 °C in 25 cm^2 plastic flasks, containing 5 ml of LIT medium supplemented with 10% fetal calf serum, 100 U/ml penicillin and 100 mg/l streptomycin (Camargo, 1964). Cells were counted using a hemocytometric chamber, harvested by centrifugation at 1500*g* for 10 min and washed three times with phosphate-buffered saline (PBS). Cell pellets were then resuspended in 50 mM Tris–HCl buffer, pH 7.3, containing 10 μM of the protease inhibitor trans-Epoxysuccinyl-L-leucylamido(4-guanidino)butane (E64), and lysed by five cycles of freezing and thawing.

2.2. Bioinformatics, cloning and expression of NDPKs

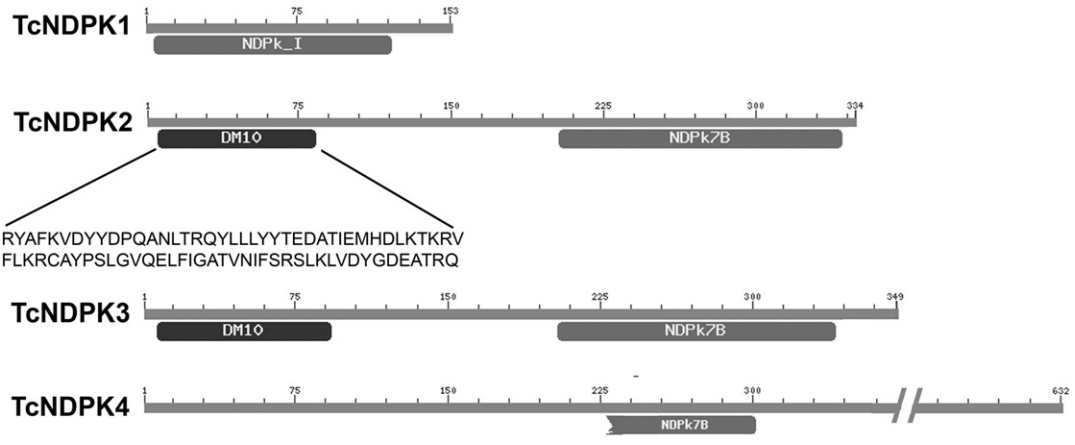
Sequences from the "Tritryps" genome projects (*T. cruzi*, *T. brucei* and *Leishmania major*) were obtained at GeneDB (http://www.genedb.org/) and TcruziDB (http://tcruzidb.org/). Assembly and analysis of the DNA sequence data, including prediction of open reading frames, were carried out using the software package Vector NTI v. 10 (Invitrogen Corporation) and the online version of BLAST at the NCBI (http://www.ncbi.nlm.nih.gov/BLAST/). Fragments carrying the entire *T. cruzi* TcNDPK1 and TcNDPK2 sequences (GeneDB systematic IDs Tc00.1047053508707.200 and Tc00.1047053508461.400) were obtained by PCR amplification (NDPK1: forward primer GGATCCATGACCAGTGAGCGTACCTT, reverse primer GGTACCTCAACGCTGTCAGAGCCATGGCACACGTTTC; NDPK 2: forward primer GGATCCATGACAACTACTTCTCGTTA, reverse primer GGTACCTTGATTACTTTTTCTCAATC) and ligated to the pRSET A expression vector (Invitrogen, Carlsbad, CA). Overexpression of the recombinant NDPKs were performed in *E. coli* strain BL21(DE3)pLysS (Invitrogen, Carlsbad, CA). Recombinant proteins were purified by affinity chromatography using a Ni^{2+}-agarose resin (Qiagen, MD, USA).

2.3. Digitonin extractions

The extraction protocol was performed as previously described (Bouvier et al., 2006). Briefly, 5.2×10^9 epimastigote cells from a 200-ml culture were washed twice and resuspended in 4 ml of 50 mM Tris–HCl buffer, pH 7.5, containing 0.25 M sucrose and 10 μM E64. Aliquots of 950 μl containing 6.5×10^8 parasites were mixed with 50 μl of the same buffer containing increasing amounts of digitonin (0–5 mg ml^{-1}). After 2.5 min of incubation at room temperature, tubes were centrifuged at 16,100*g* for 2 min. Supernatants were kept frozen for enzyme assays. Pellets were resuspended in the same buffer, lysed by five cycles of freezing and thawing and kept frozen for further assays.

2.4. Enzyme assays

Nucleoside diphosphate kinase activity was measured determining the rate of ADP formation as previously described (Ulloa et al., 1995). Briefly, a 25 μg of recombinant protein fraction was added to the reaction mixture (100 mM Tris–HCl buffer, pH 7.5, 3 mM phosphoenolpyruvate, 10 mM $MgCl_2$, 0.3 mM NADH, 25 mM KCl, 1.25 U/ml pyruvate kinase and 2.2 U/ml lactate dehydrogenase) in a cuvette to give a final volume of 0.5 ml. After 5 min at 35 °C the reaction was started by the addition of a small volume of ATP and dTDP to final concentrations of 2 mM and 0.4 mM, respectively. The activity was calculated measuring the decrease in absorbance at 340 nm due to the oxidation of NADH. When purified recombinant NDPKs were assayed for enzyme activity or SDS–PAGE, the same purification protocol



NAME	AA	pI	MW	Acc. No.	STATUS	*L.major*	*T.brucei*
TcNDPK1	153	8.52	16878	Tc00.1047053508707.200	active	LmjF32.2950	Tb11.01.7800
TcNDPK2	334	5.34	37333	Tc00.1047053508461.400	active	LmjF35.3870	Tb09.211.2560
TcNDPK3	349	5.24	39133	Tc00.1047053510879.210	putative	LmjF34.2980	Tb927.4.1720
TcNDPK4	632	6.66	69836	Tc00.1047053508989.100	putative	-	Tb927.8.4510

Fig. 1. Summary of features and predicted properties from each TcNDPK. Schematic representation of the four *T. cruzi* NDPK isoforms. Boxes indicate conserved domains predicted using the "CD-Search Service" at the NCBI: NDPk_I (cd04413), NDPk7B (cd04412) and DM10 (smart00676) (top). The table shows main features of each predicted *T. cruzi* NDPK: gene name, amino acid length, isoelectric point, molecular weight, GeneDB systematic IDs, status (biochemically active or putative), GeneDB systematic IDs of the sequence orthologs from *L. major* and *T. brucei* (bottom).

was applied to induced wild-type *E. coli* extracts and used as controls.

Pyruvate kinase and hexokinase activities were determined using a lactic dehydrogenase and glucose-6-phosphate dehydrogenase coupled assay, respectively (Cazzulo et al., 1989; Cáceres et al., 2003). $NADP^+$-dependent isocitrate dehydrogenase activity was determined as previously described (Denicola et al., 2002).

All assays were performed at least in triplicates and data are representative of at least three independent experiments.

2.5. Western blot analysis

Nucleoside diphosphate kinase antisera were obtained from BALB/c mice immunized with recombinants TcNDPK1 or TcNDPK2. Extract samples from *T. cruzi* epimastigotes, trypomastigotes and amastigotes were resolved by SDS–PAGE. Proteins were electrotransferred from polyacrylamide gels to PVDF membranes (Pierce). For reaction with the antibody, transferred membranes were blocked with 5% (w/v) non-fat milk suspension for 60 min. TcNDPK1 and TcNDPK2 antibodies were added and incubated for 12 h at a dilution of 1:1000, 1:2500, respectively. Antiserum detection was carried out by incubating with a 1:5000 dilution of anti-mouse Ig conjugated with horseradish peroxidase (Vector Laboratories). The latter was developed with Supersignal™ West Pico Chemiluminescent Substrate (Pierce).

3. Results and discussion

3.1. Nucleoside diphosphate kinases in Trypanosomatids

Using the recently released data from the "Tritryps" genome projects a database screening was performed at the GeneDB (http://www.genedb.org) followed by a bioinformatic sequence analysis. Four putative NDPK genes were found in the *T. cruzi* genome (TcNDPK1-4) coding for predicted polypeptides between 153 and 602 aa long. All the sequence orthologs were also found in *T. brucei*, however *L. major* lack the gene corresponding to the putative NDPK4. A summary of NDPK sequences is shown in Fig. 1. Amino acid identities between the different *T. cruzi* predicted polypeptides were in the range of 6–28% and similarities between 18 and 48%, being TcNDPK4 the most divergent sequence of the group. TcNDPK1 is the shorter enzyme isoform with the highest isoelectric point (8.5), all the others are acidic polypeptides and present an N-terminal extension (≈200 aa), additionally TcNDPK4 shows a C-terminal extension (≈350 aa). Interestingly, TcNDPK2 presents a conserved DM10 motif (≈88 aa) at the N-terminal domain. The presence of a single DM10 at the N-terminus was found only in a subfamily of NDPK, but the function of such motif remains unknown. Even so, other unrelated proteins have three DM10 motifs at the C-terminus that probably participates in axonemal targeting (King, 2006). The same motif is also present in the predicted TcNDPK3.

The domain called "NDPk_I" is a canonical NDPK motif whereas the "NDPk7B" is associated with NDPKs that contain a DM10 motif (Fig. 1).

3.2. Biochemical properties of TcNDPK1 and TcNDPK2

Two putative NDPK genes corresponding to the unique "short" isoform (TcNDPK1) and one of the "long" DM10-contanining isoform (TcNDPK2) were chosen for biochemical characterization. Both genes were amplified by PCR, cloned, overexpressed in *E. coli* and purified by affinity chromatography (Fig. 2A). Obtained polypeptides showed NDPK activity in the presence of ATP and

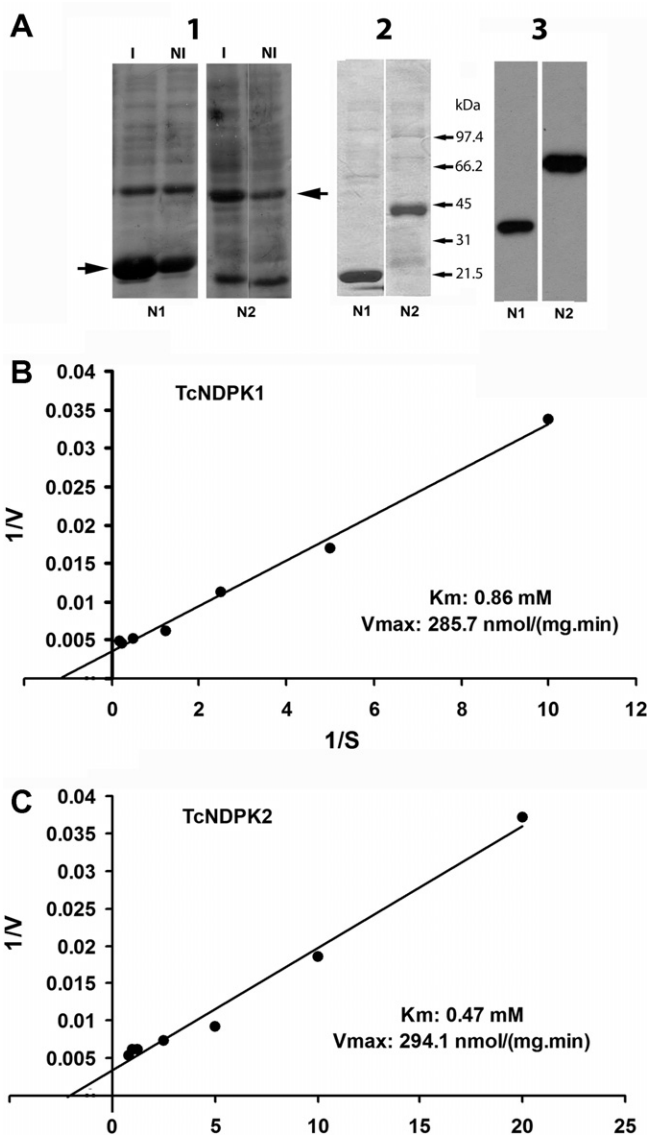


Fig. 2. Biochemical properties of TcNDPK1 and TcNDPK2. Recombinant TcNDPK1 (N1) and TcNDPK2 (N2) were overexpressed in *E. coli* (A1); affinity purified (A2), and analyzed by Western blot using anti-6x-his antibodies (A3). Initial rates of NDPK activity (V_0) measured as a function of dTDP concentration. Apparent Michaelis–Menten constants (K_m) values were calculated using Lineweaver–Burk plots for TcNDPK1 (B) and TcNDPK2 (C).

dTDP. Kinetics parameters for both enzyme isoforms were obtained. Michaelis–Menten constants (K_m) for dTDP of 0.86 mM and 0.47 mM were calculated using Lineweaver–Burk plot for TcNDPK1 and TcNDPK2, respectively (Fig. 2B and C). TcNDPK1 showed no inhibition by substrate at concentrations up to 4 mM. Interestingly, TcNDPK2 was strongly inhibited at high dTDP concentrations suggesting the presence of a second, inhibitory and low-affinity, substrate binding site which could be act as a regulatory mechanism in vivo (Fig. 3A).

3.3. Subcellular localization of NDPK activity

To preliminary determine the subcellular localization of the NDPK activity, digitonin extractions experiments were carried out. NDPK activity was detected in different cellular fractions of the replicative, non-infective, *T. cruzi* epimastigote stage. The pattern of extraction of NDPK activity was compared with those

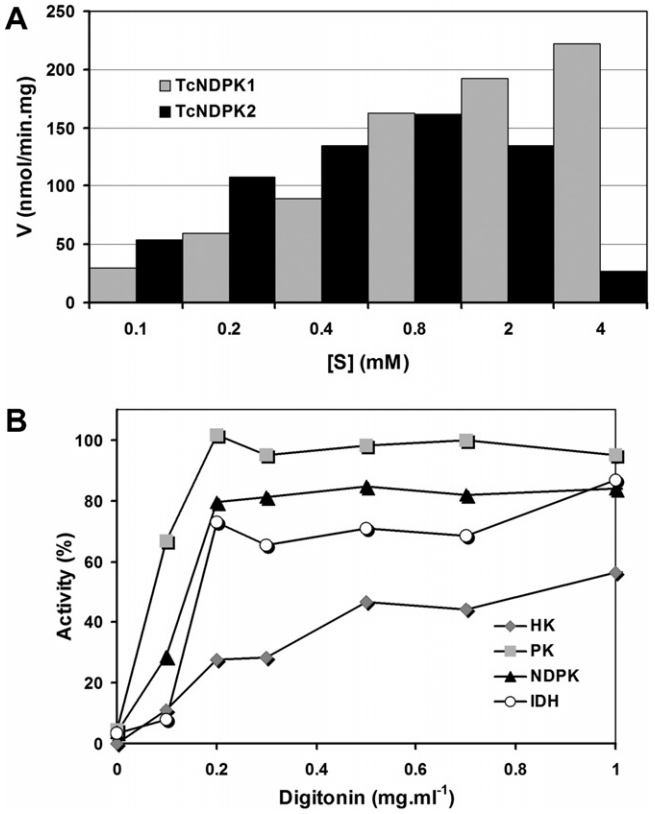


Fig. 3. Substrate regulation and digitonin extraction of NDPKs activities. TcNDPK1 and TcNDPK2 initial velocities were calculated as a function of substrate concentration in the range 0.1–4 mM (A). Digitonin extractions were carried out as described under Section 2 using *T. cruzi* epimastigotes samples. The measured activities represent the release of the indicated enzyme markers of intracellular compartments (HK, hexokinase; PK, piruvate kinase; IDH, $NADP^+$-dependent isocitrate dehydrogenase) and the nucleoside diphosphate kinase activity (NDPK) by selective permeation with digitonin in the range 0–1 mg ml^{-1} (B).

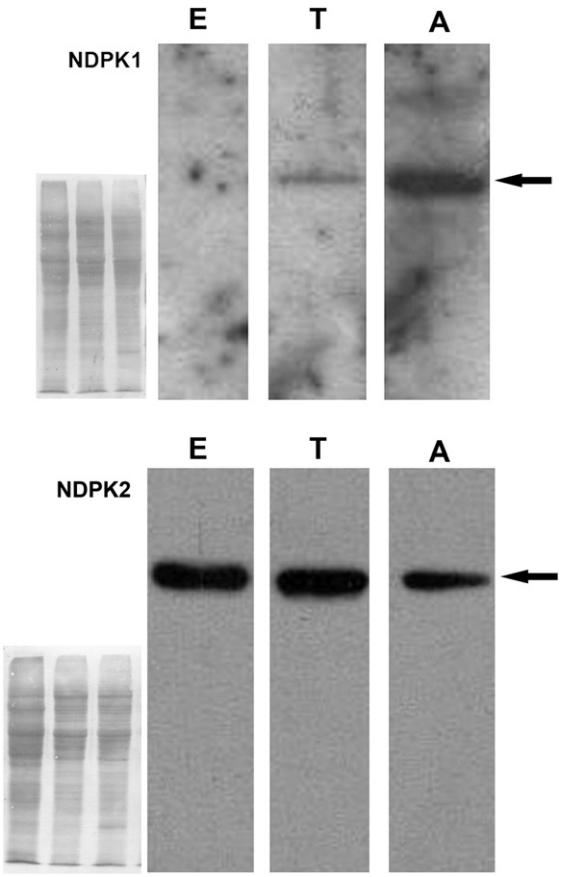


Fig. 4. Expression analysis of TcNDPK1 and TcNDPK2. Western blot analyses were performed using mouse polyclonal anti-TcNDPK1 and TcNDPK2 antibodies and *T. cruzi* extract samples from epimastigotes (E), trypomastigotes (T) and amastigotes (A). Total protein profiles stained using Coomassie brilliant blue are showed in the left side of each figure. Arrows indicates the position of TcNDPK1 and TcNDPK2.

measured for cytosolic, mitochondrial and glycosomal enzyme markers (pyruvate kinase, $NADP^+$-dependent isocitrate dehydrogenase and hexokinase, respectively). At 0.1 mg ml^{-1} of digitonin, 67% of the pyruvate kinase activity was released, and 29% of the NDPK activity was detected. At a digitonin concentration of 0.2 mg ml^{-1}, when the pyruvate kinase activity was completely extracted and the IDH activity began to be released the NDPK activity increased up to 80%. It could suggests a cytosolic and also a mitochondrial localization of NDPK activity. However, no additional NDPK activity was detected at higher digitonin concentrations (Fig. 3B), even at concentration of 5 mg ml^{-1} where the IDH and HK activities reached 100% (data not shown). This result suggests the existence of a main cytosolic NDPK activity, since most of the activity was extracted at low digitonin concentrations, in addition to other 20% of NDPK activity which probably remains associated to some organelle or subcellular structure up to 5 mg ml^{-1} digitonin concentration (Fig. 3B). The mentioned activity detected at low digitonin concentration could be attributed to the NDPK previously described by Ulloa et al. (1995). Moreover, the enzyme purified by these authors has similar kinetic properties and molecular weight than TcNDPK1 (16 kDa). However, the presence of such other NDPKs in the soluble fraction could not be discarded.

To address this issue, TcNDPK1 and TcNDPK2 mouse polyclonal antisera were produced. Western blot analyses were performed using *T. cruzi* extracts from different stages. Similar levels of TcNDPK2 were observed in the three stages (Fig. 4, lower panel). On the other hand, TcNDPK1 was found only in trypomastigotes and amastigotes (Fig. 4, upper panel) but probably these antisera could not be able to detect low levels of TcNDPK1. Due to the low antigenicity of this protein, seven independent mouse TcNDPK1 antisera were tested, but TcNDPK1 remained undetectable in epimastigotes.

NDPKs, during long time it have been considered house-keeping enzymes involved only in cellular energy homeostasis. However, additional functions in signaling pathways and in the interaction, and also modulation of the activity of other proteins were postulated during the last years (Kimura et al., 2003; Muimo et al., 2006).

Trypanosomatid organisms have an unusual large number of phosphotransferases enzymes involved in wide variety of cellular functions, including nucleotide recycling and interconversion, energy transport and balance. The large number of different NDPK isoforms is in agreement with those previously observed for other *T. cruzi* phosphotransferases, such as adenylate kinases and seems to be a common feature for these enzymes in trypansomatids organisms.

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