Purification and characterization of a soluble nucleoside diphosphate kinase in *Trypanosoma cruzi*

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**Abstract**

A soluble nucleoside diphosphate kinase (NDP kinase) was purified and characterized in epimastigote forms of *Trypanosoma cruzi*. The enzyme was purified by affinity chromatography on Blue-agarose and Q-Sepharose columns and by FPLC on a Superose 12 column. A membrane-associated NDP kinase was identified which accounts for 30% of total enzymatic activity. Western blot analysis of the soluble NDP kinase revealed a 16.5 kDa monomer recognized by polyclonal antibodies to NDP kinase from *Dictyostelium discoideum*, *Candida albicans* or human. Most of the *T. cruzi* NDP kinase is found in the cell as a hexamer composed of 16.5-kDa monomers. The *Kₐ* values of the enzyme for ATP, GDP and dTDP were 0.2 ± 0.008 mM, 0.125 ± 0.012 mM and 0.4 ± 0.009 mM, respectively. The parasite enzyme was stable, remained active at 65°C and was found to tolerate up to 2.5 M urea. The 16.5-kDa subunit was phosphorylated with [γ-³²P]ATP or thio phosphorylated with [³⁵S]GTPyS. The incubation of the ³²P-labelled phosphoenzyme with unlabelled nucleoside 5'-diphosphates resulted in the formation of ³²P-labelled nucleoside 5'-triphosphates without strict base specificity, indicating that the reaction mechanism of the *T. cruzi* enzyme is the same as reported for other NDP kinases. When the phosphoenzyme was incubated with a mixture of nucleoside 5'-diphosphates, GTP was preferentially formed.

**Keywords:** Nucleoside diphosphate kinase; *Trypanosoma cruzi*; Phosphoenzyme intermediate

1. Introduction

Nucleoside diphosphate kinase (NDP kinase) is a major component of the pathway for the synthesis of nucleoside triphosphates other than ATP. This enzyme catalyzes the transfer of the γ-phosphate of nucleoside 5'-triphosphates to nucleoside 5'-diphosphates by a ping-pong mechanism involving a phosphoenzyme intermediate. Ribose- and deoxyribonu-
cleotides with a purine or pyrimidine base structure can be used as phosphate acceptors, as well as phosphate donors, although their availability to the enzyme is different [1].

NDP kinases have been purified from cytosols of a number of eukaryotic species including human erythrocyte [2], Drosophila [3] and Dictyostelium [4]. Eukaryotic NDP kinases are hexamers composed of 17-kDa subunits [2,3,5]. A nuclear encoded mitochondrial NDP kinase has also been demonstrated in Dictyostelium discoideum, and is likely to occur in other species [6].

NDP kinase has also been involved in biological processes not necessarily linked to its biosynthetic role. Thus mammalian NDP kinase was described as associated with tubulin [7] or G proteins [8] and to participate in signal transduction in Dictyostelium [9]. While a direct role in phosphorylation of GDP bound to G proteins has been disproved [10,11], it remains possible that NDP kinase synthesizes GTP for G protein activation in a channeling mechanism. The two isozymes of NDP kinase present in human, NDP kinase A and NDP kinase B have been identified as the products of the genes nm23-H1 and nm23-H2, respectively [2]. Nm23-H1 was proposed to be a putative metastasis suppressor gene [12], while the product of nm23-H2 has recently been demonstrated to activate the transcription of c-myc in vitro [13]. The exact role of NDP kinase in these processes is not known and may be distinct of the catalytic activity carried by the enzyme [14].

In protozoan parasites, the metabolism of purines is significantly different from that in mammals where purines are synthetized de novo from non-nucleotide precursors. Nucleotides can also be formed from free purines and purine nucleosides. These routes, regarded as salvage pathways, are present both in mammals and pathogenic protozoa. In the latter, they are the only means of synthesizing purine nucleotides [15]. NDP kinases are involved in the salvage pathway by which free purines are converted to nucleosides and these to nucleotides [16].

In this article, a soluble NDP kinase from epimastigote forms of Trypanosoma cruzi was purified, characterized and its reaction mechanism analyzed as a first step to study the role of this enzyme in the parasite. Also it describes the presence of a particulate form of the enzyme in this protozoa.

2. Materials and methods

2.1. Materials

Superose 12 and Sephadex G-50 were from Pharmacia (Uppsala, Sweden), pyruvate kinase and lactate dehydrogenase were from Boehringer-Mannheim (Mannheim, Germany), 2,4-Dinitrophenylhydrazine was from K&K Laboratories (Plainview, NY, USA) and Tween-20 was from Merck. Aprotinin was a kind gift of Gador Laboratories (Argentina). Prestained SDS-PAGE standards were from Bio-Rad Laboratories (Hercules, CA, USA). All other reagents were of analytical grade. [γ-32P] ATP and [35S]GTPγS were from New England Nuclear (Boston, MA, USA).

The polyclonal antibody against NDP kinase from Candida albicans was a kind gift of Dr. S. Passeron (Fac. Agronomía, Universidad de Buenos Aires, Buenos Aires, Argentina). The polyclonal antibodies against NDP kinase from Dictyostelium discoideum and human were obtained as in Refs. 17 and 18, respectively.

2.2. Cell cultures

Unless otherwise indicated, all operations were performed at 2–5°C. Trypanosoma cruzi epimastigote forms (Tulahuen 2 and Tulahuen 0 strains) were cultured for 7 days at 28°C up to the late exponential phase in monophasic axenic medium, as described [19]. After disruption, cell debris were separated by centrifugation at 1000×g for 10 min. The supernatant was adjusted to 0.1 mM PMSF/25 units ml⁻¹ Trasylol/1 µg ml⁻¹ antipain/2 µg ml⁻¹ soybean trypsin inhibitor/10 µg ml⁻¹ leupeptine/1 mM benzamidine, and further centrifuged at 105,000×g for 60 min. The cytosolic fraction, designated S100, was immediately processed to avoid proteolytic degradation.

2.3. Subcellular fractionation

T. cruzi epimastigote forms from a 50-ml late exponential phase culture were collected by centrifugation at 7500 rpm for 15 min. Cells (5×10⁸ parasites) were washed with 1 ml of 0.25 M saccharose/50 mM KCl containing the above-mentioned pro-
tease inhibitors and centrifuged at the same speed. Cell pellets were lysed by 5 cycles of freezing and thawing and resuspended in 0.5 ml of 10 mM Tris-HCl pH 7.4 containing 2 mM MgCl₂/1 mM EDTA/1 mM DTT/0.25 mM PMSF (TMEDP buffer). The homogenate (H) was subjected to three successive centrifugations at 121 × g for 15 min, 1000 × g for 15 min and 105000 × g for 60 min. Sediments from each centrifugation corresponded to 'nuclear' (N), 'flagellar' (F) or 'microsomal' (M) enriched fractions, respectively, as demonstrated by Ogueta et al. [20]. Malic enzyme activity, reported to be soluble by Cannata et al. [21], was monitored in the various subcellular fractions for its distribution.

2.4. Purification of the soluble NDP kinase

The NDP kinase activity was purified according to Kimura and Shimada [8], modified as follows: The S100 (90 ml, 1.88 mg ml⁻¹) was fractionated by the addition of saturated ammonium sulfate. The precipitate (between 45 and 70% saturation), designated S.A.70, was collected by centrifugation at 27000 × g for 20 min, dissolved in 90 ml (0.91 mg ml⁻¹) of TMEDP buffer and overnight dialysis against TMEDP. The enzyme fraction was applied to a Blue-agarose column (0.6 × 14 cm) equilibrated with the same buffer. After washing with TMEDP, the column was rinsed with the same buffer containing 0.3 M NaCl. The enzyme was eluted with TMEDP containing 0.3 M NaCl/2 mM ATP. The pooled fractions with enzymatic activity were dialyzed overnight against 40 mM Tris-HCl pH 7.5 containing 1 mM DTT/0.1 mM PMSF/activated charcoal to remove free ATP, and loaded onto a Q-Sepharose column (0.6 × 2 cm) equilibrated with TMEDP. After washing, the column was eluted stepwise with 0–0.3 M NaCl in the same buffer.

The NDP kinase activity which was not retained by the Q-Sepharose column was extensively dialyzed against 40 mM Tris-HCl pH 8, and loaded onto a second Q-Sepharose column equilibrated with 40 mM Tris-HCl pH 8/1 mM DTT. The enzyme was once again recovered in the flow through fraction (0.016 mg protein ml⁻¹) and was concentrated 10-fold by centrifugation (3000 rpm, 90 min) with an Amicon centrprep (MW cut-off: 12000). This fraction, referred to as T. cruzi NDP kinase, was stored at −20°C. Kinetic and electrophoretic assays were performed with this preparation.

2.5. FPLC chromatography on Superose 12

An aliquot of the concentrated Q-Sepharose fraction (0.165 mg protein) was injected onto a Superose 12 column, equilibrated with TMEDP buffer containing 0.15 M NaCl. Elution was performed with the same buffer and 0.25 ml fractions were collected. Protein markers used as internal standards were: aldolase (158 kDa), malic dehydrogenase (70 kDa), ovalbumin (43 kDa), chymotrypsinogen A (25 kDa) and cytochrome c (12 kDa). NDP kinase was followed by enzymatic activity and was concentrated against PEG 8000. The concentrated fraction was analyzed in 12% denaturing gels.

2.6. Enzyme assay

The NDP kinase activity was assayed using the pyruvate kinase-lactate dehydrogenase coupled enzyme system [22]. The assays were performed at room temperature in a 1-ml reaction mixture containing 80 mM Tris-HCl pH 7.5/3 mM phosphoenolpyruvate/2 mM ATP/10 mM MgCl₂/25 mM KCl/0.3 mM NADH⁺/0.4 mM dTDP/1.25 units of pyruvate kinase/2.2 units of lactate dehydrogenase. The reaction was started with the addition of purified NDP kinase (0.16–0.5 μg of protein). A 28000 × g liver supernatant from male Wistar rats was used as a positive control of NDP kinase activity.

Alternatively, NDP kinase activity was assayed following the formation of pyruvic acid in a colorimetric reaction coupled to pyruvate kinase [23] modified as follows by Lic. Biondi (personal communication). Assays were performed at 30°C for 15 min in a reaction mixture (100 μl) containing 50 mM Tris-HCl pH 7.5/50 mM KCl/10 mM MgCl₂/1 mM ATP/1 mM PEP/10 mM potassium phosphate/2 mM dTDP/2 units of pyruvate kinase. Absorbance at 520 nm was measured after reaction with 2,4-dinitrophenylhydrazine.

2.7. [35S]GTP incorporation

Thiophosphorylation of proteins in T. cruzi extracts was performed in a final volume of 50 μl
during 5 min at 30°C. The samples (100–200 μg protein per assay) were incubated with 25 μl of 20 mM Tris-HCl pH 8/1 mM EDTA/1 mM DTT/0.1 M NaCl/0.1% Lubrol/10 mM MgCl₂ containing [³⁵S]GTPγS (1.5 × 10⁶ cpm assay⁻¹). The reaction was stopped by the addition of 2% SDS/5% 2-mercaptoethanol/20% glycerol/60 mM Tris-HCl pH 6.8/0.002% Bromophenol blue (cracking buffer) and the samples were boiled for 2 min at 100°C. Electrophoresis was carried out in 12% denaturing gels which were then incubated in 22% 2,5-diphenyloxazole (PPO) in DMSO prior to X-ray film exposition as described in Ref. 24.

2.8. Autophosphorylation assays

The T. cruzi fractions or rat liver extracts were phosphorylated for 2 min at room temperature with 5 μM [γ³²P]ATP (2 × 10⁶ cpm assay⁻¹) in 20 mM Tris-HCl pH 7.5/1 mM EDTA/1 mM DTT/10 mM MgCl₂/25 mM KCl/0.1 M NaCl/50 μM CaCl₂ in a final volume of 50 μl. Reactions were stopped by the addition of cracking buffer and boiled for 2 min. Samples were resolved by SDS-PAGE and the dried gels were autoradiographed.

An aliquot of the pure NDP kinase fraction (180 μl, 290 μg protein) was incubated with this same mixture for 3 min at room temperature and the preparation was immediately passed through a Sephadex G-50 column, previously equilibrated with 10 mM Tris-HCl pH 7.5/1 mM EDTA/1 mM DTT/0.1 M KCl, to remove free [γ³²P]ATP. The labelled phosphoenzyme was used to study the ³²P-labeled nucleoside 5'-triphosphate formation from the corresponding nucleoside 5'-diphosphate.

Characterization of various 5'-diphosphates as phosphate acceptors from the [³²P]phosphoenzyme was done as described in Ref. 25 with minor modifications. The labelled phosphoenzyme (10 μg) was added to a reaction mixture containing 20 mM Tris-HCl pH 7.5/0.1 M KCl/0.25 mM CaCl₂/4 mM DTT/5 μM of the following nucleoside 5'-diphosphates ADP, GDP, CDP, UDP in a final volume of 25 μl. Each reaction (3 min in an ice bath) was arrested by the addition of 25 mM EDTA and was spotted on a PEI-cellulose F thin-layer plate. ³²P-Labelled nucleoside triphosphates formed were detected by autoradiography after development in 0.75 M potassium phosphate at room temperature.

2.9. Analytical methods

Proteins were determined by the method of Lowry et al. [26] using bovine serum albumin as standard. Malic enzyme was determined as described in Ref. 21. After SDS-PAGE [27] gels (12% polyacrylamide) were fixed and stained with Coomassie blue and the samples were boiled for 2 min at 100°C. Electrophoresis was carried out in 12% denaturing gels which were then incubated in 22% 2,5-diphenyloxazole (PPO) in DMSO prior to X-ray film exposition as described in Ref. 24.

3. Results

A soluble NDP kinase activity was purified from the parasite Trypanosoma cruzi. Aliquots of the purification steps, were electrophoresed and the gel was stained with Coomassie blue (Fig. 1, lanes a–d). In addition, an aliquot of the flow-through fraction from the Q-Sepharose column was electrophoresed and the gel was silver-stained. A single 16.5-kDa band was revealed by both staining procedures (Fig. 1, lanes d and e). The enzyme was purified 800-fold with a 47% yield (Table 1). The yield was evaluated by considering the total activity present in the S.A.70 precipitate.

The presence of the enzyme was detected by the formation of ADP as a result of phosphate transfer from ATP to dTDP. The kinetic parameters of the parasite NDP kinase were determined by the double-reciprocal plot of Lineweaver-Burk. The data were analyzed by linear regression using the Grafit
Fig. 1. SDS-PAGE analysis of the sequential steps followed to purify the soluble NDP kinase activity from T. cruzi. The gel was stained with Coomassie blue (lanes a–d) or was silver stained (lane e). Lane a, S100 fraction; lane b, S.A.70 precipitate; lane c, Blue-agarose fraction; lanes d and e, flow-through fraction from Q-Sepharose column (32 and 15 µg, respectively). Molecular mass standards are indicated.

program from Sigma. The $K_m$ values were 0.2 ± 0.008 mM for ATP, 0.125 ± 0.12 mM for GDP and 0.4 ± 0.009 mM for dTDP. These values are similar to those reported for NDP kinases from higher eukaryotic systems [7,8,21]; however, the protozoa enzyme had a lower $K_m$ for ATP. The optimum concentrations for Ca$^{2+}$ and Mg$^{2+}$ were 50 µM and 10 mM, respectively.

The S100, S.A.70 precipitate and Blue-agarose fractions were incubated with $[^{35}S]GTPyS$ and electrophoresed on SDS-PAGE. These same fractions were immunoblotted with a polyclonal antibody against the D. discoideum NDP kinase. A liver extract was used as a positive control in both experiments. As shown in Fig. 2A the fluorography (lanes b–d) revealed a 16.5 kDa polypeptide which was strongly $^{35}$S-labelled, while two bands of 18 and 16 kDa were present in the liver extract (lane a). The NDP kinase reaction mechanism is characterized by transient autophosphorylation prior to transfer to a diphosphate acceptor and is known to use thio-phosphate, as well as orthophosphate donors as substrates [29]. Immunoblotting with the anti-NDP kinase antibody detected a 16.5-kDa peptide in the Blue-agarose preparation and a 18-kDa band in the liver extract (Fig. 2B, lanes a, b) while a faint band of 16.5 kDa was also detected in the crude T. cruzi extracts (lanes c, d). The enhancement of the 16.5-kDa band in the Blue-agarose preparation was due to the specific enrichment achieved with this purification step (lane a). The anti-NDP kinase antibody revealed other

Fig. 2. (A) Thio-phosphorylation with $[^{35}S]GTPyS$; (B) Immuno-detection with anti-NDP kinase antibody from D. discoideum (1:250). Lane a, rat liver extract; lane b, Blue-agarose fraction; lane c, S.A.70 precipitate; lane d, S100 fraction. Molecular mass standards: α-lactalbumin (14.2 kDa); trypsin inhibitor (20.1 kDa); trypsinogen (24 kDa); glyceraldehyde-3-phosphate dehydrogenase (36 kDa); ovalbumin (45 kDa); albumin (66 kDa).
bands (lanes c and d) which were also detected with the preimmune serum (data not shown). These experiments established an apparent molecular mass of 16.5 kDa for the *T. cruzi* enzyme, very similar to that reported for the monomers of the NDP kinase from *Dictyostelium* [4], yeast [30], *Pisum sativum* [31], rat liver [32], bovine brain [7] and human erythrocytes [2].

To further purify the NDP kinase activity, two sequential Q-Sepharose columns, equilibrated at pH 7.5 and pH 8, were used. Most of the activity (85%) eluted in the flow-through fraction while the remaining 15% eluted at 0.1 M NaCl. Both the flow-through and the included fraction were subjected to phosphorylation with \( \gamma^{32}P \)ATP in the presence or absence of 20 mM ADP and analyzed by SDS-PAGE. A 16.5-kDa labelled polypeptide was obtained when the flow-through fraction was phosphorylated with \( \gamma^{32}P \)ATP (Fig. 3A, lanes a–d). Maximum labelling was achieved after a 2-min incubation. No band was detected when unlabelled ADP was present in the reaction mixture (Fig. 3A, lanes e–g), indicating that it contained a high-energy phosphate intermediate that could be removed from the \( ^{32}P \)-labelled enzyme. A similar result was obtained using the 0.1 M NaCl fraction (data not shown), although the label was extremely faint.

The flow-through fraction was also analyzed by Western blot with anti-NDP kinase antibodies from three different sources, *D. discoideum*, *C. albicans* and human. A preimmune serum was used as a negative control (Fig. 3B, lane d). All the antibodies recognized the 16.5-kDa monomer from the parasite enzyme (Fig. 3B, lanes a–c). These antibodies could also recognize the included fraction (data not shown).

### Table 1

<table>
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<th>Fraction</th>
<th>Protein (mg)</th>
<th>Volume (ml)</th>
<th>Specific activity (Units mg⁻¹)</th>
<th>Total activity (Units)</th>
<th>Yield (%)</th>
<th>Purification (fold)</th>
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<td>90</td>
<td>50.4</td>
<td>8019.6</td>
<td>100</td>
<td>1</td>
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<tr>
<td>S.A.70</td>
<td>77.13</td>
<td>90</td>
<td>230.7</td>
<td>17794</td>
<td>100</td>
<td>4.6</td>
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<tr>
<td>Blue-agarose</td>
<td>6.53</td>
<td>96</td>
<td>1384.4</td>
<td>9090</td>
<td>51</td>
<td>27.5</td>
</tr>
<tr>
<td>Q-Sepharose</td>
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<td>13</td>
<td>42278</td>
<td>8455</td>
<td>47.5</td>
<td>838</td>
</tr>
</tbody>
</table>

*One unit is defined as the amount of enzyme that catalyzes the production of 1 nmol ADP min⁻¹.*
Characterization of various nucleoside 5'-diphosphates as phosphate acceptors of 3'P-labelled phosphates from the labelled phosphoenzyme. The phosphoenzyme (20 µg protein, 8 × 10^4 cpm) was added to the reaction mixture (final volume 25 µl) containing the following nucleoside 5'-diphosphates (10 µM each): ADP (lane b); GDP (lane c); UDP (lane d); CDP (lane e) or a mixture of the four diphosphates, 5 µM each (lane f), or a mixture of ADP and GDP (lane g). The labelled triphosphates formed were detected by autoradiography after development in 0.75 M potassium phosphate.

The dual elution of NDP kinase suggests the presence of two isoforms of the enzyme differing in their isoelectric point. Further studies are necessary to confirm this hypothesis.

The phosphate transfer between the high-energy phosphate of the phosphoenzyme and the nucleoside 5'-diphosphates was tested in vitro. The autoradiography of the PEI-cellulose chromatography is shown in Fig. 4. The 32P-labelled phosphoenzyme remained at the origin and the residual [γ-32P]ATP from the phosphorylation reaction was scanned in order to substrack it from the newly synthesized nucleotide (lane a). Incubation of the phosphoenzyme with any of the nucleoside 5'-diphosphates (ADP, GDP, CDP, UDP) resulted in the formation of the corresponding 32P-labelled nucleoside 5'-triphosphate (lanes b-e). ADP and GDP (lanes b, c) were the best acceptors. But the phosphoenzyme did not transfer all of its phosphates to CDP or UDP as indicated by the remaining label at the origin. When the 32P-labelled phosphoenzyme was added to a reaction mixture containing the four nucleoside 5'-diphosphates, only [γ-32P]ATP and [γ-32P]GTP were produced (lane f). If the mixture contained ADP and GDP, there was a preferential formation of [γ-32P]GTP (lane g) as expected from the K_\text{m} value of the enzyme for GDP.

When T. cruzi membranes were incubated with the labelled phosphoenzyme, without the addition of diphosphates, [32P]ATP and [32P]GTP were formed (data not shown). These results are in agreement with the reports that phosphorylation of GDP by NDP kinase may constitute the major source of GTP in vivo [32].

Studies regarding the thermal stability of the enzyme were carried out using purified NDP kinase. Aliquots of the enzyme were preincubated 10 min at increasing temperatures (30–80°C) and were immediately placed on ice to avoid renaturation. Subsequently, the enzymatic assay was performed for 10 min at 30°C or, alternatively, the enzyme was labelled for 2 min with [γ-32P]ATP and analyzed by SDS-PAGE. Both methods demonstrated that the parasite enzyme remained active up to 65°C (Fig. 5A), similar to other NDP kinases [3]. The incubation with increasing concentrations of urea (0–7 M) showed that the enzyme remained stable up to 2.5 M, but its activity dropped at 3 M urea (Fig. 5B), showing that the T. cruzi enzyme was less stable than other NDP kinases which usually tolerate 5.5 M urea [3]. Moreover, the inactivation curve does not show a sharp transition as does the urea inactivation curve of Drosophila and Dictyostelium NDP kinases [3,33].

The quarternary structure of T. cruzi NDP kinase was determined by FPLC. The purified Q-Sepharose fraction was analyzed on a Superose 12 column (Fig. 6A). Two peaks with NDP kinase activity, designated I and II, were resolved (Fig. 7A). Peak I corresponded to a molecular mass of 90 ± 6 kDa while peak II to a molecular mass of 45 ± 3 kDa. Peak II eluted either as a shoulder or was clearly resolved, suggesting a partial dissociation of the enzyme. The contaminating proteins, eluting near the V_c of the column, could correspond to proteolytic fragments of NDP kinase or to small peptides. Both peaks were concentrated and analyzed by SDS-PAGE (Fig. 6B). A purified NDP kinase from D. discoideum (16.8 kDa) was used as positive control (lane a). A slightly smaller monomer (16.5 kDa) was stained in both T. cruzi fractions (lanes b and c). These results indicate that the native NDP kinase from T. cruzi is a hexamer.

Preliminary studies regarding the membrane-associated NDP kinase were undertaken. Subcellular
fractions were obtained and NDP kinase activity was assayed (Table 2). The particulate enzyme accounted for 30% of total NDP kinase activity and was localized mainly in the enriched flagellar and microsomal fractions (48.3 and 38.3% of the particulate activity, respectively). To eliminate the possibility that soluble NDP kinase was trapped in these pelletable fractions and could be responsible for the detected activity, a cytosolic enzymatic activity in T. cruzi, the malic enzyme [21], was assayed in the subcellular fractions. As shown in Table 2, this activity was meaningless in the flagellar (2.5%) and microsomal (2.5%) fractions, confirming that the NDP kinase activity present in these fractions was a particulate bound enzyme.

All subcellular fractions were immunoblotted with the anti-NDP kinase antibody from Candida albi-
Fig. 7. SDS-PAGE analysis of subcellular fractions (100 μg each) from T. cruzi. (A) Western blot analysis of nuclear, flagellar, microsomal and cytosolic fractions (lanes N, F, M and S) with anti-NDP kinase antibody from C. albicans (1:250). A purified NDP kinase from D. discoideum (50 μg) was used as positive control (lane Dd). (B) Fluorography; nuclear, flagellar, microsomal and cytosolic fractions were thiophosphorylated with [35S] GTPyS (1.5 × 10⁶ cpm assay⁻¹), (lanes N, F, M and S). A rat liver extract was used as positive control (lane L).

cans (Fig. 7A). The 16.5-kDa monomer reacted with the anti-NDP kinase antibody in all particulate fractions (lanes N, F and M) though the signal was stronger in the soluble fraction (lane S). The antibody revealed the 16.8-kDa monomer of the homogeneous D. discoideum NDP kinase preparation used as positive control (lane Dd). The preimmune serum (negative control) did not recognize the band (data not shown).

Moreover, these fractions were thiophosphorylated with [35S]GTPyS and subjected to SDS-PAGE. The 35S-labelled phosphoenzyme could be detected in the fluorography of these fractions (Fig. 7B, lanes N, F, M and S) while two bands (18 and 16 kDa) were detected in the liver extract (lane L) used as positive control. These results strongly suggest the presence of a particulate NDP kinase activity in T. cruzi as demonstrated in higher eukaryotic cells [7,8].

4. Discussion

NDP kinase is a key enzyme in the synthesis of non-adenine nucleoside triphosphates. The gene for NDP kinase has been cloned from D. discoideum and found highly homologous to its counterparts in higher eukaryotes [17], such as the gene awd involved in Drosophila development, and the human genes nm23-H1 and nm23-H2 [12,34]. The involvement of Nm23 in proliferation [35] and possibly in metastasis [12] points to a role for NDP kinase in cellular regulation that may be distinct to its role in the biosynthesis of nucleotides.

The NDP kinase from T. cruzi was purified and characterized. The purified soluble enzyme is a hexamer composed of 16.5-kDa monomers as determined by FPLC, SDS-PAGE, Western blots and thiophosphorylation experiments. The biochemical results described in this paper indicate that only one type of NDP kinase is present in T. cruzi. There is only one gene coding for a cytosolic NDP kinase in Dictyostelium discoideum [4] and most probably in Drosophila melanogaster (A. Shearn, personal communication). In contrast, two closely related but distinct isozymes have been described in several mammalian species, including the NDPK-A and NDPK-B isoforms found in human erythrocytes [2]. The presence of a single NDP kinase in T cruzi argues for a late appearance of isozymes of NDP kinase in evolution, possibly related to non-metabolic function of the enzyme (see below).

All eukaryotic NDP kinases known to date, including those from Dictyostelium [36], Drosophila [37] and human [2], are hexamers. In contrast, the Myxomycetes enzyme is a tetramer made of subunits
with a very similar fold to that of the *Dictyostelium* enzyme [38]. The study of mutant NDP kinases from *Dictyostelium* and *Drosophila* [3] with altered stability has shown that increased sensitivity to urea denaturation corresponds to a decreased stability of the hexameric structure. Thus, a mutant with a Pro → Ser substitution in the *Dictyostelium* NDP kinases dissociates at low urea concentration into folded inactive monomers [33,39].

The heat stability of *T. cruzi* NDP kinase (Tₘ 65°C) was similar to that of other NDP kinases. In contrast, it was much less stable towards urea inactivation with a half maximum inactivation at 2.5 M as compared with 6 M for the *Dictyostelium* enzyme [33]. Moreover, the curve of urea inactivation did not show a sharp transition, but rather a gradual decrease (Fig. 5B). This might indicate weaker inter-subunit contacts within the oligomer than in other NDP kinases.

The reaction mechanism of the parasite NDP kinase involves a phosphoenzyme intermediate. Ohtsuki et al. [25] reported that NDP kinase from Ehrlich ascites tumor formed approximately 5 times more ATP than GTP, whereas GTP formation was remarkably increased when the phosphoenzyme was incubated together with ADP and GDP in the presence of G-protein. This is apparently not the case of the parasite enzyme, where the formation of GTP is privileged independent of the presence of a G-protein. Moreover, following incubation of the parasite phosphoenzyme with membranes, labelled GTP was detected suggesting that NDP kinase is the source of GTP in the parasite. Uridine kinase remains undetected in extracts from trypanosomatids [15]; however, *T. cruzi* NDP kinase can form labelled UTP from the corresponding nucleoside 5’ diphosphate.

The association of membrane NDP kinase in *T. cruzi* with the flagellar fraction is interesting. Although no direct role in tubulin polymerization could be demonstrated in vitro using brain tubulin [40], NDP kinase has been found to be associated with microtubules [7]. NDP kinase could be involved in flagellar movement and therefore in the pathology of the parasite. Further study of this enzyme during the life cycle of the parasite could lead to important information about the energetic requirements of the different forms of *T. cruzi* and help elucidate the role of NDP kinase in proliferation.

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