



Polyphosphate kinase from *Leishmania donovani* amastigotes : a polyphosphate driven generator of ATP

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Abstract:

We have identified the presence of polyphosphate kinase (PPK) for the first time in *Leishmania donovani* amastigotes, the causative pathogenic form of leishmaniasis. Digitonin permeabilized *L. donovani* amastigote cells in presence of short chain polyphosphate and ADP or GDP produced either ATP or GTP, respectively. ATP and GTP were quantified by coupling with the enzymes hexokinase and glucose-6-phosphate dehydrogenase. Maximum PPK activity was observed for wild-type, sodium stibogluconate resistant and paromomycin resistant AG83 *L. donovani* amastigotes when ADP was the phosphate group acceptor from polyphosphate. This activity was 29 times higher than the PPK activity when GDP was the phosphate group acceptor from polyphosphate. When AMP was the phosphate group acceptor from polyphosphate, the activity of PPK was 97 times lesser than the activity of PPK when ADP was the phosphate group acceptor from polyphosphate. Existence of both PPK1 and PPK2 is probable in *L. donovani* amastigotes.

Keywords: polyphosphate kinase, *Leishmania*, ATP, GTP

Introduction

Inorganic polyphosphate (poly P) is a linear polymer compound of tens to hundreds of orthophosphate residues (P_i) linked by the energy-rich phosphoanhydride bonds, and it is found in all prokaryotes and eukaryotes (1-3). Poly P has numerous biological functions that include substitution for ATP in kinase reactions, acting as an energy source or storage reserve of P_i, chelating metals, and adjusting cellular physiology during growth, development, stress, starvation, virulence (1,4), activation of enzymes (5,6) and regulation of chromatin condensation, gene expression and translation (1, 7,8). Several specialized enzymes are involved in poly P metabolism. Polyphosphate kinase 1 (PPK1) is responsible for reversible synthesis of the majority of poly P in the cell (1,9). PPK2 enzyme uses poly P as a substrate to generate GTP from GDP (10).

PPK2 shows no sequence similarity to PPK1 and is distinguished by much higher poly P utilization activity (11). In this report, we describe the characteristics of PPK from *Leishmania donovani* amastigotes based on its activity as a nucleotide diphosphate kinase (NDK). Despite its universal occurrence and its broad functions, to our knowledge, there have been no studies addressing the function and occurrence of PPK in *L. donovani* amastigotes.

Materials and methods

Materials

Standard glass wears of Borosil® were used for experimental purposes. All chemicals unless otherwise mentioned were purchased from Sigma-Aldrich (St. Louis, MO). Sodium stibogluconate (SSG) was a generous gift from

Albert David Ltd. (Kolkata, India). GraphPad Prism 5.01 was used for the data analysis purposes.

Parasites and culture conditions

Promastigotes of *Leishmania donovani* clones, AG83 (MHOM/IN/83/AG83) and GE1 (MHOM/IN/80/GE1F8R) were VL isolates obtained as a gift from Indian Institute of Chemical Biology, Council of Scientific and Industrial Research, Kolkata, India. Antimony-sensitive strain, AG83 and antimony-resistant isolate, GE1 were characterized earlier (12, 13). AG83 is used to consider as reference standard strain of *L. donovani* in India. Parasites were routinely grown as promastigotes in medium 199 supplemented with 10% heat-inactivated fetal calf serum (FCS) at 24°C.

Resistance selection to sodium stibogluconate and paromomycin on promastigotes and their transformation into drug resistant amastigotes

The drug sensitive AG83 and drug resistant GE1 promastigote cells were cultured in medium 199, in the presence of drug concentration corresponding to the 50% inhibitory concentration (IC₅₀) of the strain. The cultures were stabilized by three subcultures before increasing the drug concentration. Drug concentration was increased in such a way that the cell population was decreased approximately 20% for each batch. Finally when 90% cell population of the initial count was reduced, the phenotype so generated was plated on medium 199 agar plates in the presence of same drug concentration, and a single colony was picked for culture in medium 199 liquid media at the same drug concentration (14, 15). Stability of resistance was checked at four, eight and sixteen weeks after removal from

drug pressure. Evidence for the generation of drug resistant *Leishmania donovani* cells had already published (16, 17).

Generation of axenic amastigotes

Leishmania donovani amastigote forms were grown and maintained as described by Debrabant *et al.* (18). Axenically grown amastigotes of *L. donovani* were maintained at 37°C in 5% CO₂ /air by weekly sub-passages in MMA/20 at pH 5.5 in petri dishes (19). Under these conditions, promastigotes differentiated to amastigotes within 120 hours. Cultures were maintained by 1:3 dilutions once in a week.

Preparation of digitonin permeabilized *Leishmania* cells

Leishmania donovani promastigote and/or amastigote cells were collected, washed once by buffer A (140 mM NaCl, 20 mM KCl, 20 mM Tris, 1 mM EDTA, pH 7.5), and resuspended in isolation buffer (20 mM MOPS-NaOH, 0.3% BSA, 350 mM sucrose, 20 mM potassium acetate, 5 mM magnesium acetate, 1 mM EGTA, pH 7.0). Cells were permeabilized by 200 µg digitonin per mg of protein and incubated in ice for 10 minutes. After incubation, the cells were centrifuged at 6000x g for 7 minutes. Pellets were re-suspended in assay buffer.

Assay for ppk as a poly P-dependent ATP/GTP generator

Poly P-dependent synthesis of ATP/GTP was determined by using a modified enzyme-coupled assay with hexokinase and glucose-6-phosphate dehydrogenase (20). Assay mixture (500µl) contained 4mM Mg(Ac)₂, 12µg digitonin permeabilized amastigote cells, 0.4µmole ADP/GDP/AMP, 50 nmoles poly P₁₅. Assay mixture

was incubated in 50 mM KCl, 300 mM sucrose, 50 mM Tris-HCl and 2 mM EGTA, pH 7 at 37°C for 20 minutes. (Amastigote cells were permeabilized by 200 µg digitonin/ mg protein in 140 mM NaCl, 20 mM KCl, 20 mM Tris-HCl, 1 mM EGTA, pH 7.5 and incubated in ice for 10 minutes. After incubation, the cells were centrifuged at 6000xg for 7 minutes. Pellet was resuspended in assay buffer). Reaction was terminated by the addition of 0.14 ml 2.5(N) HClO₄ followed by addition of 0.1 ml 8(N) KOH to maintain pH 7. Terminated mixture was centrifuged at 15000 xg for 10 minutes. 0.1 ml aliquot was transferred to 0.3 ml 50 mM Tris-HCl, 10 mM MgCl₂, 5 mM EGTA, pH 7.4, 1.5 mM NADP⁺, 1 mM glucose, 3 U yeast hexokinase, 1.5 U *Leuconostoc mesenteroides* glucose-6-phosphate dehydrogenase. Hexokinase converts ATP/GTP generated by PPK to ADP/GDP and glucose-6-phosphate using glucose as the phosphate acceptor and the glucose-6-phosphate will be converted to 6-phosphogluconate by NADP⁺-dependent glucose-6-phosphate dehydrogenase. During this reaction, NADP⁺ will be converted to NADPH which was measured spectrophotometrically at 340 nm (2340 nm = 6.22 mM⁻¹ cm⁻¹). NADPH formed was measured upto 10 minutes and the enzyme activity was expressed as V_{max}. The enzyme kinetics was analyzed by nonlinear curve fitting using GraphPad Prism 5.01 software.

Results

It appears from Table 1 that in digitonin permeabilized *L. donovani* amastigotes, ATP and GTP synthetic activity, which is PPK dependent and driven by short-chain poly P, has been observed. PPK dependent ATP formation has been found to be highest in wild-type, SSG

resistant and PMM resistant strains, compared to GTP formation. However, ATP formation from AMP showed lowest activity. Polyphosphate kinase (PPK) dependent ATP formation showed linearity with time (Figure 1) and cell protein concentration (Figure 2).

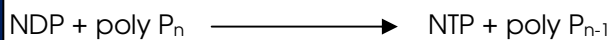
Discussion

A potent activity in *L. donovani* amastigotes converts ADP to ATP and GDP to GTP by using poly P as donor. The study was designed to identify PPK1 and PPK2 activity. Volulin or poly P granules were also found in a number of eukaryotic microbes. These granules were later identified as the acidic, calcium rich components of trypanosomatids known as acidocalcisomes (21). One of the major function of poly P is the energy source and ATP substitute. PPK converts poly P to ATP by catalyzing an ADP attack on the termini of the poly P chain. An aggregate of poly P associated with this membrane-bound enzyme could generate large amount of ATP at that very spot. Another source of ATP could come from an AMP attack on poly P by AMP-phosphotransferase to produce ADP, which is readily converted to ATP by coupling with PPK or adenylate kinase:



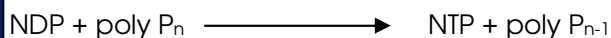
One of the important enzyme in biosynthesis and degradation of poly P_s is polyphosphate: ADP phosphotransferase, referred to as polyphosphate kinase (PPK). This enzyme is referred to as polyphosphate kinase 1 (PPK1). It has been discovered that the enzyme accepts all nucleotide diphosphates (NDPs) and uses a

poly P chain as a phosphate donor; it shows preference to purine nucleotides (22).



NDP: ADP > GDP > UDP > CDP

It was also revealed that a novel enzyme PPK2, which phosphorylates GDP to GTP by using poly P as donor. It was also found that PPK2 could use GTP or ATP in the synthesis of poly P chains, differing the PPK1, which exclusively use ATP (15).



NDP: GDP, ADP

Our previous study proved that substrate level phosphorylation is essential for the survival of amastigote forms of *L. donovani* (23). It is evident from Table 1 that either PPK1 or both PPK1 and PPK2 are present in *L. donovani* amastigotes. Kinetic characterization of PPK1 and PPK2 will reveal the relative importance of PPK in *L. donovani* amastigote metabolism.

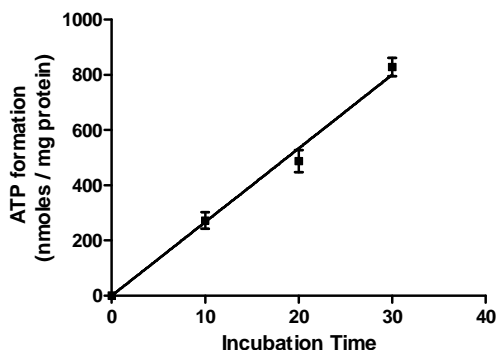


Figure 1: ATP formation in relation to incubation time in PPK Assay

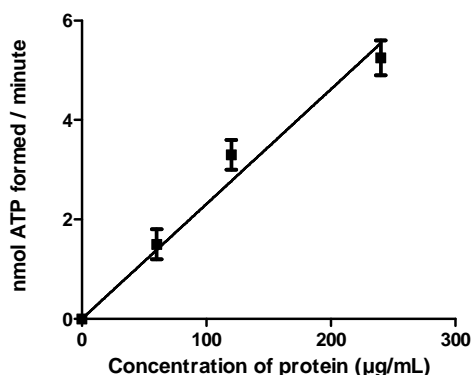


Figure 2: ATP formation in relation to cell concentration in PPK assay.

Table 1: Comparison of poly P-utilizing activity of PPK1, PPK2 and AMP phosphotransferase in *L. donovani* amastigotes ^a

Source of enzyme	Rate of nucleoside triphosphate formation (nmol/min/mg protein)		
	P _i acceptor		
	ADP	GDP	AMP
AG83 (Wild-type)	44.16±5.28	0.67±0.07	0.47±0.05
AG83 (SSG resistant)	36.88±4.68	1.20±0.15	0.50±0.06
AG83 (PMM resistant)	33.50±4.62	2.08±0.28	0.20±0.02

^a For PPK1, poly P₁₅ and ADP were used as a donor and acceptor, respectively. For PPK2, poly P₁₅ and GDP were used as a donor and acceptor, respectively. For AMP phosphotransferase, poly P₁₅ and AMP were used as a donor and acceptor, respectively. Poly P-dependent ATP and GTP synthesis were determined spectrophotometrically using a modified enzyme coupled assay. Assays were repeated three times and the data were expressed as mean ±S.E.

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Conflict of interest

The authors have declared that no competing interests exist.

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