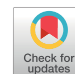
Parasitology International 72 (2019) 101929

Contents lists available at ScienceDirect

Parasitology International

journal homepage: www.elsevier.com/locate/parint

Leishmania donovani adenylate kinase 2a prevents ATP-mediated cell cytolysis in macrophages



Kulkarni P.G.[a], Shah N.[a], Waghela B.N.[b], Pathak C.M.[b], Pappachan A.[a,c,*]

[a] Department of Bioinformatics and Structural Biology, Indian Institute of Advanced Research, Koba, Gandhinagar 382007, Gujarat, India
[b] Department of Cell Biology, Indian Institute of Advanced Research, Koba, Gandhinagar, 382007, Gujarat, India
[c] School of Life Sciences, Central University of Gujarat, Gandhinagar 382030, Gujarat, India

A R T I C L E I N F O

Keywords:
Putative AK
Leishmania donovani
Secreted enzymes
Extracellular ATP
Macrophages

A B S T R A C T

In *Leishmania* spp. ATP utilizing enzymes serves as a key role in preserving integrity of host cells for survival of parasite. Earlier reports suggested that Adenylate kinase (AK) a phosphotransferase enzyme released by *Leishmania donovani* secretome, involved in modulating levels of NTPs. In the present study, we cloned, expressed and characterized recombinant putative AK. Based on a sequence and phylogeny analysis, we identified the prominent features of the seven AK isoforms of *Leishmania donovani* and assigned our putative AK as LdAK2a. The K_m value of LdAK2a for ATP and AMP substrate were 204 μM and 184 μM, respectively and V_{max} was calculated as 1.6 μmol min^{-1} mg^{-1} protein. Ap5A, a known inhibitor of AK inhibited LdAK2a with estimated K_i values of 280 nM and 230 nM for ATP and AMP respectively. CD spectral studies were carried out to estimate its structural stability. Recombinant LdAK2a was found to prevent ATP mediated cell cytolysis of Raw 264.7 macrophages in vitro, which was determined by LDH assay and MMP assay. This is the first report which validates that Leishmanial AK2a can prevent ATP mediated cytolysis of macrophage cells and thereby probably play a role in preserving integrity of host cells for survival of parasite.

1. Introduction

AK (E.C.2.7.4.3) is a ubiquitous, phosphotransferase enzyme, which catalyzes the reversible transfer of ϒ- phosphoryl group from ATP to AMP and converts it into two molecules of ADP. It acts as a metabolic monitor, which regulates cellular homeostasis of adenine nucleotides and thereby plays a key role in energy metabolism and nucleic acid synthesis. Apart from this, it is proposed that AK acts as a virulence factor in a number of pathogenic bacteria, where it contributes to the modulation of ATP levels during macrophage death [1–3]. In humans, isoforms of AK exists in cytosol, mitochondrial matrix and mitochondrial intermembrane space [4]. In case of kinetoplastids (*Leishmania*, trypanosomes and other pathogenic parasites) AK is known to maintain ATP levels in mitochondria like kinetoplast organelle and in glycosomes i.e. energy producing microbodies [5]. In such flagella containing parasites it supports agility and energetics of the parasite.

Earlier studies have revealed that AK is one of the enzymes that are released by many pathogenic microorganisms to modulate levels of NTPs and some of these released enzymes can utilize eATP as its substrate [6–10]. eATP is known to accumulate at the site of inflammation and induce death of immune cells. Extracellular pathogenic bacteria such as *Pseudomonas aeruginosa* [1,7,9], *Mycobacterium tuberculosis* [11], *Vibrio cholera* [2] have evolved strategies to escape the phagocytic activity by releasing enzymes that increases eATP level which will result in apoptosis of these phagocytes. However, in intracellular parasites like *Leishmania*, where pathogenicity of the parasite also depends on their ability to prolong their survival in their host macrophage cells, they might be resorting to a reverse strategy for which they release eATP utilizing enzymes so that it becomes unavailable for cytolysis of the host cells. A proteomics study of *Leishmania* secretome confirms the presence of ATP utilizing enzymes [12,13]. Studying the role of such proteins in survival and death of their host cells is important for development of therapeutic strategies of leishmaniasis. In *Leishmania amazonensis*, it was shown that nucleoside diphosphate kinase, a

Abbreviations: AK, Adenylate Kinase; LdAK, *Leishmania donovani* adenylate kinase; IPTG, Isopropyl β-D-thiogalactopyranoside; Ap5A, P_1, P5 – bis (adenosine) - 5′-pentaphosphate; AMP, Adenosine monophosphate; ADP, Adenosine diphosphate; ATP, adenosine triphosphate; CD, circular dichorism; MMP, Mitochondrial membrane potential permeability; LDH, Lactate dehydrogenase; eATP, extracellular adenosine triphosphate; NDK, Nucleoside diphosphate kinase; NTP, Nucleoside triphosphate; Enzyme: AK, (ATP: AMP phosphotransferase EC 2.7.4.3)

* Corresponding author at: School of Life Sciences, Central University of Gujarat, Gandhinagar 382030, Gujarat, India.
E-mail address: anju.p@cug.ac.in (A. Pappachan).

https://doi.org/10.1016/j.parint.2019.101929
Received 29 January 2019; Received in revised form 12 April 2019; Accepted 16 May 2019
Available online 17 May 2019
1383-5769/ © 2019 Elsevier B.V. All rights reserved.

housekeeping gene was released at the time of infection, which helped to prevent ATP-mediated cytolysis of their host cells. AK is reported as a one of the secreted protein of *Leishmania donovani* [12,13]. In *Leishmania*, whether AK, like NDK, also serves in the survival of the parasite in host macrophages by preventing ATP-mediated cell lysis has not yet been investigated.

Most organisms have many isoforms of adenylate kinase and trypanosomatids has a larger AK network. In *T. brucei* and *T. cruzi,* seven variants of AK were reported targeted to different subcellular structures, such as flagellum, glycosomes, mitochondrion and cytosol [14–16]. In *Leishmania donovani*, the causative agent of visceral leishmaniasis, which is the most fatal form of leishmaniasis, genome analysis shows the presence of seven AK isoforms. Of these, only one of them- AK2 has been characterized till date [17]. This study reports the functional and molecular characterization of a putative AK (LdAK2a) from *L. donovani*. We successfully cloned, expressed and purified AK gene of *Leishmania donovani*. Detailed kinetic analysis of the enzyme with its substrates ATP and AMP, and the known inhibitor Ap5A was carried out. Further biophysical characterization of LdAK2a was carried out using CD spectroscopy and homology modeling to understand the protein structure. Based on a sequence and phylogeny analysis, we tried to identify the prominent features of the seven AK isoforms of *Leishmania*. We also demonstrated that recombinant LdAK2a protein prevents eATP mediated cell cytolysis of RAW 264.7 macrophages. Based on these results we propose that *Leishmania* AK must be one of the secreted proteins at the site of inflammation that can prevent ATP-mediated cytolysis of their host cells by decreasing eATP, which is expected to accumulate at the site of infection.

2. Experimental procedures

2.1. In silico sequence and structure analysis

The amino acid sequences of AK from *Leishmania donovani, Trypanosoma brucei* and *Homo sapiens* were retrieved from Swiss-UniProt gene database [18]. The multiple sequence alignment of retrieved sequences was performed using Clustal Omega. The phylogenetic analysis was performed using MEGA 7.0 [19]. The maximum likelihood method with statistical boot strapping (100 tests) was used for phylogenetic tree analysis. Sequence analysis and identification of nuclear localization sequence (NLS), nuclear exportation signal, was carried out using online programs like NetNES (prediction server of DTU Bioinformatics), MitoProt, and PSORT (Protein localization sites in cells).

Three-dimensional homology model of LdAK2a was built using I-tasser server with closest human homolog protein template [20]. The X-ray crystal structure of Human AK 2 (PDB code: 2C9Y) was used to generate homology model, which was then subjected to model refinement which was done using Moderefiner program. The energy minimization of generated model was carried out using Charmming-gui server [21]. Stereochemical analysis of LdAK2a homology model was performed using PROCHECK [22]. The overall quality factor analysis of homology model was done by Verify 3D and ERRAT analysis. Chimera program was used for visualization of homology model [23]. The program ALIGN was used to superpose LdAK2a model with Human AK (PDB ID: 2C9Y) and analyze the RMSD of corresponding C-alpha positions of both structures [24].

2.2. Cloning, expression and purification of LdAK2a protein

L. donovani (Ag83 strain) genomic DNA was used as a template for PCR based amplification of 657 bp long putative AK gene (UniProt ID: E9BQG6). The sense primer 5′-GTA GGA TCC ATG AAG ATT GTG CTC ATG GGC-3′ introduced with BamHI restriction site and antisense primer 5′-GTA CTC GAG CTA CTT GAG ACC CGT TGC AAT-3′ introduced with XhoI restriction site were used for PCR amplification. The restriction digested PCR amplified product was cloned into pET30a expression vector (Novagen). The successful clone construct pET 30a-LdAK2a was checked using sequencing. Since the cloned gene did not give soluble protein expression, in order to sub clone this LdAK2a gene into pET 28a expression vector (Novagen), the pET 30a-LdAK2a clone was digested with BamHI and XhoI and the purified digested gene product was ligated into BamHI and XhoI restriction digested pET 28a plasmid. The successful cloned construct was confirmed through sequencing.

The pET28a-LdAK plasmid was transformed in Rosetta DE3 *E.coli* cells (Invitrogen). The transformed *E.coli* cells was grown in Luria broth medium supplemented with 34 μg/ml chloramphenical and 50 μg/ml kanamycin at 37 °C shaking at 220 r.p.m. At an $OD_{600} = 1$, induction was given to cells with 0.5 mM IPTG (Sigma Aldrich) after which the cells were grown for 12 h at 16 °C and harvested by centrifugation at 5000*g*. The pellet was further resuspended in lysis buffer (50 mM tris, 200 mM NaCl, 1% TritonX-100, 10 mM $MgCl_2$) and lysed using sonication for 5 min with 10 s burst and 10 s cooling (Sonics, Vibra cell). The lysed cells were centrifuged at 8000 r.p.m. The supernatant was loaded on equilibrated Ni-NTA agarose resin (Sigma Aldrich) column (equilibration buffer 50 mM Tris-HCl, 200 mM NaCl, 15 mM Immidazole, pH 8.5). It was further washed with wash buffer (50 mM Tris-HCl, 200 mM NaCl, 20 mM Immidazole, pH 8.5). The protein elution was carried out using elution buffer (50 mM Tris-HCl, 200 mM NaCl, 250 mM Immidazole, pH 8.5). Subsequent eluted fractions were analyzed by SDS PAGE. LdAK2a protein fractions were pooled together and further dialyzed at 4 °C overnight in 50 mM Tris-HCl pH 8.5 containing EDTA (0.1 mM), 2-mercaptoethanol (0.1%). LdAK2a protein was concentrated using 10,000 MW cutoff centricon (Amicon) and were analyzed by SDS-PAGE and quantified by Bradford method.

2.3. Protein identification by MALDI-TOF analysis

For identification of protein, LdAK2a protein band (33 kDa) was excised from the destained SDS PAGE gel followed by trypsin digestion. The digested sample was then subjected to MALDI TOF MS/MS using AB SCIEX QSTAR elite LC-MS/MS system. (AB SCIEX, USA).

2.4. Enzyme kinetic analysis

AK enzyme activity was studied by measuring rate of NADH disappearance spectrophotometrically at 340 nm with addition of varying concentrations of ATP (Sigma Aldrich) and AMP (Sigma Aldrich) to the reaction mixture at 30 °C. Assay reaction mixture (0.5 ml) contained 0.1 mM Tris-HCl (pH 8.5), 120 mM KCl, 1 mM Phosphoenol pyruvate (Sigma Aldrich, St. Louis, Mo), 0.2 mM NADH (Sigma Aldrich), 0.125 mM ATP, 0.125 mM AMP, 4 U pyruvate kinase and 5 U LDH (Sigma Aldrich), varying concentrations of ATP (0.025 mM - 1.5 mM) and AMP (0.025 mM - 2.0 mM The K_m and V_{max} were analyzed by double reciprocal Lineweaver–Burk plot. Enzyme inhibition by Ap5A (Sigma Aldrich) was carried out using different concentrations of Ap5A (50,100, 250, 500, 750 nM), 3 μg of enzyme and three different concentrations of one of the nucleotides (AMP (0.5 mM,1.25 mM, 2 mM) and ATP (1 mM, 2 mM, 3 mM). The *Ki* value for Ap5A inhibitor was calculated and analyzed by using Dixon plot.

2.5. Analysis of effect of temperature, pH and divalent cations on enzyme activity

In order to study the effect of pH on LdAK2a activity, the LdAK2a protein (3 μg) was incubated with buffers of varying pH (4.5–9). The buffers used for this study were Sodium acetate (pH 4.5, 5, 5.5), phosphate (6, 6.5) and Tris HCl (pH 7, 7.5, 8, 8.5, 9). 0.5 ml of assay reaction mixture contained 0.1 mM buffer, 120 mM KCl, 1 mM Phosphoenol pyruvate, 0.2 mM NADH, 0.125 mM ATP, 0.125 mM AMP, 4 U pyruvate kinase and 5 U lactate dehydrogenase, ATP (3 mM) and

AMP (2 mM).

Effect of temperature on LdAK2a activity was monitored by incubating purified LdAK2a samples (3 µg) at diverse range of temperatures i.e. from 5 °C to 95 °C and measuring the activity of the enzyme.

The metal dependence of activity was measured in the presence of various metals ($CaCl_2$, $CuCl_2$, $CoCl_2$, $MnCl_2$ and $MgCl_2$) at 5 mM concentration along with the reaction mixture and activity was measured.

2.6. CD spectroscopic studies

Far-UV measurement of LdAK2a was carried out using Jasco-J715 spectropolarimeter. 500 mM of LdAK2a in 50 mM Tris-HCl buffer (pH 8.5) was used for the CD spectral measurements. The measurement was taken at 16 °C within 190 to 260 nm range. The collected data points were analyzed using K2D3 software for estimation of the secondary structure. To determine effect of temperature on structural changes of LdAK protein thermal denaturation was performed with increase in temperature from 25 °C to 95 °C (1 °C/min) and data were collected at 222 nm. The effect of ligands on secondary structural conformation of LdAK2a was analyzed by using CD spectroscopy after incubating the protein (5 µg) with 3 µg of ligands such as ATP, AMP, ATP + AMP, ADP and inhibitor Ap5A.

2.7. Cell culture and enzyme treatment

The RAW 264.7 mouse macrophage cell line was obtained from NCCS, Cell repository, Pune, India. Cells were cultured in DMEM (GIBCO, Invitrogen) containing L-Glutamate. The culture medium was supplemented with 10% FBS and PSN antibiotic solution (GIBCO, Invitrogen). The cells were maintained at 5% CO_2 at 37 °C in CO_2 incubator. Exponentially grown cells were used in experiments. For all experiments, cells in exponentially growing conditions were used. For *in vitro* experiments LdAK2a was purified. The buffers used during purification were maintained at pH 7.4. The cells were primed with 100 ng of LPS for 12 h. The reactants used for *in vitro* experiments were ATP (3 mM), AMP (2 mM) and Mg^{2+} (0.4 mM). The final concentration of 1 µg/ml LdAK2a enzyme was used with or without reactants. The incubation period or treatment time of cells with reactants was kept constant i.e. 6 h for each experiment.

2.8. Cell viability assay (MTT assay)

Cell viability was analyzed by MTT assay. RAW 264.7 (1.5×10^4 cells/200 µl DMEM/ well) were cultured in 96 wells plate for 24 h, which was then treated with 100 ng of LPS (Sigma Aldrich, St. Louis, Mo) to activate P2X7 receptor expression and incubated for 12 h at 37 °C in CO_2 incubator. After incubation, cells were washed with DPBS (GIBCO, Invitrogen). The primed cells were treated with all reactants in all possible combinations with or without recombinant LdAK2a protein and were incubated for 6 h. Upon completion of incubation period, 0.5 mg/ml MTT solution was added to the total volume of culture medium and incubated for 3 h at 37 °C in a CO_2 incubator. After that, MTT solution was removed and 0.1 ml of DMSO was added to each well. Finally, the absorbance was recorded at 570 nm with a reference wavelength of 650 nm using a Multimode microplate reader (SpectraMax M2^e, Molecular devices, USA).

2.9. Lactate dehydrogenase assay

In brief, RAW 264.7 were seeded into 96 well plate at 1.5×10^4 cells/200 µl DMEM/ well and primed with 100 ng of LPS (Sigma Aldrich) for 12 h at 37 °C with 5% CO_2. The LPS primed macrophage cells were then incubated for 6 h with all possible combinations of 3 mM ATP, 2 mM AMP, 0.4 mM Mg^{2+} with or without recombinant LdAK2a protein. Upon 6 h incubation with the reactants, cell suspension was collected and LDH release was measured using Multimode microplate reader (SpectraMax M2^e, Molecular devices, USA). The ratio of LDH release was calculated. Cells treated with Triton-X-100 (Sigma Aldrich, St. Louis, Mo) were taken as positive control and untreated cells as negative control.

2.10. Mitochondrial membrane potential permeability assay

JC-1, a lipophilic cationic fluorescent dye was used to monitor change in mitochondrial membrane potential [25]. 1×10^5 RAW 264.7 cells were cultured in 24 well plate on poly L-lysine coated coverslips. The cells were primed with 100 ng/ml LPS followed by incubation for 12 h at 37 °C in CO_2 incubator. The primed cells were washed with DPBS (pH 7.4) and treated with 3 mM ATP alone and or with reactant mixture (3 mM ATP + 2 mM AMP + 0.4 mM Mg^{2+}) in presence of activated and heat inactivated protein for 6 h. Further, residual medium was replaced with DPBS containing JC-1 dye (5 µg/ml) and incubated for 20 min in the dark. Thereafter, the cells were counterstained with DAPI and mounted with fluoromount mounting medium (Sigma-Aldrich, USA). The cells were observed under fluorescence microscope. > 150 cells from three random fields were analyzed by Image J software (NIH, USA). For quantitative analysis of MMP assay, 2×10^4 cells were cultured in 96 black well plate and subjected to treatment as described earlier. After completion of incubation, the cells were stained with JC-1 dye (5 µg/ml) and incubated for 20 min in the dark. The fluorescence was recorded at 527 nm (green fluorescence) and 590 nm (red fluorescence) using Multimode microplate reader (SpectraMax M2^e, Molecular devices, USA). The results for changes in mitochondrial membrane potential were interpreted by comparing ratio of red to green fluorescence.

2.11. Statistical analysis

All the cell biology experiments were performed in triplicates. The values of results were means of triplicate samples with standard errors. Data analysis was carried out using one way analysis of variance (ANOVA) followed by Newman Keuls using GraphPad Prism 5.0 software. Differences between values were considered statistically significant when $*P \leq .05$, $**P \leq .010$, $***P \leq .010$.

3. Results

3.1. Sequence analysis of L. donovani AK isoforms

A phylogeny analysis of LdAK isoforms were performed with human and *Trypanosoma brucei* AK isoforms using the maximum likelihood method (Fig. 1). *Leishmania* AK6 grouped together with the Human AK6 and *T. brucei* AK6. Both these isoforms had a characteristic NLS sequence, which is generally found in AKs associated with the nucleus. The LdAK isoform E9BI02 which possessed a large LID domain grouped with human AK4 and AK3. Human AK3 and AK4 are mitochondrial AKs. Leishmanial AK- E9BTK0 was grouped with *T. brucei* Q38B28. Each of these AKs had high pI values indicating association of their activities within glycosomal matrix. Leishmanial AK- E9BFD5 was clubbed together with several *T. brucei* AKs that possessed long leader sequence at upstream of phosphate binding loop. It has been reported that these AKs have role in flagellar activity. *Leishmania* putative AK (E9B861) with a short LID domain grouped with Human AK1, whose activity was associated with cytosol.

Multiple sequence analysis of several isoforms of AKs from human, *Trypanosoma brucei* and *Leishmania donovani* was carried out to identify conserved and functionally important domains, amino acid residues as well as to distinguish putative AK isoforms from each other. All the three domains i.e. LID domain, CORE domain and NMP binding domain was found to be conserved within all the isoforms. Critical analysis of all these sequences reveal that *L.donovani* putative AK E9BIO2 is the only Leishmanial AK isoform that has the characteristic Zinc binding

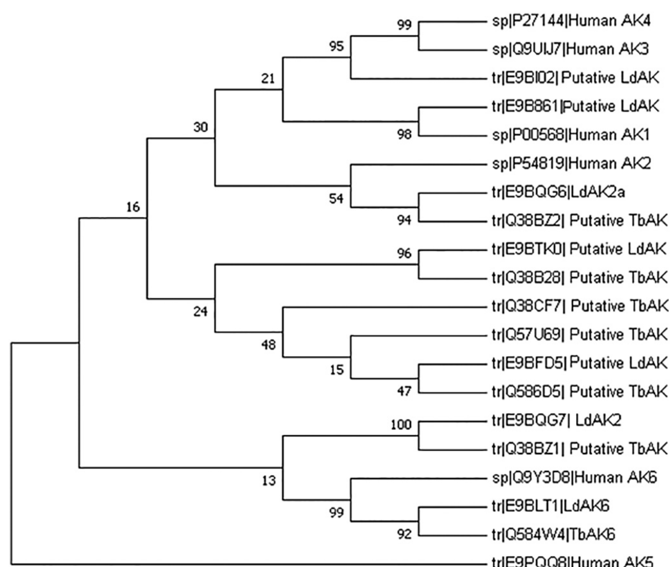


Fig. 1. Phylogenetic tree of Adenylate Kinase isoforms of *Homo sapiens, Trypanosoma brucei, Leishmania donovani.*
Sequences were retrieved from Swiss-Prot gene data base and phylogenetic tree was constructed using MEGA 5.0 software. The maximum likelihood method with statistical boot strapping (100 tests) was used.

motif as well as the mitochondrial import sequence at N-terminal 11-amino acid residues, which is very close to P-loop.

The multiple sequence alignment of LdAK2a (E9BQG6) with other AKs confirms the presence of three conserved domains i.e. NMP binding domain, LID domain and a CORE domain (Supplementary Fig. 1). A decapeptide walker motif (P loop), G-X-X-X-G-L-T/S related to ATP binding site was conserved in position 7–16 amino acids. A 38 amino acid large Lid domain was found to be conserved within 121–159 amino acid residues. The zinc motif sequence i.e. C-X_2-C-X_n-C-X_2-C whose function is to provide stability to protein, was not found in LdAK2a sequence. The NMP binding domain i.e. AMP binding site ranges from 30 to 61 amino acids. Catalytically important amino acids residues Lys 13, Arg 36, 88,119 were found to be conserved in LdAK2a.

3.2. Cloning, expression and purification of LdAK2a

The LdAK2a gene was cloned in pET28a expression vector and expression in *E.coli* DE3 Rosetta cells was obtained with 0.5 mM IPTG induction. The expressed soluble LdAK2a protein was further purified using Ni-NTA affinity chromatography. The purified protein showed a single polypeptide band in SDS PAGE corresponding to a molecular mass of 33 kDa (Fig. 2.A and B). The identity and molecular mass of *Leishmania donovani* AK2a protein was confirmed by MALDI TOF MS/MS analysis.

3.3. Molecular characterization of recombinant LdAK2a

Enzyme activity of the purified LdAK2a was assayed as described in methodology. The K_m value estimated for ATP and AMP was 204 μM and 184 μM respectively. V_{max} i.e. maximum enzyme activity of LdAK2a measured at saturation concentration for both ATP and AMP substrates (i.e. 3 mM and 2 mM, respectively) was 1.6 μmol ADP formed $min^{-1}mg^{-1}$protein. The stability of the recombinant protein was also checked by measuring its time dependent activity at a temperature of 30 °C. Though there was a time dependent reduction in the AK activity, it remained active up to approximately 30 days after purification. (Fig. 2.C).

The pH dependence of the enzyme was checked by measuring its activity in buffers with varying pH ranges. LdAK2a was active over a pH range (4.5 to 9) with maximum activity at pH 8.5. (Fig. 2.E) Thermo stability studies showed that the enzyme is active up to approximately 35 °C, beyond which it started losing its activity. (Fig. 2.D).

The effect of divalent metal ions on LdAK2a activity was checked with Ca^{2+}, Co^{2+}, Cu^{2+}, Mg^{2+} and Mn^{2+}. The enzyme did not exhibit its activity with Co^{2+} and Mn^{2+} divalent cations. The maximum activity was seen with Mg^{2+}.The metal ion activation was in the order of $Mg^{2+} > Ca^{2+} > Cu^{2+}$. (Fig. 2.F).

3.4. Inhibition of recombinant LdAK2a by AP5A inhibitor

The effect of Ap5A, which is a known nucleotide analogue inhibitor of AK, on the activity of LdAK2a, was studied under standard assay conditions. Analysis of LdAK2a inhibition was carried out using varying concentration of Ap5A at saturating concentrations of the other substrate [ATP (3 mM) and AMP (2 mM)]. K_i values were calculated as 280 nM and 230 nM for ATP and AMP respectively, (Fig. 3.A and B) using Dixon plot.

3.5. Comparative modeling of LdAK2a

In the absence of *L. donovani* crystal structure, the tertiary structure of LdAK2a was predicted using I-tasser server using the closest human homolog- Human AK 2 (PDB code: 2C9Y) X-ray crystal structure as the template (Fig. 4.A). The energy minimization of the best fit model was carried out using Charmm-gui server. Ramachandran plot analysis of the predicted structure was carried out using online tool PROCHECK, which showed predicted protein with 87% residues in most favored regions, 9.2% in additional allowed regions and 3.8% in generously allowed regions. ERRAT plot analysis indicates that overall quality factor for LdAK2a model was about 98.068.

LdAK2a model is α/β domain protein comprising of eleven α helices (α1-α11) and three β strands (β1-β3). The structure has the three characteristic sub domains- LID, CORE and NMP binding domain. The CORE domain consists of a decapeptide Walkers motif i.e. P loop binding site (7–16 amino acid residues). The NMP binding site consists of α2 and α3 helixes. This NMP binding site is flexible and is involved in binding of AMP substrate. The LID domain comprising of α8 and α9, is the active site of LdAK2a involved in binding of ATP.

The human homolog structure of AK (PDB ID: 2C9Y) was superposed over the modeled LdAK2a with an RMSD of 1.14 A°. (Fig. 4. B.). RMSD of C-alpha positions of the LdAKa model and the human AK structure was plotted after superposing these structures using the program ALIGN keeping the LdAK2a model as the fixed coordinate set and the Human AK as the moving co-ordinate set. (Supplementary Fig. 2). There was maximum deviation in the LID region corresponding to residues 121–159. Residues corresponding to the NMP binding region (residues 30- to 61) was absent in the Human AK structure.

3.6. Circular dichorism spectroscopy of LdAK2a

The CD spectroscopic analysis was used to determine secondary structure and folding patterns of the LdAK2a protein (Fig. 5.A). The CD spectra of LdAK2a showed minima at 208 nm and 222 nm. Analysis of CD data through K2D3 program showed that the recombinant LdAK2a secondary structure comprises of 51% α-helices and 9% β-sheets consistent with the predicted LdAKa model. Thermal melting study showed that the protein remained intact to around 37 °C after which it started unfolding. (Fig. 5.B).

The effect of ligands on secondary structural conformation of LdAK2a was analyzed by using CD spectroscopy. There were no significant changes in the secondary structure on incubating the protein with ATP / AMP / ADP / Ap5A alone. Largest secondary structure conformational change was observed in the presence of both the substrates- ATP and AMP together. (Fig. 5.C) There was a decrease in β-sheet content with an increase in α-helix content.

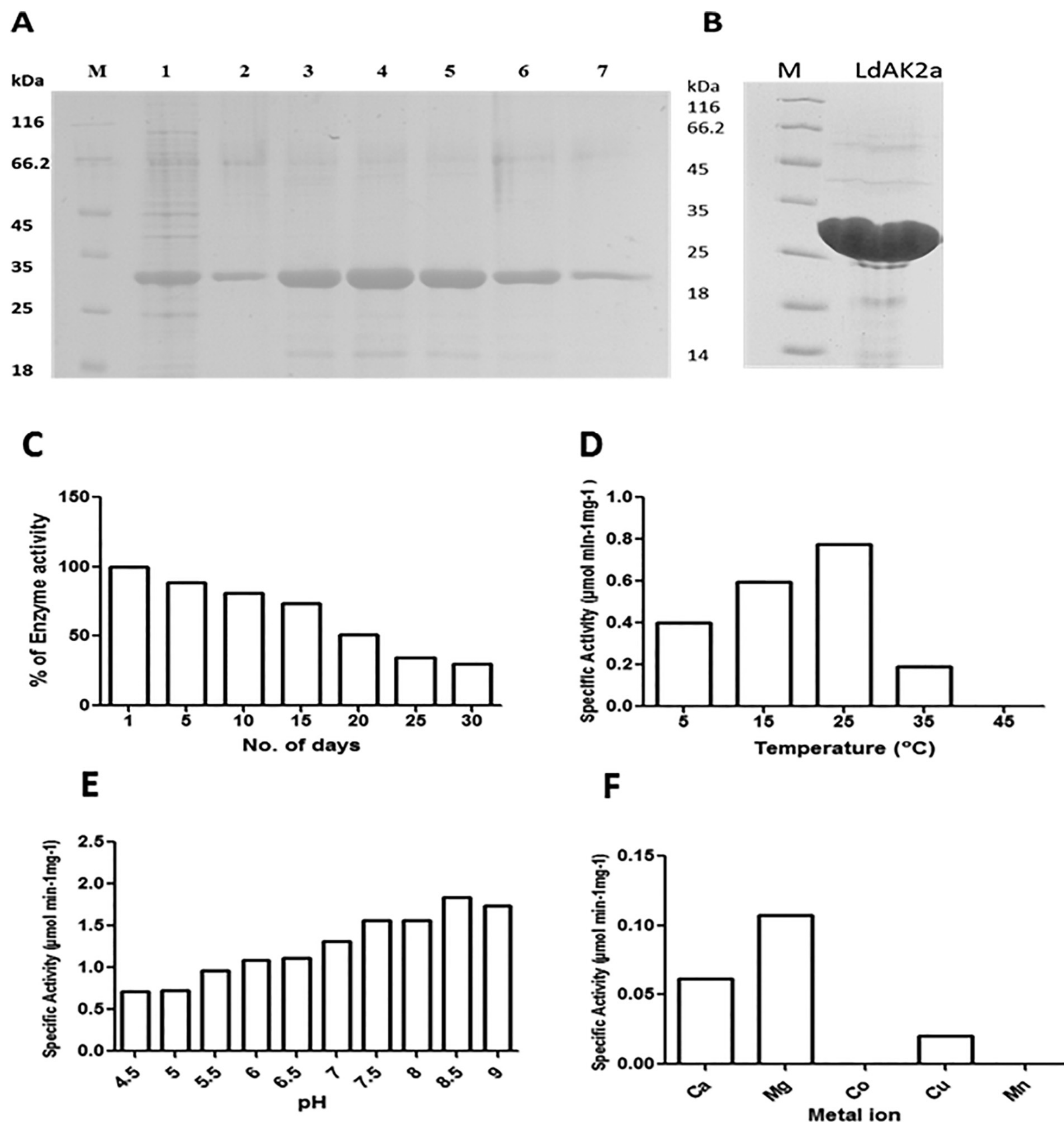


Fig. 2. Heterologous expression and molecular characterization of recombinant LdAK2a.
SDS PAGE analysis of expression and purification of LdAK2a (A) *E.coli* DE3 Rosetta cells containing pET28a-LdAK2a were induced with 0.5 mM IPTG. Lane M: Molecular weight marker, Lane 1: Total bacterial cells sample after IPTG induction and cell lysis, Lane 2: Supernatant; Lane 3–7: eluted factions of LdAk2a (B) SDS PAGE analysis for purity of concentrated recombinant LdAK2a protein. Lane 1: Molecular weight marker and Lane 2: purified concentrated protein. The concentrated purified protein shows approximately 95% purity after purification.
Kinetic analysis of purified recombinant LdAK2a (C) Time dependent enzyme activity (D) Effect of temperature (E) Effect of pH (F) Effect of metal ions (Manganese, Calcium, Copper, Magnesium) on LdAK2a enzyme activity.

3.7. Leishmanial recombinant AK2a mediates increase in cell viability and prevents ATP induced release of LDH from RAW 264.7 macrophages

Earlier reports suggest that eATP exposure to macrophages resulted in their cytolysis and that AK and other ATP utilizing enzymes were found to be released by Leishmanial parasites at the site of inflammation in macrophages. To examine whether LdAK2a has any role in accumulation of eATP and prevent eATP induced macrophage cell death, we initially investigated the macrophage cell viability in presence of different nucleotides with or without purified recombinant LdAK2a enzyme. We have analyzed cell viability in LPS primed macrophage cells in presence of different concentrations of ATP (1 mM, 2 mM, 3 mM and 5 mM) and AMP (0.5 mM, 1.5 mM, 2 mM) and Magnesium (0.05 mM, 0.1 mM, 0.2 mM, 0.4 mM) (Data not shown). The increase in concentration of ATP and incubation treatment time resulted in macrophage cell death. It was observed that AMP itself does not have any effect on viability of cells. The ATP and AMP concentration was selected on the basis of the enzyme activity studies. In order to assess cell viability of macrophages in presence of LdAK2a, we used 3 mM ATP, 2 mM AMP and 0.4 mM Mg^{2+} with 1 µg/ml of purified recombinant LdAK2a for an incubation period of 6 h in the MTT assay. A significant 70% decrease in cell viability was observed in 3 mM ATP treated LPS-primed macrophage cells. While AMP and Mg^{2+} individually do not have any effect on change in cell viability, it was significantly increased in the

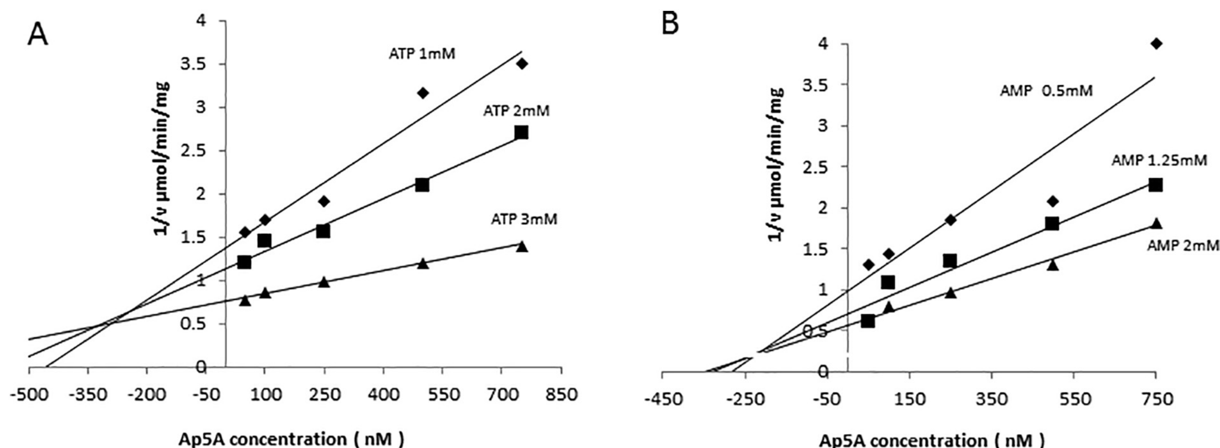


Fig. 3. Dixon Plot of inhibition of recombinant LdAK2a by inhibitor Ap5A Inhibition kinetics study was carried out using 3 μg of LdAK2a incubated with different concentrations of Ap5A (50,100, 250, 500, 750 nm) and three different concentrations of (A) ATP (1 mM, 2 mM, 3 mM) and (B) AMP (0.5 mM, 1.25 mM, 2 mM). Each value represents mean of triplicate determinants.

presence of LdAK2a incubated with both nucleotides- ATP and AMP. Addition of cofactor Mg^{2+} along with ATP and AMP nucleotide modulates LdAK2a activity, which results in percentage increase of cell viability from 55% to 86% in ATP, treated LPS primed macrophages. Enzymatically active LdAK2a increases the cell viability in eATP treated LPS macrophage cells (Fig. 6.A). To further investigate the role of LdAK2a in reducing ATP mediated cytolysis, we performed the LDH release assay with LPS primed RAW 264.7 macrophages in the presence of LdAK2a, ATP, AMP and Mg^{2+} with suitable concentrations. In the presence of 3 mM of eATP, increase in LDH release was observed compared to the control. The LdAK2a protein with other combinations of ATP, AMP and Mg^{2+} was used in this study. In the presence of ATP, AMP and Mg^{2+}, the enzymatically active LdAK2a decreases LDH release which confirmed that LdAK2a utilizes eATP and prevents eATP induced cytolysis of macrophages (Fig. 6.B).

3.8. Recombinant LdAK2a impede ATP induced changes in mitochondrial membrane potential permeability of RAW264.7 macrophages

The role of LdAK2a in preventing early events of eATP induced changes in mitochondrial membrane potential permeability of macrophages was performed by observing mitochondrial membrane potential. JC-1 incorporates into mitochondria of LPS primed RAW 264.7 cells i.e. control cells and forms J aggregates which results in emission of red fluorescence, indicative of healthy non apoptotic macrophage cells. In apoptotic cells, it remains in monomeric form within cytoplasm and emits green fluorescence. The exposure to 3 mM ATP for 6 h resulted in apoptosis of the macrophages cells as indicated by the green fluorescence. The addition of enzymatically active LdAK2a restricted the macrophages cells to undergo changes in mitochondrial membrane potential permeability event that may lead to cell death. (Fig. 7.A) On the other hand, when the cells were treated with LdAK2a which was heat inactivated by boiling (BrLdAK2a); it resulted in the emission of green fluorescence indicating apoptosis of the cells. The quantitative analysis of mitochondrial membrane potential was done through spectrofluorometry, which replicated the same results. (Fig. 7.B).

4. Discussion

Adenylate kinase is a highly conserved ubiquitous and essential P-transfer enzyme, whose primary functions are related to maintenance of

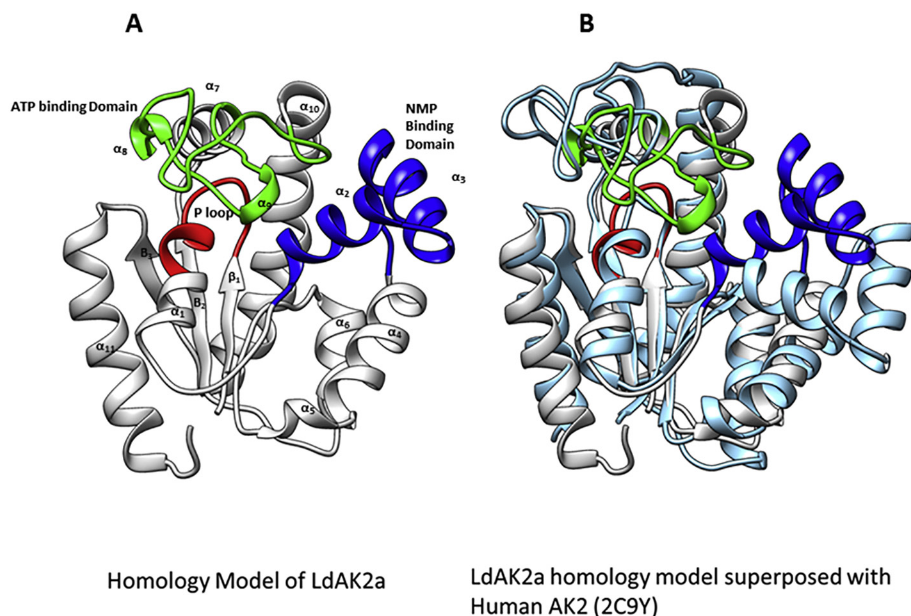


Fig. 4. Homology model and superposition of LdAK2a
(A) Homology model of LdAK2a showing P-loop, NMP binding domain and ATP binding domain.
(B) Superposition of LdAK2a model with crystal structure of Human AK2 (PDB code: 2C9Y) shows a superposition with RMSD of 1.140 A°. LdAK2a model and Human AK2 are indicated in grey and cyan color respectively.

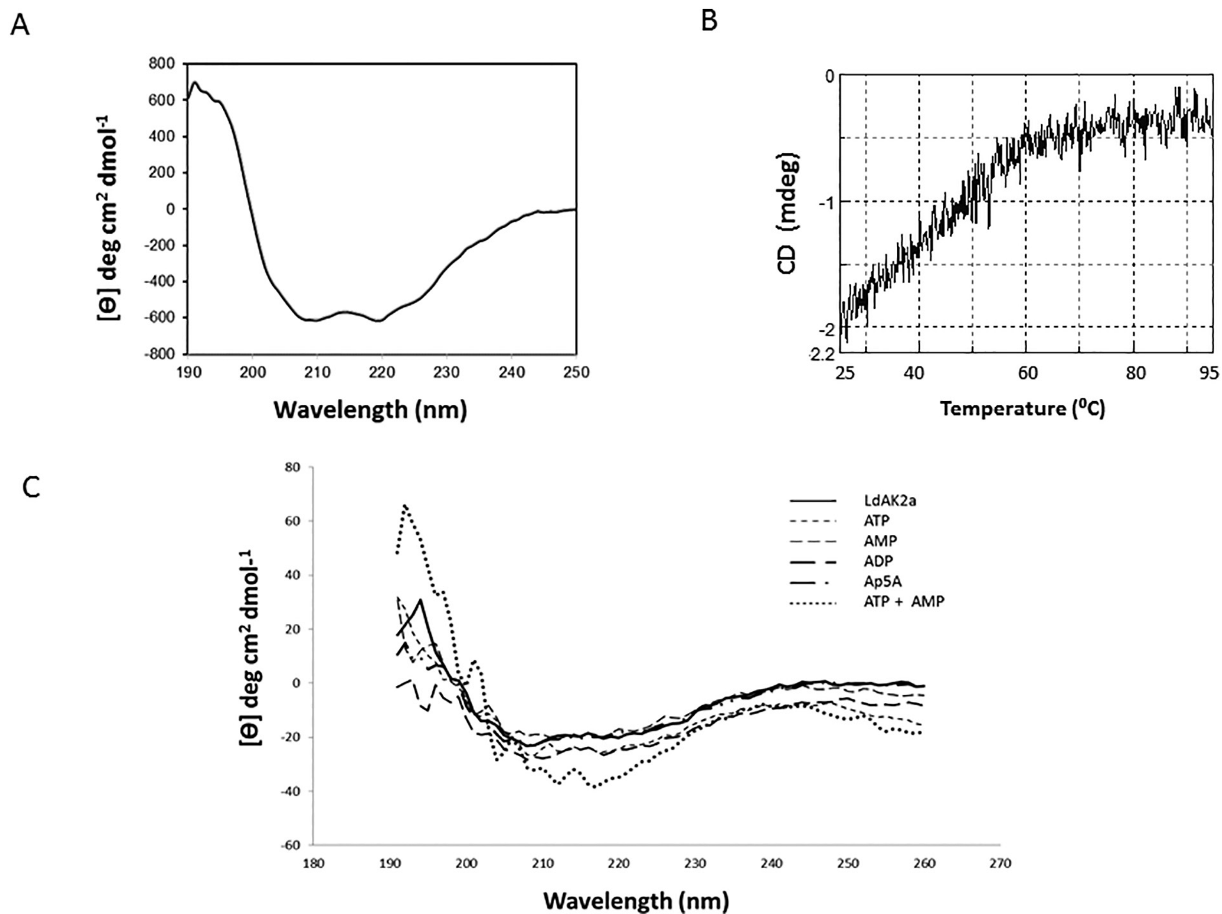


Fig. 5. Secondary structural conformational profiles of LdAK2a analyzed by far-UV CD spectroscopy.
(A) Far UV CD spectra of LdAK2a shows LdAK2a is comprised of both α-helices and β-sheets
(B) Thermal denaturation curve showing that LdAK2a is stable till ~ 37⁰C.
(C) Effect of substrates and inhibitor (Ap5A) on secondary structure of Ap5A. LdAK2a in the presence of ATP and AMP substrates showed maximum secondary structural changes.

adenine nucleotide pool and energy metabolism within cells. In addition to this, AK, secreted enzyme was found to be released along with other ATP-related enzymes which are known to modulate ATP levels during macrophage death and has been proposed as a virulence factor in many bacteria [1–3]. Not much work has been done in this interesting group of enzymes from the deadly protozoan parasite *Leishmania* sp. The only earlier characterization done on AKs from *Leishmania* sp. was that of adenylate kinase 2 [17].

A network of AK isoforms was reported within human (AK1-AK9) and in *Trypanosoma sp.* (AK1-AK7) [4,14]. Trypanosomatids have an expanded AK family with *T. brucei* having seven variants targeted to different subcellular structures, such as flagellum, glycosome, mitochondrion and cytosol [14,15] and *T. cruzi* having six variants targeted to different predicted subcellular compartments [26,27]. Another nuclear AK variant in *T. cruzi* was located in the cell nucleus whose functions associated with ribosomal RNA processing [16]. AK activity in kinetoplastids has been found to be associated with the membrane of glycosomes. Presence of several putative *Leishmania donovani* AK sequences in NCBI nucleotide database arises question about why these distinct isoforms within *Leishmania donovani* are needed in diverse cellular compartment and whether there is a functional similarity between LdAK isoforms with human and *Trypanosoma* species AK. To get insight into these questions we performed phylogenic and sequence analysis.

Our critical sequence analysis of all of these LdAK sequences identifies some interesting information about presence of AK family isoforms within *Leishmania*. Genome analysis showed that *Leishmania major* presents seven putative variants. Sequence comparisons and phylogeny analysis of these isoforms with Human and *Trypanosoma brucei* isoforms gave us insight about the probable subcellular localization of these AK isoforms. All the three characteristic domains of AK i.e. NMP binding domain, LID domain and CORE domain were conserved in these isoforms. In mammalian cells, it is reported that a large LID domain is characteristic of AKs showing activity in mitochondria while AKs with small LID domain show cytoplasmic activity [28]. Among the seven AK isoforms of *L. donovani*, E9B861 might be the only cytosolic LdAK, since it encoded for a shorter form of AK, with a LID domain of 18 amino acids. In *T. brucei* AK, it was reported that AK isoforms with N-terminal flagellar localization signals were found inside axoneme or extra axonemal structure called paraflagellar rod [14]. The presence of similar N-terminal extension within E9BFD5 might be indicative of its activity within flagellar axoneme of leishmania promastigotes. Presence of mitochondrial import signal and nuclear exportation signal in E9BIO2 sequence reveals its association with leishmanial mitochondria.

The putative LdAK2a (E9BQG6) that we cloned and characterized in the present study showed significant homology with Human AK (46%) and *L. donovani* AK2 (36%). This had a long LID domain of 38 amino acids showing high sequence similarity with LdAK2. The Phosphate binding loop which is responsible for ATP binding was found to be conserved. The Zn binding motif, which provides stability to certain AKs was absent in LdAK2a, which also suggested that it uses only Mg^{2+}

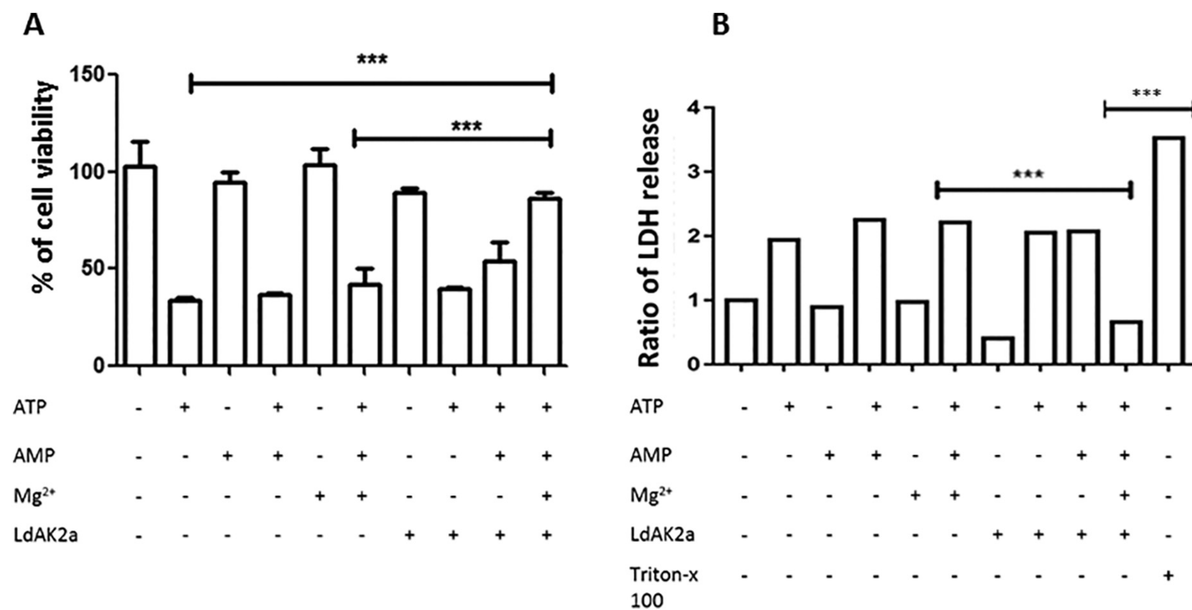


Fig. 6. LdAK2a mediates increase in cell viability and prevents ATP induced release of LDH.
(A) Determination of LPS primed Raw 264.7 macrophage cells viability in presence of ATP (3 mM), AMP (2 mM) nucleotides and Mg^{2+} (0.4 mM) cofactor with or without purified recombinant LdAK2a (1 μg) was carried out through MTT assay. The obtained value were plotted into graph by using Graphpad Prism. (B) ATP mediated cytolysis of LPS-primed Raw 264.7 macrophage cells were assayed by determining activities of cytosolic LDH release. LDH release from cells treated with Tritox X-100 was set as 100%. The graph shows prevention of LDH release in presence of Triton-X-100, recombinant LdAK2a enzyme (1 μg), ATP (3 mM), AMP (2 mM) and Mg^{2+} (0.4 mM) cofactor,. The values of results were means of triplicate samples with standard errors. Data analysis was carried out using one way analysis of variance (ANOVA) followed by Newman Keuls using Graphpad Prism 5.0 software. Differences were considered statistically significant at $*P \le .05$, $**P \le .010$, $***P \le .010$.

as a cofactor to accelerate its activity. The catalytically important residues such as R36, 88,119 were found conserved in LdAK2a.

To further characterize the enzyme, we cloned LdAK2a (UniProt Id: E9BQG6) from *L. donovani* genomic DNA (ag83) and expressed it as a His-tagged recombinant protein in *E. coli* Rosetta DE3 cells. Purified LdAK2a was further used for its characterization. Kinetic characterization was carried out. Maximum activity has been observed at pH 8.5. LdAK2a showed activity till ~ 37 °C. This was consistent with the CD spectroscopy studies of the recombinant protein, which showed that the enzyme loses its secondary structure at its T_m of ~ 37 °C. Among the divalent ions tested, LdAK2a showed maximum activity in the presence of Mg^{2+} and there was no activity in the presence of Mn^{2+} and Co^{2+}. The enzyme was inhibited by Ap5A, which is a substrate analogue that mimics the simultaneous binding of both the substrates i.e. ATP and AMP. Ap5A is a proven inhibitor of several AKs including Leishmanial AK2. It has been shown that Ap5A inhibits leishmanial growth, which was partially reversed by the addition of ADP [17]. Structural characterization of the protein and comparative studies with human homologous AK structures is required to evaluate its potential as a suitable anti-Leishmanial drug target.

Homology modeling of LdAK2a and comparison of the predicted structure with human AK structure showed that there is substantial difference in the LID domain region. Presence of several isoforms of AK in different compartments of the cell led to the suggestion that intracellular enzymatic phosphotransferase network that communicates the spatially separated intracellular ATP consumption and production processes is a promising starting point for drug design against human trypanosomatids like *Leishmania* and *Trypanosoma* [29]. Further evaluation of LdAK2a as a drug target is possible only after a critical analysis of the crystal structure of this enzyme with various ligand complexes and our current studies are focused on obtaining crustal structures of this protein.

It is well studied that many pathogenic microorganisms evolved strategy to modulate the level of NTPs by secreting NTP utilizing enzymes. Extracellular ATP (eATP), an important signal nucleotide which is found to accumulate at the site of inflammation and trigger the death of immune cells, by binding to the purinergic receptor of the P2X family such as P2Z, P2X7 receptors [30]. This activation of puringenic receptors is responsible for pore formation in macrophage membranes, which results in cell death. Extracellular pathogenic bacteria like *Pseudomonas*, *Mycobacterium*, *Vibrio* etc. have evolved strategies to promote macrophage apoptosis by releasing enzymes that increase eATP levels. Intracellular pathogens like *Leishmania* spp. should follow a reverse strategy that support invasion and persistence within host macrophages cells, by releasing eATP utilizing enzymes that prolong their survival in the host cells. Very little information is available about such molecules that mediate apoptosis / necrosis of host cells from intracellular pathogens. It has been reported that NdK is released in *L. amazonensis* capable of decreasing eATP and both native and recombinant NdK could prevent ATP induced cytolysis of macrophage cells *in vitro* [31].

Proteomics analysis of secretome of leishmania identified adenylate kinase as a secreted protein whose activity is found be associated with the glycosomes [12,13]. In order, to investigate whether LdAK2a have any functional importance as an evasive determinant for the successful infection of macrophages, in the present study, recombinant adenylate kinase from *Leishmania donovani* was analyzed for its role in infection within mouse macrophage cells. Our analysis showed that enzymatically active recombinant LdAK2a along with its substrates i.e. ATP, AMP and cofactor Mg^{2+} enhanced the percentage of cell viability within macrophages and lowered the ATP-induced LDH release from macrophages. Enzymatically active LdAK2a could also lower the mitochondrial membrane permeability of macrophage cells whereas heat inactivated LdAK2a had no effect. The change in membrane potential plays crucial role in many physiological processes and the loss of mitochondrial membrane potential is associated with apoptosis. Based on these results, we propose that in *Leishmania donovani*, secreted AK might be one of the ATP utilizing enzymes that when released at the site of infection can prevent ATP mediated cytolysis of their host cells by decreasing eATP which accumulates at the site of inflammation,

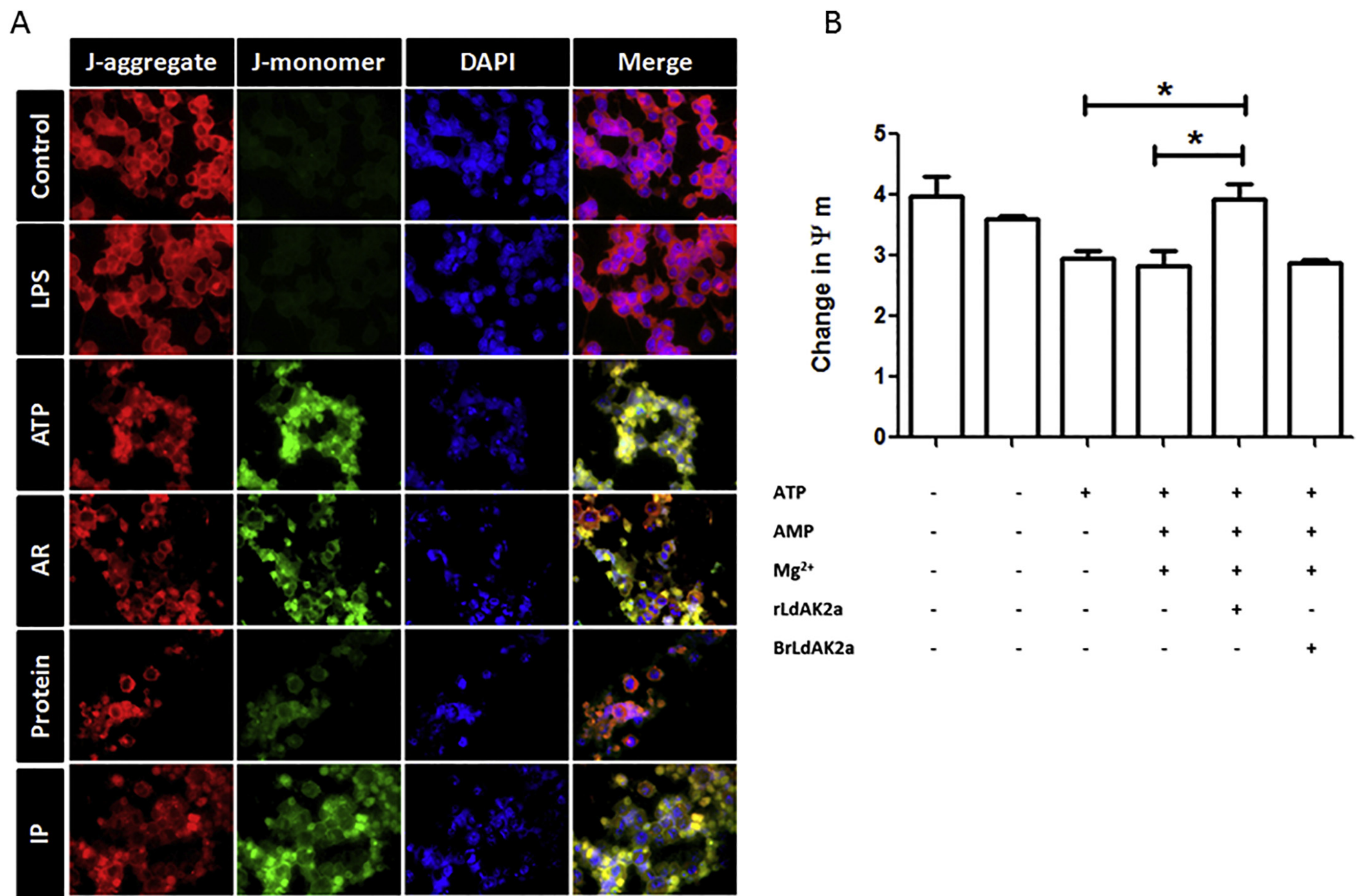


Fig. 7. Recombinant LdAK2a prevents ATP induced changes of RAW264.7 macrophages in mitochondrial potential permeability.
(A) LPS primed Raw 264.7 macrophage cells were treated with 3 mM ATP alone, as well with reactants mixture (3 mM ATP + 2 mM AMP + 0.4 mM Mg^{2+}) in presence of activated and heat inactivated LdAK2a (Boiled recombinant (Br) LdAK2a) (1 μg) for 6 h. After incubation period, cells were treated with JC-1 dye (5 μg/ml in DPBS medium) and incubated for 30 min in dark. Upon completion of incubation, cells were counterstained with DAPI and thereafter cells were mounted under fluorescence microscope. (IP: Inactive protein, AR: all reagents)
(B) Quantitative analysis of mitochondrial membrane potential assay was performed. Fluorescence was recorded at 527 nm (green fluorescence) and 590 nm (red fluorescence) using multimode microplate reader and results were interpreted with comparing ratio of red to green fluorescence. The values of results were means of triplicate samples with standard errors. Data analysis was carried out using one way analysis of variance (ANOVA) followed by Newman Keuls using Graphpad Prism 5.0 software. Differences were considered statistically significant at *P ≤ .05, **P ≤ .010, ***P ≤ .010. (rLdAK2a: recombinant LdAK2a, BrLdAK2a: boiled or heat inactivated LdAK2a). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

helping them in their survival as an intracellular parasite.

In conclusion, our data suggests the AK2A activity is likely to be necessary for survival of macrophages infected by *L. donovani*, and therefore survival of *L. donovani*. Additional genetic knockout and RNA knockdown experiments of the AK2A gene are necessary to test this hypothesis which would further strengthen the idea about its potential as a drug target in *Leishmania*.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.parint.2019.101929.

Author contribution

PGK carried out protein expression and purification, biochemical and biophysical characterization and bioinformatics analysis, cell biology experiment designing and overall data interpretation. PGK and NS have carried out cloning experiment. BW performed cell biology experiments. CMP was involved in critical review of cell biology experiments. PGK and AP were involved in conceptualizing this work and writing this manuscript.

Acknowledgement

This work was supported by DBT, Gov. of India and Puri Foundation for Education in India. The authors are thankful to Dr. Shriram Kanvah, IIT Gandhinagar, for allowing the use of CD spectrophotometer. We also thank to Prof. J.K. Pal, Savitribai Phule Pune University for providing us genomic DNA of *Leishmania donovani*.

References

[1] A. Markaryan, O. Zaborina, V. Punj, A.M. Chakrabarty, Adenylate kinase as a virulence factor of *Pseudomonas aeruginosa*, J. Bacteriol. 183 (2001) 3345–3352.
[2] V. Punj, O. Zaborina, N. Dhiman, K. Falzari, M. Bagdasarian, A.M. Chakrabarty, Phagocytic cell killing mediated by secreted cytotoxic factors of *Vibrio cholerae*, Infect. Immun. 68 (2000) 4930–4937.
[3] A. Melnikov, O. Zaborina, N. Dhiman, B.S. Prabhakar, A.M. Chakrabarty, W. Hendrickson, Clinical and environmental isolates of *Burkholderia cepacia* exhibit differential cytotoxicity towards macrophages and mast cells, Mol. Microbiol. 36 (2000) 1481–1493.
[4] C. Panayiotou, N. Solaroli, A. Karlsson, The many isoforms of human adenylate kinases, Int. J. Biochem. Cell Biol. 49 (2014) 75–83.
[5] G.H. Coombs, Intermediary metabolism in parasitic protozoa, Int. J. Parasitol. 17 (1987) 97–104.
[6] S. Kamath, M.L. Chen, A.M. Chakrabarty, Secretion of nucleoside diphosphate kinase by mucoid *Pseudomonas aeruginosa* 8821: involvement of a carboxy-terminal

motif in secretion, J. Bacteriol. 182 (2000) 3826–3831.
[7] O. Zaborina, N. Misra, J. Kostal, S. Kamath, V. Kapatral, M.E. El-Idrissi, B.S. Prabhakar, A.M. Chakrabarty, P2Z-independent and P2Z receptor-mediated macrophage killing by *Pseudomonas aeruginosa* isolated from cystic fibrosis patients, Infect. Immun. 67 (1999) 5231–5242.
[8] A. Melnikov, O. Zaborina, N. Dhiman, B.S. Prabhakar, A.M. Chakrabarty, W. Hendrickson, Clinical and environmental isolates of *Burkholderia cepacia* exhibit differential cytotoxicity towards macrophages and mast cells, Mol. Microbiol. 36 (2000) 1481–1493.
[9] O. Zaborina, N. Dhiman, M. Ling Chen, J. Kostal, I.A. Holder, A.M. Chakrabarty, Secreted products of a nonmucoid *Pseudomonas aeruginosa* strain induce two modes of macrophage killing: external-ATP-dependent, P2Z-receptor-mediated necrosis and, Microbiology. 146 (Pt 10) (2000) 2521–2530, https://doi.org/10.1099/00221287-146-10-2521.
[10] O. Zaborina, X. Li, G. Cheng, V. Kapatral, A.M. Chakrabarty, Secretion of ATP-utilizing enzymes, nucleoside diphosphate kinase and ATPase, by *Mycobacterium bovis BCG*: sequestration of ATP from macrophage P2Z receptors, Mol. Microbiol. 31 (1999) 1333–1343.
[11] P. Chopra, A. Singh, A. Koul, S. Ramachandran, K. Drlica, A.K. Tyagi, Y. Singh, Cytotoxic activity of nucleoside diphosphate kinase secreted from *Mycobacterium tuberculosis*, Eur. J. Biochem. 270 (2003) 625–634.
[12] J.M. Silverman, S.K. Chan, D.P. Robinson, D.M. Dwyer, D. Nandan, L.J. Foster, N.E. Reiner, Proteomic analysis of the secretome of *Leishmania donovani*, Genome Biol. 9 (2008) R35, https://doi.org/10.1186/gb-2008-9-2-r35.
[13] J.P.B. Menezes, T.F. Almeida, A.L.O.A. Petersen, C.E.S. Guedes, M.S.V. Mota, J.G.B. Lima, L.C. Palma, G.A. Buck, M.A. Krieger, C.M. Probst, P.S.T. Veras, Proteomic analysis reveals differentially expressed proteins in macrophages infected with *Leishmania amazonensis* or *Leishmania major*, Microbes Infect. 15 (2013) 579–591, https://doi.org/10.1016/j.micinf.2013.04.005.
[14] M.L. Ginger, E.S. Ngazoa, C.A. Pereira, T.J. Pullen, M. Kabiri, K. Becker, K. Gull, D. Steverding, Intracellular positioning of isoforms explains an unusually large adenylate kinase gene family in the parasite *Trypanosoma brucei*, J. Biol. Chem. 280 (2005) 11781–11789, https://doi.org/10.1074/jbc.M413821200.
[15] T.J. Pullen, M.L. Ginger, S.J. Gaskell, K. Gull, Protein targeting of an unusual, evolutionarily conserved adenylate kinase to a eukaryotic flagellum, Mol. Biol. Cell 15 (2004) 3257–3265.
[16] M. de los M. Camara, L.A. Bouvier, G.E. Canepa, M.R. Miranda, C.A. Pereira, Molecular and functional characterization of a *Trypanosoma cruzi* nuclear adenylate kinase isoform, PLoS Negl. Trop. Dis. 7 (2013) e2044, , https://doi.org/10.1371/journal.pntd.0002044.
[17] H. Villa, Y. Perez-Pertejo, C. Garcia-Estrada, R.M. Reguera, J.M. Requena, B.L. Tekwani, R. Balana-Fouce, D. Ordonez, Molecular and functional characterization of adenylate kinase 2 gene from *Leishmania donovani*, Eur. J. Biochem. 270 (2003) 4339–4347.
[18] S. Pundir, M.J. Martin, C. O'Donovan, UniProt protein knowledgebase, Methods Mol. Biol. 1558 (2017) 41–55, https://doi.org/10.1007/978-1-4939-6783-4_2.
[19] S. Kumar, G. Stecher, K. Tamura, MEGA7: molecular evolutionary genetics analysis version 7.0 for bigger datasets, Mol. Biol. Evol. 33 (2016) 1870–1874.
[20] Y. Zhang, I-TASSER server for protein 3D structure prediction, BMC Bioinforma. 9 (2008) 40, , https://doi.org/10.1186/1471-2105-9-40.
[21] B.T. Miller, R.P. Singh, J.B. Klauda, M. Hodoscek, B.R. Brooks, H.L. Woodcock 3rd, CHARMMing: a new, flexible web portal for CHARMM, J. Chem. Inf. Model. 48 (2008) 1920–1929, https://doi.org/10.1021/ci800133b.
[22] R.A. Laskowski, J.A.C. Rullmann, M.W. MacArthur, R. Kaptein, J.M. Thornton, AQUA and PROCHECK-NMR: Programs for checking the quality of protein structures solved by NMR, J. Biomol. NMR 8 (1996) 477–486, https://doi.org/10.1007/BF00228148.
[23] E.F. Pettersen, T.D. Goddard, C.C. Huang, G.S. Couch, D.M. Greenblatt, E.C. Meng, T.E. Ferrin, UCSF chimera–a visualization system for exploratory research and analysis, J. Comput. Chem. 25 (2004) 1605–1612, https://doi.org/10.1002/jcc.20084.
[24] G. Cohen, E., ALIGN: a program to superimpose protein coordinates, accounting for insertions and deletions, J. Appl. Crystallogr. 30 (1997) 1160–1161, https://doi.org/10.1107/S0021889897006729.
[25] M. Reers, T.W. Smith, L.B. Chen, J-aggregate formation of a carbocyanine as a quantitative fluorescent indicator of membrane potential, Biochemistry. 30 (1991) 4480–4486.
[26] M. de L. Milagros Camara, L.A. Bouvier, M.R. Miranda, C.A. Pereira, Identification and validation of *Trypanosoma cruzi*'s glycosomal adenylate kinase containing a peroxisomal targeting signal, Exp. Parasitol. 130 (2012) 408–411, https://doi.org/10.1016/j.exppara.2012.01.020.
[27] L.A. Bouvier, M.R. Miranda, G.E. Canepa, M.J.M. Alves, C.A. Pereira, An expanded adenylate kinase gene family in the protozoan parasite *Trypanosoma cruzi*, Biochim. Biophys. Acta 1760 (2006) 913–921, https://doi.org/10.1016/j.bbagen.2006.02.013.
[28] K. Fukami-Kobayashi, M. Nosaka, A. Nakazawa, M. Go, Ancient divergence of long and short isoforms of adenylate kinase: molecular evolution of the nucleoside monophosphate kinase family, FEBS Lett. 385 (1996) 214–220.
[29] C.A. Pereira, L.A. Bouvier, L. Cámara Mde, M.R. Miranda, Singular features of trypanosomatids' phosphotransferases involved in cell energy management, Enzym. Res. 2011 (2011), https://doi.org/10.4061/2011/576483 576483.
[30] D. Ferrari, P. Chiozzi, S. Falzoni, M. Dal Susino, L. Melchiorri, O.R. Baricordi, F. Di Virgilio, Extracellular ATP triggers IL-1 beta release by activating the purinergic P2Z receptor of human macrophages, J. Immunol. 159 (1997) 1451–1458.
[31] B.K. Kolli, J. Kostal, O. Zaborina, A.M. Chakrabarty, K.-P. Chang, Leishmania-released nucleoside diphosphate kinase prevents ATP-mediated cytolysis of macrophages, Mol. Biochem. Parasitol. 158 (2008) 163–175, https://doi.org/10.1016/j.molbiopara.2007.12.010.