Molecular cloning and expression of adenosine kinase from *Leishmania donovani*: identification of unconventional P-loop motif

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The unique catalytic characteristics of adenosine kinase (Adk) and its stage-specific differential activity pattern have made this enzyme a prospective target for chemotherapeutic manipulation in the purine-auxotrophic parasitic protozoan *Leishmania donovani*. However, nothing is known about the structure of the parasite Adk. We report here the cloning of its gene and the characterization of the gene product. The encoded protein, consisting of 345 amino acid residues with a calculated molecular mass of 37173 Da, shares limited but significant similarity with sugar kinases and inosine–guanosine kinase of microbial origin, supporting the notion that these enzymes might have the same ancestral origin. The identity of the parasite enzyme with the corresponding enzyme from two other sources so far described was only 40 %. Furthermore, 5′ RNA mapping studies indicated that the Adk gene transcript is matured post-transcriptionally with the trans-splicing of the mini-exon (spliced leader) occurring at nt −160 from the predicted translation initiation site. The biochemical properties of the recombinant enzyme were similar to those of the enzyme isolated from leishmanial cells. The intrinsic tryptophan fluorescence of the enzyme was substrate-sensitive. On the basis of a multiple protein-alignment sequence comparison and ATP-induced fluorescence quenching in the presence or the absence of KI and acrylamide, the docking site for ATP has been provisionally identified and shown to have marked divergence from the consensus P-loop motif reported for ATP- or GTP-binding proteins from other sources.

Key words: nucleoside kinase, nucleotide-binding motif, parasitic protozoan, purine salvage.

INTRODUCTION

Leishmania donovani, a dimorphic parasitic protozoan, is the causative agent of visceral leishmaniasis in human populations [1]. This group of organisms cannot synthesize purines *de novo* and therefore, to survive, have developed a unique series of stage-specifically expressed purine-salvage-pathway enzymes [2,3]. In an earlier study it was demonstrated that the differential pattern of adenosine (Ado) utilization observed between the intracellular pathogenic (amastigote) and extracellular (promastigote) vector forms of *L. donovani* is accompanied by a marked increase in the specific activity of adenosine kinase (Adk) in the amastigote stage [4].

Although Adk has been purified and characterized from a number of sources, there is still a lack of consensus about its kinetic properties [5–8]. Furthermore, several newly discovered novel characteristics of the enzyme from higher eukaryotes, namely its ability to perform an ADP-stimulated Ado/AMP exchange reaction and a complete dependence for its catalytic activity on the presence of a pentavalent ion such as phosphate, arsenate or vanadate, still remain unexplained [9–12]. The gene for this enzyme, cloned recently from human and murine sources, was shown to be more than 95 % conserved [13–15].

Extensive studies performed in this laboratory indicated distinctly unique catalytic characteristics and immunochemical specificity of the parasite Adk compared with Adks isolated from other sources [16–18]. By using group-specific chemical modifiers it was shown that the *L. donovani* enzyme, in contrast with other Adks, harbours at least one essential arginine residue and two conformationally vicinal cysteine residues at or close to the active site [19,20] and is not subject to inhibition by Ado [21]. The response of the enzyme to ATP was also intriguing and suggested possible differences in ATP binding from the corresponding enzyme from other sources [16]. Because the importance of Adk in the maintenance of the AMP pool, especially in purine auxotrophs, is well documented, the enzyme clearly would be a viable target in drug design [5]. However, key information for the design of new analogues or in the improvement of existing analogues is virtually non-existent because of a lack of knowledge about the active site of the enzyme and the unavailability of X-ray structural data on a substrate (or substrate analogue) complex. Clearly, the poor yield of the enzyme and the unavailability of the cloned gene have constrained further biochemical and structural studies.

To overcome these difficulties and to delineate specifically the common and distinguishing features that differentiate the parasite Adk from the counterpart enzyme from higher eukaryotes, we undertook cloning of the gene, analysis of some of its structural features and characterization of the gene product. Because the activity pattern of the parasite enzyme displayed anomalous kinetic characteristics in the presence of various concentrations of $MgATP^{2-}$ [16], our initial studies have focused on the identification of the nucleotide-binding domain (P-loop), shown to be critical to many enzymic activities that bind ATP or GTP.

MATERIALS AND METHODS

Reagents, cells and libraries

All chemicals and reagents were of the highest quality available. DNA modification and restriction enzymes, *Thermus aquaticus*

Abbreviations used: Adk, adenosine kinase; Ado, adenosine; DTT, dithiothreitol; IPTG, isopropyl β-D-thiogalactoside; ORF, open reading frame; RT–PCR, reverse transcriptase-mediated PCR.

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The nucleotide sequence data reported will appear in DDBJ, EMBL and GenBank Nucleotide Sequence Databases under the accession number AF 056937.

(*Taq*) polymerase and Titan™ one-tube reverse transcriptase-mediated PCR (RT–PCR) kit were from Boehringer Mannheim. Oligonucleotides were obtained from Bangalore Genei. *L. donovani* (D1700), a clone of the 1S Sudanese strain, and its genomic library, made in bacteriophage λGEM-11, were a gift from Professor Buddy Ullman [22]. The production and characterization of the rabbit antiserum against *L. donovani* (MHOM/Ind/78/UR6)-coded Adk have been described earlier [16,18]. Unless otherwise stated, for all transformation experiments with plasmids, *Escherichia coli* JM109 strain was used.

Nucleic acid isolation, cloning and sequencing

Preparation of nucleic acids, ligation and transformation were performed as described [23]. Nucleotide sequencing on both strands was performed with an automated DNA sequencer.

Peptide sequencing

Initial attempts at Edman degradation of the purified protein revealed a blocked N-terminus. Purified protein was therefore subjected to either digestion with CNBr or cleavage by endopeptidase LysC. The generated peptide fragments were separated by reverse-phase HPLC; well-resolved peaks were analysed by automated sequencer. Sequences obtained were as follows: peptide 1, GIFEELEQHPNVTYVPGG*GLNTA*VAQ; peptide 2, DISLVANLSAANLLSA; peptide 3, AAPYLDVIFGNEVEAK; peptide 4, NYVLQVAEAARASGGQ. Of these, the first three peptides were generated by endopeptidase LysC cleavage, whereas the fourth one cleaved with CNBr. An asterisk denotes that no amino acid was identified in that cycle. Amino acid sequencing of the endopeptidase LysC- and CNBr-cleaved fragments was performed at the Macromolecular Facility at the University of Kentucky (Lexington, KY, U.S.A.) and at the Albert Einstein College of Medicine (New York, NY, U.S.A.) respectively.

Southern and Northern blot analyses

All the analyses were performed in accordance with procedures described previously [22,23]. Hybridizations were performed either with a nick-translated or random-primed ^{32}P-labelled appropriate probe for 12–14 h [22]. The filters were washed twice in 2×SSC buffer (1×SSC is 0.15 M NaCl/0.015 M sodium citrate) plus 0.1% SDS at room temperature, followed by one wash in 1×SSC/0.1% SDS at 42 °C for 1 h. The final washings were performed at 50 °C with 0.1×SSC/0.1% SDS for 30 min. ^{32}P-labelled oligonucleotides were prepared by a standard method with T4 polynucleotide kinase. Oligonucleotide hybridizations were perfomed by using the procedure of Sambrook et al. [23].

Amplification of Adk-encoding fragment

To generate a DNA probe, PCR was employed using degenerate oligonucleotides in both forward and reverse orientations corresponding to two sequence regions IFEELEQ and FGNEVEA. The reaction mixture (20 μl), containing 150 ng of genomic DNA, 60 mM KCl, 25 mM Tris/HCl, pH 8.5, 1.5 mM $MgCl_2$, each dNTP at 200 μM, 25 pmol of each oligonucleotide primer and 2.5 units of *Taq* polymerase, was incubated in a thermocycler at 94, 50 and 72 °C for 60 s, 60 s and 2 min respectively for 30 cycles. The amplified product (540 bp), produced with the combination of sense primer 5′-CTCGGAATTCCA-TITT(T/C)GA(A/G)GA(A/G)CTIGA(A/G)CA-3′ and the anti-sense primer 5′-CTCGGGATCCCGC(C/T)TCIAC(C/T)-TC(G/A)TTICC(G/A)AA-3′, corresponding to the sequences IFEELEQ and FGNEVEA respectively, was subcloned into the *Eco*RI/*Bam*HI site of pUC19 and sequenced.

Isolation of the full-length gene

To isolate the full-length clone, a leishmanial genomic library was screened as follows: bacteriophage (10^5)-infected *E. coli* P2392 cells were blotted on nitrocellulose papers and were further treated at 50 °C for 4 h in solutions containing 4×Denhardt's reagents, 0.1×SDS, 5×SSC and 100 μg/ml single-stranded calf thymus DNA. Filters were hybridized at 50 °C in the same solution containing 10^6 c.p.m./ml nick-translated ^{32}P-labelled 540 bp long DNA probe (PCR-generated). After stringent washings and autoradiography, three positive recombinants were obtained. Purification of the recombinant phage was performed through rescreening.

The DNA isolated from one of the bacteriophages was digested to completion with *Kpn*I/*Xho*I, electrophoresed on a 0.8% agarose gel, transferred to nitrocellulose and hybridized with ^{32}P-labelled DNA probe as above. An approx. 3.2 kb fragment that hybridized with the probe was isolated and ligated into the *Kpn*I/*Sal*I site of pBS vector and transformed. The relevant portion of the insert was sequenced by using the dideoxy chain-termination method.

Mapping the 5′ terminus of mature Adk mRNA

To determine the 5′ terminus of the *L. donovani* Adk transcript, the following reaction was performed. The one-tube RT–PCR reaction mixture (50 μl) contained 1.0 μg of total RNA, each dNTP at 200 μM, 1 mM $MgCl_2$, 50 pmol of each oligonucleotide primer and 1 μl of Expand™ high-fidelity enzyme mixture supplied by Boehringer Mannheim. The sense primer, 5′-CTCGGAATTCCAACGCTATATAAGTATCAGTTTCTG-TACTTTATTG-3′, containing a short leader sequence with an *Eco*RI site followed by 36 nt of the 39 nt mini-exon that is trans-spliced on the 5′ end of the transcript [24,25], and the degenerate anti-sense primer, 5′-CTCGGGATCCCTG(C/T)TCIAG(C/T)-TC(C/T)TC(G/A)AAIAI-3′, corresponding to residues 46–52, IFEELEQ, with a 5′ *Bam*HI site, were used. The PCR programme consisted of an initial reverse transcription at 41 °C for 2 h, followed by amplification of the cDNA product for 35 cycles at 94, 50 and 72 °C for 60, 60 and 120 s respectively. The RT–PCR product (approx. 360 bp) was subcloned into the pBS vector at *Eco*RI/*Bam*HI sites and sequenced.

Bacterial expression of *L. donovani* Adk

The 1035 bp open reading frame (ORF) was amplified with a 5′ sense primer, 5′-CTCGAATTCATATGTCCGCGCTTCCG-CAGCTC-3′, and a reverse complement primer, 5′-ACAC GGATCCTCACGGAGAGATGGACGGT-3′. Amplified DNA was subcloned into the *Nde*I/*Bam*HI site of pEt3a and transformed into *E. coli* strain BL21 (DE-3) pLysS. Induction was performed for 2–6 h in the presence of 1 mM isopropyl β-D-thiogalactoside (IPTG) at 37 °C.

Purification of the recombinant protein

IPTG-induced plasmids containing BL21(DE3) pLysS cells were suspended in buffer [20 mM Tris/HCl (pH 7.5)/1 mM EDTA/5 mM dithiothreitol (DTT)/1 mM PMSF/1% (v/v) Triton X-100/50 μg/ml lysozyme] and sonicated. After centrifugation, the clear supernatant was subjected to 40–80%-satd.

$(NH_4)_2SO_4$ fractionation. The precipitate collected was dialysed extensively against buffer containing 20 mM Tris/HCl, pH 7.5, 1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 1 mM PMSF and 5% (v/v) glycerol. The enzyme was purified and assayed with the use of procedures described previously [16].

Fluorescence measurements and ATP-binding assay

Ligand-induced fluorescence-quenching experiments were performed with purified Adk (0.8 μM) in buffer containing 20 mM Tris/HCl, pH 7.5, 10% (v/v) glycerol and 1 mM DTT in 1 ml quartz cuvettes at 25 °C, as described previously [19,20]. The binding of ATP to the enzyme was monitored by ligand-induced fluorescence-quenching assays with the assumption that fluorescence change was directly proportional to the concentration of the enzyme–ligand complex and that the molar fraction v of the enzyme bound at each concentration of the ligand was given by [26,27]:

$$v = [EL]/[E]_{total} = \Delta F/\Delta F_{max}$$

where the enzyme concentration is expressed in normality (i.e. active-site concentration), ΔF is the actual fluorescence change at a given concentration of the ligand and ΔF_{max} is the maximum fluorescence change at saturating concentration of the ligand. The correct evaluation of ΔF_{max} was obtained from a double-reciprocal plot of ΔF against ligand concentration. K_d and the stoichiometry of association were calculated from a Scatchard analysis.

Quenching experiments in the presence of KI and acrylamide were analysed by using a Stern–Volmer plot. On the basis of the nature of the curve of F_0/F against [Q], it was concluded that the mode of quenching was collisional with heterogeneity, thus reducing the original Stern–Volmer equation to:

$$F_0/F = 1 + K_{SV}[Q]$$

The quenching parameters were determined from the modified Stern–Volmer equation, described by Lehrer as:

$$F_0/\Delta F = 1/f_a + (1/f_a K_{SV})(1/[Q])$$

where F_0 and F are the fluorescence in the absence and the presence of quenchers respectively, $\Delta F = F_0 - F$, [Q] is the concentration of the quenchers, f_a is the fraction of total emission accessible to the quencher and K_{SV} is the Stern–Volmer constant.

Computer analysis

The search for Adk-related sequences was performed with BLAST programs [28]. The statistical significance of sequence similarity between protein sequences was determined with the program PCOMPARE [29] of the PCGENE package. The nucleotide sequence analysis of the cloned fragment was done with program PC-DOS H1B10 DNASIS™ (fifth version). Similarity was determined with the program FASTA against the SWISS-PROT data bank [30].

RESULTS

Isolation and analysis of the Adk gene

On the basis of a partial amino acid sequence and PCR (see the Materials and methods section), a probe for screening the *L. donovani* λGEM-11 genomic library was developed initially. Translation of the 540 bp PCR fragment in all possible reading frames revealed that several stretches of the predicted peptide sequence from one of the six reading frames matched exactly with the experimentally determined amino acid sequence of the peptide fragments obtained from the purified protein digest (Figure 1). To isolate the full-length clone, the PCR product was used to screen the parasite DNA library [22]. Out of three positive plaques, one was used for subsequent analyses: a *Kpn*I/*Xho*I-digested approx. 3.2 kb fragment from the chimaeric phage DNA that hybridized with the PCR fragment seemed to contain the complete ORF and was subcloned and sequenced. Southern blot hybridization of the genomic DNA and gene titration experiments indicated that the gene was not tandemly repeated and was present as a single copy (results not shown).

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                     ▼              TGGTAAACGTGTCTCCTTCCTCGTGCCTCGC   -181
CGCTCTGTGGGACTCCGCAGAAGCTGCACAGGAAGTCCATCGCATCTACGTACACATAAG   -121
CCCGCAAGTATCTTGTTTCCGTTATATTGTGATCCGTCATTACAGTTGTACCTTCACCAA    -61
CCTCCCCGCCGCGCCTGCCTTTCTCCCTTCTTCAACTGCCCTTTCCTACCCTCTTCGACC     -1
ATGTCCGCGCTTCCGCAGCTCTACATTCAGTGCAACCCGCTCCTCGACGTGTCTGCCCCT     60
 M  S  A  L  P  Q  L  Y  I  Q  C  N  P  L  L  D  V  S  A  P      20
GTCGATGACGCGTTCTTAGAGAAGTACAAGGTGCAGAAGACGTCTGCCTGTCTGATGGAG    120
 V  D  D  A  F  L  E  K  Y  K  V  Q  K  T  S  A  C  L  M  E      40
GAGATCCATAAGGGCATCTTCGAGGAGCTAGAGCAGCACCCCAACGTGACCTACGTCCCC    180
 E  I  H  K  G  I  F  E  E  L  E  Q  H  P  N  V  T  Y  V  P      60
GGCGGCTCTGGCCTCAACACCGCCCGCGTGGCGCAGTGGATCGCGCAGGCCCCCAAGAGC    240
 G  G  S  G  L  N  T  A  R  V  A  Q  W  I  A  Q  A  P  K  S      80
AGTTTGTTCAACTACGTCGGCTGCGCTTCGGACGACAAGTACGGCAAAATACTCAAGGAA    300
 S  L  F  N  Y  V  G  C  A  S  D  D  K  Y  G  K  I  L  K  E     100
GCCGCGGAGAAGAACGGTGTGAACATGCACCTTGAGTACACAACAAAGGCTCCCACCGGC    360
 A  A  E  K  N  G  V  N  M  H  L  E  Y  T  T  K  A  P  T  G     120
TCGTGCGCCGTGTGCATCTCAGGCAAGGATCGCTCGCTGGTGGCGAACTTGTCCGCAGCG    420
 S  C  A  V  C  I  S  G  K  D  R  S  L  V  A  N  L  S  A  A     140
AATTTGCTCTCCGCGGATCACATGCACAGCAGCGATGTCGTTGAGACGCTGAAGGGCTGC    480
 N  L  L  S  A  D  H  M  H  S  S  D  V  V  E  T  L  K  G  C     160
CAGCTCTACTACCTCACCGGCTTCACGCTGACGATCGACGTGAACTACGTGCTTCAGGTG    540
 Q  L  Y  Y  L  T  G  F  T  L  T  I  D  V  N  Y  V  L  Q  V     180
GCGGAGGCGGCCCGTGCATCGGGTGGGCAGTTCATGATGAACCTCTCCGCCCCCTTCGTG    600
 A  E  A  A  R  A  S  G  G  Q  F  M  M  N  L  S  A  P  F  V     200
CTGCAGTACTTCACGGAGAGCTTCAACAAGGCCGCGCCGTACCTCGACGTCATCTTTGGT    660
 L  Q  Y  F  T  E  S  F  N  K  A  A  P  Y  L  D  V  I  F  G     220
AACGAGGTCGAGGCTAAGGCACTTGCGGACGCCATGAAGTGGAACCCCGCCAGCACCCAC    720
 N  E  V  E  A  K  A  L  A  D  A  M  K  W  N  P  A  S  T  H     240
AATTTGGCTAAAAAGGCAGCGATGGAGCTGCCGTACAGCGGCACTCGCGACCGCCTCGTC    780
 N  L  A  K  K  A  A  M  E  L  P  Y  S  G  T  R  D  R  L  V     260
GTCTTCACGCAGGGCAGCCAACCGACGGTGTACGCCACCCGCAGTGGCAAGACCGGCTCA    840
 V  F  T  Q  G  S  Q  P  T  V  Y  A  T  R  S  G  K  T  G  S     280
GTCACTGTGCAACCCATCGCGCATGACATCATTGTGGACCTGAACGGCGCCGGTGACGCC    900
 V  T  V  Q  P  I  A  H  D  I  I  V  D  L  N  G  A  G  D  A     300
TTCGTTGGCGGCTTCCTTGCCGCGTACGCAATGAGCTGCAGCATCCAACGGTGCTGCGAA    960
 F  V  G  G  F  L  A  A  Y  A  M  S  C  S  I  Q  R  C  C  E     320
GTGGGCAATTACGCCGCCGGTGTCATCATCCAACACAACGGCTGCACCTATCCCGAAAAA   1020
 V  G  N  Y  A  A  G  V  I  I  Q  H  N  G  C  T  Y  P  E  K     340
CCGTCCATCTCTCCGTGAATGTGAAGGGCATCCTTTTATCTGGAGCTAANAAGGAACGGC   1080
 P  S  I  S  P  *                                                345
GAAGGCCCTGCGGTGCACATCCACACATCGCAACATATGCCGAGCCTTCCCTTGCTTCAC   1140
TTTGCGCTTT                                                      1150
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Figure 1 Nucleotide and deduced amino acid sequences of Adk from *L. donovani*

The solid underlines indicate the amino acid sequence of peptides obtained from the purified protein digest. Amino acids indicated in bold do not conform to the amino acid sequence of the peptides. The arrowhead indicates the site of mini-exon splicing.

The nucleotide sequence of the *L. donovani* Adk clone was found to contain a single ORF 1035 bp long encoding a protein of 345 amino acid residues with a calculated molecular mass of 37173 Da, in agreement with the experimentally determined value of 38 kDa [16]. Further analysis of the ORF revealed that, as with other leishmanial genes, the Adk-encoding sequence preferentially preserved a fairly high G or C codon bias at the wobble position [31,32]. Approx. 82% of the codon used contained either a G or a C residue at the third position.

Mapping the 5′ terminus of matured Adk mRNA

Northern blotting of the total *L. donovani* RNA with the PCR-generated probe revealed a single low-abundance transcript of approx. 2.0 kb (results not shown). However, Northern analysis did not provide any information on the 5′ end of the transcript. Therefore an experiment was designed to determine accurately the 5′ end of the Adk mRNA. Owing to the phenomenon of

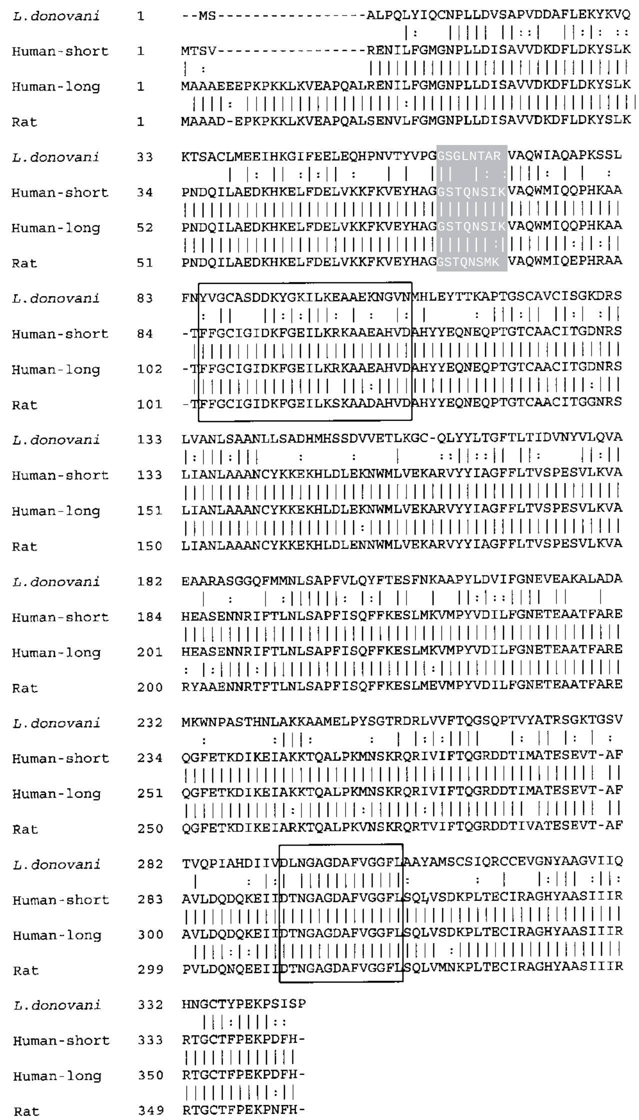

Figure 2 Comparison of the predicted amino acid sequences of the clone with human and rat Adks

Comparison of the predicted amino acid sequences of the clone with human and rat Adks [23–25]. Amino acid positions are indicated on the left. Dashed lines indictate gaps that were introduced to increase similarities between proteins. The dark box indicates the P-loop motif, whereas the open boxes represent amino acids that are similar to those found in the equivalent positions in sugar kinases and inosine–guanosine kinases.

trans-splicing, the 5′ termini of matured mRNA species from parasitic protozoa are known to contain an identical sequence of 35 nt (spliced-leader sequence) [24,25]. We took advantage of this observation and used a combination of the mini-exon sequence (sense) with another anti-sense sequence from the encoding portion of the gene and performed an RT–PCR amplification of total RNA isolated from *L. donovani*. Electrophoresis of the RT–PCR product on agarose gel and hybridization of the blotted gel with an appropriate ^{32}P-labelled oligonucleotide probe designed against the internal amino acid sequence ($P^{20}VDDAFL^{26}$) revealed a product approx. 360 nt in length (results not shown). Cloning and sequencing of the RT–PCR product demonstrated that the site for trans-splicing of the mini-exon was located at nt −160 from the predicted translation initiation site (Figure 1).

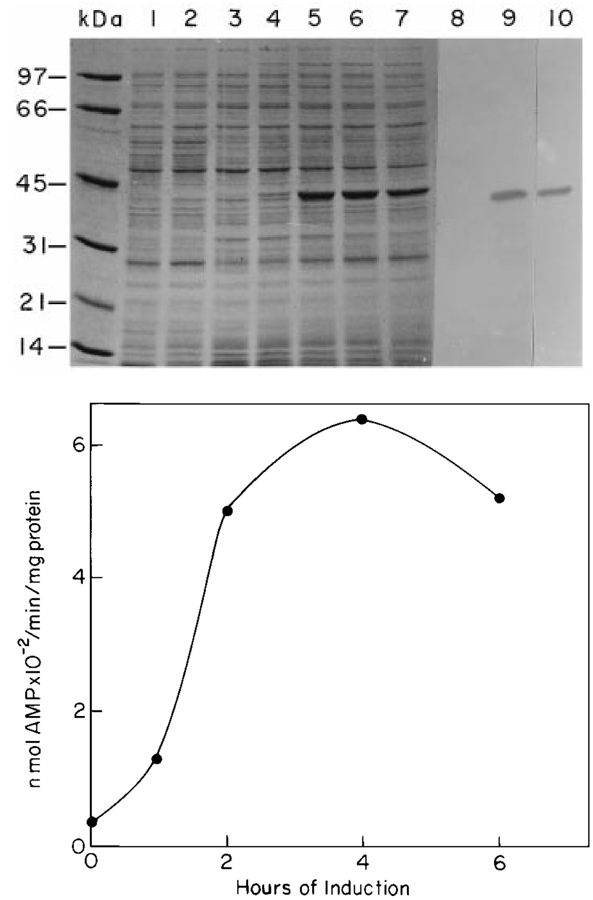

Figure 3 Induction and characterization of *L. donovani* Adk synthesized in *E. coli*

Top panel: total cellular protein (25 μg in each lane) was separated on an SDS/10% polyacrylamide gel and stained with Coomassie Blue. Lanes: 1, clone (−IPTG, 6 h), 2, pEt3a (6 h), 3–7, clone (+IPTG) for 0, 1, 2, 4 and 6 h. Lanes 8 and 9 are immunoblots of lanes 2 and 5, while lane 10 shows an immunoblot of the purified recombinant enzyme: Lower panel: the graph shows time-dependent induction of Adk following exposure of the expression clone to IPTG.

Homology studies

The Adk sequences from two mammalian sources reported so far show more than 90% amino acid identity with each other [13–15]. Most of the changes observed between these species are conservative substitutions. An initial BLAST search of the protein database revealed that the predicted protein sequence had significant similarity to Adk sequences from other sources (Figure 2). However, the extent of identity of *L. donovani* Adk sequence with other Adk sequences was comparatively low (40%). The most interesting region of sequence similarity detected from the search was the presence of an ATP- and/or GTP-binding loop-like sequence known to be present in all nucleotide-binding proteins and/or enzymes. Further homology searches indicated that despite the low identity, two stretches (residues 85–108 and 293–306) of the *L. donovani* Adk sequence, like the Adk sequence from other sources, bore a notable level of similarity to sugar kinases and inosine–guanosine kinase of microbial and plant origin [33–35]. However, the sequence did not show any significant similarity with other nucleoside kinases including deoxycytidine or thymidine kinases. A sequence simi-

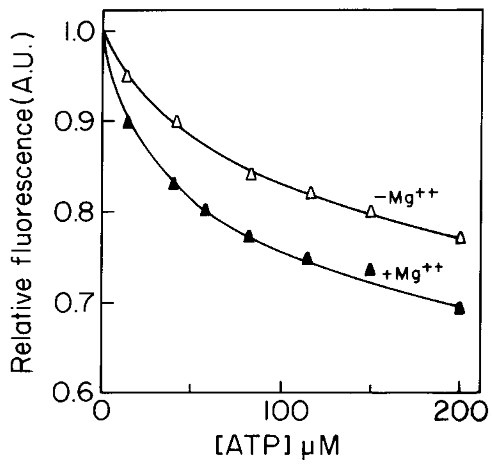

Figure 4 Binding of ATP to *L. donovani* Adk monitored by intrinsic fluorescence quenching

Incubation of the enzyme with increasing concentrations of ATP was performed at room temperature in the absence (△) and the presence (▲) of 1 mM $MgCl_2$. $MgCl_2$ (1 mM) did not have any appreciable effect.

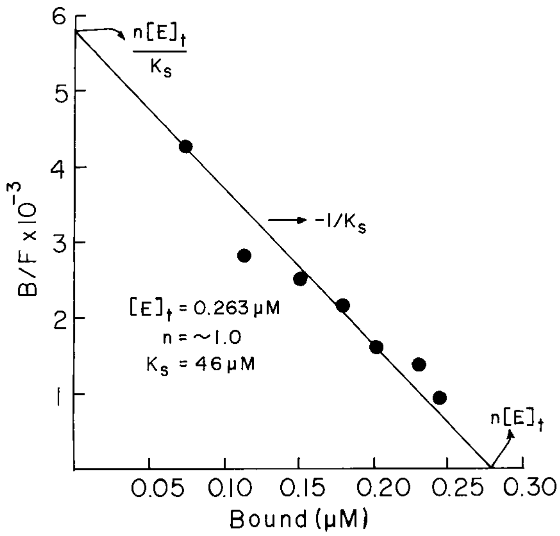

Figure 5 Scatchard analysis of $MgATP^{2-}$ binding

The results of the experiments shown in Figure 4 were used to deduce the binding constant and stoichiometry of binding (see the text for details).

larity search of the published *L. major* genome database and a recent BLAST P search of GenBank also did not identify any putative Adk-like sequence in this species [36].

Biochemical characterization of the bacterially expressed protein

To authenticate the reading frame and characterize the recombinant protein, the encoding sequence was cloned in-frame in pEt3a vector. Treatment with IPTG of the BL21 (DE3) pLysS cells harbouring the chimaeric plasmid resulted in time-dependent induction of a protein corresponding to a molecular mass of approx. 38 kDa (Figure 3). A Western blot analysis of the IPTG-induced cell extract with an antibody preparation raised against purified Adk of leishmanial origin, and also activity measurement assays, confirmed that the 38 kDa protein was indeed biologically active Adk. The induced Adk activity after purification to homogeneity displayed biochemical characteristics similar to those of the enzyme isolated from whole cells and showed K_m values for Ado and ATP of approx. 11.0 and 31.0 μM respectively [17,18].

Tryptophan fluorescence of *L. donovani* Adk is ATP-sensitive

Nucleotide-binding domain(s) are known to be critical to many enzyme activities that bind ATP and/or GTP. Because Adk is one of the major enzymes responsible for the maintanance of the AMP pool, especially in purine-auxotrophic parasitic protozoans, the unusual $MgATP^{2-}$-utilizing behaviour of the *L. donovani* enzyme led us to study its nucleotide-binding motif. The intrinsic tryptophan fluorescence of Adk was previously used as a tool to monitor the active-site environment of the enzyme, suggesting the presence of at least one tryptophan residue at or near the active site of Adk [19,20]. Here we report that ATP significantly quenches Adk fluorescence without changing the emission spectrum. In the presence of $MgCl_2$, ATP had, as expected, an enhanced quenching effect. The titration curve obtained with ATP and $MgATP^{2-}$ was monophasic, suggesting that there was only one binding site (Figure 4). The Scatchard analysis [26] derived from these results showed that approx. 1.0 mol of ATP was bound per mol of enzyme with a dissociation constant of 46 μM, in agreement with the K_m for $MgATP^{2-}$ (Figure 5). Further analysis of the quenching constant by using Stern–Volmer and Lehrer plots indicated that the binding of ATP significantly decreased the accessibility of tryptophan to KI and acrylamide. As expected, the effect was more pronounced with KI than with acrylamide, indicating tryptophan heterogeneity (Table 1). Because the quenching of protein fluorescence by ATP occurs without a change in maximal emission spectrum, the most likely explanation would be a resonance energy transfer between the tryptophan residue and the adjacent adenine chromophore, as observed by various workers [37–39]. Taken together, these results suggest that, of the two tryptophan residues (Trp-73 and Trp-234) present in the Adk of *L. donovani*, at least one tryptophan residue must be strategically located at or near the ATP-docking site (P-loop).

Table 1 Accessibility of tryptophan in Adk to quenchers

The concentrations of acrylamide and KI were varied from 0 to 0.4 M and from 0 to 0.5 M respectively. ATP concentration was maintained at 200 μM where applicable. The fluorescence emission was measured at 330 nm, with excitation at 295 nm. See the text for details.

Quencher	Addition to Adk	K_{SV} (M^{-1})	f_a
Acrylamide	−ATP	10.00	1.0
	+ATP	7.00	0.74
KI	−ATP	6.2	0.72
	+ATP	6.0	0.45

DISCUSSION

Amino acid sequence information on two peptide fragments obtained from the purified protein digest and PCR enabled us to amplify and clone the encoding sequence of Adk from *L. donovani*. Three independent lines of evidence clearly indicate that the clone does indeed represent the correct encoding sequence: (1) several predicted amino acid sequence stretches matched exactly with the amino acid sequences obtained directly from the purified protein; (2) the deduced amino acid sequence showed appreciable similarity with Adk sequences cloned recently from other eukaryotic sources; and (3) the bacterially expressed protein displayed properties similar to those of the enzyme isolated from the leishmanial cells.

Multiple protein alignment studies revealed that although the parasite enzyme shares some identity with Adks from human and rat sources, there are several distinct differences (Figure 2). Furthermore, unlike all other Adks, which are highly conserved among themselves, the parasite enzyme showed only approx. 40 % identity, vindicating our earlier prediction that the parasite enzyme was different from the analogous enzyme from other sources.

The non-coding regions of Adk transcript have not yet been analysed in detail. However, amplification of the 5′ terminus of the *L. donovani* Adk mRNA by RT–PCR and sequence analysis of the resultant DNA product confirmed that, like other kinetoplastida genes, the Adk gene of *L. donovani* is processed post-transcriptionally after the addition of the mini-exon at the 5′ end of the mRNA [40]. Furthermore, analogously with other kinetoplastida genes, no consensus eukaryotic promoter sequences such as TATA or CCAAT could be identified upstream of the initiation codon.

One of the most important structural features of all kinases is the presence of an ATP- or GTP-binding motif (P-loop) [41]. This motif is a highly conserved structure in most nucleotide-binding proteins and kinases and has been variously described as (G/A)XXXXGK(T/S), GXXGXXK, GXXXXGKS or GXXGXGKS [42–45]. In view of the anomalous and differential response of *L. donovani* Adk to ATP in comparison with other Adks, we decided to identify and characterize the ATP-binding site and examine the nucleotide binding properties of the parasite enzyme [16]. Several experimental observations were analysed for this purpose. First, alignment of the human and rat Adk sequences with the *L. donovani* Adk sequence and their comparison with the consensus Walker motif resulted in the identification of an *L. donovani* sequence GSGLNTAR (residues 62–69), similar to the suggested ATP-binding motif GSTQNSIK (residues 64–71) of human and other Adks [13,15,41,42] (Figure 2); secondly, the quenching of tryptophan fluorescence emission by ATP implicated the presence of at least one tryptophan residue proximal to the ATP-binding motif; thirdly, the eight-residue (GSGLNTAR or GSTQNSIK) putative ATP-binding motif-like sequence is invariably followed by a totally conserved four-residue sequence VAQW (residues 70–73) in Adk from all sources; fourthly, the chemical modification of a single arginine residue of *L. donovani* Adk is accompanied by inactivation of the enzyme with a concomitant loss of tryptophan fluorescence [19]; fifthly, the said motif is located near the N-terminal end of the encoding sequence, the characteristic position of the ATP-binding motif of all kinases analysed so far [41]. Because no other P-loop-like sequences were detected in other parts of the encoding sequence, these observations collectively suggest that the sequence GSGLNTAR of *L. donovani* is indeed the motif for ATP binding and that Trp-73, owing to its fixed close proximity to this motif, is most probably responsible for ATP-dependent fluorescence quenching. Although an alternative explanation such as substrate-induced conformational change resulting in fluorescence quenching of the other distal Trp-234 residue of *L. donovani* can still be argued, the contribution of Trp-234 seemed unlikely owing to its variable positioning in different Adks and the sequences surrounding it.

The most striking difference to be noted in the P-loop of the Adk from *L. donovani* is the presence of Arg-69 in place of the

Table 2 Alignment of P-loop sequence of various kinases and nucleotide-binding proteins

Consensus was determined on the basis of 70% or more occurrences among sequences. Sequences were obtained from the SWIS-PROT or PIR databases.

Adenylate Kinase	G	G	P	G	S	G	K	G	
Myosin Kinase	G	E	S	G	A	G	K	T	
Guanylate Kinase	G	P	S	G	T	G	K	S	
Rec A	G	P	E	S	S	G	K	T	
Actin	N	G	S	G	L	C	K	A	
ras p21	G	A	G	G	V	G	K	S	
EF-Tu	G	H	V	D	H	G	K	T	
Thymidine Kinase	G	P	M	F	S	G	K	S	
Phosphoglycerate Kinase	G	A	K	V	A	D	K	I	
ATP Synthase	G	G	A	G	V	G	K	D	
Deoxycytidine Kinase	G	N	I	A	A	G	K	S	
ATPase (F-I)	G	G	A	G	V	G	K	T	
Consensus	G	X	X	X	X	G	K	(T/S)	
Adenosine Kinase (Human)	G	S	T	Q	N	S	I	K	V
Adenosine Kinase (*L. dono.*)	G	S	G	L	N	T	A	(R)	V

invariant Lys present within the ATP-binding loop of most kinases, including Adk from eukaryotic sources and other nucleotide-binding proteins (Table 2). Detailed studies from various laboratories have shown that, whereas some variability can be permitted in the intervening residues of the P-loop, the C-terminal Lys residue is absolutely conserved and cannot be changed even to Arg [46–49]. To explain the detrimental effects of the replacement of Lys by Arg, it was postulated that although in principle Arg, because of its ability to form hydrogen bonds, could replace Lys in chemical terms, the bulky nature of the Arg side chain possibly disrupts the NTP-binding pocket and prevents such substitution in practice [50]. Therefore the present observation on the occurrence of an Arg residue at the ATP-binding loop of the Adk from *L. donovani* is novel and should provide a platform for reassessing ideas of the nature and function of the critical P-loop of *L. donovani* Adk.

The rationale for the development of a chemotherapeutic agent against infectious organisms clearly hinges on the exploitation of differential biochemical pathways between the host and the infecting agents. Although mutant analysis and gene knock-out experiments seem to suggest the non-essentiality of some of these purine-salvage enzymes at the extracellular promastigote stage of *L. donovani* growth, their roles in the growth of the pathogenic intracellular parasite are not known [51]. The availability of an Adk clone from *L. donovani* will certainly help us to address this question. Furthermore, site-specific mutants can be generated with a view to delineating the role of its uncommon structural features that set apart the parasite Adk from the analogous enzyme from other sources.

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