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Isolation and Characterization of Adenosine Kinase from *Leishmania donovani**

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Adenosine kinase (ATP:adenosine 5′-phosphotransferase, EC 2.7.1.20) has been purified 3250-fold from *Leishmania donovani* promastigotes using ion-exchange, gel filtration, and affinity chromatography techniques. Both native and sodium dodecyl sulfate-gel electrophoresis of the enzyme revealed a single polypeptide of around 38,000 molecular weight.

Biophysical and biochemical analyses of the enzyme reveal unique characteristics different from those of adenosine kinases from other eukaryotic sources. The isoelectric pH of the enzyme is 8.8. In native acrylamide gels the enzyme moves with an R_F of about 0.62. The enzyme displays a maximum activity at pH between 7.5 and 8.5 and is dependent upon an optimum ATP/Mg^{2+} ratio. ATP at high concentration inhibits the reaction. Adenosine and Mg^{2+} are not inhibitory. EDTA completely knocks off the activity. Enzyme activity is dependent upon the presence of active thiol group(s) at or near the active center.

Under a defined set of conditions the enzyme exhibited an apparent K_m for adenosine and ATP of 33 and 50 μM, respectively. Of the nucleoside triphosphates tested ATP and GTP were the most effective phosphate donors. Marginal inhibition of activity was detected with other nucleosides as competitors. However, adenosine analogs, such as 7-deaza-adenosine (tubercidin) and 6-methylmercaptopurine riboside at very low concentrations, were found to be excellent inhibitors and substrates as well. *S*-Adenosylhomocysteine does not inhibit the reaction even at very high concentration.

Adenosine kinase (ATP:adenosine 5′-phosphotransferase, E.C. 2.7.1.20) catalyzes the phosphorylation of adenosine to its monophosphate form by the following reaction (1, 2).

$$\text{Adenosine} + \text{ATP} \xrightarrow{Mg^{2+}} \text{adenosine 5′-monophosphate} + \text{ADP}$$

Purified enzymes from several sources have been shown to possess broad substrate specificities (3–7). Because of this property the enzyme has been implicated in the phosphorylation of many potentially pharmacologically active purine nucleoside analogs (8, 9).

The systemic endotheliosis commonly known as kala azar is caused by the parasitic protozoa *Leishmania donovani*. The flagellated protozoan has a digenic life cycle; in the alimentary tract of its insect vector, the sandfly, it exists extracellularly as the motile promastigote form (10), whereas in the phagolysosomal system of mammalian macrophages, it exists intracellularly as the non-motile amastigote form (11).

Reports from various laboratories indicate that parasitic protozoa are incapable of *de novo* synthesis of the purine rings and are, therefore, obligate auxotrophs of purines (12–17). Parasitic protozoa utilize a unique series of purine salvage enzymes to scavenge host and exogenous purines for their own growth and reproduction. Several purine-metabolizing enzymes from promastigotes have been studied in detail (18–21). Cohen *et al.* (22) have shown that, in mammalian cells, the adenylate nucleotide pool, by and large, constitutes the major source of host purines. As nucleotides do not enter the cells readily, adenine nucleoside probably permeates the plasma membrane first and then is converted to its nucleotides and other nucleotides. Using mutant cells of *L. donovani* lacking adenosine kinase, Iovannisci and Ullman (23) demonstrated that 75% of the incorporated adenosine is directly phosphorylated by *L. donovani* promastigotes, whereas the remainder is cleaved to adenine. Another report indicates that, during transformation of promastigote to amastigote, adenosine kinase is stimulated almost 50-fold (24) and thus can be considered as a stage-specific peak function enzyme (25, 26). Thus, adenosine kinase probably plays a key function in the process of transformation. Unfortunately, very little is known regarding the properties and biochemical characteristics of the parasite enzyme because of its unavailability in pure form. We have, therefore, decided to study the enzyme and its regulation in greater detail in order to explore the possibility of whether the differential properties of the enzyme (if any) could be utilized to design specific antileishmanial agents. As a first step toward the goal we have purified the enzyme to apparent homogeneity and studied some of its properties. Since hamsters are one of the natural hosts for *L. donovani*, some of the properties of the enzyme have been compared with adenosine kinase isolated from hamster liver.

EXPERIMENTAL PROCEDURES

Materials—Tubercidin (7-deaza-adenosine), 6-methylmercaptopurine riboside and N^6-linked 5′-AMP-Sepharose 4B with a 6-carbon spacer were purchased from Sigma. Sephadex G-100 was obtained from Pharmacia Fine Chemicals, DE81 paper and DE52 cellulose were the products of Whatman. Molecular weight marker proteins were from Boehringer Mannheim. [2,8-^{3}H]Adenosine (32 Ci/mmol) were obtained from New England Nuclear. Erythro-9-(2-hydroxyl-3-nonyl)adenine (EHNA)[1] and acycloguanosine triphosphates were obtained from Burroughs Wellcome Company. All other chemicals used were of reagent grade.

Source and Growth of L. donovani Promastigotes—Our strain of *L.*

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[1] The abbreviations used are: EHNA, erythro-9-(2-hydroxyl-3-nonyl)adenine; BSA, bovine serum albumin; DTT, dithiothreitol; NEM, *N*-ethylmaleimide; EGTA, [ethylenebis(oxyethylenenitrilo)] tetraacetic acid; TEMED, *N,N,N′,N′*-tetramethylethylenediamine; SDS, sodium dodecyl sulfate.

donovani (UR6) was isolated from an Indian patient with kala azar. The organisms were maintained and grown on agar slants or Petri dishes prepared in Ray's medium as described elsewhere (27). Usually after 72 h of growth at 22–26 °C the cells were scraped carefully and suspended in cold phosphate-buffered saline containing 1 mM each phenylmethylsulfonyl fluoride; benzamidine-HCl, iodoacetic acid, and EGTA. The cells were washed three times with the same buffer and kept frozen until used.

Enzyme Assays—The enzyme was assayed routinely by determining adenine nucleotide formation from [2,8-^{3}H]adenosine at 30 °C. The time of incubation in different reactions was varied in order to maintain the reaction strictly in the linear range. Therefore, counts incorporated over time of incubation were directly proportional to reaction velocity. Standard reaction mixture (50 μl) contained 50 mM Tris-HCl, pH 7.5, 1.5 mM $MgCl_2$, 1 mM ATP, 50 mM KCl, 0.5 mM DTT, 0.005 mM EHNA, 0.5 mM NaF, 100 μg/ml BSA, 50 μM [2,8-^{3}H] adenosine (25–250 cpm/pmol), and the required amount of enzyme. The reaction was terminated with the addition of 10 μl of 0.1 M EDTA, pH 6.0. Reaction mixture (50 μl) was spotted on DE81 paper, dried, washed, and counted as previously described (28). Unless otherwise stated all the assays were carried out at pH 7.5 and at 50 μM adenosine concentration. For initial velocity measurements reactions were incubated for 10 min. Adenylate kinase, adenosine deaminase, and ATPase were assayed as previously described (29, 30).

Spectrophotometric Assays—The activity was determined by a modification of the method of Lindberg *et al.* (3) using a pyruvate kinase/lactate dehydrogenase-coupled assay. Reaction mixtures in 1-cm path length cuvettes contained 50 mM Tris-HCl, pH 7.5, 1.5 mM $MgCl_2$, 1 mM ATP, 50 mM KCl, 0.5 mM DTT, 0.005 mM EHNA, 0.5 mM NaF, 10 μg/ml BSA, 50 μM adenosine or its analogs, 1 mM phosphoenolpyruvate, 0.77 A_{340} equivalent NADH, 18 IU/ml pyruvate kinase, and 48 IU/ml lactic dehydrogenase. Myokinase was omitted from the reaction as there was no contamination of myokinase in the preparation. The reaction was started with the addition of adenosine kinase. The formation of nucleotide monophosphate with its concomitant oxidation of an equimolar amount of NADH was monitored by reduction in the absorbance at 340 nm. Rate of reaction was proportional with time up to at least 15 min.

Buffers—The following buffers were used. Buffer A (250 mM potassium phosphate, pH 7.5, 1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 1 mM each phenylmethylsulfonyl fluoride, benzamidine-HCl, iodoacetic acid), Buffer B (same as Buffer A, but with 20 mM potassium phosphate, pH 7.5, and 5% glycerol), Buffer C (same as Buffer B, but with 100 mM potassium phosphate, pH 7.5), Buffer D (same as Buffer B with the difference that 100 mM Tris-HCl, pH 9.0, was included instead of potassium phosphate, and glycerol concentration was raised to 20%).

Purification of the Enzyme—Unless otherwise stated, all the operations were carried out at 2–4 °C.

Preparation of the Cell Extract—Frozen packed cells (6–7 ml) were suspended in 30 ml of Buffer A, and the viscous suspension was homogenized. The resultant suspension was sonicated two or three times at maximum output for 30 s on a Labsonic 2000 sonifier with 1-min intervals between each sonication. The suspension was centrifuged at 20,000 × *g* for 30 min. The supernatant obtained was called "crude extract." To the crude extract (37 ml), 16.1 g of ammonium sulfate was added with constant stirring. The resultant precipitate was collected by centrifugation, dissolved in 15 ml of Buffer B, and dialyzed overnight against the same buffer (fraction A.S.(0–70)I).

DE52 Cellulose Column—The dialyzed sample (24 ml) was loaded onto a DE52 cellulose column (3 × 18 cm) pre-equilibrated with Buffer B. Adenosine kinase activity was not absorbed onto the column and eluted out in the flow-through fraction. Fractions containing the major activity were pooled (100 ml) and were further concentrated by addition of 51.6 g of ammonium sulfate. The precipitate formed was collected by centrifugation and dissolved in 2.5 ml of Buffer C (fraction A.S.(0–80)II).

Sephadex G-100 Gel Filtration—The A.S.(0–80)II fraction (3.1 ml, Table I) was layered onto a Sephadex G-100 column (1.6 × 90 cm) pre-equilibrated with Buffer C. The column was washed with the same buffer, and fractions (approximately 5 ml) were collected at an interval of 15 min (Fig. 1*A*).

5′-AMP-Sepharose 4B Affinity Column—The pooled fraction (25 ml) from Sephadex G-100 was loaded on a 5′-AMP-Sepharose 4B column (0.8 × 5 cm) pre-equilibrated with Buffer C at a flow rate of 10 ml/h. Fractions (approximately 2 ml) were collected. After initial wash with 25 ml of Buffer C, nonspecifically bound protein was eluted with 20 ml of Buffer C containing 1 M KCl. The column was then successively washed with 10 ml each of Buffer D, Buffer D containing 10 mM ATP, and again Buffer D. Final washing of the column with Buffer D containing 5 mM adenosine led to elution of adenosine kinase activity in a sharp peak (Fig. 1*B*). The peak fractions were pooled. Part of the fraction was used for polyacrylamide gel electrophoresis and isoelectric focusing experiments. To the rest of the portion BSA and ATP were added to final concentrations of 1 mg/ml and 100 μM, respectively. This fraction was further dialyzed against Buffer C containing 100 μM ATP to remove adenosine and was distributed in 50–100 μl aliquots. When kept at −20 °C very little loss of activity was observed after 2 months. This fraction was used in all the experiments.

Calculations of units and specific activities in the final preparation were carried out after dialysis of the enzyme (Table I). A 3250-fold purification was obtained with 24% recovery.

Polyacrylamide Gel Electrophoresis—SDS-polyacrylamide gel electrophoresis was performed according to the method of Laemmli (31) with 12.5% cross-linked gels.

Electrophoresis of native enzyme was carried out in a tris glycine buffer system according to the method described by Gabriel (32). Disc gel electrophoresis was performed in glass tubes (0.6 × 9 cm) containing acrylamide monomer (5% w/v), bisacrylamide (0.25% w/v), and TEMED (0.028% w/v). Ammonium persulfate was used instead of riboflavin for gel polymerization, and the stacking gel was omitted. Presence of riboflavin in the gel interfered with the silver staining procedure (33). Ammonium persulfate was removed by pre-electrophoresis, and duplicate gels were either stained or sliced and assayed according to the published procedure (34) using the assay condition described above.

Gel Filtration—Gel filtration was performed on a Sephadex G-100 column (1.6 × 90 cm) pre-equilibrated with Buffer C. A two-milliliter sample was applied and the column was run as described before. The column was standardized with BSA, ovalbumin, chymotrypsinogen A, and lysozyme. Dextran blue and orange G were used to determine the void volume and total column volume, respectively. Molecular weight and Stokes' radii were calculated according to published methods (35–37).

Protein Determination—Protein was estimated according to the method of Lowry *et al.* (38). Protein estimation of the pooled 5′-AMP-Sepharose 4B fraction was done after trichloroacetic acid-deoxycholate precipitation.

Electrofocusing—Isoelectric focusing of homogeneous preparation of enzyme was carried out in tube polyacrylamide gels following the procedure of O'Farrell (39) with the following modifications. Gels contained 7.5% acrylamide, 0.375% bisacrylamide, 0.5% of each ampholine interval (pH 3–10, 4–6.5, 5–8, and 6.5–9.0), and 0.05% TEMED. The gels in duplicate were pre-run for 30 min using 1 M sodium hydroxide and 1 M phosphoric acid in cathode and anode chambers, respectively. Electrophoresis was continued for about 5 h. After the run, one gel was used for measurement of pH and the other gel was stained with silver nitrate after overnight fixing and washing.

RESULTS

Enzyme Purification—The first three steps did not produce appreciable purification. However, it was necessary to retain the steps. Results (Table I) indicate that the bulk of the unnecessary proteins were eliminated with about 44% recovery of units. Gel filtration through Sephadex G-100 resulted in another 3-fold purification and removed most of the large molecular weight proteins, some of which were found to possess AMP-binding properties (Fig. 1*A*).[2] The affinity chromatography step was the key to the purification. About 130-fold purification was achieved in a single step. Elution of *L. donovani* adenosine kinase from the 5′-AMP-Sepharose 4B column was found to be very specific. A combination of high ionic strength, elevation of pH from 7.5 to 9.0, and high adenosine concentration was necessary to elute the enzyme. ATP was ineffective in this respect (Fig. 1*B*). The final preparation had a specific activity of 11 μmol/min/mg of protein. There was no detectable adenylate kinase, ATPase, and adenosine deaminase activity in the preparation.

[2] A. K. Datta, D. Bhaumik, and R. Chatterjee, unpublished observation.

TABLE I

Purification of adenosine kinase from L. donovani promastigotes

Fraction	Protein	Total protein	Total units[a]	Recovery	Specific activity	Purification
	mg/ml	*mg*		*%*	*units/mg*	*-fold*
Crude extract	6.34	235	794	100	3.38	1
Dialyzed A.S. (0–70)I	6.92	166	554	70	3.34	0.98
DE52 pooled	0.6	60	476	60	7.93	2.3
A.S. (0–80)II	4.48	14	349	44	25.9	7.6
Sephadex G-100 pooled	0.1	2.4	202	25	84	25
5′-AMP-Sepharose 4B	0.003	0.018	197	24	11000	3250

[a] 1 unit = nmol of AMP formed/mg of protein/min under standard assay conditions.

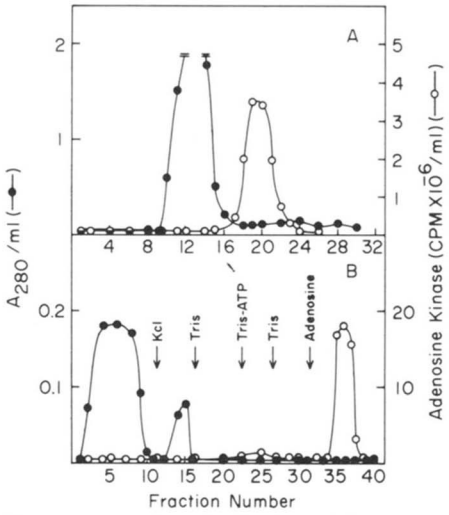

FIG. 1. **Chromatography of adenosine kinase on Sephadex G-100 (*A*) and 5′-AMP-Sepharose 4B (*B*) columns.** Elution profiles of protein (●) and adenosine kinase activity (○) from respective columns are presented. Fractions containing the activity were pooled and processed as discussed under "Experimental Procedures." Approximate A_{280} units of each fractions were derived after subtracting absorbancies contributed by the respective elution buffers.

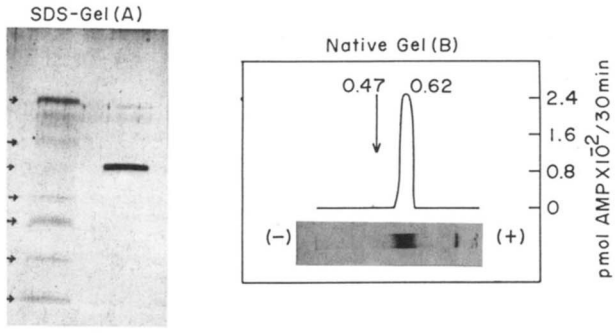

FIG. 2. **Electrophoresis of adenosine kinase on polyacrylamide gels in the presence (*A*) and absence (*B*) of SDS.** *Left panel A* shows molecular weight standards applied on the gel. Markers were BSA (68,000), ovalbumin (45,000), glyceraldehyde-3-phosphodehydrogenase (36,000), carbonic anhydrase (29,000), trypsinogen (24,000), trypsin inhibitor (20,000), and α-lactalbumin (14,000). Enzyme protein (150 ng) is shown on the *right panel* of the gel. *B* represents electrophoretic mobility of the enzyme (300 ng) under nondenaturing condition. One gel was stained with silver nitrate, whereas the other one was sliced and assayed for the enzyme activity. *Arrow* indicates the mobility of affinity column purified adenosine kinase isolated from hamster liver. Relative mobilities (R_F) are displayed on *top* of the activity peaks. Denatured proteins were found to take silver stain less efficiently.

The enzyme exhibited a single stainable band on both native and SDS-polyacrylamide gels (Fig. 2). Silver nitrate and Coomassie Blue (not presented) staining procedures produced identical results. In native gel, enzyme activity corresponds with the protein band (Fig. 2*B*). Relative electrophoretic mobility (R_F) was calculated as 0.62, whereas affinity-purified adenosine kinase from hamster liver shows an R_F of about 0.47. It should be mentioned here that the crude preparations of adenosine kinases from both the sources move with identical R_F values as compared to their purified forms (data not shown).

Molecular Weight Determination—SDS-gel electrophoresis indicated a molecular weight of around 38,250, whereas gel filtration yielded a molecular weight of about 37,700 (Fig. 3). From these results we conclude that the enzyme is a monomer molecule. Stokes radius of the enzyme was about 25.8 Å.

Isoelectric pH—The isoelectric pH of *L. donovani* enzyme as determined by electrofocusing was 8.8.

Dependence of Enzyme Activity on pH, ATP, and Mg^{2+}—Adenosine kinase from rabbit liver displays maximal activity at pH 4.5 with an ATP/Mg^{2+} ratio of 0.1 (29), whereas enzyme from human placenta is optimally active at pH 6.5, with an ATP/Mg^{2+} ratio at 0.5 (40). Results presented in Fig. 4 indicate that *L. donovani* adenosine kinase has broad pH optima from 7.5 to 8.5 and is also dependent upon ATP/Mg^{2+} ratio. However, the response of the enzyme to varying ATP/Mg^{2+} ratio follows unique characteristics of its own. At a high ATP/Mg^{2+} ratio there is a pH-dependent lag in the reaction rate at low pH values. With decrease in the ATP/Mg^{2+} ratio the lag in the reaction rate was abolished. Maximum activity was attained when the ATP/Mg^{2+} ratio was fixed at 0.66. There was inhibition of enzyme activity at pH values above 8.5 with a high ATP/Mg^{2+} ratio. Further increase in pH

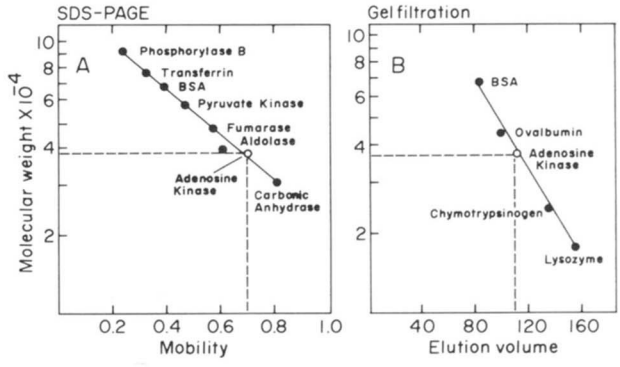

FIG. 3. **Molecular weight determination.** *A*, standard curve for adenosine kinase subunit molecular weight determination by SDS-polyacrylamide gel electrophoresis (*SDS-PAGE*). The subunit molecular weight in this determination was 38,000; the mean molecular weight from three experiments was 38,250. The enzyme used for the experiment was the purified adenosine kinase after 5′-AMP-Sepharose 4B chromatography. *B*, determination of molecular weight by Sephadex G-100 gel filtration using the A.S.(0–80)II fraction (Table I). Average molecular weight from three determinations was 37,700. This particular experiment yielded molecular weight of 37,000.

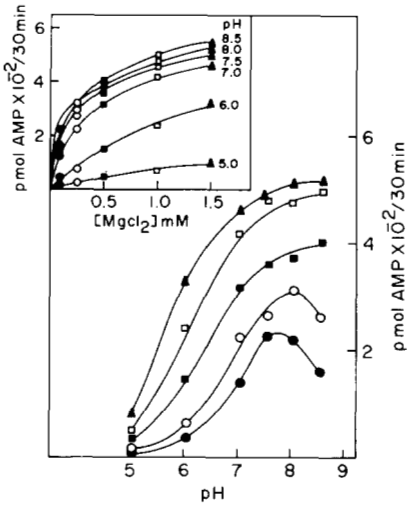

FIG. 4. **Effect of pH on adenosine kinase activity at different ATP/Mg^{2+} ratios.** Purified enzyme (1.5 ng) was used in each assay. ATP concentration was fixed at 1 mM. *Inset* shows the same results when plotted against varying Mg^{2+} concentrations. The ratios of ATP to Mg^{2+} were 0.66 (▲), 1 (□), 2 (■), 4 (○), and 10 (●). Buffers of pH 5–6 (sodium acetate), 7–9 (Tris-HCl), and 10.0 (glycine-KOH) were used.

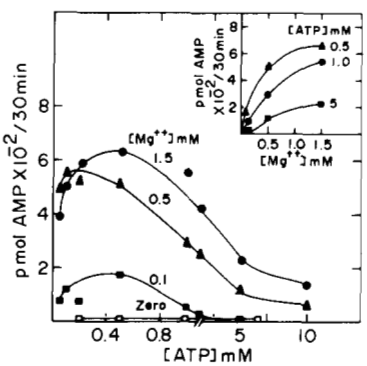

FIG. 5. **Effect of ATP concentration on the adenosine kinase activity at several fixed concentrations of Mg^{2+} ranging from 0 to 1.5 mM.** In the *inset* the same results are plotted against Mg^{2+} concentration.

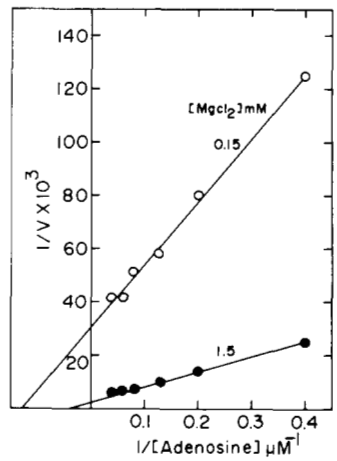

FIG. 6. **Double-reciprocal plot of the effect of adenosine concentration on enzyme activity at two $MgCl_2$ concentrations.** ATP concentration was kept at 1 mM. Reaction velocity (*V*) is represented as picomoles of AMP formed/10 min under the assay condition.

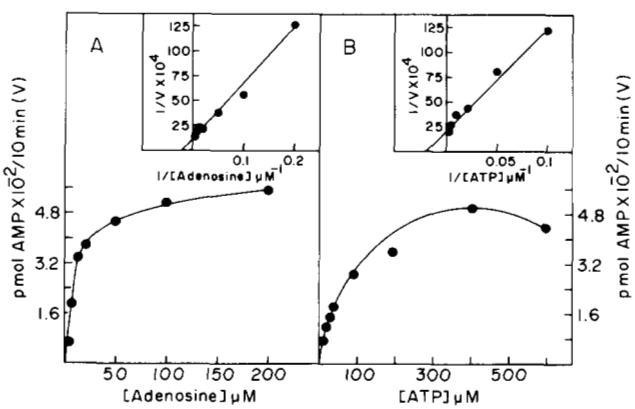

FIG. 7. **Double-reciprocal plots of adenosine kinase-catalyzed reaction with (*A*) adenosine and (*B*) ATP as the variable substrates.** In *A*, concentrations of ATP and Mg^{2+} were 1 and 1.5 mM, respectively, whereas the experiment presented in *B* contained 50 μM adenosine and 1.5 mM Mg^{2+}. Calculations were as described in the legend to Fig. 6.

inhibits enzyme activity at all ATP/Mg^{2+} concentrations tested (not shown).

L. donovani enzyme is inhibited by ATP. Mg^{2+} does not inhibit the reaction. Increasing concentration of ATP above 0.5 mM led to an increase in the inhibition. The inhibition is discernible at all Mg^{2+} concentrations tested (Fig. 5 and *inset*). Absolute requirement of Mg^{2+} is shown by the fact that addition of EDTA in moderate concentration completely knocks off the activity (Fig. 5, *inset*). The functions of Mg^{2+} can be substituted equally well by Mn^{2+}, whereas Ca^{2+} and Ba^{2+} were not so effective in this respect. That the reaction is dependent upon Mg^{2+} is further shown in Fig. 6. Lowering of Mg^{2+} concentration at a fixed concentration of ATP decreases both V_{max} and affinity of the enzyme for adenosine. This observation is exactly opposite to what has been found with rabbit liver enzyme (29). We also failed to detect any inhibition by excess adenosine under this condition. In this bisubstrate reaction keeping concentrations of ATP and adenosine at saturating levels we have determined the K_m for adenosine and ATP of 33 and 50 μM, respectively (Fig. 7).

Results of these experiments thus differ from the observations that have been documented with enzymes from other sources (3, 4, 29, 40).

Effect of p-Hydroxymercuribenzoate and NEM—Treatment of the enzyme with *p*-hydroxymercuribenzoate (0.1 mM) totally inactivates the enzyme. This inactivation can be fully reversed by treatment of the inactivated enzyme with DTT. NEM cannot inhibit the enzyme as efficiently as *p*-hydroxymercuribenzoate. Fifty percent inhibition occurs with 1 mM NEM. The inhibition is also not totally reversible by DTT. Approximately 50% of the NEM-inhibited activity could be restored by DTT treatment. Probably differences in the hydrophobicity between two inhibitor molecules is the reason for this differential effect.

Substrate Specificity—In view of the reports that the substrate specificity of purified adenosine kinase included a broad range of compounds (4, 8, 9), we checked the effect of different nucleosides, nucleosides analogs, and nucleoside triphosphates on their abilities to inhibit or stimulate adenosine kinase activity, respectively. Results (Table II) indicate that the parasite enzyme utilizes ATP and GTP most effectively. Of the nucleosides tested, none except deoxyadenosine were found to have noticeable effect on the phosphorylation (Table III). Deoxyadenosine at 1 mM concentration inhibits adenosine phosphorylation by about 26%. However, adenosine analogs such as tubercidin (7-deaza-adenosine) and 6-methylmercaptopurine riboside have strong inhibitory effects on the

TABLE II

Nucleoside triphosphate donor specificity

Nucleoside triphosphate (1 mM)	Relative activity
	%
ATP	100
dATP	21
Ara-ATP	5
CTP	12
dCTP	0
Ara-CTP	2
GTP	60
dGTP	16
UTP	8
TTP	4
Acycloguanosine triphosphate	0

TABLE III

Effect of nucleosides and nucleoside analogs on adenosine kinase activity

Each compound, except 7-deaza-adenosine and 6-methylmercaptopurine riboside at a concentration of 1 mM, was used in reaction mixture. 7-Deaza-adenosine and 6-methylmercaptopurine riboside concentrations were maintained at 100 μM. [^{3}H]Adenosine concentration was maintained at 50 μM. Relative activity was calculated with respect to control taken as 100%.

Compounds	Relative activity
	%
2′-Deoxyguanosine	92
2′-Deoxyadenosine	74
2′-Deoxycytidine	95
Guanosine	89
Uridine	96
Thymidine	94
Cytidine	95
S-Adenosylhomocysteine	100
7-Deaza-adenosine	40
6-Methylmercaptopurine riboside	15

phosphorylation of adenosine by adenosine kinase. Spectrophotometric assays revealed that both tubercidin and 6-methylmercaptopurine riboside act as substrates for the enzyme. At 50 μM substrate concentrations of each, reaction rates were about 130 and 45% of the rate observed with adenosine. K_m for tubercidin and 6-methylmercaptopurine riboside were determined as 83.3 and 3.84 μM, respectively.

Palella *et al.* (40) reported that adenosine kinase from human placenta is strongly inhibited by *S*-adenosylhomocysteine and the inhibition is noncompetitive with respect to adenosine. Interestingly, our results do not show inhibition of adenosine kinase by *S*-adenosylhomocysteine (Table III) even at an adenosine concentration (12.5 μM) below its K_m.

DISCUSSION

The procedure of purification described here is very simple and reproducible and can be readily scaled up to yield quantities suitable for detailed structural and mechanistic studies. Homogeneous preparation of the enzyme has allowed us to study some of its properties and compare it with adenosine kinases isolated from various other mammalian sources (Table IV).

During the chromatographic procedure of purification we encountered differential behavior of the enzyme in comparison to enzymes from other sources (28, 29). The enzyme could not be eluted from the 5′-AMP-Sepharose column by high pH buffer as has been reported earlier (29). Inclusion of ATP in the buffer was also not effective (Fig. 1). However, addition of 5 mM adenosine in buffer alone was very efficient in eluting the enzyme. The results indirectly show that AMP competes with adenosine for the same binding site, whereas ATP has a different binding site. Kinetic studies also support this contention (data not shown). Molecular weight of the enzyme was found to be similar with enzymes from other sources (28, 41–43). However, the isoelectric pH, pH optima, and electrophoretic mobility (R_F) under nondenaturing conditions differ from the values reported by others using enzymes from different sources. Isoelectric pH and pH optima of adenosine kinase from all other sources have been reported to be around 5.9 and 4.8–6.8, respectively (28, 29). Thus, the differences in the isoelectric pH and electrophoretic mobility clearly point to extensive differences in the amino acid composition of the protein. Results of our isoelectric pH data are in close agreement with the previous results using crude enzyme preparation from *L. donovani* (23).

pH-dependent optimal activity at a fixed ATP/Mg^{2+} ratio (Fig. 4) suggests that the ATP·Mg^{2+} complex is probably the substrate for the enzyme. This has been also demonstrated with adenosine kinases from other sources (6, 28, 40). However, the differential response observed with leishmanial enzyme might be due to the following reason. As opposed to adenosine kinases from other sources, which are reported to be inhibited by both ATP and Mg^{2+} (4, 24, 28, 40), leishmanial enzyme is inhibited by ATP only (Fig. 4). In the presence of 1 mM ATP, Mg^{2+} did not exert any inhibitory effect, even if its concentration was raised to 4 mM (data not presented). Since the pK of ATP has been shown to be around pH 6.5 (44), the observed lag in the reaction rate (Fig. 4) at acidic pH values and abolition of the same at alkaline pH values is probably dependent upon sufficient and efficient formation of an ATP·Mg^{2+} complex at alkaline pH. At pH values above 6.5, ATP is more efficiently dissociated and thus forms an ATP·Mg^{2+} complex in the presence of excess Mg^{2+} ions. In this context it is worth mentioning that purified enzyme from hamster liver, under our assay condition, responds identically as the enzymes from Ehrlich's ascites tumor cells (8) and rabbit liver (29) with respect to ATP·Mg^{2+} concentration (data not shown). Thus, the differences observed are not due to the differences in assay conditions employed by various other workers. The physiological implication of this intricate interplay of ATP·Mg^{2+} on different adenosine kinases is not known yet. It is possible that the fluctuations of intracellular Mg^{2+} and ATP concentrations resulting in varying concentrations of free ATP or Mg^{2+} or ATP·Mg^{2+} complex might play a critical role in the regulation of adenosine kinase activity. Recently it has been reported that the metabolic activities of amastigotes (intracellular form) are facilitated at acidic pH, whereas promastigotes (culture form) grow more efficiently in alkaline pH (45). Analysis of purine uptake studies at different pH values by the two morphological forms of *L. donovani* might throw some light onto this question. Moreover, a detailed peptide mapping or amino acid sequence analysis of the enzyme protein is a prerequisite for a clear biochemical understanding of this phenomenon.

The enzyme behaves similar to adenosine kinases from other sources in terms of phosphate donor specificities. We are aware of the recent report (46) that human placental adenosine kinase has a deoxyadenosine phosphorylating activity when assayed under optimal condition. However, at this stage we cannot say whether the inhibition of adenosine kinase by deoxyadenosine is due to an analogous phenomenon. Uridine has been shown to inhibit placental adenosine kinase strongly (40); in contrast, the parasite enzyme is not affected by uridine. Likewise, *S*-adenosylhomocysteine, which also has been shown to be a strong inhibitor of human placental adenosine kinase (40), does not inhibit the parasite

TABLE IV

Comparative properties of adenosine kinases from different sources

Source of enzyme	pH optima	pI	Molecular mass	Adenosine inhibition	Mg^{2+} inhibition	ATP inhibition	K_m for adenosine	K_m for ATP	Effective substrates	Reference
			kDa				*μM*	*μM*		
Rabbit liver	4.8–5.5	<6.2	51 ± 2	Yes	Yes	Yes	0.4	600	Adenosine > 8-aza-adenosine > toyocamycin	9, 29
Human placenta	5.5–6.5	5.9	37.25	Yes	Yes	Yes	0.4	75	Adenosine > tubercidin = 6-MeMPR[a]	28, 40
Human tumor cells type H.Ep.No.2	6.2–6.8			None	Yes		1.8		Tubercidin > 6-MeMPR > adenosine	4
Human erythrocyte	5.5–6.2			Yes	Yes	Yes	1.9	0.04	Adenosine	6
Sarcoma 180 cells	≤5.5			Yes	Yes		0.5	200	Tubercidin > 6-MeMPR > toyocamycin > adenosine	5
L. donovani	7.5–8.5	8.8	38	None	None	Yes	33	50	Tubercidin > adenosine > 6-MeMPR	This paper

[a] 6-MeMPR = 6-methylmercaptopurine riboside.

enzyme. Thus, the mechanism of regulation of adenosine metabolism by *S*-adenosylhomocysteine as proposed to be operative (40) in cells deficient in adenosine deaminase (47–49) may not be applicable in *L. donovani* cells. However, the strong competitive inhibition exerted by tubercidin and 6-methylmercaptopurine riboside is interesting. Comparison of the "substrate status" property of each compound indicated that the rates of monophosphorylation of tubercidin, adenosine, and 6-methylmercaptopurine riboside are inversely proportional to the affinities of the enzyme for the substrates. Moreover, rabbit muscle myokinase used tubercidin monophosphate as substrate but failed to utilize 6-methylmercaptopurine riboside monophosphate (data not shown).

We have, therefore, characterized adenosine kinase from *L. donovani* with respect to molecular weight, substrate specificity, divalent cation requirement, pH optima, isoelectric pH, electrophoretic mobility, etc., and have shown some of its differential properties from enzymes isolated from other sources. Our additional kinetic data (not shown) also indicate that the enzyme is indeed different from other eukaryotic cell adenosine kinases (50) in regard to its mode of action. Whether these differential properties will be helpful in designing new metabolic inhibitors is an open question. Nevertheless, the data presented here contribute to the information currently available about the enzymology of *L. donovani* which is very scanty at present. Availability of homogeneous preparation of the enzyme will also facilitate studies on the regulation of adenosine metabolism in *L. donovani*.

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