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ADENOSINE KINASE FROM TRYPANOSOMA CRUZI

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Summary: Adenosine kinase was demonstrated in the soluble fraction of Trypanosoma cruzi. Magnesium is required for activity. ATP and GTP are efficient phosphate donors while p-nitrophenylphosphate is without activity. The pH optimum is high (8.0), it is heat labile and is stabile to freezing (-20° or -80°). It is substrate inhibited, does not survive dialysis and its stability to gel filtration is enhanced by the presence of ATP or GTP. Time curves are parabolic only if the enzyme is preincubated with ATP (or GTP); sigmoid when preincubated with adenosine.

INTRODUCTION

Adenosine kinase carries out the phosphorylation of adenosine to adenosine-5'-phosphate (AMP). In many organisms or cell lines this reaction is difficult to demonstrate in crude extracts due to the presence of very active ribonucleosidase (EC 3.2.2.1) and adenosine deaminase (EC 3.5.4.4) both of which effectively remove the substrate from the phosphorylating activity. While adenosine deaminase can be inhibited by coformycin or deoxycoformycin (1) the adenosine kinase activity may also be inhibited, as it is with the enzyme from Trypanosoma cruzi (Table 1). Henderson (2) circumvented the difficulty by substituting 6-methylmercaptopurine riboside for adenosine, as it is an alternate substrate for adenosine kinase but not for adenosine deaminase (3).

The purpose of the present investigation was to determine the presence or absence of adenosine kinase in the Trypanosoma cruzi, the causative organism in Chagas disease. This trypanosomatid flagellate is a favorable organism for the study of this kinase because it has a very weak nucleosidase (inactive at high pH) and does not possess an adenosine or adenine deaminase (4,5). It is possible, therefore, by carrying out the reaction

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at the optimum pH for the enzyme from this organism (pH 8.0) to study adenosine phosphorylation with no competing reactions.

MATERIALS AND METHODS

The radioactive purine ribonucleosides were [^{14}C]-labelled in position 8; were 45-50 mCi/mM and were obtained from Amersham/Searle (Arlington Heights, IL). The ingredients of the semi-defined medium of Berens, et al. (6) were purchased from GIBCO Laboratories (Grand Island, N.Y.). Special chemicals, including unlabelled nucleosides, were purchased from Sigma Chemical Co. (St. Louis, MO), except for coformycin (3-β-D-ribofuranosyl-6,7,8-trihydroimidazo [3,4-d] [1,3] diazopin-8-(R) ol) and 2'-deoxycoformycin (kindly supplied by H. W. Dion, Warner-Lambert/Parke-Davis) and 8-azaguanosine (a gift from Morris Friedkin, University of California at San Diego). Trypanosoma cruzi (epimastigotes, ATCC 30013, Culbertson strain) was originally obtained from V. Iralu (Philadelphia College of Osteophathic Medicine).

The flagellates were grown in low profile flasks at 28° in an atmosphere of 5% CO_2/95% air. For favorable yields of log phase organisms, 25 ml of six day old cultures were inoculated into 200 ml of medium and incubated 3 days. Two types of media were used; the filter-sterilized HOSMEM medium of Berens, et al (6), which contains hemin and heat inactivated fetal calf serum, and heat sterilized Trypticase-Soy broth (BBL Microbiological Systems, Cockeyville, MD) to which filter-sterilized hemin (2 μg/ml) and 8% heat inactivated fetal calf serum had been added. Little difference of yield of enzyme from the two media was found.

The cells were harvested, washed twice in 0.05 M Tris HCl buffer (pH 7.5) containing 1mM dithioerythritol. They were then suspended in 3-5 ml of the buffer and disrupted by brief sonication (15 sec.) under ice cold conditions, as previously described (4,7). After sonication the cell debris was removed by centrifugation at 40,000 x g for 1 hr. The supernate was passed through glass wool to remove the flaky lipid layer and this extract was used without further treatment for the majority of the experiments reported. Re-centrifugation at 100,000 x g resulted in a preparation of similar nature to the 40,000 x g supernate, but with higher specific activity.

Adenosine kinase was routinely assayed as follows: 0.02 ml of cell extract, diluted to contain 1-4 mg protein was preincubated for 20 min. at 22° with ATP, 3.6 mM; $MgSO_4$, 0.4 mM; EDTA, 0.18 mM; Tris buffer, 0.05 M, pH 8.0; dithioerythritol, 1 mM. The above was then equilibrated at 35° and the radiolabelled adenosine was added to bring the total volume to 0.1-0.14 ml and the substrate concentration to 40-50 μM. The reaction was stopped, usually after 3 min., by the addition of a drop of glacial acetic acid and aliquots were subjected to paper electrophoresis, as previously described (8). The radioactive peaks, resulting from scans (Tracer-Lab 4 π scanner) were quantified by planimetry. The product formation was linear with time, up to eight min. As T. cruzi possesses only a very weak adenosine nucleosidase (inactive at pH 8.0) and no adenosine deaminase, only residual radiolabelled adenosine, AMP, ADP and ATP were present on the paper strips. All separated satisfactorily when formic acid (8:300) was used as the electrolyte at 400 V. The di- and triphosphates of adenosine appeared sequentially only after a longer incubation period than that usually employed; when formed their values were added to those for AMP.

When inhibitors were used they were added to the preincubation mixture and, after 20 min. at 22°, the reaction was started by the

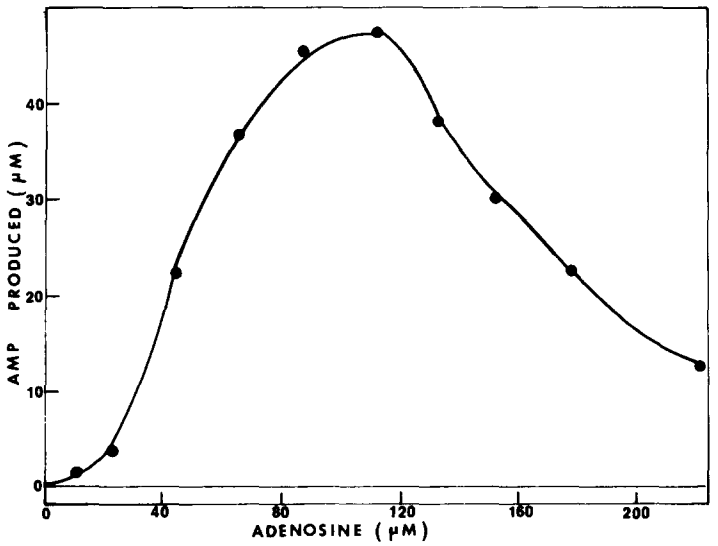

Fig. 1. Adenosine kinase activity at varying concentrations of 8-[^{14}C]-adenosine. The reaction mixture contained, in a final volume of 0.1 ml, Tris HCl 22 mM (pH 8.0); dithioerythritol, 1.1 mM; ATP, 4 mM; $MgSO_4$, 0.4 mM; EDTA, 1.8 mM; cell extract, 0.3 mg protein. Incubation time 5 min. at 35°. Average values of 5 experiments.

addition of 8-[^{14}C]-adenosine. In experiments to test the effects of preincubation with substrate, 8-[^{14}C]-adenosine was added to the preincubation mixture from which the ATP and Mg^{++} had been omitted. In this case the reaction was initiated by the addition of ATP-Mg^{++}.

Gel filtration on Sephadex G-200 which had been equilibrated with the Tris-dithioerythritol buffer (pH 8.0) resulted in vanishingly low yields of adenosine kinase activity. Fair yields were obtained, however, when the column was equilibrated with the buffer containing 1 mM ATP or GTP. The columns were 2.5 x 35 cm, the flow rate was 15 ml/hr, the void volume was 81 ml and 3 ml fractions were collected.

Protein determinations were made using the method of Bradford (9) as outlined in the Bio-Rad Technical Bulletin 1051 (Richmond, CA).

RESULTS AND DISCUSSION

p-Nitrophenylphosphate (used at pH 6.5, 7.0 or 8.0) was without activity as a phosphate donor which rules out a purine nucleoside phosphotransferase, reported from leishmanias (10). ATP and GTP were equal in phosphate donor efficiency, while ITP was less efficient (Fig. 1). This corresponds to the finding of Miller, et al. (11) for the enzyme from rabbit liver.

The optimum pH for adenosine kinase activity in T. cruzi extracts is high (pH 8.0) as compared with pH 5.8 for the enzyme from mouse erythrocytes (12), and pH 5-6 (depending upon the ratio of ATP/Mg^{++}) from rabbit

liver (11). Workers on this enzyme from various tissues frequently use 6-methyl mercaptopurine instead of adenosine, following the finding of Schnebli, et al (3) that this analog is a substrate for the kinase but not for the competing adenosine deaminase. This makes pH optima comparisons of little value.

The adenosine kinase activity from extracts of T. cruzi is heat labile, being entirely destroyed after one min. at 55°. It withstands freezing at -20° or -80°, losing only ∿2% of its activity after 6 months. At 4°, however, 90% of the activity is lost after 4 days even in the presence of dithioerythritol or mercaptoethanol. Only minimal protection is afforded by the inclusion of ATP or GTP. In practice, therefore, small aliquots of fresh cell extracts are quick-frozen for future use.

Substrate inhibition of the adenosine kinase from T. cruzi is very pronounced. Linear response is only achieved below a concentration of ∿ 80 µM of adenosine (Fig. 2). Neither ATP nor Mg^{++} are inhibitory at over 100 times this concentration. This means that only sub-saturation levels of adenosine can be used and apparent K_m values (derived from Lineweaver-Burke plots) have grave limitations. However, at low concentrations of adenosine the apparent K_m for adenosine was found to be 20-28 µM. Adenosine inhibition of adenosine kinase has been reported from Sarcoma 180 cells (13) and from rabbit liver (11). Substrate inhibition of adenosine kinase was not found, however, in H. Ep2 cells or human red blood cells (14).

The enzyme from T. cruzi does not withstand dialysis. No activity is recovered after 12 hrs. dialysis at 12° against the Tris-dithioerythritol buffer (pH 8.0) either with or without 1 mM ATP. The addition of ATP or GTP to the equilibration and elution buffer during gel filtration (Sephadex G-200) appreciably protected the enzyme. The enzyme eluted as a single peak at ∿ 156 ml, indicating a rather small particle size.

Henderson, et al. (2) proposed a sequential mechanism for the adenosine kinase from Ehrlich ascites cells in which ATP binds to the free

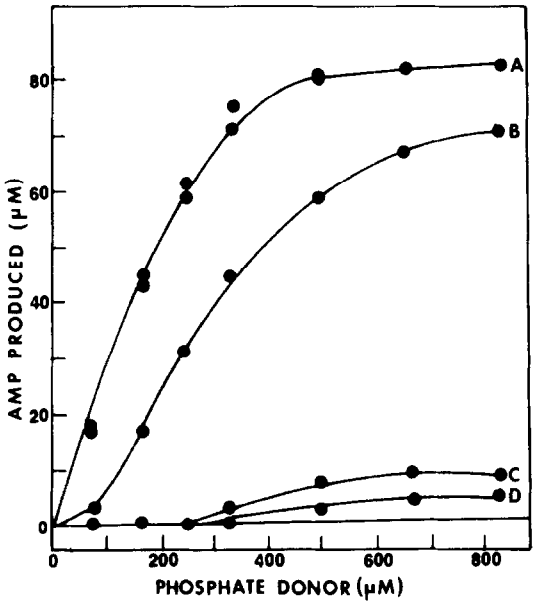

Fig. 2. Comparison of efficiencies of phosphate donors in the adenosine kinase reaction. A = ATP or GTP; B = ITP; C = fructose -1, 6-diphosphate; D = phosphoenolpyruvate. The reaction mixture contained, in a volume of 0.14 ml, 8-[^{14}C] adenosine, 80 μM; Tris HCl, 20 mM (pH 8.0); dithioerythritol, 1 mM; $MgSO_4$ 0.36 mM; EDTA, 2.4 mM; cell extract 0.3 mg protein. Average of 3 experiments.

enzymes, and the nucleoside monophosphate (they used 6-methylmercaptopurine as the substrate) was the last to dissociate. This suggestion found support from the observation of Miller et al. (11). They observed that the adenosine kinase from rabbit liver binds to both ATP- and AMP-agarose gels. Further support for the binding order suggested by Henderson, et al (2) is indicated for the T. cruzi enzyme by order-of-preincubation experiments. When the enzyme is preincubated with the phosphate donor (ATP or GTP) time curves are parabolic (there is no lag), but sigmoid curves are obtained if the enzyme is preincubated with adenosine, the reaction being initiated with ATP (Fig. 3).

If the intracellular pH for the adenosine kinase of T. cruzi is high (by compartmentalization ?) then no competition for the substrate exists as the flagellate possesses no adenosine deaminase and no adenosine nucleosidase activity at high pH. The enzyme may very well function as a trapping mechanism for adenosine salvage, as is suggested by Schmidt, et al. (12), in these purine-requiring organisms. An equally attractive hypo-

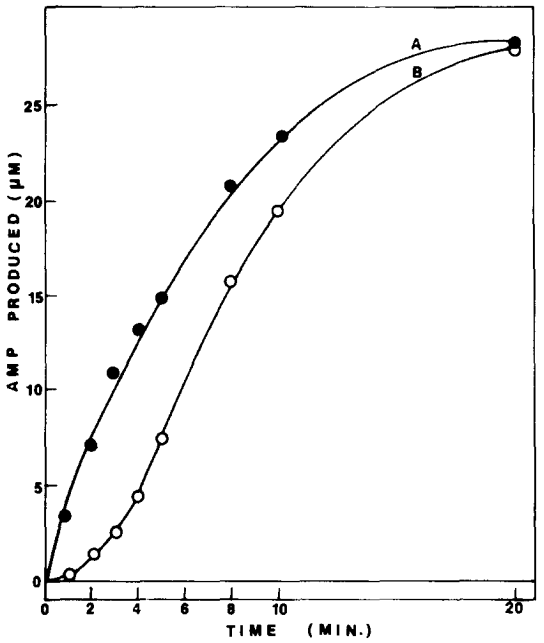

Fig. 3. Time course of the adenosine kinase activity with different orders of preincubation. A = preincubation (20 min/22°) of adenosine kinase with all components of the reaction mixture except 8-[^{14}C]-adenosine, which was added to initiate the reaction. B = preincubation with all components of the reaction mixture except ATP and $MgSO_4$ a mixture of which was added to initiate the reaction. The reaction mixture was the same as given for Fig. 1, with 8-[^{14}C]-adenosine at 45 μM. Average of 4 experiments.

thesis is that the enzyme acts as a detoxifying mechanism by phosphorylating adenosine, the free nucleoside causing cell toxicity (15). These two hypotheses are not mutually exclusive.

A number of purine and purine ribosides were tested for inhibition of adenosine kinase. The free bases were without activity. The nucleosides tested which proved inhibitory ranged from potent inhibitors (i.e. 6-methylaminopurine riboside) to those which are almost inert (Table I). The mode of inhibition is unclear since only guanosine and xanthosine (both poor inhibitors) were tested for nucleotide formation, as these were the only radiolabelled ones available. Neither they, nor inosine (non-inhibitory), acted as substrate for the enzyme. Some may be competing with adenosine for the active site on the enzyme without being themselves phosphorylated, or they may be competing as true substrates for the kinase, resulting in the production of analog nucleotides. These results are

TABLE I

The effects of the addition of adenosine analogs on the activity of adenosine kinase

Analog nucleoside	Concentration (mM)	% Inhibition
6-Methylaminopurine riboside	0.04	98
deoxyCoformycin	0.11	98
Tubercidin	0.80	95
6-Methylmercaptopurine riboside	0.60	67
6-Dimethylaminopurine riboside	1.00	85
deoxyAdenosine	1.40	85
deoxyGuanosine	1.40	46
4-Amino-5-imidazolecarboxamide riboside	2.40	45
8-Azaguanosine	0.60	29
Guanosine	1.40	28
Xanthosine	1.40	28
6-Mercaptopurine riboside	1.10	17

The reaction mixture contained (in 0.12 ml) 8-[^{14}C]-adenosine, 50 μM; Tris HCl, 18 mM; dithioerythritol, 0.9 mM; ATP, 3.6 mM; $MgSO_4$, 0.36 mM; EDTA, 1.8 mM; cell extract to product 50% conversion of substrate in 3 min. at 35°. Average of 6 experiments.

preliminary at best but they do suggest that certain nucleosides (especially 6-methylaminopurine riboside) warrant attention as possible therapeutic agents. It was earlier reported that 6-methylaminopurine inhibited the growth of epimastigotes of T. cruzi (4) and specifically its guanine aminohydrolase (EC 3.5.4.3), the ribonucleoside being inactive as a guanase inhibitor. The reverse is true for the adenosine kinase of the flagellate. The degree of substrate inhibition of the adenosine kinase made it difficult to obtain meaningful kinetic data for the inhibitors.

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