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PURIFICATION AND PROPERTIES OF ADENOSINE KINASE FROM RAT BRAIN

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Summary

Adenosine kinase (ATP:adenosine 5′-phosphotransferase, EC 2.7.1.20) has been purified to apparent homogeneity from rat brain by $(NH_4)_2SO_4$ fractionation, affinity chromatography on AMP-Sepharose 4B, gel filtration with Sephadex G-100, and DE-52 cellulose column chromatography. The yield was 56% of the initial activity with a final specific activity of 7.8 μmol/min per mg protein. The molecular weight was estimated as 38 000 by gel filtration with Sephadex G-100 and 41 000 by acrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS). The enzyme catalyzed the phosphorylation of adenosine, deoxyadenosine, arabinoadenosine, inosine and ribavirin. The activity of deoxyadenosine phosphorylation was 20% that of adenosine phosphorylation. The pH optimum profile was biphasic; a sharp pH optimum at pH 5.5 and a broad pH optimum at pH 7.5—8.5. The K_m value for adenosine was 0.2 μM and the maximum activity was observed at 0.5 μM. At higher concentrations of adenosine, the activity was strongly inhibited. The K_m value for ATP was 0.02 mM and that for Mg^{2+} was 0.1 mM. GTP, dGTP, dATP and UTP were also proved to be effective phosphate donors. Co^{2+} was as effective as Mg^{2+}, and Ca^{2+}, Mn^{2+} or Ni^{2+} showed about 50% of the activity for Mg^{2+}. The kinase is quite unstable, but stable in the presence of a high concentration of salt; e.g., 0.15 M KCl.

Introduction

Adenosine kinase (ATP:adenosine 5′-phosphotransferase, EC 2.7.1.20) catalyzes the phosphorylation of adenosine to AMP according to the following reaction; Adenosine + ATP $\xrightarrow{Mg^{2+}}$ AMP + ADP. The enzyme has a broad sub-

strate specificity [1—6] and exists at relatively high levels in tissue [7,8]. Decreased levels of adenosine kinase have been associated with resistance to the antiproliferative effects of a number of adenosine analogs [9], increased purine excretion [10] and primary gout [11].

Adenosine kinase was first discovered in yeast [12,13] and in mammalian tissue [12]. It had been partially purified from a number of mammalian sources [1—4,14—20] and purified to homogeneity from brewer's yeast [21] and most recently from rabbit liver [5].

We are interested in adenosine metabolism in brain since adenosine is an effective agent for stimulating the formation of cyclic AMP [22], and it decreases the amplitude of postsynaptic potentials evoked by electrical stimulation [23]. Adenosine kinase from rat brain was only studied by Shimizu et al. [15]. This paper describes the first extensive purification of rat brain adenosine kinase and reports several properties of the enzyme.

Materials and Methods

Materials

[8-^{14}C]Adenosine was purchased from The Radiochemical Centre Amersham. Nucleosides, nucleotides, phospho*enol*pyruvate, NADH, pyruvate kinase and lactate dehydrogenase were obtained from Sigma Chemical Co. and Boehringer Mannheim. AMP-Sepharose 4B and Sephadex G-100 were obtained from Pharmacia. Other reagents were commercial preparations of the highest purity available.

Enzyme assay and protein determination

Standard radiochemical assay was prepared in a final volume of 0.1 ml and contained 64 mM Tris-HCl (pH 7.5), 1 μM [8-^{14}C]adenosine (59 Ci/mol), 40 mM KCl, 1 mM ATP, 0.5 mM $MgCl_2$, 0.5 mM phospho*enol*pyruvate, 5 units/ml pyruvate kinase and appropriate amounts of adenosine kinase. After incubation of reaction mixtures for 2 min at 30°C, the reaction was initiated by addition of [8-^{14}C]adenosine. Reaction mixtures were incubated for 5 min at 30°C and then in boiling water bath for 1 min. After cooling, 0.05 ml of the reaction mixtures were transferred to DE-81 discs. The discs were air-dried and washed several times in 2 mM ammonium formate (pH 7), to remove unphosphorylated nucleosides, and air-dried. The discs were counted in 5 ml of toluene scintillation fluid which consisted of 0.4% 2,5-diphenyloxazole and 0.01% 2,2'-*p*-phenylene-bis(5-phenyl-oxazole), using an Aloka LSC-651 liquid scintillation spectrometer.

The kinase activity for other nucleosides and the activity in the presence of high concentration of adenosine were assayed by enzymatically coupling the formation of ADP to NADH oxidation. NADH oxidation was measured by following decrease in absorbance at 340 nm ($\Delta\epsilon = -6220\ M^{-1} \cdot cm^{-1}$) minus 400 nm on a Hitachi 356 two-wavelength double-beam spectrophotometer at 30°C. The assay mixture contained 64 mM Tris-HCl (pH 7.5), 180 mM KCl, 1 mM ATP, 0.5 mM $MgCl_2$, 0.5 mM phospho*enol*pyruvate, 0.1 mM NADH, 5 units/ml pyruvate kinase, 13.8 units/ml lactate dehydrogenase, appropriate nucleoside and the enzyme in a final volume of 1 ml. Reactions were initiated by addition

of nucleoside. 1 unit of the enzyme activity is defined as the amount catalyzing the phosphorylation of 1 μmol nucleoside/min. Specific activity was defined as units/mg protein. Protein concentration was determined by the method of Lowry et al. [24], using bovine serum albumin as a standard.

SDS electrophoresis

SDS-polyacrylamide gel electrophoresis was carried out by the method of Weber and Osborn [25]. The samples, prior to electrophoresis, were incubated for 3 min in boiling water in 0.01 M sodium phosphate (pH 7) containing 1% SDS and 0.5% 2-mercaptoethanol. Electrophoresis was performed in 7.5% acrylamide gel. As the protein standards, Pharmacia's electrophoresis calibration kit was used. The kit gives six calibration points covering the molecular weight range 14 400—94 000; phosphorylase *b* (94 000), bovine serum albumin (67 000), ovalbumin (43 000), carbonic anhydrase (30 000), soybean trypsin inhibitor (20 100), and α-lactalbumin (14 400).

Results

Purification of enzyme

Fresh rat brain (50 g) was homogenized in 150 ml of buffer A which consisted of 10 mM Tris-HCl (pH 7.5) and 0.15 M KCl. The homogenate was centrifuged at 20 000 × *g* for 30 min. The supernatant was saved and solid $(NH_4)_2SO_4$ was added to 45% saturation. After centrifugation, solid $(NH_4)_2SO_4$ was added to the supernatant to 80% saturation. The solution was centrifuged and the pellet was dissolved in buffer B which consisted of 10 mM potassium phosphate (pH 7.0) and 1 mM dithiothreitol.

The solution was dialyzed against buffer B overnight, and applied to a column of AMP-Sepharose 4B (0.75 cm^2 × 10 cm) equilibrated with buffer B. Most of proteins were passed through AMP-Sepharose 4B, but the kinase activity was adsorbed. After washing with 100 ml of buffer B, the kinase activity was eluted with buffer B containing 5 mM adenosine. The column was further eluted with buffer B containing 2 M KCl. Some proteins were eluted in this fraction, but no kinase activity was detected (Fig. 1). In this step, specific activ-

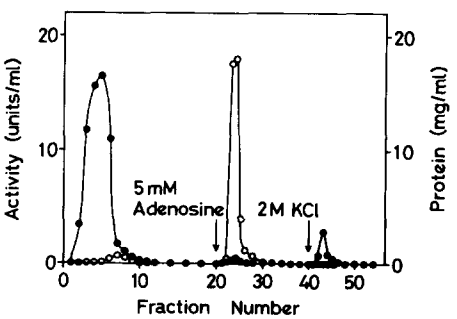


Fig. 1. Chromatography of rat brain adenosine kinase on AMP-Sepharose 4B. 20 ml of the $(NH_4)_2SO_4$ fraction was applied to a column of AMP-Sepharose 4B (0.75 cm^2 × 10 cm) equilibrated with buffer B. The column was eluted with buffer B containing 5 mM adenosine, and further eluted with buffer B containing 2 M KCl. Nos. 1—20 were collected in 5-ml fractions, and Nos. 21—54 were collected in 3-ml fractions. ○———○, adenosine kinase activity in the presence of 25 μM adenosine; ●———●, protein.

TABLE I

PURIFICATION OF RAT BRAIN ADENOSINE KINASE

1 unit equals 1 μmol of [8-^{14}C]AMP formed per min under standard assay conditions. The ratio of deoxyadenosine phosphorylation to adenosine phosphorylation was calculated from the respective maximum activities.

Fraction	Total volume (ml)	Total activity (units)	Total protein (mg)	Specific activity (units/mg protein)	Deoxyadenosine/ adenosine
Crude extract	140	1.94	974	0.002	
$(NH_4)_2SO_4$ (45—80%)	20	2.24	276	0.008	
AMP-Sepharose 4B	6	1.42	1.15	1.24	0.22
Sephadex G-100	30	1.42	0.25	5.66	0.20
DE-52 cellulose	9	1.09	0.14	7.79	0.20

ity rose about 150-fold over $(NH_4)_2SO_4$ fraction. The fractions containing the kinase activity were pooled and concentrated by the addition of solid $(NH_4)_2SO_4$ to 90% saturation. After centrifugation, the pellet was dissolved in 4—5 ml of buffer A.

The solution was then applied to a column of Sephadex G-100 (5.2 $cm^2 \times 45$ cm) equilibrated with buffer A. The column was eluted with the same buffer. The fractions containing the kinase activity were pooled and dialyzed against buffer C which contained 10 mM Tris-HCl (pH 7.5) and 1 mM dithiothreitol.

The Sephadex G-100 fraction was applied to a column of DE-52 cellulose (0.75 $cm^2 \times 10$ cm) equilibrated with buffer C. After washing with 100 ml of buffer C, the column was eluted with a linear gradient of 0—0.1 M KCl in buffer C (50 ml each). The kinase activity was eluted at about 40 mM KCl concentration.

The stepwise purification of adenosine kinase is summarized in Table I. The specific activity of the final preparation is about 7.8 μmol/min per mg protein and represents a 3900-fold purification over the original supernatant and recovery of 56% of the initial activity.

Enzyme properties

The final preparation was tested for purity and appeared to be homogeneous, since only a single protein band was observed by acrylamide gel electrophoresis in the presence of SDS. When the native enzyme preparation was applied to a Sephadex G-100 column, enzyme activities toward adenosine and deoxyadenosine were eluted from the column as a single peak and a molecular weight of 38 000 was estimated. The molecular weight of the kinase was also determined by electrophoresis in 7.5% acrylamide gel carried out in the presence of 0.1% SDS. The kinase migrated as a single electrophoresis species and a molecular weight of 41 000 was obtained. These results indicate that adenosine kinase has a monomeric structure.

During purification, the ratios of the ability to phosphorylate deoxyadenosine remain constant at a value of around 0.2 after the AMP-Sepharose step (Table I). When the final preparation was assayed for the phosphorylation of other nucleosides, arabinoadenosine, inosine and ribavirin were also phos-

TABLE II

SUBSTRATE SPECIFICITY

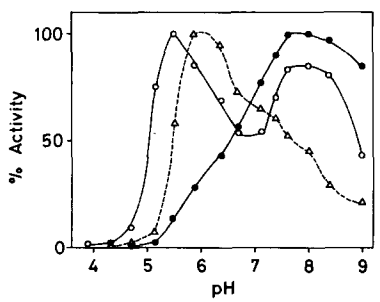
The enzyme activity was assayed at 1 mM ATP and 0.5 mM $MgCl_2$ by enzymatically coupling the formation of ADP to NADH oxidation, except at 0.0005 mM adenosine which was assayed radiochemically.

Substrate	Concentration (mM)	Relative activity
Adenosine	0.0005 *	100
	0.025	27.2
	0.25	15.5
Deoxyadenosine	0.025	1.9
	1.0 *	20.3
Arabinoadenosine	0.025	2.0
	0.5 *	10.8
Inosine	0.025	1.8
	1.0 *	9.2
Guanosine	0.25	0.0
Deoxyguanosine	0.25	0.0
Cytidine	1.0	0.0
Deoxycytidine	1.0	0.0
Uridine	1.0	0.0
Deoxythymidine	1.0	0.0
Ribavirin	0.025	2.4
	1.0	59.4

* Optimal concentration to obtain the maximum activity.

phorylated (Table II). The maximum activities of arabinoadenosine and inosine phosphorylations were about 10% of adenosine phosphorylation. The maximum activity of ribavirin phosphorylation was not tested, but 1 mM ribavirin showed about 60% of the activity of adenosine phosphorylation. Guanosine, deoxyguanosine, cytidine, deoxycytidine, uridine and deoxythymidine were not phosphorylated.

Effects of pH on the activity are summarized in Fig. 3. The pH optimum profile for adenosine kinase, using adenosine as a substrate, was biphasic; a sharp pH optimum at pH 5.5 and a broad pH optimum at pH 7.5—8.5. When high concentration of adenosine (25 μM) was used, the pH optimum profile



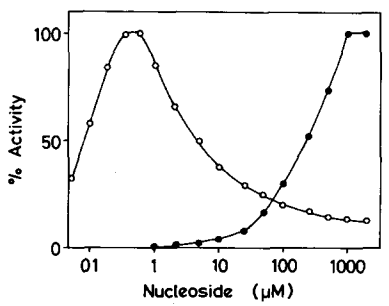


Fig. 2. Effects of pH on enzyme activity. Acetate buffers at pH 3.9—5.2, cacodylate buffers at pH 5.2—7.2, and Tris-HCl buffers at pH 7.4—9.0 were used. 1 μM adenosine (○———○), 25 μM adenosine (△———△), and 1 mM deoxyadenosine (●———●) were used as substrates.

Fig. 3. Effects of adenosine or deoxyadenosine concentration on enzyme activity. Adenosine (○———○), and deoxyadenosine (●———●) were used as substrates.

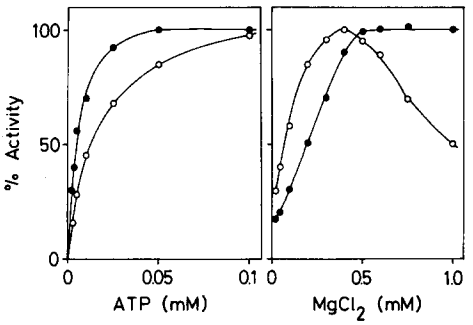


Fig. 4. Effects of ATP or Mg^{2+} concentration on the phosphorylation of adenosine and deoxyadenosine. 1 μM adenosine (○———○), and 1 mM deoxyadenosine (●———●) were used as substrates.

was monophasic with one peak at pH 6.0. The pH optimum, using deoxyadenosine as a substrate, was pH 7.5—8.5 (Fig. 2).

Effects of adenosine or deoxyadenosine concentration on enzyme activity are shown in Fig. 3. An apparent K_m value for adenosine at pH 7.5 was 0.2 μM and the maximum activity was observed at 0.5 μM. At higher concentrations of adenosine, the activity was strongly inhibited; 50% and 75% inhibition at 5 and 50 μM adenosine, respectively. An apparent K_m value for deoxyadenosine at pH 7.5 was 360 μM.

Effects of ATP or Mg^{2+} concentration on the phoshorylation of adenosine and deoxyadenosine are shown in Fig. 4. When adenosine phosphorylation was studied under standard conditions, apparent K_m values for ATP and $MgCl_2$ were 0.02 and 0.1 mM, respectively, and a concentration of Mg^{2+} higher than 0.4

TABLE III

NUCLEOSIDE TRIPHOSPHATE DONOR SPECIFICITY AND METAL ION REQUIREMENT

The enzyme activity was assayed under standard conditions.

Substance added	Relative activity	Substance added	Relative activity
Nucleoside triphosphate * (1 mM)		Divalent metal ion *** (0.5 mM)	
ATP	100	none	30
dATP	141	$MgCl_2$	100
AraATP	10	$CaCl_2$	51
GTP	185	$CrCl_2$	26
dGTP	175	$MnCl_2$	50
CTP	59	$FeSO_4$	28
dCTP	9	$CoCl_2$	114
UTP	94	$NiCl_2$	57
dTTP	17	$CuSO_4$	1
EDTA **		$ZnSO_4$	25
none	30	$BaCl_2$	31
0.5 mM	14		
5.0 mM	2		

* Assayed at 1 μM [8-^{14}C]adenosine; 0.5 mM $MgCl_2$.

** Enzyme was pretreated with EDTA for 5 min at 30°C, and 10 μl was used for assay. Reaction was carried out in the absence of $MgCl_2$.

*** Assayed at 1 μM [8-^{14}C]adenosine; 1 mM ATP.

mM was inhibitory. Using deoxyadenosine as a substrate, apparent K_m values for ATP and $MgCl_2$ were 0.006 and 0.25 mM, respectively, and the inhibition at higher concentration of Mg^{2+} was not observed. Neither adenosine nor deoxyadenosine phosphorylation was inhibited by higher concentration of ATP.

Adenosine kinase had a fairly broad specificity for the naturally occurring nucleoside triphosphate. ATP, dATP, GTP, dGTP and UTP proved to be effective phosphate donors (Table III). GTP, dGTP and dATP were more effective than ATP. In general, the pyrimidine triphosphates were less effective than ATP, except that UTP was as effective as ATP. AraATP was not as effective.

Divalent metal ion requirement is also summarized in Table III. Co^{2+} was as effective as Mg^{2+}. Ca^{2+}, Mn^{2+}, and Ni^{2+} were also capable of replacing Mg^{2+} in the reaction mixture but resulted in reduced reaction rate to about 50% of the rate of Mg^{2+}. Cr^{2+}, Fe^{2+}, Zn^{2+}, and Ba^{2+} were not effective and in the presence of Cu^{2+} the activity was inhibited. In the absence of added metal ion, the reaction proceeded at 30% of the rate observed in the presence of an optimal Mg^{2+} concentration. However, by the preincubation of the enzyme preparation with EDTA, the activity in the absence of added metal ion was reduced; 50% and 90% reduction at 0.5 mM EDTA and 5.0 mM EDTA, respectively (Table III). These results indirectly suggest that adenosine kinase from rat brain contains a bound metal ion.

The kinase was stable in the presence of a high concentration of salt; e.g., 0.15 M KCl. Under the condition of low concentration of salt, the enzyme was rapidly inactivated. But the activity was completely recovered, when it was redialyzed against the buffer containing dithiothreitol.

Apparently homogeneous adenosine kinase was obtained from rat liver by the same purification procedure, although rat liver contained 20-fold the enzyme activity of the brain per wet weight. However, no difference was observed between adenosine kinase from brain and the enzyme from liver, with respect to molecular weight, kinetic properties and the elution profiles from AMP-Sepharose and DEAE-cellulose.

Discussion

Adenosine kinase was purified to apparent homogeneity from rat brain. The molecular weight of the kinase was estimated as 38 000 by Sephadex G-100 gel filtration. This value was similar to the value of the enzyme from rabbit liver [5], yeast [21], mouse erythrocytes [17] and *Plasmodium chabaudi* [17], determined by the same method. The molecular weight calculated from results of SDS-polyacrylamide gel electrophoresis was 41 000, and was similar to the enzyme from yeast [21], but different from rabbit liver which showed a molecular weight of 51 000 by SDS-polyacrylamide gel electrophoresis. These data suggest that adenosine kinase from rat brain has a monomeric structure and the molecular weight is about 40 000.

We are interested in whether adenosine and deoxyadenosine are phosphorylated by separate enzyme activities or by the same enzyme activity. Previously Streeter et al. [19] copurified ribavirin, adenosine and deoxyadenosine kinase activity from rat liver, but concluded that adenosine and deoxyadenosine kinase are separate enzyme activities and that phosphorylation of

ribavirin is associated with the latter activity. The final preparation purified from rat brain appeared to be homogeneous and showed enzyme activity for adenosine, deoxyadenosine and ribavirin (Table II). In addition, during purification, the ratios of the ability to phosphorylate deoxyadenosine to adenosine remained constant. The deoxycytidine kinase from calf thymus has activity for deoxyadenosine [26], but the rat brain adenosine kinase has no activity for deoxycytidine (Table II). The requirements for optimal activity are quite different for adenosine kinase and deoxyadenosine kinase activity. However, the present observations strongly suggest that adenosine and deoxyadenosine are phosphorylated by the same enzyme.

We observed that the pH optimum profile was biphasic using adenosine as a substrate and monophasic using deoxyadenosine (Fig. 2), while a biphasic profile was not observed in previous studies [1—5,19,21]. The difference may be due to the fact that the previous studies were tested under the higher concentration of adenosine. Similarly, when we used 25 μM adenosine, the pH profile showed a single peak at pH 6.0. In order to explain these phenomenon, we take into consideration that adenosine kinase has two active sites; one site is more active at lower pH, and the other is more active at higher pH; adenosine interacts at both sites controlled by the various factors. Deoxyadenosine interacts only at the higher pH site. Furthermore, it is evident that the requirements for optimal activity are quite different for adenosine kinase and deoxyadenosine kinase.

Adenosine kinase activity is complicated by the influence of pH, and concentrations of substrate, ATP and Mg^{2+} [5]. Our K_m data for nucleosides, ATP and Mg^{2+} were only obtained under optimal conditions for other factors. Further study is necessary to determine the effect of the ATP/Mg^{2+} ratio and so forth, under various conditions.

The phosphate donor specificity of adenosine kinase appears to vary from source to source. ATP, dATP, GTP, dGTP and UTP are effective phosphate donors for the rat brain enzyme (Table III). With both the rabbit liver enzyme [5] and the enzyme from human erythrocytes [27], GTP could be substituted for ATP with no apparent loss of enzymic efficiency, but dATP and UTP were not so effective. The yeast enzyme has been reported to use ATP and dATP with equivalent efficiency [21]. On the other hand, the enzyme from Ehrlich ascites cells is relatively nonspecific with regard to the phosphate donor [14].

Effects of Mg^{2+}, Ca^{2+}, Mn^{2+} or Ba^{2+} on the activity of adenosine kinase from rat brain were similar to that from rabbit liver [5]. In our studies, Co^{2+} was found to be as effective as Mg^{2+}. In the absence of added metal ion, the enzyme had appreciable catalytic activity (Table III). Previous workers have suggested that adenosine kinase may contain a bound metal ion [2,3,14], and Miller et al. [5] showed that the higher purified enzyme contained tightly bound Mg^{2+} and Ca^{2+} by emission spectrum analysis. We presume that the enzyme from rat brain contains a bound metal ion, since the treatment of the enzyme with EDTA reduced the enzyme activity.

No difference in properties was observed between the adenosine kinase from brain and the enzyme from liver purified by the same method, at the present time. Whether adenosine kinase from brain and liver are identical or not, must await further study.

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