

Purification and Properties of Adenosine Kinase from Rat Liver: Separation from Deoxyadenosine Kinase Activity

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Ion exchange and affinity chromatography techniques, similar to those previously reported for purification of adenosine kinase from human placenta, were applied to purification of rat liver adenosine kinase. The enzyme, purified 400-fold in 41% yield, was homogeneous on SDS-polyacrylamide gel electrophoresis, with a molecular weight of 52 000. Its specific activity, 18 $\mu\text{mol}/\text{min}/\text{mg}$ protein, is the highest hitherto reported for this enzyme from mammalian sources.

Chromatography on DEAE-cellulose removed about 98% of the phosphorylating activity towards 2'-deoxyadenosine present in the initial pH-treated liver extract. The final preparation exhibited only minimal activity ($\sim 1.5\%$) under optimal conditions (pH 7.5) vs 2'-deoxyadenosine, the lowest yet reported for such a preparation, with a K_m of 670 μM , as compared to 0.3 μM for adenosine.

The residual activity towards deoxyadenosine is considered an intrinsic property of the purified adenosine kinase and, in fact, phosphorylation of adenosine was inhibited competitively by deoxyadenosine, with a K_i of 70 μM . Competitive inhibition was also exhibited by cordycepin (3'-deoxyadenosine) with a K_i of 150 μM . A more potent competitive inhibitor was tubercidin, the K_i for which was 1.9 μM .

Introduction

Adenosine kinase (ATP: adenosine-5'-phosphotransferase, EC 2.7.1.20) catalyzes the phosphorylation of adenosine to adenosine-5'-phosphate in the presence of ATP, or other nucleoside 5'-triphosphates, and a cation. Together with adenosine deaminase, it plays a critical role in regulation of adenosine levels *in vivo*, the significance of which is underlined by the fact that adenosine is toxic to mammalian and bacterial cells, and its presence is associated with inhibition of the immune response and many other biochemical effects [1]. Adenosine kinase has also been implicated in the "activation" (phosphorylation) of a number of pharmacologically active analogues of purine nucleosides, including some with antiviral activity, *e.g.* ribavirin [1–7].

The enzyme has been reported purified to apparent homogeneity from yeast [8], human placenta [9], mouse L1210 cells [10], rat brain [11], and from rat [12], rabbit [13], and human [14] livers.

It is still controversial as to whether 2'-deoxyadenosine is phosphorylated by adenosine kinase. From studies on the cytotoxicity of 2'-deoxy-

adenosine in mutant cell lines deficient in adenosine kinase activity, Ullman *et al.* [15, 16] concluded that deoxyadenosine is phosphorylated largely by adenosine kinase. A similar conclusion was reached by Ogasawara *et al.* [12] for the rat liver enzyme, based on copurification of activities vs adenosine and deoxyadenosine by their isolation procedure. On the other hand, adenosine kinase preparations from rabbit liver [13], L1210 cells [10] and human placenta [9] are reported to exhibit relatively low levels of activity vs deoxyadenosine. Furthermore, other kinases are known which actively phosphorylate deoxyadenosine, but not adenosine [17–21].

Following initial attempts to purify adenosine kinase from rat liver by a variety of methods, we noted that a procedure devised by Andres and Fox [9] for isolation of this enzyme from human placenta was based on the almost quantitative separation of activities towards adenosine and 2'-deoxyadenosine on DEAE-52 cellulose. The essential features of this method have been applied in the present study to purification of the enzyme from rat liver.

Materials and Methods

Chemicals

Nucleosides, nucleotides and analogues such as cordycepin, tubercidin and formycin A, as well as

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phosphoenolpyruvate and pyruvate kinase, were products of Sigma (St. Louis, M., USA). CM- and DEAE-celluloses were from Whatman, and 5'-AMP-Sepharose 4B and DEAE-Sepharose 4B from Pharmacia (Uppsala, Sweden). PEI-cellulose plates were from Merck (Darmstadt, GFR). Bovine serum albumin (crystalline, A grade) was from Calbiochem-Boehringer. Ribavirin was kindly provided by Dr. R. Sidwell. Standard proteins for molecular weight determinations were from the Combithek[®] kit of Boehringer (Mannheim, GFR). Other reagents were commercial preparations of analytical grade. [2-³H]adenosine (24 Ci/mmol) and 2'-deoxy-[G-³H]adenosine (40 Ci/mmol) were from Amersham and New England Nuclear, respectively. Both were purified by thin-layer chromatography, using authentic cold standards to locate them prior to elution.

Enzyme purification

Rat livers were collected and stored at -25°C . All subsequent operations were at $0-6^{\circ}\text{C}$. Minced livers were suspended in the ratio 1 g/2 ml of 10 mM Tris-HCl buffer pH 7.4 containing 0.05 mM of the proteinase inhibitor, phenyl-methylsulphonylfluoride, and homogenized $6\times$ for 30 s in an MSE Auto-Mix homogenizer, the homogenate being cooled in ice before each homogenization. The homogenate was passed through a double layer of gauze and centrifuged in a Servall SS-23 for 15 min at 15 000 rpm. The resulting supernatant was centrifuged at $100\,000\times g$ for 1 h in a MSE Superspeed 65 centrifuge. The lipid layer was removed, and the clear supernatant brought to pH 6.0 with 1 M acetic acid in an ice bath, and stirred for 30 min, following which the resulting precipitate was removed by centrifugation in a Servall SS-23 at 15 000 rpm.

The clear supernatant was then treated with CM- and DEAE-celluloses previously equilibrated with 10 mM acetate buffer pH 6.0, as described by Andres and Fox [9], and filtered. The filtrate was concentrated 15-fold in an Amicon with a UM-10 membrane, clarified by centrifugation, and deposited on a 1.6×15 cm column of 5'-AMP Sepharose 4B equilibrated with 10 mM acetate buffer pH 6.0. The column was washed overnight with the same buffer to remove non-specifically bound proteins, and then, in turn, with 100 ml acetate buffer (10 mM) pH 6.0 containing 1 M KCl, 100 ml 0.01 M Tris-HCl

buffer pH 7.4 containing 1 M KCl, and 50 ml 0.1 M Tris-HCl buffer pH 7.4 containing 5 mM ATP. Adenosine kinase was then eluted with 0.1 M Tris-HCl buffer pH 7.4 containing 5 mM ATP, 5 mM MgCl_2 and 5 mM adenosine, at a rate of 8 ml/h, with collection of 0.3 ml fractions which were assayed for protein and kinase activity. Under these conditions the active fractions formed a sharp peak. Fractions containing less than 1 mg protein/ml were concentrated in an Amicon as above. Such a preparation, when stored at -25°C , exhibited no loss of activity over a period of several months.

Because the foregoing preparation exhibited trace impurities (see Results, below), attempts at further purification were undertaken, culminating in chromatography on DEAE-Sepharose. About 1–2 mg of enzyme from the 5'-AMP Sepharose 4B column, in a volume of 0.5 ml, was dialysed against 10 mM Tris-HCl buffer pH 7.4 at 6°C until spectrophotometry demonstrated complete removal of adenosine and ATP. It was then deposited on 0.9×6 cm column of DEAE-Sepharose equilibrated with 10 mM Tris-HCl buffer pH 7.4. The column was washed with 100 ml of the same buffer to remove non-specifically adsorbed protein, and adenosine kinase eluted with a linear gradient of KCl (0–1 M) in the same buffer, with collection of 0.3 ml fractions. The enzyme eluted as a sharp peak embracing 3–5 fractions (0.9–1.5 ml) at 50–60 mM KCl, with unchanged specific activity, but further freed of traces impurities.

Adenosine kinase activity

The standard incubation medium included, in a total volume of 100 μl : 50 mM acetate buffer pH 5.5, 1 mM ATP, 1 mM MgCl_2 , 3 mM dithiothreitol, 30 μg bovine serum albumin, 100 μM [2-³H]adenosine (1 Ci/mmol), 5 mM phosphoenolpyruvate and 1.9 U pyruvate kinase. Following preincubation for 5 min at 37°C , the reaction was initiated by addition of enzyme, and terminated after the desired time interval by immersion of the sample tubes in boiling water for 2 min. The samples were transferred to an ice bath, the tubes centrifuged to collect solvent from the tube walls, and a 5 μl aliquot deposited on TLC plates along with cold reference samples of adenosine and AMP, followed by development with water ($R_f=0.53$ and 0.0 for adenosine and AMP, respectively). The spots revealed under a dark UV-

lamp were cut out, extracted with 1 ml of 1 M Tris-HCl buffer pH 7.4 containing 0.7 M MgCl₂ for 15 min [22] and 0.5 ml of the eluate transferred to a scintillation vial, to which was added 0.1 ml water and 3.5 ml toluene-triton scintillation fluid. Radioactivity was counted in a Beckman LS-9000 scintillation counter.

Under the foregoing conditions, enzyme activity was proportional to time of incubation for periods up to 30 min. Activities of crude and partially purified preparations were assayed in the presence of 20 mM NaF to inhibit phosphatases. For kinetics of adenosine phosphorylation, the preparation was diluted into 20% glycerol containing 3 mM dithiothreitol and 0.3 mg bovine serum albumin/ml. For activities vs 2'-deoxyadenosine and other analogues, the required higher concentrations of enzyme necessitated prior removal of adenosine and ATP by dialysis against 0.1 M Tris-HCl buffer pH 7.4 containing 3 mM dithiothreitol.

Deoxyadenosine kinase activity

The procedure was as for adenosine kinase activity, above, but in 50 mM Tris-HCl buffer pH 7.5 with 1 mM [2-³H]deoxyadenosine (40 Ci/mol) as substrate. The product was separated as above (*R_f* of dAMP and deoxyadenosine, 0.0 and 0.57).

Polyacrylamide gel electrophoresis

This was carried out as described by Laemmli [23], with 5–15 µg samples of protein in 10% gels. Electrophoresis was in 0.025 M Tris-HCl and 0.192 M

glycine, pH 8.6 under denaturation conditions (addition of 1% SDS). Under non-denaturing conditions (no SDS), the buffer concentration was reduced twice. Staining was with Coomassie Blue.

Inhibition studies

Inhibition of adenosine phosphorylation by adenosine kinase inhibitors was determined from the changes in initial rates of phosphorylation in the presence of two concentrations of inhibitor, with adenosine concentrations of 0.08–0.56 µM.

Protein estimation

Protein was estimated by the method of Bradford [24], using bovine serum albumin as standard.

Other estimations

The final preparation was checked for adenosine deaminase activity by means of a new fluorimetric assay method with formycin A as substrate [25], for phosphomonoesterase activity at both pH 5.5 and 7.5 with *p*-nitrophenylphosphate as substrate, for 5'-nucleotidase according to the method of Chen *et al.* [26] as modified by Walters and Loring [27], and for adenylate kinase as described by Miller *et al.* [13].

Results

Purification of adenosine kinase

As may be seen from Table I, the two ion exchange batch elutions of the pH-treated liver extract

Table I. Purification of rat liver adenosine kinase. Activities vs adenosine and deoxyadenosine were measured by radiochemical assays as described in Materials and Methods.

Purification step	Total protein [mg]	Adenosine ^a			dAdo ^b	
		Spec. act. [µmol/min/mg]	Total act. [µmol/min]	Recovery [%]	Spec. act. [µmol/min/mg]	Activity ratio dAdo/Ado
pH-treated extract	11 520	0.046	529	100	0.027	0.59
CM-cellulose treatment	3833	0.127	486	92	0.077	0.61
DEAE-cellulose treatment	2350	0.200	470	89	< 0.002	0.01
Amicon concentrate	1785	0.193	344	65	< 0.020	0.01
5'-AMP-Sepharose 4B eluate	12	18.0	216	41	0.280	0.016

^a With 100 µM adenosine, at pH 5.5.

^b With 1 mM deoxyadenosine, at pH 7.5.

led to only a 5-fold increase in specific activity, but with a yield of about 90%. This is clearly due to the fact, previously pointed out by Andres and Fox [9], that with both resins equilibrated to pH 6.0, which is close to the isoelectric point of adenosine kinase from diverse mammalian sources [9, 12], the enzyme is weakly bound, if at all, by anionic or cationic exchangers. The subsequent Amicon concentration, which decreased the yield to 65%, appeared unavoidable, despite numerous attempts to ameliorate this. The key step proved to be, as in the case of the human placenta enzyme [9], AMP-Sepharose 4B chromatography, which resulted in a more than 90-fold additional purification. The overall recovery (Table I) was 41%, as compared to 24% for the placenta enzyme.

At this point, SDS-polyacrylamide gel electrophoresis of the preparation, using a large amount of protein (15 µg), exhibited a single very intense band, and traces of an additional band. Since the latter could conceivably be ascribed to the residual activity towards 2'-deoxyadenosine, following AMP-Sepharose 4B chromatography (Table I), attempts at further purification were undertaken, culminating in the use of DEAE-Sephacel. Although this step did not affect the specific activity or yield of adenosine kinase, and only slightly reduced the residual activity of the preparation towards deoxyadenosine, it yielded a preparation which was fully homogeneous on SDS-polyacrylamide gel electrophoresis (Fig. 1). The purified preparation, even prior to DEAE-Sephacel chromatography, was completely free of adenosine deaminase, adenylate

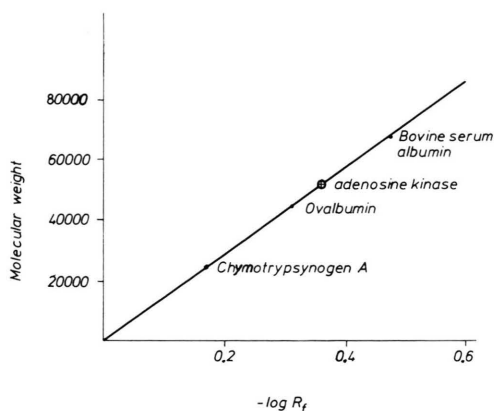


Fig. 2. Molecular weight determination of rat liver adenosine kinase, following affinity chromatography on 5'-AMP-Sepharose 4B, by SDS-polyacrylamide gel electrophoresis at pH 8.4 (Tris-glycine buffer). Standard proteins were chymotrypsinogen A (25 000), ovalbumin (45 000) and bovine serum albumin (68 000).

kinase, phosphatase and 5'-nucleotidase activities. Its specific activity, with an adenosine concentration of 100 µM at pH 5.5, is 18 µmol/min/mg, and it is totally inactive in the absence of Mg^{+2} . The molecular weight, in SDS-polyacrylamide gels, is 52 000 (Fig. 2). When stored at $-25^{\circ}C$ at a concentration of 1 mg/ml, it is fully stable even after frequent thawing.

Activity of purified enzyme vs 2'-deoxyadenosine

Relative to the activity of the enzyme towards adenosine at a concentration of 100 µM at pH 5.5, the activity towards deoxyadenosine at a concentration of 1 mM was 0.7% at pH 5.5 and about 1.5% at pH 7.5. This is the lowest reported activity towards deoxyadenosine for an adenosine kinase preparation of mammalian origin, and suggests that adenosine kinase itself may recognize deoxyadenosine as a feeble substrate. This is supported by the results of gel electrophoresis, particularly following DEAE-Sephacel chromatography (Fig. 1). An examination was therefore made of the behaviour of deoxyadenosine as a potential inhibitor of adenosine kinase activity. The results in Fig. 3 do, in fact, demonstrate that deoxyadenosine is a competitive inhibitor of adenosine kinase, with a $K_i = 70$ µM at pH 7.5, and consistent with the inference that the residual activity towards deoxyadenosine is an intrinsic property of the enzyme.

Fig. 1. SDS-polyacrylamide gel electrophoresis of adenosine kinase (6 µg protein), following DEAE-Sephacel chromatography, in a 10% gel.

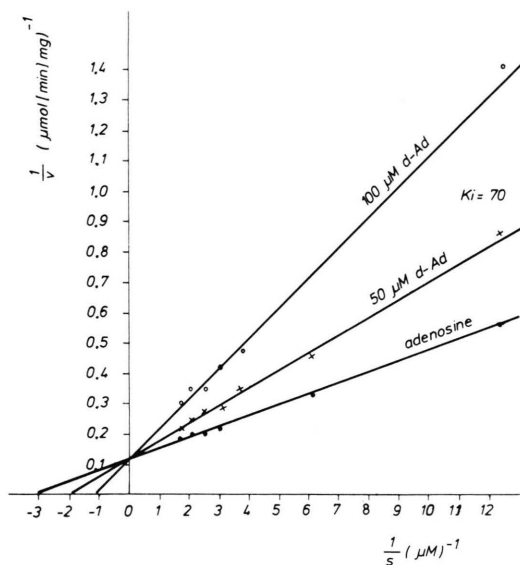


Fig. 3. Double-reciprocal plot for inhibition of adenosine kinase by 2'-deoxyadenosine in 0.05 Tris-HCl buffer pH 7.5, in the presence of 1 mM each of ATP and Mg^{+2} . Initial velocities are expressed as μmol AMP formed per mg protein per min.

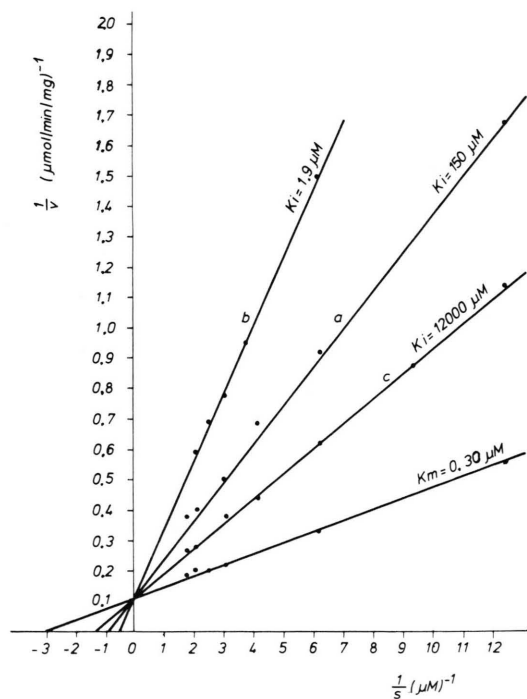


Fig. 4. Double-reciprocal plots for determination of inhibition of adenosine kinase by: a) cordycepin, 375 μM ; b) tubercidin, 9.35 μM ; c) ribavirin, 15 mM, all in 0.05 M Tris-HCl buffer pH 7.5, in presence of 1 mM each of ATP and Mg^{+2} . Initial velocities are in μmol AMP formed per min per mg protein. For the sake of clarity, results with only one concentration of inhibitor are presented.

The foregoing was extended to measurements of inhibition constants for previously established substrates of adenosine kinase [6], such as tubercidin, cordycepin and ribavirin. All three of these competitively inhibited the enzyme (Fig. 4), with K_i values of 1.9 μM , 150 μM and 12 mM, respectively, suggesting that the same active site is involved in all these instances.

pH-Dependence of adenosine kinase activity

From Fig. 5 it will be seen that the pH-dependence of enzyme activity varies appreciably with the substrate concentration. The optimum pH, which is 5.5 at 100 μM substrate, is slightly shifted to more alkaline pH with a decrease in adenosine concentration. The rapid decrease in activity below about pH 5.5 may conceivably be due, at least in part, to protonation of the ring N [3] of adenosine, for which the pK_a is 3.7. Above pH 5.5 the appreciable decrease in activity at high (10–100 μM) substrate concentrations is probably due to inhibition by substrate, since at a very low concentration (1.5 μM) the activity remains virtually unaltered over the pH range 6–8. The influence of pH and substrate concentration for our purified enzyme is similar to that reported for the enzyme from rabbit liver [28] and human placenta [29].

Effect on enzyme activity of adenosine concentration and ratio of ATP/ Mg^{+2}

Figure 6A exhibits the effect of adenosine concentration on enzyme activity at pH 5.5 in the presence

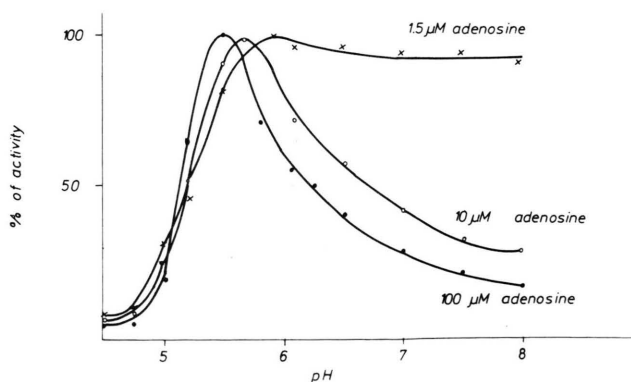


Fig. 5. pH-dependence of phosphorylation of adenosine, at various concentrations as indicated, by purified adenosine kinase in the presence of 1 mM each of ATP and Mg^{+2} . Buffers were: acetate, pH 4.5–6.0; Hepes, pH 6.1–6.9; Tris-HCl, pH 7.0–8.0, each at a concentration of 50 mM.

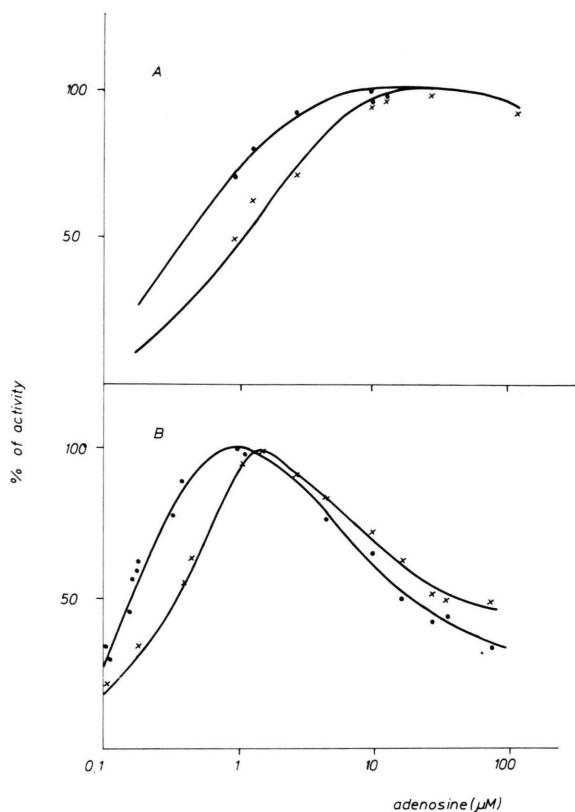


Fig. 6. Effect of adenosine concentration on adenosine kinase activity, determined at: A) pH 5.5; B) pH 7.5; (××××), $\text{ATP/Mg}^{+2} = 1$; (●●●●), $\text{ATP/Mg}^{+2} = 2$.

of 1 mM ATP and two concentrations of Mg^{+2} , 0.5 mM and 1 mM. At low substrate concentrations, activity is much lower with $\text{ATP/Mg}^{+2} = 2$ than with $\text{ATP/Mg}^{+2} = 1$. Once the adenosine concentration attains a saturation level, the maximal rate of phosphorylation levels off up to a concentration of 100 μM . The observed differences in affinity of substrate for the enzyme, with two different Mg^{+2} concentrations, are reflected by the difference in K_m values. The K_m values, determined from the initial reaction rates, are 0.6 μM and 2.3 μM for ATP/Mg^{+2} ratios of 1 and 2, respectively (Fig. 7 A).

Similarly, at pH 7.5, enzyme activity is much lower with an ATP/Mg^{+2} ratio of 2 than with the ratio of 1, while maximal activities are the same. The K_m values are lower than at pH 5.5, being 0.3 and 0.6 μM with ATP/Mg^{+2} ratios of 1 and 2 respectively (Fig. 7 B). Our apparent K_m values are in agreement with those reported for the enzyme from other sources [13, 29].

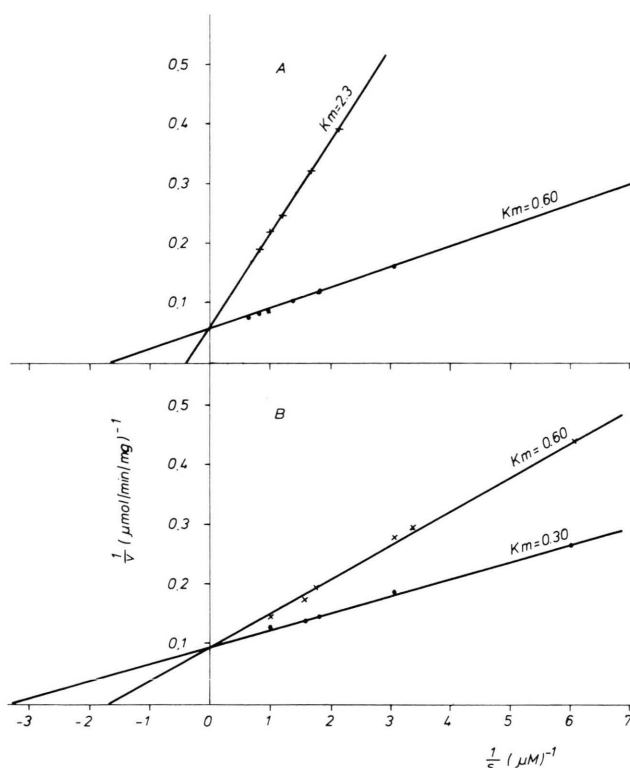


Fig. 7. Double-reciprocal plots of initial adenosine concentration with respect to reaction rate. Enzyme activity was determined at: A) pH 5.5; B) pH 7.5, in the presence of (××××), 1 mM ATP + 0.5 mM Mg^{+2} , or (●●●●) 1 mM ATP + 1 mM Mg^{+2} , with various adenosine concentrations.

Adenosine at concentrations above 1 μM with $\text{ATP/Mg}^{+2} = 1$, and above 2 μM with $\text{ATP/Mg}^{+2} = 2$, appreciably inhibits the reaction. At an adenosine concentration of 100 μM , activity is only 35–45% of the maximum (Fig. 6 B).

At pH 7.5 a similar dependence of K_m values on the ATP/Mg^{+2} ratio was observed with ribavirin as substrate (unpublished observation).

Discussion

It is of undoubted interest that the procedure of Andres and Fox [9] for isolation of adenosine kinase from human placenta proved even more efficacious for purification of the same enzyme from rat liver. Recoveries at the various steps (Table I) are similar except for the final affinity chromatography, which gave an overall yield of the rat liver adenosine kinase of 41% as compared to 27% for the human

placenta enzyme. Furthermore, following affinity chromatography, our preparation was close to homogeneity, whereas that from human placenta was estimated to be 68% pure. The specific activity of our preparation was also considerable higher, 18 $\mu\text{mol}/\text{min}/\text{mg}$, as compared to 3.5 $\mu\text{mol}/\text{min}/\text{mg}$ for the placental adenosine kinase. Bearing in mind that the isoelectric point of adenosine kinase from various sources is about 6.0, and is presumably well removed from the isoelectric point of enzyme(s) involved in the phosphorylation of deoxyadenosine, it appears to us that the DEAE-cellulose step of Andres and Fox [9] might profitably be applied to removal of such activities from crude extracts of the enzyme from other tissues and physiological fluids.

One apparent significant difference between the two preparations is in the molecular weight, 52 000 $\pm$ 2000 for the rat liver enzyme, and 41 000 for the placental preparation [9]. A value of 41 000 has also been reported for the enzyme from rat brain [11]. By contrast, our value is close to that for the rabbit liver enzyme, 51 000 [13] and for the enzyme from L1210 cells, 56 000 [10].

A molecular weight of 40 000 for rat liver adenosine kinase was reported by Ogasawara *et al.* [12], but their preparation differed from the present one in a very important respect, viz. during the 5-step procedure employed, the ratio of deoxyadenosine kinase to adenosine kinase activities was 0.7 throughout. It is perhaps not coincidental that this is close to the ratio (0.6, Table I) found for our first two steps. But in our procedure this ratio decreased dramatically following DEAE-cellulose treatment (Table I), as for the placenta enzyme [9], indicating that the deoxyadenosine phosphorylating activity is due to an enzyme with a different electrical charge.

By contrast, Ogasawara *et al.* [12] concluded that a single enzyme is responsible for phosphorylation of both adenosine and deoxyadenosine. Notwithstanding the reported homogeneity of their preparation, it clearly has little in common with that described in this study.

Some controversy still exists regarding the activity of adenosine kinase towards deoxyadenosine. Earlier studies demonstrated that deoxyadenosine kinase [18, 29] differs from adenosine kinase. Calf thymus deoxyadenosine kinase was initially reported to also phosphorylate deoxyguanosine [17]. Subsequently so-called deoxycytidine kinase from the same source was reported to phosphorylate

deoxyadenosine, deoxyguanosine and cytidine. More significantly, none of the foregoing preparations, active against deoxyadenosine, phosphorylated adenosine [17–20, 30].

In a study on mouse S49 lymphoma and human lymphoblastoid cells and some adenosine kinase-deficient mutants, Ullman *et al.* [15, 16] reported the presence of two enzymes which phosphorylated deoxyadenosine, adenosine kinase and deoxycytidine kinase. In attempts to clarify the role of each of these enzymes in phosphorylation of deoxyadenosine, appropriate mutants were isolated from WI-L2 lymphoblasts. The adenosine kinase-deficient mutant was resistant to deoxyadenosine and exhibited a much lower level of dATP than the parent strain. By contrast, a deoxycytidine kinase-deficient mutant was sensitive to deoxyadenosine and accumulated dATP from exogenous deoxyadenosine, whereas cell-free extracts of the adenosine kinase-deficient mutant phosphorylated deoxyadenosine like the parent strain. It was concluded by the authors [16], that measurements of phosphorylating activity towards deoxyadenosine in mammalian cell extracts may give misleading results regarding activity of enzymes phosphorylating deoxyadenosine in cells. In our opinion, the disagreement between activities of intact cells and cell extracts is not consistent with such a conclusion, and requires further clarification of the source of observed differences. Furthermore, in contrast to the foregoing, Willis *et al.* [32] found an unchanged level of activity vs 2'-deoxyadenosine in an adenosine kinase deficient mutant of the same cells.

As mentioned above, there are enzymes other than adenosine and deoxycytidine kinases which may be involved in phosphorylation of deoxyadenosine [17–20]. A recently described deoxyguanosine kinase from human placenta has been reported to phosphorylate also deoxyadenosine, but not adenosine or deoxycytidine [21]. Our observations indicate that rat liver contains enzyme(s) other than adenosine kinase capable of phosphorylating deoxyadenosine. Such activity, in crude rat liver extracts, is about 60% of the activity responsible for phosphorylation of adenosine, and our procedure virtually eliminates such activity (Table I). In addition, the low V_{max} and high K_m of our purified adenosine kinase preparation for deoxyadenosine *cf.* ref. [6], relative to the K_m for adenosine, suggests that phosphorylation of deoxyadenosine by adeno-

sine kinase under physiological conditions does not occur.

Furthermore, although we made no attempt to extend previous published observations on the regulation of adenosine kinase activity our findings are consistent with those of other authors [10, 12–14, 28]. The much lower value of K_m with an ATP/Mg²⁺ ratio of 1, relative to that prevailing with an excess of ATP, is in agreement with the postulated mechanism of phosphorylation in which the complex

MgATP⁻² is one of the substrates [29, 33], the observed decreased affinity of enzyme for substrate in the presence of free ATP, and the lack of phosphorylation in the absence of Mg²⁺.

Acknowledgments

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