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Properties of rat heart adenosine kinase

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1. Adenosine kinase was purified 870-fold from rat heart by a combination of gel filtration and affinity chromatography. The preparation was free of purine-metabolizing enzymes that could interfere in the assay of the kinase. 2. A study of the properties of the purified enzyme showed that it is activated by Na^+ and K^+, it possesses a broad pH optimum between 6 and 8, MgATP is the nucleotide substrate, free Mg^{2+} is an inhibitor with respect to both MgATP and adenosine, and the enzyme is subject to substrate inhibition by adenosine. The severity of this inhibition increases as the concentration of free Mg^{2+} increase. 3. The K_m for MgATP was calculated to be 0.8mM and that for adenosine, at likely physiological concentrations of MgATP and free $MgCl_2$, was about 0.2μM. *In vivo* the enzyme is likely to be saturated with both MgATP and adenosine. Indeed, the adenosine concentration in rat heart *in vivo* is probably sufficient to cause substrate inhibition, and this would be increased by an increase in free Mg^{2+} concentration. Changes in the concentrations of adenosine and free Mg^{2+} may play a role in modifying the activity of the enzyme *in vivo*.

Under certain circumstances, coronary blood flow may be controlled by the concentration of adenosine in the heart (for review, see Berne, 1980). It has been proposed that the adenosine concentration in tissues is regulated by the concerted activities of the enzymes that produce and utilize adenosine, 5'-nucleotidase (EC 3.1.3.5), adenosine deaminase (EC 3.5.4.4) and adenosine kinase (EC 2.7.1.20) (Arch & Newsholme, 1978*a*). Adenosine kinase phosphorylates adenosine, and the participation of the enzyme in cardiac adenosine metabolism has been demonstrated by the finding that radiolabelled adenosine is incorporated into the adenine nucleotide pool of the heart (Namm, 1973; Wiedmeier *et al.*, 1972). Knowledge of the properties of adenosine kinase may provide information on the role of this enzyme in the regulation of adenosine concentration. Although the enzyme has been the subject of several studies (e.g. Miller *et al.*, 1979; Chang *et al.*, 1980; Palella *et al.*, 1980; Yamada *et al.*, 1980), little work has been reported on the enzyme from heart. This paper reports a detailed study of the properties of adenosine kinase partially purified from rat heart.

Materials and methods

Animals, chemicals and enzymes

Male Wistar rats (200–300g) were obtained from OLAC (1976) Ltd., Blackthorn, Bicester, Oxon, U.K. All substrates and enzymes were obtained from Boehringer Corp. (London), Lewes, East Sussex, U.K. Ion-exchange resin (AG1-X8, 200–400 mesh) was supplied by Bio-Rad Laboratories, St. Albans, Herts., U.K. Whatman DEAE-cellulose discs (DE 81) were from W. and R. Balston (Modified Cellulose), Maidstone, Kent, U.K. Sephadex G-100 (40–120μm bead size) and 5'-AMP–Sepharose 4B were from Pharmacia (G.B.), London W.5, U.K. Radiochemicals were obtained from The Radiochemical Centre, Amersham, Bucks., U.K. Tris/maleate and bovine serum albumin (fraction V) were from Sigma, Poole, Dorset, U.K. All other chemicals were from BDH, Poole, Dorset, U.K.

Enzyme assays

Adenosine kinase, ATPase and adenylate kinase were assayed by a continuous spectrophotometric

method (Lindberg *et al.*, 1967), the products of each reaction being linked to the oxidation of NADH. The standard incubation medium (total volume 1ml) consisted of 20mM-Tris/maleate buffer, pH5.8, 1mM-$MgCl_2$, 1mM-ATP, 0.25mM-phosphoenolpyruvate, 0.2mM-NADH, 50μg of pyruvate kinase and 25μg of lactate dehydrogenase. This standard incubation medium was used for the assay of ATPase. For the assay of adenosine kinase, the incubation medium also contained (final concn.) 12μM-adenosine and 25μg of adenylate kinase. Adenosine was omitted when assaying column fractions which contained the nucleoside. For the assay of adenylate kinase, the standard incubation medium contained, in addition, 1mM-AMP. AMP deaminase was assayed by the method of Chaney & Marbach (1962), adenosine deaminase by the method of Kalckar (1947) and 5'-nucleotidase by the method of Baer *et al.* (1966). Protein was assayed by the method of Bradford (1976).

Purification of adenosine kinase

Rat heart adenosine kinase was partially purified by a combination of gel filtration (Namm & Leader, 1974) and affinity chromatography (De Jong *et al.*, 1980). During preliminary work it was observed that considerable loss of enzyme activity occurred when adenosine kinase was present in a solution containing a low protein concentration. Hence, for purification, a procedure was developed which directly combined the elution of adenosine kinase from a gel-filtration column with the adsorption of the enzyme on to an affinity column.

The buffer used throughout the purification procedure consisted of 4mM-NaH_2PO_4, 2mM-EDTA and 5% (v/v) glycerol at pH7.0. All procedures were carried out at 4°C. The methods for the preparation of the high-speed supernatant of rat ventricle and its subsequent gel filtration on a Sephadex G-100 column (2.5cm × 40cm) were as described by Namm & Leader (1974). During the elution of the heart extract from the gel-filtration column, the passing of the void volume was monitored by the activity of an ATPase that was found to be eluted with the excluded protein fraction. The included protein fraction of the column, which contained the activity of adenosine kinase, was then passed directly from the gel-filtration column on to the affinity column (1cm × 7cm) containing 5'-AMP–Sepharose 4B (De Jong *et al.*, 1980). After the elution of the included protein fraction through the coupled column system, the affinity column was disconnected, and unbound protein present in the column was washed out. Adenosine kinase was eluted from the affinity column with buffer containing 0.6mM-adenosine. Pooled fractions from the affinity column were supplemented with bovine serum albumin (0.1mg/ml) and dialysed to equilibrium against buffers containing 5, 10, 15 and 20% (v/v) glycerol. Dialysed enzyme free of adenosine was stored in batches at −65°C.

The specific activity of adenosine kinase in peak activity fractions from the affinity column was about 2μmol/min per mg of protein, a purification of 870-fold from the high-speed supernatant. The enzyme preparation was free of purine-metabolizing enzymes that could interfere in the assay of adenosine kinase; AMP deaminase and 5'-nucleotidase were present in the void volume of the gel-filtration column, whereas adenosine deaminase, adenylate kinase and some of the ATPase activity were readily washed through the affinity column.

Adenosine kinase assays

At saturating adenosine concentration, adenosine kinase was assayed by the radiochemical method of De Jong & Kalkman (1973). The reaction was started by the addition of 5μl of suitably diluted enzyme solution to 15μl of assay medium, and the assay was conducted at 37°C. The composition of the incubation medium, varied by additions to the standard condition, was 50mM-triethanolamine, 0.0625–2.0mM-ATP, 0.125–2.0mM-$MgCl_2$, 4μM-adenosine containing 0.3μCi of [U-^{14}C]adenosine/ml, at pH7.0. The reaction was stopped by removing a small volume (17μl) and spotting it on DEAE-cellulose filter discs (2.5cm diameter). The discs were washed three times with 1mM-ammonium formate (20ml/disc) by decantation, dehydrated in ethanol, dried at 70°C, and the radioactivity on discs was measured in 5ml of scintillation fluid [4g of 2,5-diphenyloxazole and 0.1g of 1,4-bis-(5-phenyloxazol-2-yl)-benzene per litre of toluene]. In substrate-selectivity studies, labelled adenosine was replaced by [U-^{14}C]deoxyadenosine or [U-^{14}C]inosine, both at final concentrations of 4μM (0.3μCi/ml), or ATP was replaced by other nucleoside triphosphates at 1mM.

At sub-saturating adenosine concentration, adenosine kinase was assayed radiochemically by using small columns of ion-exchange resin to separate product from substrate (Sullivan & Alpers, 1971). The reaction was started by the addition of 25μl of suitably diluted enzyme solution to 175μl of assay medium, and the assay was conducted at 37°C. The assay medium consisted of 50mM-triethanolamine, 2.0–5.5mM-$MgCl_2$, 4.5mM-ATP, 0.1–13μM-adenosine and 0.06μCi of [U-^{14}C]adenosine/ml at pH7.0. Incubation was for 2–8min, the shorter times being used at the lower adenosine concentrations. The reaction was stopped by the addition of 200μl of

industrial methylated spirit, after which the mixture was transferred to small resin columns, followed by two 0.4ml washes of the incubation tube with deionized water. The columns consisted of disposable glass Pasteur pipettes plugged with cotton wool and containing the resin AG1-X8 (Cl^- form) to a height of 2cm. Labelled adenosine was washed from the columns with 4.5ml of deionized water, with a recovery from a standard amount applied of 99%. Labelled AMP was then eluted from the column directly into scintillation vials by the application of 2.5ml of 0.1M-HCl. The recovery from a standard amount of labelled AMP applied was 98% in these conditions. Radioactivity in vials was measured after addition of 15ml of scintillation fluid [containing 6g of 2,5-diphenyloxazole and 0.2g of 1,4-bis-(5-phenoxazol-2-yl)-benzene per litre of toluene/Triton X-100 (2:1, v/v)].

Repeated freezing and thawing of the enzyme preparation caused loss of activity, and thus a separate frozen sample was used for each experiment. To prevent loss of activity on dilution before assay, the stock enzyme solution was diluted into buffer containing 0.1mg of bovine serum albumin/ml.

Expression of results

Adenosine kinase activity is expressed in units of pmol/min per μl of undiluted enzyme solution. The concentrations of MgATP and of free Mg^{2+} present in assays were calculated from the ATP and $MgCl_2$ concentrations by the method of Wong (1975), by using the value of 0.022mM for the dissociation constant of MgATP (Philips *et al.*, 1966). Some data have been replotted as double-reciprocal plots of activity against substrate concentration. Where substrate inhibition was indicated, plots of reciprocal activity against adenosine concentration were used to calculate the substrate inhibition constant (Laidler & Bunting, 1973).

Results

Substrate selectivity and pH optimum

Adenosine kinase showed no activity with deoxyadenosine or inosine as the nucleoside substrate. Similarly, no activity was observed with CTP or UTP as substrates, but CTP and ITP produced 74% and 35% respectively of that with ATP as substrate. Adenosine kinase exhibited a broad pH optimum between 6 and 8 and retained activity even at pH10 (Fig. 1).

Effects of NaCl and KCl

The activity of adenosine kinase was increased in a hyperbolic manner by increases in concentration of NaCl (Fig. 2). The maximum increase of

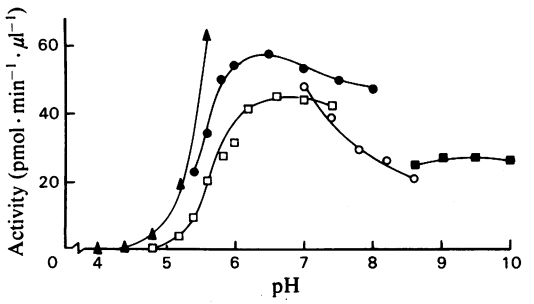


Fig. 1. *Effect of pH on the activity of rat heart adenosine kinase*

The kinase was assayed at 4μM-adenosine, 0.75mM-$MgCl_2$ and 2mM-ATP in various buffers (concn. 50mM) at the pH values indicated; ▲, acetate; □, citrate/phosphate; ●, Tris/maleate; ○, triethanolamine; ■, glycine.

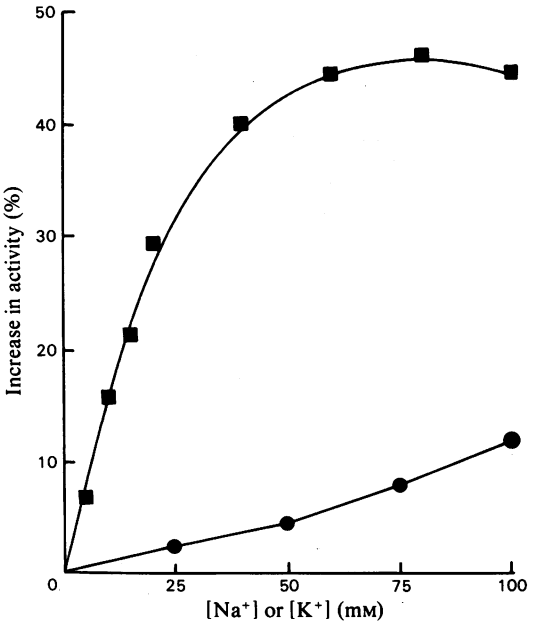


Fig. 2. *Effects of Na^+ and K^+ concentrations on the percentage increase in activity of rat heart adenosine kinase*

The kinase was assayed at 4μM-adenosine, 1mM-ATP and 0.5mM-MgCl. ■, Na^+; ●, K^+.

46% occurred in the range 60–100mM-NaCl. The activity of the enzyme was also increased by KCl, but by only 12% at the highest concentration (100mM-KCl). At these concentrations other ions (choline) had no effect, so that the stimulation by Na^+ is unlikely to be an effect of ionic strength.

Effects of $MgCl_2$ and ATP

There was no apparent activity of adenosine kinase in the absence of Mg^{2+} (Fig. 3). The enzyme

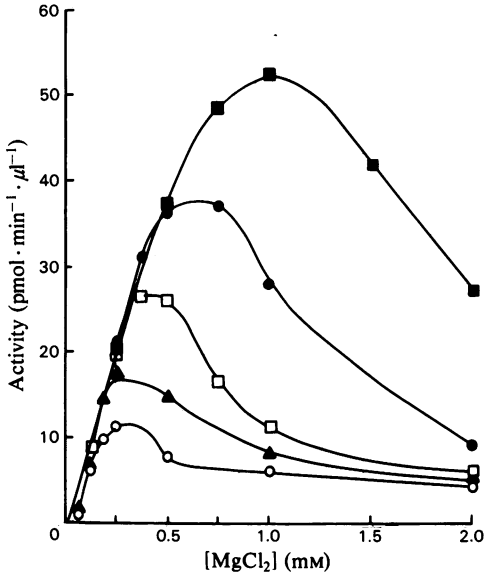


Fig. 3. *Effect of $MgCl_2$ concentrations on the activity of rat heart adenosine kinase at various ATP concentrations*
The kinase was assayed at 4 μM-adenosine and various concentrations of $MgCl_2$ in the presence of (○) 0.125mM-, (▲) 0.25mM-, (□) 0.5mM-, (●) 1.0mM- and (■) 2.0mM-ATP.

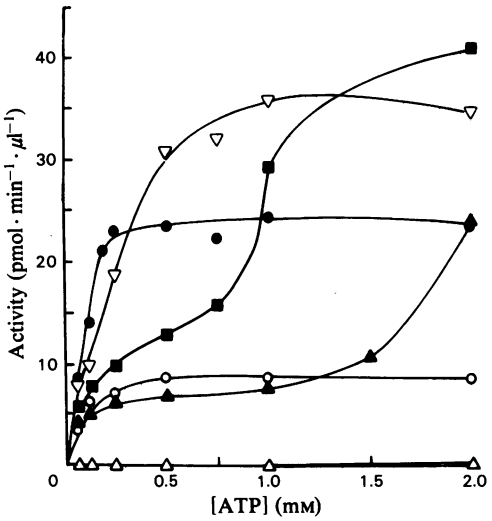


Fig. 4. *Effect of ATP concentration on the activity of rat heart adenosine kinase at various $MgCl_2$ concentrations*
The kinase was assayed at various concentrations of ATP in the absence of (△) and in the presence of (○) 0.125mM-, (●) 0.25mM-, (▽) 0.5mM-, (■) 1.0mM- and (▲) 2.0mM-$MgCl_2$.

was active in the presence of various fixed $MgCl_2$ concentrations, but the relationship between activity and the ATP concentration was found to vary in a way that was dependent on the fixed $MgCl_2$ concentration (Fig. 3). At fixed $MgCl_2$ concentrations of 0.125, 0.25 and 0.5mM the activity of adenosine kinase increased in a hyperbolic manner with that of ATP (from 0.0625 up to 2mM). However, in the presence of 1mM- and 2mM-$MgCl_2$, the activity increased very little at low ATP concentrations, but rose markedly as the ATP concentration increased from 0.75 up to 2mM.

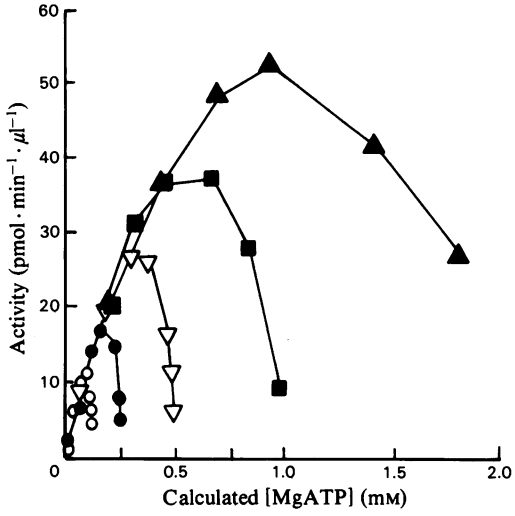


Fig. 5. *Effect of MgATP concentration (calculated) on the activity of rat heart adenosine kinase at various concentrations of ATP*
The data of Fig. 4 are replotted as activity of adenosine kinase against the calculated MgATP concentration present in the assay. For the data in this replot, the points at the various fixed ATP concentrations given in Fig. 4 are joined together; these concentrations are indicated by 0.125mM (○), 0.25mM (●), 0.5mM (▽), 1mM (■) and 2.0mM (▲).

When $MgCl_2$ concentration was varied in the presence of various fixed ATP concentrations, there appeared to be a specific $MgCl_2$ concentration that gave an optimum activity at each fixed ATP concentration (Fig. 4). The activity of adenosine kinase at each fixed ATP concentration increased with increasing $MgCl_2$ concentration until the optimum $MgCl_2$/ATP concentration ratio was reached, and thereafter decreased with increasing concentrations of $MgCl_2$.

The lack of activity of adenosine kinase in the absence of $MgCl_2$ (Fig. 3) suggested that the true nucleotide substrate for the enzyme is MgATP. To examine this possibility, the data of Fig. 4 were replotted as activity against calculated MgATP concentration. In this replot (Fig. 5) some of the points now lie on or close to a common curve, supporting the view that MgATP is the true substrate. The free Mg^{2+} concentrations at these points of the common curve were calculated and in

all cases appeared to be less than 0.22mM, whereas the calculated free Mg^{2+} concentrations for the remaining data ranged from 0.1 up to nearly 2mM. A double-reciprocal plot of activity against calculated MgATP concentration for the data of the common curve was linear and gave values for MgATP of K_m 0.8mM and V of 100pmol/min per μl. From inspection of the calculated free ATP concentrations present, there appeared to be no correlation between these and the activity of the enzyme. Thus it is considered that MgATP is the true nucleotide substrate of adenosine kinase and that free ATP has no effect on the activity of the enzyme. The decreases in the activity at similar calculated MgATP concentration correlated with an increasing calculated free Mg^{2+} concentration present (see above), suggesting that free Mg^{2+} is an inhibitor of adenosine kinase (Fig. 6). This can also be deduced from the data of Fig. 4, where, at the lower fixed ATP concentrations, the activity of the enzyme is inhibited when the $MgCl_2$ concentration was in excess of the ATP concentration. The data in Figs. 4, 5 and 6 suggest that the degree of inhibition by free Mg^{2+} increases with increasing MgATP concentration. These findings suggest that the inhibition caused by free Mg^{2+} is either uncompetitive or mixed with respect to MgATP concentration.

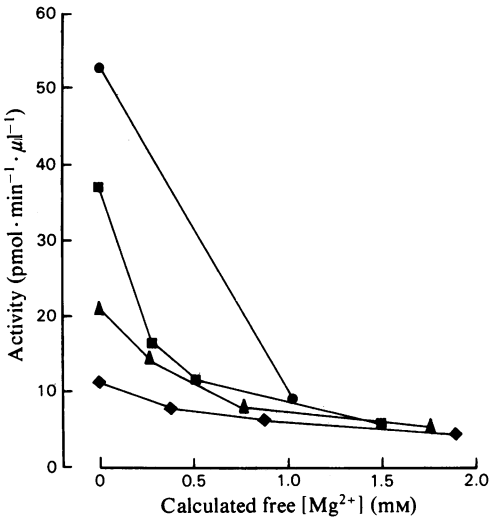
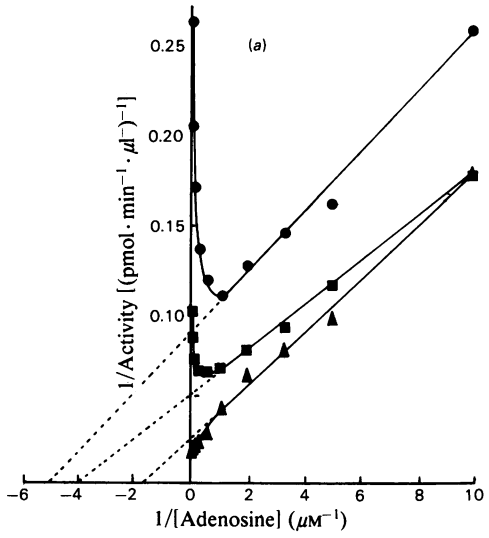


Fig. 6. *Effect of free Mg^{2+} (calculated) on the activity of rat heart adenosine kinase at various MgATP concentrations (calculated)*

The activity of adenosine kinase at similar calculated MgATP concentrations of 0.10–0.12mM (♦), 0.23–0.25mM (▲), 0.47–0.49mM (■) and 0.98mM (●) are plotted against increasing calculated free Mg^{2+} concentrations. These are selected data replotted from the results presented in Fig. 4.

Effects of adenosine

Double-reciprocal plots of the activity of adenosine kinase against adenosine concentration obtained at 4.5mM-ATP and in the presence of 2mM-, 4.5mM- and 5.5mM-$MgCl_2$ are shown in Fig. 7(*a*). In the presence of 2mM- and 4.5mM-$MgCl_2$, the



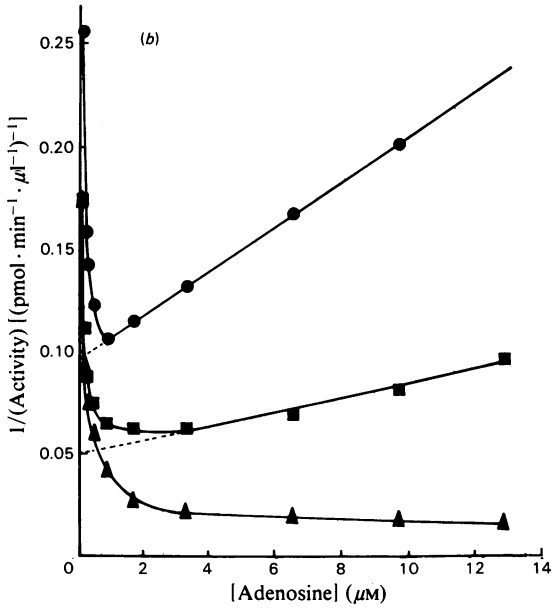


Fig. 7. *Effect of adenosine concentration on the activity of rat heart adenosine kinase at various concentrations of $MgCl_2$*

(*a*) Double-reciprocal plot; (*b*) adenosine concentration versus reciprocal of activity. The kinase was assayed at various concentrations of adenosine and at 4.5mM-ATP in the presence of (▲) 2.0mM-, (■) 4.5mM- and (●) 5.5mM-$MgCl_2$.

Table 1. *Summary of the values of K_m, V and K_{IS} of adenosine kinase at various concentrations of MgATP and free Mg^{2+}*
The kinetic constants tabulated below were calculated from the plots presented in Fig. 7, and the methods for calculation of the concentrations of free Mg^{2+} and MgATP are given in the Materials and methods section. The total ATP concentration was 4.5mM.

	Calculated concentrations		Kinetic constants		
Total [$MgCl_2$] (mM)	MgATP (mM)	Free Mg^{2+} (mM)	K_m (adenosine) (μM)	V (pmol·min^{-1}·μl^{-1})	K_{IS} (μM)
2.0	2.0	0.02	0.59	38.1	∞
4.5	4.2	0.3	0.25	19.7	13.8
5.5	4.4	1.0	0.20	11.4	8.3

plots curved upward at high adenosine concentration, indicating substrate inhibition by adenosine. The kinetic constants calculated from these plots and the substrate inhibition constants calculated from plots of reciprocal of activity against adenosine concentration (Fig. 7*b*) are given in Table 1. It is important to note that the occurrence of substrate inhibition correlated with the presence of high calculated free Mg^{2+} concentrations. In fact, the severity of substrate inhibition was increased with the 3-fold increase in free Mg^{2+} concentration (this is indicated by the lowering of the substrate inhibitor constant from 13.8 to 8.3 μM; see Table 1). It should be noted that, at low concentrations of free Mg^{2+}, high concentrations of adenosine appear to activate the enzyme (Fig. 7*a*). The reason for this effect is not known, but it is unlikely to be of physiological importance.

Discussion

Properties of adenosine kinase

It is considered that three important properties of rat heart adenosine kinase was established in the present work: MgATP is the nucleotide substrate; free Mg^{2+} is an inhibitor (uncompetitive or mixed) of the enzyme with respect to both MgATP and adenosine; and the enzyme is subject to substrate inhibition, the occurrence and severity of which are dependent on the concentration of free Mg^{2+}.

That MgATP is the nucleotide substrate and that free Mg^{2+} is inhibitory have also been shown for adenosine kinase from other tissues (Miller *et al.*, 1979; Kyd & Bagnara, 1980; Palella *et al.*, 1980). The present work has identified the pattern of inhibition with respect to both adenosine and MgATP concentration. Substrate inhibition by adenosine has also been reported for the rat heart enzyme (Arch & Newsholme, 1978*b*; De Jong & Keijzer, 1979), but the present work identifies the importance of the concentration of free Mg^{2+}.

To test further the relationship between the activity of adenosine kinase and the concentrations of ATP and of $MgCl_2$ proposed here, some of the data have been compared with predicted activities which have been calculated by using a simple kinetic model based on the current findings. It is assumed that the activity of adenosine kinase, at a fixed concentration of adenosine, is given by the following equation (see Laidler & Bunting, 1973):

$$v = \frac{V[\text{MgATP}]}{K_m + \left(1 + \frac{[Mg^{2+}]}{K_i}\right)\cdot[\text{MgATP}]}$$

The constants were derived for the fixed adenosine concentration of 4 μM and were as follows: K_m, 0.8mM, V, 100pmol/min per μl, K_i, 0.1mM; in addition, the concentrations of MgATP and free Mg^{2+} were calculated as described in the Results section. For the results described in Fig. 4, the calculated activities were compared with the corresponding observed activities, and there was a strong correlation ($r = 0.987$, $n = 35$, $P < 0.001$). A similar correlation was observed with the results in Fig. 3 ($r = 0.964$, $n = 29$, $P < 0.001$).

Previous studies of cardiac adenosine kinase have not been sufficiently detailed to enable the precise relationship between activity, $MgCl_2$ and ATP to be determined. De Jong (1977) was able to show that concentrations of $MgCl_2$ in excess of ATP were inhibitory, but only one fixed ATP concentration was studied: free ATP was regarded as the nucleotide substrate, since in the presence of low fixed concentrations of $MgCl_2$ the activity of the enzyme increased in an hyperbolic manner with increasing ATP concentration. This was also found in the present study (Fig. 3), but can now be recognized as fortuitous. At higher fixed $MgCl_2$ concentrations, the hyperbolic relationship is lost, owing to inhibition by the high free Mg^{2+} concentrations present. Free ATP has also been considered to be inhibitory for cardiac adenosine kinase (Alma & Feinberg, 1971; Olsson *et al.*, 1972), but there was no evidence in the present work to substantiate this.

The presence of Na^+ increased the activity of adenosine kinase. This property of the enzyme differed at similar pH but in different buffers. Tris/maleate buffers contain Na^+ ions that are introduced when adjusting to the required pH with NaOH, whereas triethanolamine is adjusted with acid. Thus the higher activity in the former buffer than in the latter could be due to Na^+ (and see Arch & Newsholme, 1978*b*). While considering buffers, it is important to note that P_i, dicarboxylic and tricarboxylic acids can bind Mg^{2+} (O'Sullivan, 1969).

Physiological activity of adenosine kinase

Adenosine kinase catalyses a non-equilibrium reaction in heart (Arch & Newsholme, 1978*a*). Thus the physiological activity of the enzyme can be deduced from a comparison of its properties with the concentrations of substrates and inhibitor measured for the heart (see Newsholme & Crabtree, 1976). Since these properties include the effects of ATP, it is important to know the concentration of this compound that would be available to the enzyme. However, although the problem of adenine nucleotide compartmentation is of considerable interest and relevance (Ottaway & Mowbray, 1977), firm data on the precise concentrations of ATP at specific sites in the cell are not yet available.

The ATP content of rat heart is about 4.5 μmol/g fresh wt. (Williamson, 1966). Phosphorus-n.m.r. studies indicate that at least 95% of the ATP of muscle is complexed with Mg^{2+} (Hoult *et al.*, 1974), and so the ATP content of heart can be taken as an approximation of the MgATP content. We have calculated a K_m value of adenosine kinase for MgATP of 0.8 mM, which is 4-fold less than the concentration of this substrate in heart. The K_m value for MgATP could in fact be lower *in vivo*, since free Mg^{2+} in heart would decrease this constant by uncompetitive inhibition. The precise free Mg^{2+} content of heart is unknown, but it is likely to be in the order of 1 mM (Veloso *et al.*, 1973). The current estimate of the inhibitor constant for free Mg^{2+} is 0.1 mM, which is 10% of the value for the Mg^{2+} content of the heart, and thus the effect of free Mg^{2+} will be significant *in vivo*. Hence it is suggested that adenosine kinase is saturated with MgATP *in vivo*, and changes in MgATP content will have little effect on the activity of the enzyme.

The basal adenosine content of rat heart is about 4 nmol/g fresh wt. (Frick & Lowenstein, 1976; Foley *et al.*, 1978), most of which is probably located intracellularly (see Schrader *et al.*, 1977). The K_m value of adenosine kinase for adenosine, 0.20–0.25 μM, obtained at concentrations of MgATP and free Mg^{2+} that may be present *in vivo* (see Table 1), is considerably lower than the basal adenosine content. Thus adenosine kinase will be saturated with substrate *in vivo*. However, since the enzyme is inhibited by concentrations of substrate above saturation, the activity should fall when the adenosine content of the heart rises (e.g. after short periods of ischaemia; Berne & Rubio, 1974). Substrate inhibition was not apparent at low free Mg^{2+} concentration, but was present and increased in severity with increases in free Mg^{2+}. It was suggested above that the free Mg^{2+} concentration of heart is 1 mM, a concentration at which substrate inhibition did occur (see Table 1).

The physiological activity of adenosine kinase could be decreased in a manner that was independent of changes in adenosine concentration. An increase in free Mg^{2+} concentration in heart should decrease the adenosine concentration at which substrate inhibition occurs and should decrease the maximal activity. As yet, there is no direct evidence for changes in free Mg^{2+} concentration in heart. The decrease in the physiological activity of adenosine kinase is predicted by the data presented here, and this decrease in the rate of adenosine utilization in the heart may contribute to the large increases in adenosine content that occur after ischaemia and hypoxia (Berne & Rubio, 1974; Rubio *et al.*, 1974).

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