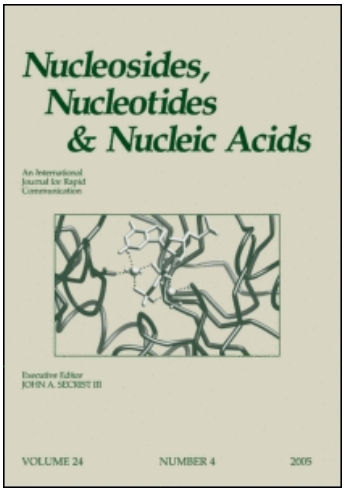

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ADENOSINE KINASE FROM RAT LIVER: NEW BIOCHEMICAL PROPERTIES

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□ *Adenosine kinase is a well-known enzyme which catalyzes the phosphorylation of adenosine to AMP: Its metabolic and kinetic properties are well studied. Here, we report new properties of rat liver enzyme, demonstrating a new reaction: ADP can be a phosphate donor instead ATP, according to the reaction: adenosine + ADP → 2AMP) demonstrating the efficiency of AdK to phosphorylate adenosine, also starting from ADP. Cells could exploited this property in situations in which ATP levels are strongly decreased and ADP decreases slowly.*

Keywords Adenosine kinase; Purine metabolism; Adenosine; ADP

INTRODUCTION

It is well known that adenosine (ADO) is present both in cell cytosol, plasma, and extracellular spaces and plays important roles in the life of cells: changes in ADO concentrations regulate a number of important physiological processes, such as rate of blood flow in the heart, skeletal muscle and brain, rate of lipolysis in adipose tissue, and neurotransmission in the brain.[1] Many reports also have indicated general immunosuppressive and anti-inflammatory properties,[2] underlying its role in regulation of the immune system, stimulation of angiogenesis and inhibition of inflammatory reactions at the site of injury.[3] Such actions are carried out through protein-coupled receptors: A_1AR, $A_{2a}AR$, $A_{2b}AR$, A_3AR.

Adenosine is produced both in the cell and at the cell surface from AMP respectively by soluble 5'nucleotidase (e-Ns) or membrane-bound 5'nucleotidase (ecto 5'-NT) (EC 3.1.3.5); its internal levels are controlled by four enzymes: e-Ns, S-adenosyl homocysteine hydrolase (EC 3.3.1.1), adenosine deaminase (ADA, EC 3.5.4.4), which splits it to inosine and

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adenosine kinase (AdK, EC 2.7.1.20) which transforms adenosine into AMP, regulating the AMP-ADP-ATP pool, according to the following reaction (Reaction 1):

$$\text{Adenosine} + \text{ATP} \rightarrow \text{AMP} + \text{ADP}$$

Magnesium also is required in this reaction and is probably associated with the substrate nucleotide.[4,5]

AdK is abundant and ubiquitous in eukaryotes and is a key enzyme in the regulation of both extra cellular adenosine and intracellular concentrations of adenine nucleotides. In addition, it is responsible for the phosphorylation and consequent clinical activity of several therapeutically useful nucleosides, including the antiviral drug ribavirin and the immunosuppressive drug mizoribine.[6,7] AdK has been purified from a number of sources[7–11] and extensively characterized at the kinetic level.[1] The enzyme is strongly inhibited by adenosine concentrations above 5 μM; substrate specificity has also been studied: Deoxyadenosine, arabinoadenosine, and inosine are phosphorylated, whereas other nucleosides such as guanosine, cytidine, and uridine and their deoxy correspondents are not; ATP, dATP, GTP, dGTP, and UTP are phosphorous donors and GTP, dGTP, and dATP were more effective than ATP in rats.

The enzyme has been cloned from both rat and human tissues and expressed in *E. coli*.[12,13] Two distinct forms of AdK mRNA were identified with variations restricted to the extreme 5'-end, consistent with differential splicing of a single transcriptional product.[13]

Here we describe a new properties of rat liver AdK: ADP can be a phosphate donor according to the reaction (Reaction 2):

$$\text{adenosine} + \text{ADP} \rightarrow 2\text{AMP}$$

MATERIALS AND METHODS

We purified AdK to homogeneity from rat liver according to slight modifications of Yamada et al. procedure.[7]

Supernatant, derived from ultracentrifugation of fresh homogenized rat liver, was submitted to a cationic exchange chromatography on a CM-sepharose column; active fractions were pooled and solid $(NH_4)_2SO_4$ was added to 45% saturation. After centrifugation, solid $(NH_4)_2SO_4$ was added to the supernatant at 80% saturation. The solution was centrifuged, the pellet dissolved in buffer, dialyzed and applied to an affinity chromatography column of AMP-Sepharose. The AdK activity was eluted adding 5 mM adenosine to the buffer and active fractions were applied to a gel-filtration Superdex column. The last step of the procedure consisted in an anionic exchanged chromatography, on a DE-52 cellulose column.

The final fraction shows only a single protein band by silver stained SDS-PAGE[14] or analysed by nano ESMS (MW 38,345): because of the high sensitivity of the methods used for the detection (less than 50 ng), we can exclude any contamination with other related activities such as ADA, 5'nucleotidase (5'NT) which catalyzes the dephosphorylation of AMP to adenosine or adenylate kinase which generates ATP starting from 2ADP.

The pure enzyme was active when assayed for conventional AdK reaction (Reaction 1) showing specific activity, Vmax and Km values for substrates in the same order of magnitude than the literature.[15]

Reaction 2 was assayed incubating the enzyme with Mg^{+2}, ^{14}C adenosine, and ADP (we purified the substrates by HPLC to avoid the presence of contaminant triphosphates). The ^{14}C AMP formed was separated on HPLC column and quantified on the base of the peak area and radioactivity content (the specific activity of the product was compatible with the stoiochiometry of the reaction). The addition of 0.05 mM HENA and 0.1 mM AMP-CP (potent inhibitors respectively of ADA and 5'NT) to the mixture was negligible. The stoichiometry of the reaction was verified: two moles of AMP were produced for mole of ADP consumed.

The reaction was characterized for time course, linearity with protein concentrations, effect of substrates and Mg^{+2} concentrations and the corresponding Km values.

RESULTS

Kinetic properties of AdK from several sources are reported in the literature: rat liver or rat brain enzymes shows the same apparent Km value, that is 0.2 μM for adenosine and 0.02 mM for ATP.[15] This value is much higher in *Lupinus luteus*[16] or in rat heart[17] (0.3 and 0.8 mM respectively).

No data are reported in literature about the possibility that ADP can be a phosphate donor.

Figure 1 (A, B, C) reports the Michaelis–Menten plots for Adenosine, ADP and Mg^{+2}. We demonstrate that, using purified preparation of rat liver AdK, the affinity between the enzyme and adenosine does not vary so much if ADP substitutes ATP in the reaction. In this condition the Km value for adenosine, evaluated at fixed ADP concentration was calculated to be 0.83 μM; the Km value for ADP (1.92 mM) evaluated at fixed adenosine concentration was higher than ATP (about 95 times, but only 2.4 times higher than in rat heart). The affinity versus Mg^{+2} was also evaluated incubating concentrations of Mg^{+2} ranging from 0.5 to 8 mM at fixed concentration of Adenosine (2 μM) and ADP (5 mM). The corresponding Km value was 2.1 mM.

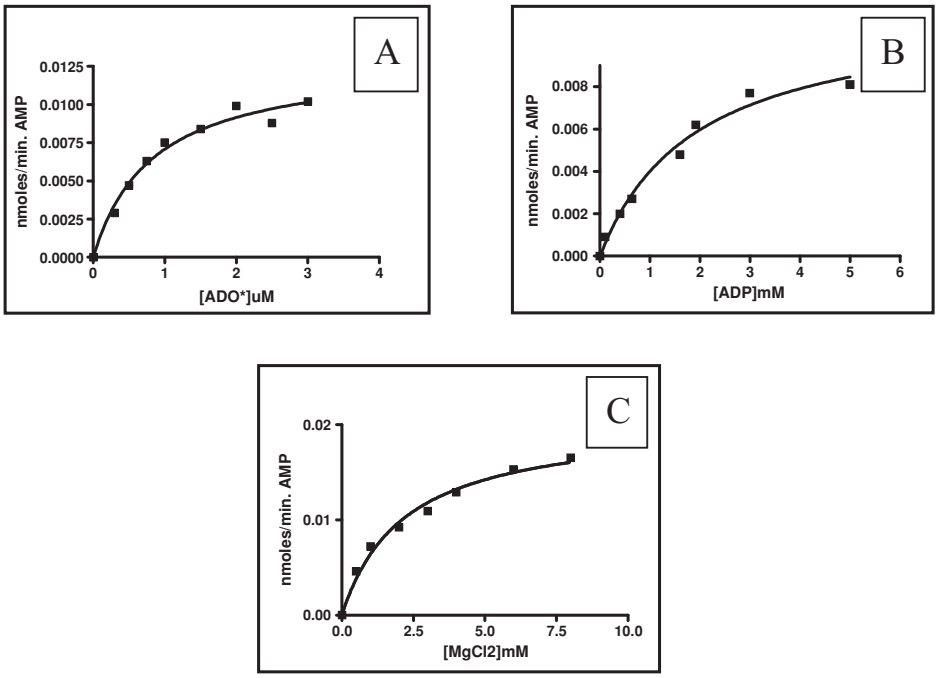

FIGURE 1 Michaelis-Menten plots obtained incubating in the assay mixture containing 0.2 ug protein: **A**: several adenosine concentration (0.3–3 μM) and fixed ADP concentration (5 mM). Km value for adenosine: 0.83 μM; **B**: several ADP concentrations (0.1–5 mM) and fixed Adenosine concentration (2 μM). Km value for ADP: 1.92 mM; **C**: several Mg^{+2} concentrations and fixed Adenosine (2 μM) and ADP (5 mM) concentrations. Km value for Mg^{+2} : 2.1 mM.

Reaction 2 was linear in the range of 0.1–0.5 μg protein and time and ADP could be substitutes by other nucleotides diphosphate. We demonstrated that UDP, GDP, CDP, and dADP can be phosphate donors, more or less efficiently than ADP (Table 1).

TABLE 1 Substrates Alternative to ADP in AdK Reaction 2

Nucleotide	AdK activity (IU)	% respect to ADP
ATP	860	
ADP	12.46	100
dADP	31.78	255
GDP	29.91	240
CDP	3.49	28
UDP	4.98	40

5 mM of each nucleotide diphosphate was added to the incubation mixture. 1 IU corresponded to the nmoles of AMP formed / min/mg protein.

DISCUSSION

Our results deserve attentive scrutiny and some considerations on the reaction:

$$\text{ADP} + \text{adenosine} \rightarrow 2\ \text{AMP}$$

It is well known that the ratio of adenosine phosphorylation to adenosine deamination is important in determining the fate of adenosine and that there is active cycling between adenosine and AMP in many cells: AdK plays an important role in such regulation.[2]

From our results we may deduce that such regulation may be sometime partially exerted by ADP. Such control could be important in all the circumstances when ATP is decreased at high extent because of the block of its synthesis or its high requirement and at the same time the decrease of ADP is much less relevant. This may occur under several circumstances, such as during particularly strong muscular work, fructose-induced hyperuricemia with ATP depletion,[18] or severe nucleotide depletion as in rheumatoid arthritis.[19] A striking example is represented by temporary anoxia subsequent cardiac surgery, when it was demonstrated that during aortic cross –clamping and successive clamp removal, ATP and AMP concentrations vary significantly, while ADP levels remain in the range of basal levels[20] and might be sufficient to contribute to eliminate adenosine by phosphorylation.

In all this temporary ATP-deficient situations, AdK can, using residual ATP and through our reaction, continue to exploit its critical role in regulation of cellular levels of adenosine.

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