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GENERAL AND KINETIC PROPERTIES OF PIG HEART MITOCHONDRIAL ADENYLATE KINASE

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Summary

The precise localization of adenylate kinase (ATP:AMP phosphotransferase, EC 2.7.4.3) has been studied in pig heart mitochondria. This enzyme, which was distinct from the cytoplasmic enzyme, was insensitive towards SH reagents and exhibited a relatively weak inhibition by the specific inhibitor P_1, P_5-diadenosine-5′-pentaphosphate. The enzyme has been partially purified from isolated mitochondria. In the forward reaction adenylate kinase was very specific for AMP and less specific for the ATP site. Kinetic studies showed that in the forward direction, K_{MgATP} and K_{AMP} the dissociation constants of the substrates from the binary complexes were lower than the dissociation constants from the ternary complexes. In the reverse direction K_{MgADP} was higher than K_{ADP}, but these values were not modified by the binding of the other substrate. In the forward direction, the enzyme was inhibited by excess of substrate when AMP concentrations were greater than 1 mM. This inhibition could prevent the phosphorylation of AMP to ADP and thus decrease the amount of adenine nucleotides available for oxidative phosphorylations.

Introduction

Adenylate kinase (ATP:AMP phosphotransferase, EC 2.7.4.3) plays an important role in the energetic metabolism of the cell. When energy expenditure exceeds energy synthesis as in myocardial ischemia [1] it allows utilisation of energy from the β-phosphate group of ADP and transient synthesis of ATP. As reported in a review by Noda [2] adenylate kinase is an ubiquitous enzyme,

Abbreviations: DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); Ap_5A, P_1, P_5-diadenosine 5′-pentaphosphate.

which is mainly cytoplasmic in muscle and mitochondrial in liver. However, in pig heart, we have shown [3] that a significant percentage of adenylate kinase activity was associated with mitochondria. In the present paper we describe the localization and some properties of mitochondrial adenylate kinase, in situ; this enzyme has been found to be identical to the acidic pig heart adenylate kinase described by Itakura et al. [4]. If detailed kinetic studies have been reported on cytosolic adenylate kinase from muscle or mitochondrial enzyme from liver [5] no information was available so far on the kinetic properties of mitochondrial adenylate kinase from muscle in general. In this work, we have thus carried out a detailed study of equilibrium constant, specificity and kinetic properties of adenylate kinase purified from isolated pig heart mitochondria.

Material and Methods

Mitochondria were isolated from pig heart homogenate by differential centrifugation, washed and resuspended in 0.25 M sucrose, 10 mM Tris-HCl, pH 7.4.

Fractionation of mitochondria and isolation of inner and outer membranes has been performed by swelling in hypotonic medium followed by sucrose gradient centrifugation as previously described in this laboratory [6].

Oxygen consumption and respiratory control ratio of mitochondrial suspension have been measured by oxypolarography at 30°C in the following medium: 16 mM Tris-HCl, pH 7.4, 112 mM KCl, 5 mM P_i, 10 mM glutamate, 2 mg mitochondrial protein, final volume 2 ml. State 3 was induced by addition 0.2 mM ADP.

Protein in mitochondrial suspension was determined by the quick biuret method [7] and in dilute enzyme solutions by a modification of the procedure of Lowry et al. [8].

Adenylate kinase activity. To measure initial velocity the enzyme (about 0.05 U) was incubated 1 min at 30°C in 1 ml of a medium containing: 50 mM Tris-HCl, pH 7.5, 50 mM KCl, $MgSO_4$ and adenine nucleotides as indicated. When enzymic activities were measured with mitochondrial fractions 2 μg oligomycin were added to inhibit the reversible ATPase-ATP synthase of oxidative phosphorylation. The reaction was stopped by addition of 0.1 ml of ice-cold 50% trichloroacetic acid. After centrifugation trichloroacetic acid was removed from the supernatant fluid by four extractions with diethyl ether at 0°C. The amount of ADP produced in the forward direction (ATP + AMP → 2 ADP) was determined according to Adam [9]. ATP production in the reverse reaction (2 ADP → ATP + AMP) was evaluated as described by Lamprecht and Stein [10]. Results were expressed as μmol of ATP produced/min per mg protein (reverse reaction) or half the amount of ADP in μmol produced/min per mg protein (forward reaction). The concentrations of chelated and unchelated ATP and ADP were calculated as follows from the appropriate stability constants of the Mg^{2+} complexes, respectively, 70 000 M^{-1} and 4000 M^{-1} (Table 16 of Ref. 11): in the forward direction we have added an equimolecular concentration of total ATP and magnesium, and then we have calculated from the appropriate stability constant (70 000 M^{-1}), the concentration of the

MgATP complex assuming that, in such conditions the formation of MgAMP complex can be neglected. In the reverse direction we have added a total ADP concentration calculated from the desired MgADP and free ADP concentrations. Then from the appropriate stability constant ($4000\ M^{-1}$) we have calculated the concentration of total magnesium to be added to obtain the concentration of MgADP and free ADP previously defined.

Inhibition studies of the mitochondrial enzyme. In the forward direction when inhibition towards ATP was studied, AMP concentration was 0.2 mM, and the total ATP concentrations were in the range 0.5—2 mM. When inhibition towards AMP was studied in the presence of 2 mM ATP, the concentrations of AMP were in the range 0.2—0.05 mM. In the reverse direction the total ADP concentrations were 0.2, 0.5 or 1 mM. In all the experiments (forward and reverse direction) the total magnesium concentration was 5 mM and Ap_5A concentrations were 1, 2.5, 5, 7.5, 10 and 15 μM, and adenylate kinase activity was measured as previously described.

Partial purification of mitochondrial adenylate kinase. The detailed purification of the enzyme will be subsequently published and is briefly summarized in this paper. After isolation of mitochondria, adenylate kinase was solubilized by a 10-fold dilution of mitochondrial suspension in hypotonic medium. After centrifugation, the supernatant which contained the solubilized enzyme in 20 mM potassium phosphate (pH 7.2) was applied on a column of Sephadex-Cibachron Blue F3GA. The enzyme was retained and then eluted with a linear gradient of KCl in 20 mM potassium phosphate. The active fractions were dialysed against 10 mM potassium citrate (pH 5) and applied on a CM-Sephadex column, and eluted with a linear gradient of KCl in 10 mM potassium citrate, pH 5. The pH of the active fractions was adjusted to 7.5 and after dialysis against 50 mM Tris-HCl, pH 7.5, the purified enzyme was lyophilised. With this procedure a purification of 200-fold was obtained. The purified enzyme which was devoided of ATPase activity had a mean specific activity of 55 units/mg protein. By disc electrophoresis our preparation showed only one major contaminant.

Results

Properties of the mitochondrial enzyme in situ

In a first set of experiments we have studied some properties of the mitochondrial enzyme in situ, when this enzyme was an integral part of the mitochondria.

Localization. During the fractionation and purification procedure of pig heart mitochondrial membranes it was found that adenylate kinase activity was mainly recovered with the intermembrane space fraction. The De Duve plot [12] applied to the first steps of purification indicates that the relative specific activity of adenylate kinase was maximum in the intermembrane space (16.6) and that it was very low in fractions that contain outer or inner membranes (0.1 and 0.2, repectively).

Enzymic activity was not significantly affected when mitochondria were preincubated with Lubrol WX, 0.67 mg/mg protein, or when 0.1 mM carboxy-

atractyloside, which fully prevented the stimulation of respiration by ADP was added to the mitochondrial suspension.

Sensitivity towards SH reagents. The sensitivity of the mitochondrial enzyme towards SH reagents was tested and compared with that of the cytoplasmic enzyme extracted as previously described [3]. The cytoplasmic enzyme, the mitochondria-bound enzyme or the purified mitochondrial enzyme (about 1 unit) were incubated at 30°C in 50 mM Tris-HCl, 50 mM KCl, pH 7.5, with or without 0.2 mM 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB). 20 and 40 min after the addition of DTNB or H_2O in the control, enzyme activity was measured in aliquots, in the reverse direction. After 40 min of incubation (arrow in Fig. 1) 140 μmol of β-mercaptoethanol were added and the activity measured 20 min later. As shown in Fig. 1 in the presence of 0.2 mM DTNB, the cytoplasmic enzyme was completely inhibited and the inhibition can be reversed by addition of an excess of the thiol. In contrast, DTNB did not alter significantly the activity of mitochondrial adenylate kinase (bound or purified).

Ap_5A inhibition of the mitochondrial enzyme. The effect of P_1, P_5-diadenosine-5′-pentaphosphate (Ap_5A) on the mitochondrial adenylate kinase has been studied as described in Material and Methods, and the inhibition constants determined by Dixon plots. Our results show that in the forward direction Ap_5A acts as a competitive inhibitor towards MgATP and AMP; the K_i values are respectively: 2.5 μM and 6.5 μM and about two orders of magnitude greater than those observed for cytoplasmic enzyme from muscle [13,14] (range $5—20 \cdot 10^{-9}$ M). In the reverse direction, when magnesium concentration was in excess towards ADP concentration, we also observed a competitive inhibition with a K_i of 5 μM. This last result does not agree with those obtained by Kuby et al. [14]. This inhibition by Ap_5A was rather specific for adenylate kinase as compared to other mitochondrial kinases, creatine kinase and hexokinase, for which it remained below 10% when mitochondrial adenylate kinase was 95% inhibited by 50 μM Ap_5A.

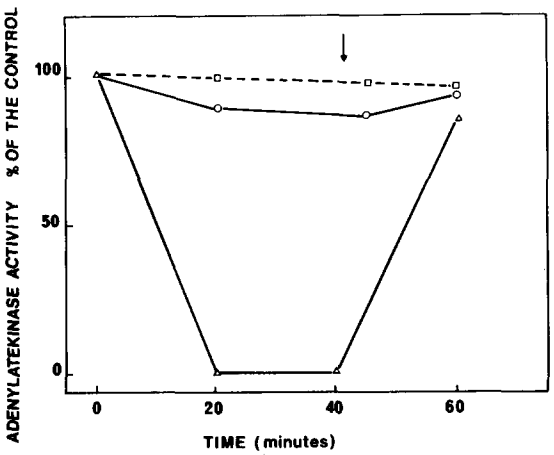


Fig. 1. Influence of 0.2 mM DTNB on the activity of cytoplasmic (△———△), mitochondria-bound (○———○), and purified mitochondrial (□- - - - -□) enzymes. At the time indicated by the arrow 140 μmol of β-mercaptoethanol were added.

Detailed studies of the partially purified adenylate kinase

In a second set of experiments we have carried out a more detailed study of the isolated purified mitochondrial adenylate kinase.

Effect of pH on enzymic activity. The effect of pH on the activity was measured over the pH range of 4—10. In the forward direction, the enzyme exhibited a broad optimum pH between 6 and 7.5. In the reverse direction, maximal activity occurred at pH 6.5. In the forward and reverse direction, initial velocity sharply decreased when pH dropped from 6 to 5.5; for both reactions, enzymic activity did not differ significantly at pH 5.5 in acetate/acetic acid buffer or in Tris/maleate buffer; however, some discrepancies appeared at pH 8.5 between initial rate measured in Tris/maleate or in boric acid/KOH buffer.

Equilibrium constants of adenylate kinase, effects of H^+ and magnesium concentration. The equilibrium constant of the reaction was calculated from observed concentration of the three nucleotides following 60 min incubation at 30°C, after addition of the purified enzyme.

Results in Table I show that the equilibrium constant decreased with the pH of the medium, and that at neutral pH it was about 1. Magnesium concentration also affected the equilibrium constant of the enzyme (Fig. 2). When initial concentration of ADP was 1 mM, the equilibrium constant sharply increased from 0.5 to 1.2 when magnesium concentration rose from 0.1 ot 1.5 mM, then it decreased slowly when magnesium concentration increased from 1.5 to 10 mM.

Specificity of the mitochondrial enzyme. The specificity of the mitochondrial enzyme towards nucleotides has been tested in the forward direction. Initial concentrations of mono- and triphosphates were, respectively, 0.4 and 2 mM. The mononucleotide site was very specific for AMP since, in the presence of ATP as cosubstrate the mitochondrial enzyme was inactive when GMP, UMP or CMP was added. With AMP as cosubstrate the enzyme exhibited only 10—20% of control activity when either CTP or GTP or UTP was used as the other substrate. Similar results were obtained when the adenylate kinase was associated with mitochondria.

Kinetic constants of the purified enzyme. Previous results on adenylate kinase from other sources [5,15—17] agree with a rapid equilibrium random mechanism, and with the existence of two distinct binding sites, one for the

TABLE I

EFFECT OF pH ON THE EQUILIBRIUM CONSTANT $[ATP] \cdot [AMP]/[ADP]^2$ OF PURIFIED MITOCHONDRIAL ADENYLATE KINASE

The enzyme was incubated 60 min in a medium containing 50 mM Tris/maleate at different pH, 50 mM KCl, 1 mM $MgSO_4$. Initial concentration of ADP was 2 mM. Mean of two experiments.

pH:	5.5	6.5	7.5	8.5
mM ATP	0.560	0.582	0.608	0.613
mM ADP	0.678	0.627	0.616	0.608
mM AMP	0.662	0.681	0.695	0.700
K_{eq}	0.81	1.01	1.12	1.16

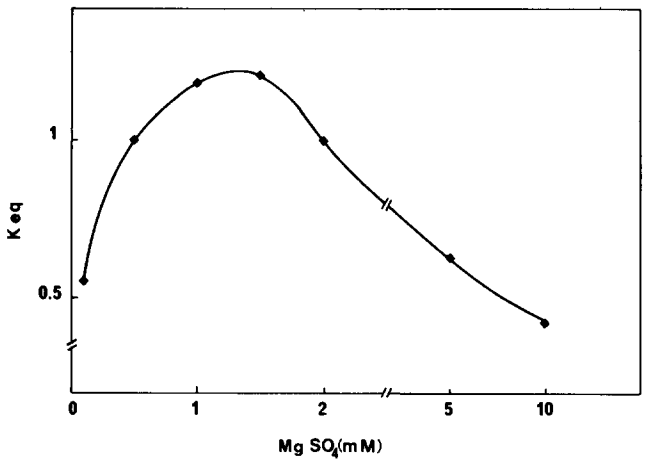
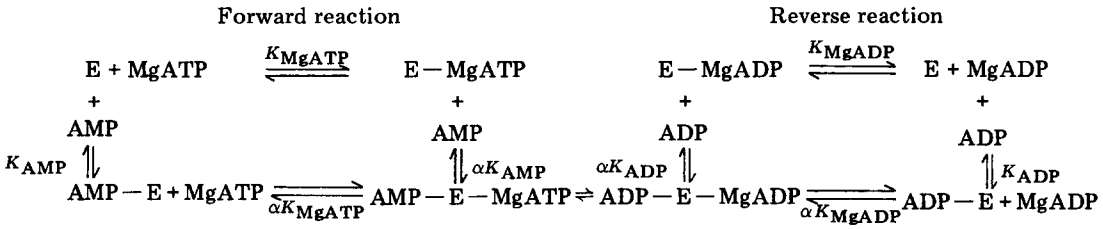


Fig. 2. Evolution of observed equilibrium constant of mitochondrial adenylate kinase with Mg^{2+} concentration. Conditions are described in the text. Mean of two determinations.

chelated nucleotide (MgATP or MgADP) and the other for the unchelated nucleotide (AMP or ADP). The system can be described by the equilibria shown in Scheme I, where K represent the dissociation constant of the sub-



$$K_{MgATP} = \frac{[E] \cdot [MgATP]}{[E-MgATP]} \qquad \alpha K_{MgATP} = \frac{[AMP-E] \cdot [MgATP]}{[AMP-E-MgATP]}$$

$$K_{AMP} = \frac{[E] \cdot [AMP]}{[AMP-E]} \qquad \alpha K_{AMP} = \frac{[E-MgATP] \cdot [AMP]}{[AMP-E-MgATP]}$$

$$K_{MgADP} = \frac{[E] \cdot [MgADP]}{[E-MgADP]} \qquad \alpha K_{MgADP} = \frac{[ADP-E] \cdot [MgADP]}{[ADP-E-MgADP]}$$

$$K_{ADP} = \frac{[E] \cdot [ADP]}{[E-ADP]} \qquad \alpha K_{ADP} = \frac{[E-MgADP] \cdot [ADP]}{[ADP-E-MgADP]}$$

Scheme I.

strate from the binary complex and αK the dissociation constant for the ternary complex, if the binding of one substrate changes the dissociation of the other substrate by a factor α.

Initial rate studies have been performed in the forward and reverse direction as a function of one substrate concentration with fixed concentrations of the

other substrate, as described under Material and Methods. Initial velocity in reciprocal form is given by Eqn. 1.

$$1/v = \frac{\alpha K_{MgATP}}{V}\left(1 + \frac{K_{AMP}}{[AMP]}\right) \cdot \frac{1}{[MgATP]} + \frac{1}{V}\left(1 + \frac{\alpha K_{AMP}}{[AMP]}\right) \quad (1)$$

Secondary plots of intercept vs. 1/[MgATP] or 1/[AMP] can be used to determine αK_{MgATP}, and αK_{AMP} and V. K_{MgATP} and K_{AMP} can be calculated from the replots of the slope vs. 1/[MgATP] or 1/[AMP]. An analogous equation was used to determine αK_{MgADP}, αK_{ADP}, K_{MgADP} and K_{ADP}.

Our results show that double-reciprocal plots of initial velocity vs. MgATP concentrations (range 0.037—1.84 mM) at four different AMP concentrations gave a family of straight lines intersecting at a common point below the x-axis. The secondary plots of intercepts and slopes vs. 1/AMP concentration are linear. The same results are obtained with AMP as the variable substrate (range 0.015—0.20 mM). In the conditions of these experiments, there was no inhibition by excess substrate.

In the reverse direction with ADP (0.004—0.02 mM) or MgADP (0.02—0.4 mM) as the variable substrate double-reciprocal plots were linear and had common points of intersection close to the x-axis. Kinetic constants obtained from four different experiments are given in Table II.

Our results show that in the forward direction dissociation constants from the ternary complex (αK_{MgATP}, αK_{AMP}) are higher by a factor of about five, than dissociation constants from the binary complexes (K_{MgATP}, K_{AMP}).

In the reverse direction the dissociation constant of uncomplexed nucleotide from the binary complex (K_{ADP} = 0.006 mM) is much lower than that of the complexed nucleotide (K_{MgADP} = 0.112 mM), but these values are not significantly modified when the other substrate is bound to the enzyme

Inhibition by excess substrate. In the forward direction, the double-reciprocal plots of initial velocity ($1/v = (f)\ 1/[AMP]$) were no more linear and an inhibition by excess substrate occurred when AMP concentrations were higher than 1 mM. A more detailed study of this inhibition has been carried out. The results of a typical experiment are given in Fig. 3 which shows the double-reciprocal plots of initial velocity versus variable MgATP concentrations with two low (0.025—0.1 mM) or two high (2—5 mM) AMP concentrations. With low AMP concentrations a classical representation is obtained. In contrast,

TABLE II

KINETIC CONSTANTS OF PIG HEART MITOCHONDRIAL ADENYLATE KINASE

Kinetic constants (mM) are defined in Scheme I. Maximum speeds in the forward (V^f) and reverse direction (V^r) are expressed as units/mg protein. Mean of four experiments ± S.E.

	K_{MgATP}	αK_{MgATP}	K_{AMP}	αK_{AMP}	V^f
Forward reaction	0.039 ± 0.005	0.178 ± 0.027	0.026 ± 0.003	0.141 ± 0.034	29 ± 1
	K_{MgADP}	αK_{MgADP}	K_{ADP}	αK_{ADP}	V^r
Reverse reaction	0.112 ± 0.020	0.155 ± 0.040	0.006 ± 0.002	0.005 ± 0.001	10 ± 3

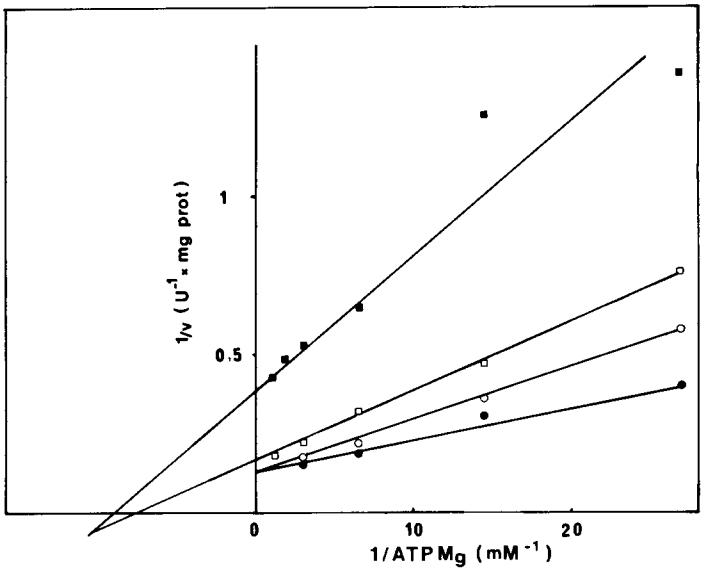


Fig. 3. Effect of MgATP concentration on the initial velocity in the forward direction. Fixed non-inhibitory concentrations of AMP were 0.025 mM (■———■) or 0.1 mM (□———□) and inhibitory concentrations were 2 mM (●———●) or 5 mM (○———○).

with high AMP concentrations the substrate in excess acts as a competitive inhibitor towards MgATP. For the calculation of K_i the following two dead-end complexes must be added to Scheme I:

$$E + AMP \overset{K_i}{\rightleftharpoons} E - \overset{*}{AMP}$$

$$AMP - E + AMP \overset{\beta K_i}{\rightleftharpoons} AMP - E - \overset{*}{AMP}$$

$\overset{*}{AMP}$, AMP molecule bound to the MgATP site.

With these inhibitory concentrations of AMP, initial velocity equation [18] in double-reciprocal form with MgATP as variable substrate was given by Eqn. 2.

$$\frac{1}{v} = \frac{\alpha K_{MgATP}}{V}\left(1 + \frac{K_{AMP}}{[AMP]} + \frac{K_{AMP}}{K_i} + \frac{[AMP]}{\beta K_i}\right)\frac{1}{[MgATP]} + \left(1 + \frac{\alpha K_{AMP}}{[AMP]}\right)\frac{1}{V} \qquad (2)$$

and the slope by eqn. 3.

$$\text{Slope} = \frac{\alpha K_{MgATP}}{V}\left(1 + \frac{K_{AMP}}{K_i}\right) + \frac{\alpha K_{MgATP} \cdot K_{AMP}}{V} \cdot \frac{1}{[AMP]} + \frac{\alpha K_{MgATP}}{V \beta K_i}[AMP] \qquad (3)$$

When [AMP] was low towards K_{AMP} and βK_i, the plot of slopes versus the reciprocal of AMP concentration was linear; when [AMP] was high towards K_{AMP}, the plot of slopes versus AMP concentration was linear. Such plots can be used to determine K_i and βK_i. Values of 1.07 and 8.87 mM have been determined for pig heart mitochondrial adenylate kinase. With the physiological range of concentrations used in our experiments, inhibition by excess substrate did not occur with ADP, MgADP or MgATP, but only with AMP.

Discussion

Our results show that during fractionation of pig heart mitochondria, adenylate kinase relative specific activity was the highest in the first soluble fraction which corresponded to the intermembrane space and thus proved the same localization of the enzyme as in liver mitochondria [19]. Furthermore these results are in agreement with the solubilisation data recently reported by Watanabe et al. [20]. This conclusion is strengthened by the observation that Lubrol WX did not significantly increase and that carboxyactractyloside did not reduce the activity of the enzyme associated with mitochondria. Since the mitochondrial enzyme exhibits properties different from those of the cytoplasmic enzyme: insensitivity towards SH reagents, relatively weak inhibition by Ap_5A, our results prove that adenylate kinase associated with mitochondria was a distinct isoenzyme and did not result from contamination of the mitochondrial suspension by the cytoplasmic enzyme. This enzyme corresponds to the acidic adenylate kinase from pig heart homogenate [4,21] which has two disulfide bonds but not free SH.

The observed equilibrium constant of the partially purified enzyme $[ATP] \cdot [AMP]/[ADP]^2$ was about 1 at pH 7.5. According to the hypothesis of Atkinson [22] in vivo concentrations of ATP, ADP and AMP are controlled by the adenylate kinase equilibrium. However, this equilibrium and thus the balance between ATP, ADP and AMP were affected by H^+ and Mg^{2+} concentrations. This effect has been previously reported by Su and Russell on adenylate kinase from baker's yeast [23]. However, with a higher Mg^{2+}/nucleotide ratio than that used in our experiments they observed a decrease of the equilibrium constant when pH increased from 5.0 to 10.0, and they have not observed the initial increase of K_{eq} when the magnesium concentration increased. An estimation of the true equilibrium constant $[MgATP] \cdot [AMP]/[MgADP] \cdot [ADP]$, which took magnesium complexes into account, gave a mean value of 5.0 when total adenine nucleotides and total magnesium concentration were 1 mM. In these conditions the equilibrium favoured the synthesis of ATP and AMP at the expense of ADP.

The purified enzyme was highly specific for AMP since other purine nucleotides like GMP or pyrimidine nucleotide with 4-amino groups (CMP) were not substrate. The ATP binding site was less specific since the enzyme was weakly active with CTP, UTP or GTP as substrate. These results are in good agreement with those reported by Noda [2]. Initial velocity studies are consistent with a rapid equilibrium random mechanism and exclude the possibility of a ping-pong mechanism for pig heart mitochondrial adenylate kinase. However, further studies on product inhibition will be required for determining unambiguously the nature of the mechanism. Our study also show that the binding of either MgATP or AMP significantly decreases the binding of the other nucleotide on the enzyme. Conformational changes of the enzyme induced by the binding of the substrate [24,25] can explain this modification. In the reverse direction in contrast, the binding of one substrate does not modify the binding of the other substrate. Our kinetic studies also point out that AMP has an appreciable affinity for MgATP binding site since at concentration higher than 1 mM AMP acts as an inhibitor. This inhibition by

excess AMP can be easily explained since the two molecules have the adenosine moiety in their structure and thus can compete for the same binding site. This inhibition by excess AMP has been already described for adenylate kinase from other sources [5,15,16]; it is pH dependent in baker's yeast adenylate kinase [16]. Our results indicate that in vivo, when AMP concentration sharply increases as for example during ischemia [1], mitochondrial adenylate kinase can be inhibited, preventing the phosphorylation of AMP to ADP and the translocation of this latter nucleotide, thus decreasing the amount of adenine nucleotide available for oxidative phosphorylations.

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