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Location and properties of two isoenzymes of cardiac adenylate kinase

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Adenylate kinase catalyses the equilibrium $2ADP \rightleftharpoons ATP + AMP$. There are two isoenzymes of adenylate kinase in bovine ventricular tissue, one cytoplasmic, the other mitochondrial. Mitochondrial subfractionation locates this isoenzyme between the mitochondrial membranes with fatty acid–CoA ligase. The cytoplasmic and mitochondrial isoenzymes are distributed in the ratio 3:2, and both forms were purified to homogeneity. They differ principally by charge, K_m values for ATP, ADP and AMP, pH–stability and –activity profiles, and susceptibility to the inhibitor adenosine pentaphosphoadenosine. The forward and reverse reactions show similar energies of activation for the cytoplasmic enzyme, but differ for the mitochondrial enzyme. The molecular weights are indistinguishable. An integrated mechanism is formulated whereby one isoenzyme suppresses the activation of fatty acid and the other enhances carbohydrate utilization in hypoxic myocytes.

AK (ATP:AMP phosphotransferase, EC 2.7.4.3) catalyses the equilibrium $2ADP \rightleftharpoons ATP + AMP$, thereby influencing the relative pool sizes of the three adenine nucleotides in cardiac myocytes. Isoenzymes of AK were reported for pig myocardial tissue by Kubo & Noda (1974), who identified an acid-stable form (pI 9.3), in a crude cytoplasmic extract, and an acid-labile form (pI 4.7–7.5) in crude mitochondrial extracts. The acid-stable form was indistinguishable from skeletal-muscle cytoplasmic AK, by electrophoretic and tryptic-mapping techniques. The acid-labile form from total lysates of frozen pig heart resembles the liver mitochondrial isoenzyme (Itakura *et al.*, 1978).

In view of the known inhibition or stimulation of many metabolic enzymes by adenine nucleotides, we expect that AK isoenzymes have key roles in regulating different aspects of metabolism in cytoplasmic and mitochondrial compartments of cardiac myocytes. Lack of comparative structural and kinetic detail for AK has hindered interpretation of changing patterns of production and utilization of ADP in cardiac myocytes (Dow & Walker, 1981). As a first step in this study we have purified simultaneously both isoenzymes of AK from the same sample of bovine ventricular tissue, and compared properties and subcellular locations of the two forms.

Abbreviations used: AK, adenylate kinase (EC 2.7.4.3); SDS, sodium dodecyl sulphate; Ap_5A, adenosine pentaphosphoadenosine.

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Experimental

Materials

Fresh bovine hearts obtained at slaughter were immediately cooled on ice, and homogenized within 1 h of excision. Linking enzymes, substrates and coenzymes for enzyme assays, proteins for standardizing gel-filtration columns and SDS/polyacrylamide gels, dithiothreitol and phenylmethanesulphonyl fluoride were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Ap_5A was obtained from Boehringer Corp. (London W.5, U.K.). Phosphocellulose and DE52 DEAE-cellulose were from Whatman Co. (Maidstone, Kent, U.K.), Bio-Gel HTP (hydroxyapatite) was from Bio-Rad Laboratories (Watford, Herts., U.K.), and Sephadex G-75 was from Pharmacia Fine Chemicals (Uppsala, Sweden). All other chemicals were of analytical-reagent grade.

Subcellular fractionation

Bovine ventricular muscle was homogenized by the first method of Smith (1967). The homogenizing buffer contained 1 mM-phenylmethanesulphonyl fluoride and 0.2 mM-dithiothreitol, and the homogenate was filtered through several layers of muslin,

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which removes a high proportion of the myofibrils in addition to fat and connective tissue.

Further fractionation was as follows. Nuclei and remaining myofibrils (fraction N, Table 1) were pelleted from the homogenate by centrifuging for 20min at 1200*g* ($r_{av.}$ 11cm). A heavy mitochondrial pellet (fraction M, Table 1) was then obtained by centrifuging the supernatants for 8min at 6000*g* ($r_{av.}$ 11cm). Light and damaged mitochondria, together with peroxisomes and lysosomes (fraction P, Table 1), were obtained from the supernatant after centrifugation for 8min at 22000*g* ($r_{av.}$ 8cm). A supernatant containing soluble protein (fraction C, Table 1) and a pellet containing sarcoplasmic reticulum and other membrane fragments, including membrane fragments from damaged mitochondria (fraction R, Table 1), were obtained from the supernatant by centrifuging for 2h at 100000*g* ($r_{av.}$ 6.4cm). Each pellet was washed by resuspension in homogenizing buffer and collected by repeating the appropriate centrifugation.

Submitochondrial fractionation

Mitochondria from bovine ventricular muscle, harvested by the first method described by Smith (1967), with 0.2mM-dithiothreitol added to the homogenizing buffer, were subfractionated by the method of Schnaitman & Greenawalt (1968). The mitochondrial suspension contained 5mg of mitochondrial protein/ml and 1mg of digitonin/ml.

Enzyme assays

AK activity was measured spectrophotometrically at 37°C. The assay solution contained 0.06M-imidazole, 0.003M-magnesium acetate, 0.2mM-EDTA, 0.05M-KCl, pH6.9, and either (*a*) 1mM-ATP, 1mM-AMP, 0.4mM-phosphoenolpyruvate, 0.2mM-NADH, and the linking enzymes lactate dehydrogenase and pyruvate kinase (each 1.25units/ml) for assays in the direction of ADP formation, or (*b*) 0.015M-glucose, 1.5mM-ADP, 0.86mM-$NADP^+$ and the linking enzymes hexokinase and glucose 6-phosphate dehydrogenase (each 2.2units/ml) for assays in the direction of ADP utilization. A unit of enzyme activity, for AK and for assay-linking enzymes, is defined as producing 1μmol of product/min at 37°C.

Fatty acid–CoA ligase was assayed by the method of Suzue & Marcel (1972), with the substitution of Triton X-100 for Triton WR-1339, lactate dehydrogenase as described by Scopes (1977), citrate synthase by a modification of the method of Srere (1969), in an assay solution containing 75mM-Tris/HCl, pH8.1, 0.2mM-5,5′-dithiobis-(2-nitrobenzoic acid), 60μM-acetyl-CoA and 2mM-oxaloacetic acid, succinate dehydrogenase by the method of Bachmann *et al.* (1966), and monoamine oxidase by a modification of the method of Tabor *et al.* (1954), with 3mM-benzylamine as substrate.

Purification of adenylate kinase

Two approaches were used for the purification of AK. All procedures were at 4°C.

In the first, 200g of bovine ventricular muscle was finely blended in 10vol. of 0.01M-Tris/HCl (pH7.8)/0.2mM-dithiothreitol/1mM-phenylmethanesulphonyl fluoride. Both isoenzymes were purified from the supernatant obtained by centrifuging this lysate at 1500*g* ($r_{av.}$ 11cm) for 15min. A second method was used also to aid in the subcellular localization of the isoenzymes. Purified mitochondria and a 40000*g* supernatant were obtained from 200*g* of ventricular muscle homogenized in 2vol. of 0.25M-sucrose/0.01M-Tris/HCl (pH7.8)/0.2mM-dithiothreitol/1mM-phenylmethanesulphonyl fluoride with a Potter–Elvehjem homogenizer. Both cytoplasmic AK and AK from those mitochondria damaged during tissue homogenization were purified from the 40000*g* supernatant as detailed in Tables 3(*a*) and 3(*b*). Enzyme was released from washed mitochondria by homogenization in 0.2mM-dithiothreitol.

Chromatographic procedures

Separation of cytoplasmic and mitochondrial forms (step IV, Table 3) is based on the observation (Itakura *et al.*, 1978) that the enzyme with the basic pI is not adsorbed to DEAE-cellulose at pH9.0. The pH of tissue extracts was adjusted to 9.0 with 1.0M-Tris base. The final concentration of Tris in the extract was not more than 20mM. To optimize recovery, attention is given to use of dithiothreitol and phenylmethanesulphonyl fluoride and timing of pH changes in this step. DEAE-cellulose (5cm depth of resin in a 23cm-diameter Buchner funnel) was equilibrated with 0.025M-Tris/HCl/0.2mM-dithiothreitol, pH9.0. The second enzyme pool (acidic pI) was eluted with 60mM-NaCl added to this buffer. The pH of these two enzyme fractions, referred to as 'low-salt' and 'high-salt' pools respectively, was adjusted to 6.9 with 1M-KH_2PO_4, and the conductivity was decreased to 1.5mmho by dilution with 0.2mM-dithiothreitol.

Hydroxyapatite (200ml resin volume; step V, Table 3) was loaded batchwise with low-salt or high-salt fractions and eluted with a linear gradient (2000ml) of 10–80mM-phosphate/0.2mM-dithiothreitol, pH6.9, at 0.5ml/min.

AK eluted from hydroxyapatite was loaded on to phosphocellulose (600ml resin volume) equilibrated with 0.025M-potassium phosphate/0.2mM-dithiothreitol, pH6.9, and eluted with a linear gradient (800ml) of 0–1.0mM-ATP in the same buffer at 0.5ml/min (step VI, Table 3). AK eluted from phosphocellulose was concentrated by dialysis

against polyethylene glycol (mol.wt. 6000–7500) or glycerol. These substances were separated from AK by gel filtration on Sephadex G-75.

Column fractions were monitored for protein absorbance at 280 or 235nm, and enzyme specific activities were calculated in terms of protein determined by the method of Lowry *et al.* (1951), or the micro-technique described by Spector (1978). Molecular weights of AK isoenzymes were determined by using calibrated SDS/polyacrylamide gels and a Sephadex G-75 column (2.2cm × 32cm). The technique described by Laemmli (1970) formed the basis of electrophoretic methods. Native samples were electrophoresed on 4% polyacrylamide gels prerun with 0.2mM-dithiothreitol before addition of the stacking gel. Samples dissociated in 1% (w/v) SDS and mercaptoethanol or dithiothreitol were electrophoresed on 10% polyacrylamide gels. Proteins were stained with Coomassie Brilliant Blue or, for enzyme detection, NADPH produced in the linked-enzyme assay described above for the ADP-utilization reaction was detected with Nitrotetrazolium Blue and phenazine methosulphate (Gabriel, 1971).

The rate of ADP formation was measured at ATP concentrations between 0.05 and 1.0mM for both isoenzymes, and at AMP concentrations between 0.08 and 2.0mM for the mitochondrial isoenzyme, and between 0.06 and 2.0mM for the cytoplasmic isoenzyme. The rate of ADP utilization was measured at ADP concentrations between 0.12 and 1.8mM for both isoenzymes. Lines were fitted to the reciprocal plots by the least-squares regression method described by Bevington (1969), by using standard deviations as a weighting factor. In all assays the Mg^{2+} concentration was sufficient to give maximal rates.

Results

Subcellular localization of adenylate kinase and fatty acid–CoA ligase

Table 1 shows the subcellular distributions of adenylate kinase, fatty acid–CoA ligase, lactate dehydrogenase, citrate synthase and monoamine oxidase. As discussed by Smith (1967), the fibrous nature of heart muscle makes the compromise between adequate tissue disruption and preservation of subcellular organelles difficult to achieve. The subcellular fractionation shown in Table 1 was obtained only at the expense of extensive damage to the outer membrane of the mitochondria, for a high percentage (44%) of monoamine oxidase, the mitochondrial-outer-membrane marker, appears in the

Table 1. *Enzyme distribution in subcellular fractions from bovine ventricular muscle*

Enzyme activity measured in the tissue homogenate is assigned the value of 100%. For full details see the text. Abbreviations: H, tissue homogenate after filtration through muslin; N, whole cells and nuclei; M, heavy mitochondria; P, light and damaged mitochondria, peroxisomes and lysosomes; C, soluble protein; R, sarcoplasmic reticulum and membrane fragments, including mitochondria membrane fragments.

Tissue fraction ...	H	N	M	P	C	R
Lactate dehydrogenase						
Relative specific activity	1	0.046	0.007	0.014	1.36	0.25
% of total activity	100	1.2	0.3	0.3	95.9	2.2
AK (ADP→ATP)						
Relative specific activity	1	0.18	0.19	0.21	1.43	0.13
% of total activity	100	2.3	0.9	0.5	95.4	0.85
AK (ATP→ADP)						
Relative specific activity	1	0.45	0.78	0.61	1.2	0.37
% of total activity	100	6.2	4.0	1.7	85.4	2.7
AK* (ATP→ADP, $+Ap_5A$)						
Relative specific activity	1	0.94	1.31	1.55	0.81	0.88
% of total activity	100	15.0	7.9	4.5	65.0	7.0
Citrate synthase						
Relative specific activity	1	2.32	4.69	2.8	0.48	0.43
% of total activity	100	33.3	23.2	7.3	33.0	3.0
Monoamine oxidase						
Relative specific activity	1	1.45	5.18	5.24	0	6.74
% of total activity	100	19.4	23.9	12.7	0	44.0
Fatty acid–CoA ligase						
Relative specific activity	1	1.75	1.8	2.57	0.24	2.85
% of total activity	100	33.0	11.6	8.7	21.4	25.8

* The concentration of Ap_5A added to these assays was sufficient to inhibit purified cytoplasmic AK by 90%, whereas purified mitochondrial AK was inhibited by less than 20%.

membrane-fragment fraction (R). When myocardial tissue is disrupted solely for the purpose of isolating mitochondria, conditions can be adjusted to provide, at relatively lower organelle yields, mitochondria retaining more of the outer membrane and intermembrane space enzymes.

The subcellular distribution of AK, measured by the ADP-utilization assay, reflected that of lactate dehydrogenase, with most of the activity in the soluble fraction. Assayed for ADP formation, a greater proportion appeared in the mitochondrial fractions (M and P) and the membrane-fragment (R) fraction. This difference is in accord with our observation that, under the conditions of the assay, whereas purified cytoplasmic AK catalyses the two directions of reaction at the same rate, purified mitochondrial AK catalysed ADP formation more rapidly than ADP utilization. When sufficient Ap_5A was added to ADP-formation assays to inhibit cytoplasmic AK by 90% but purified mitochondrial AK by less than 20%, relative specific activity of AK in the mitochondrial fractions (M and P) was further increased.

A large proportion of both fatty acid–CoA ligase and monoamine oxidase appeared in the membrane-fragment fraction (R). However, although monoamine oxidase was barely detectable in the soluble fraction (C), enough fatty acid–CoA ligase appeared there to suggest that this enzyme is only loosely bound to the mitochondrial outer membrane. The small amount of AK in the membrane-fragment fraction indicates this enzyme is less tightly bound to the mitochondrial membrane than is fatty acid–CoA ligase.

The distributions of AK, fatty acid–CoA ligase, monoamine oxidase, citrate synthase and succinate dehydrogenase in submitochondrial fractions are shown in Table 2. AK occurs with highest specific activity in the pool of soluble proteins between the mitochondrial membranes. Ap_5A did not significantly alter the distribution of AK, suggesting the presence of only one isoenzyme of AK. With lower digitonin concentrations more AK appeared in the outer-membrane fraction, confirming that AK may be only loosely attached to the membrane. Fatty acid–CoA ligase appeared in the outer-membrane fraction, its distribution suggesting that it is less tightly bound than monoamine oxidase. Integrity of the mitochondrial inner membrane is indicated by Triton-dependent release of 85% of citrate synthase.

Proportions and location of low-salt and high-salt adenylate kinase

The relative amounts of both AK activities in crude fractions were determined by eluting 1 ml samples from columns packed with 4 ml of DEAE-cellulose. Enzyme was eluted quickly from these columns with higher recovery of activity than from preparative-scale columns. The ratio of low-salt to high-salt activity in the hypo-osmotic tissue lysate was always close to 3:2. Mitochondria contain only the high-salt form. Elution of the 40000 *g* supernatant established that, allowing for the amount of mitochondrial enzyme removed with intact mito-

Table 2. *Enzyme distribution within heart mitochondrial subfractions*

'Total' refers to the activity of each enzyme measured in a lysate of isolated mitochondria from which the subfractions were prepared and is assigned a value of 100%. Citrate synthase activity was measured in the presence of 0.5% Triton. Without Triton, 90% of heavy-mitochondrial activity and 85% of matrix activity was latent. 'Relative specific activity' means relative to the specific activity of heavy mitochondria. For other details see the text.

Mitochondria or submitochondrial fraction ...	Heavy mitochondria	Outer membrane	Inner membrane	Matrix
Citrate synthase				
Relative specific activity	1	0.49	1.07	1.15
% of total activity	100	1.7	7.2	91.0
Succinate dehydrogenase				
Relative specific activity	1	1.09	0.88	1.07
% of total activity	100	3.9	5.5	90.5
Monoamine oxidase				
Relative specific activity	1	4.1	0	0.80
% of total activity	100	18.0	0	82.0
Fatty acid–CoA ligase				
Relative specific activity	1	1.61	0.79	1.07
% of total activity	100	5.8	4.8	89.4
AK (ATP→ADP)				
Relative specific activity	1	0.49	1.73	0.90
% of total activity	100	2.0	12.0	86.0

chondria, 33–40% of myocardial AK activity was of mitochondrial origin.

Purification of cytoplasmic and mitochondrial isoenzymes of adenylate kinase

A typical purification of the cytoplasmic isoenzyme from the 40000*g* supernatant is shown in Table 3(*a*). The cytoplasmic isoenzyme, purified to homogeneity from the 40000*g* supernatant or from hypo-osmotic tissue lysate had, in five independent preparations, specific activities of 3300–5600 units/mg of protein. The final yield varied between 15 and 25% of the cytoplasmic activity. Purification of the mitochondrial isoenzyme from the 40000*g* supernatant and from lysed mitochondria derived from the same tissue sample is shown in Table 3(*b*).

The homogeneous mitochondrial isoenzyme from mitochondrial lysate had a specific activity of 680 units/mg. The final yield was 13% of mitochondrial-lysate enzyme, or approx. 3% of the tissue mitochondrial enzyme, since on average only one-quarter of the mitochondria were recovered intact. Purified high-salt isoenzyme in the 40000*g* supernatant, inferred to have leaked from mitochondria during tissue homogenization, was of lower specific activity (300 units/mg of protein). This difference is likely to arise from the lengthy purification time when both forms are purified from the 40000*g* supernatant. In four independent preparations from the hypo-osmotic tissue lysate, mitochondrial isoenzyme had specific activities of 400–1000 units/mg of protein. Mitochondrial isoenzyme was obtained at higher specific activity when the purification started from a smaller amount of myocardial tissue, and was completed within a shorter time.

Electrophoresis of purified AK and determination of molecular weight

Electrophoresis of purified AK on SDS/10%-polyacrylamide gel established a mol.wt. of 21000 for both cardiac isoenzymes. Confirmation of this value was obtained by eluting the isoenzyme through a calibrated Sephadex G-75 column. Both isoenzymes purified to step VI had two additional minor bands of apparent mol.wts. 51000 and 63000. Sephadex G-75 columns, which completely removed proteins of mol.wt. 40000 or greater from AK, failed to remove these contaminants, suggesting they may be formed from the 21000-mol.wt. species during denaturation.

On 4%-polyacrylamide gel the cytoplasmic isoenzyme, a single AK activity band at R_F 0.12, pH 8.9, co-migrated with active rabbit skeletal-

Table 3. *Purification of cardiac cytoplasmic (C, a) and mitochondrial (M, b) adenylate kinase*

Adenylate kinase was purified from 200g of fresh bovine ventricular tissue. Cytoplasmic AK was purified from the 40000*g* supernatant, and mitochondrial AK from both the 40000*g* supernatant and from isolated mitochondria. Chromatographic methods are described in the text. Assays were by the standard method in the direction of ADP formation.

Fraction	Isoenzyme	Activity (μmol/min)	Recovery (%)	Specific activity (μmol/min per mg of protein)
(*a*)				
I Homogenate	C + M	42600	100	6.42
II Cell-free supernatant	C + M	40500	95	7.04
III (*a*) 40000*g* supernatant	C + M	35800	84	7.88
(*b*) Mitochondrial lysate (This fraction taken to Table 3*b*)	M	4100	9.5	5.40
IV DE52 pools from step III(*a*)				
(*a*) Low salt	C	26600	62	86.5
(*b*) High salt (This fraction taken to Table 3*b*)	M	2670	6.2	
Cytoplasmic fraction from step IV(*a*)				
V Hydroxyapatite pools				
(*a*) DE52 low salt	C	17000	40	788
VI Phosphocellulose pool	C	8050	19	4840
(*b*)				
Mitochondrial fraction of the 40000*g* supernatant [from fraction IV(*b*), Table 3(*a*)]				
IV DE52 eluate	M	2670	6.2	1.2
V Hydroxyapatite eluate	M	1200	2.9	26
VI Phosphocellulose eluate	M	805	1.9	315
Mitochondrial lysate [from fraction III(*b*), Table 3(*a*)]				
IV DE52 eluate	M	1693	3.9	26
V Hydroxyapatite eluate	M	796	1.8	401
VI Phosphocellulose eluate	M	546	1.3	680

muscle AK (Sigma). The mitochondrial isoenzyme had R_F 0.36 under the same conditions.

pH–activity profiles of cytoplasmic and mitochondrial AK

The influence of pH on enzyme activity was measured between pH 5.8 and 8.0. The pH profile for ADP formation was similar for both isoenzymes, with maximum activity between pH 6.4 and 7.0. The pH-dependence of ADP utilization was different for the two isoenzymes, with mitochondrial AK having maximum activity in the pH range 6.1–6.7, and cytoplasmic AK having a sharper maximum at pH 7.5.

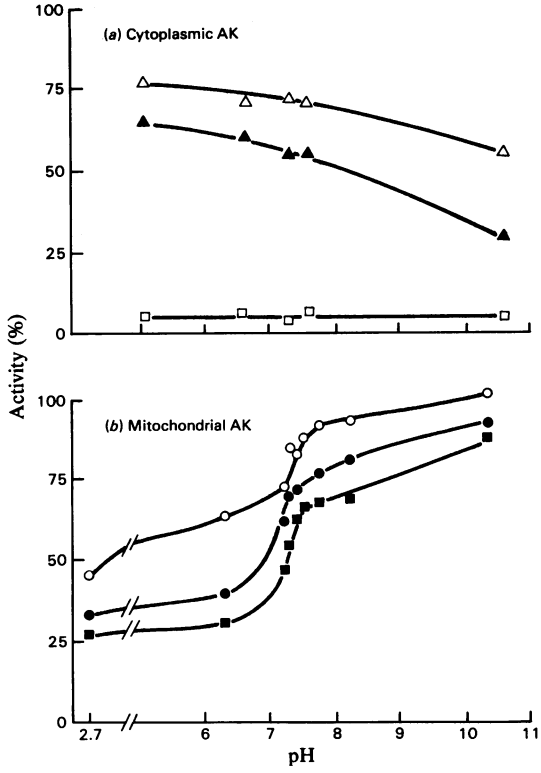

Fig. 1. *pH-stability of cytoplasmic (a) and mitochondrial (b) adenylate kinase*

Residual activity (ADP formation) of cytoplasmic (0.4 μg/ml) and mitochondrial AK (1.3 μg/ml) is expressed as a percentage of the activity at each pH immediately after dilution with potassium phosphate (10 mM). pH was adjusted to the required value by adding either KOH or HCl to the diluting buffer. (*a*) Cytoplasmic AK incubated for 15 min (△) or 40 min (▲) at 20°C, then for 16 h (□) at 4°C. (*b*) Mitochondrial AK incubated for 30 min (○) or 2 h (●) at 20°C, then for 18 h (■) at 4°C. Assay solutions are detailed in the text.

pH-stability of cytoplasmic and mitochondrial AK

The pH-stability of both enzymes after dilution with 10 mM-phosphate (pH 2.7–10.5) is shown in Fig. 1. The cytoplasmic isoenzyme is unstable at low protein concentration, having little activity after 16 h at any pH studied. The mitochondrial isoenzyme was more stable, and at alkaline pH, after 2 h at 20°C and a further 18 h at 4°C retained 88% activity. Stability of the cytoplasmic isoenzyme was improved at low pH. The mitochondrial form is acid-labile, showing a sharp decline in activity between pH 7.5 and 7.0.

Temperature-dependence of mitochondrial and cytoplasmic AK activity

The temperature-dependence of activity was determined between 5 and 40°C for both isoenzymes and for both directions of the reaction at pH 6.9. Arrhenius plots were linear, except for a marginal apparent increase in the activation energy of both isoenzymes at temperatures below 9°C, in ADP-formation assays. For ADP utilization, the activation energies for cytoplasmic and mitochon-

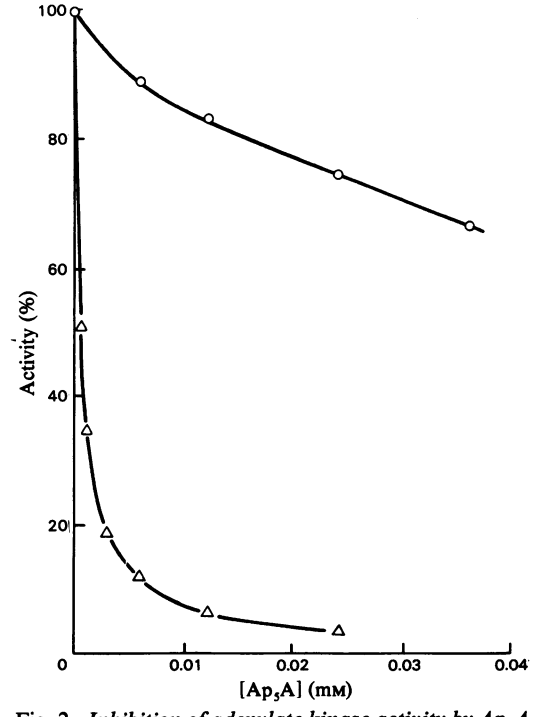

Fig. 2. *Inhibition of adenylate kinase activity by Ap_5A*

Cytoplasmic (△) and mitochondrial (○) adenylate kinase isoenzymes were assayed (ADP utilization) in the presence of Ap_5A (0–0.03 mM) by using the assay solution detailed in the text. Activity is expressed as a percentage of the activity in the absence of inhibitor.

drial isoenzymes are 48.8 and 53.9 kJ·mol^{-1} respectively. For ADP formation the activation energies differ, being 52.6 kJ·mol^{-1} for cytoplasmic and 34.4 kJ·mol^{-1} for mitochondrial isoenzymes.

Inhibition of AK by Ap_5A

Inhibition of cytoplasmic and mitochondrial AK by Ap_5A (0–0.03 mM) is shown in Fig. 2 for ADP utilization. Cytoplasmic isoenzyme was substantially more sensitive to inhibition by Ap_5A than was the mitochondrial isoenzyme.

Affinities of cytoplasmic and mitochondrial isoenzymes of adenylate kinase for ATP, AMP and ADP

The influence of Mg^{2+} concentration (0.5–15 mM) on reaction velocity was studied over the entire range of ATP, ADP and AMP concentrations used in these experiments. It was established firstly that within these concentrations Mg^{2+} does not inhibit the reaction velocity, and secondly, that 3 mM-Mg^{2+} ensures maximum reaction velocity at all substrate concentrations used.

In three preparations of cytoplasmic isoenzyme, apparent K_m values for ATP between 0.18 and 0.27 mM were obtained. For two preparations of mitochondrial isoenzyme, apparent K_m for ATP was 0.12 and 0.13 mM. All assays were in the presence of 2 mM-AMP.

The rate of ADP formation as a function of AMP concentration and at 1 mM-ATP is shown in Fig. 3. The Lineweaver–Burk plot for the mitochondrial isoenzyme is biphasic. For three preparations of mitochondrial AK, data for AMP concentrations between 0.06 and 0.25 mM intercepted between 0.083 and 0.095 mM-AMP, whereas for AMP concentrations between 0.25 and 2.0 mM, the intercept was 0.027–0.029 mM. Lineweaver–Burk plots were linear for the cytoplasmic isoenzyme, allowing estimates of apparent K_m for AMP between 0.176 and 0.184 mM.

The rate of ADP utilization is shown in Fig. 4 as a function of ADP concentration. For mitochondrial isoenzyme, the Lineweaver–Burk plot was linear between 0.06 and 0.9 mM-ADP, allowing calculation of 0.15 mM as the apparent K_m for ADP. For cytoplasmic isoenzyme, a linear relationship was

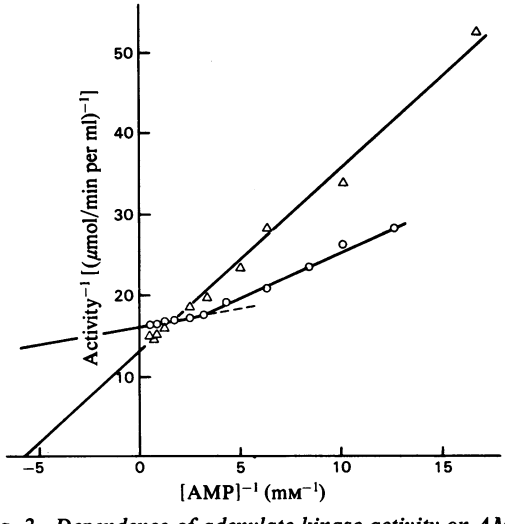

Fig. 3. *Dependence of adenylate kinase activity on AMP concentration*

For the cytoplasmic (△) and mitochondrial (○) isoenzymes, the rate of ADP formation at 37°C and pH 6.9 was measured as a function of AMP concentration. ATP concentration was 1.0 mM. AMP concentration was 0.08–2.0 mM for the mitochondrial isoenzyme, and 0.06–2.0 mM for the cytoplasmic isoenzyme. In all assays the Mg^{2+} concentration was sufficient to give maximal catalytic rates. Data are presented as plots of the reciprocal of AK activity as a function of the reciprocal of AMP concentration.

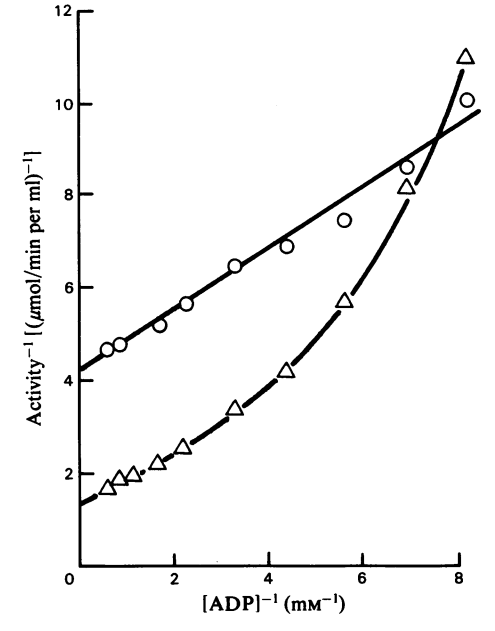

Fig. 4. *Dependence of adenylate kinase activity on ADP concentration*

For cytoplasmic (△) and mitochondrial (○) isoenzymes, the rate of ADP utilization at 37°C and pH 6.9 was measured as a function of ADP concentration, which was varied between 0.12 and 1.8 mM, with Mg^{2+} concentration sufficient to allow maximal rates. Data are presented as a plot of the reciprocal of AK activity as a function of the reciprocal of ADP concentration.

obtained between reciprocal activity and reciprocal of the square of ADP concentration. Making the assumption that the cytoplasmic isoenzyme has two sites with identical affinity for ADP, we have calculated the apparent K_m for ADP as 0.22 mM.

Discussion

Subcellular and submitochondrial fractionation shows that AK is located in cytoplasm and mitochondria of bovine heart. Mitochondrial AK is more loosely attached to the outer membrane than is fatty acid–CoA ligase. Kinetic properties of AK purified from each site indicate that these are distinct isoenzymes.

In subcellular-fractionation experiments 15% of the total AK was found in the mitochondria. However, under these fractionation conditions mitochondria retained only 43% of the fatty acid–CoA ligase, an enzyme of purely mitochondrial origin (Schnaitman *et al.*, 1967). If the same proportion of mitochondrial AK was retained, then intact mitochondria must contain 35% of the total tissue AK. This is a minimal value, and is in accord with our observation that approx. 40% of the total AK activity of the hypo-osmotic tissue homogenate occurs as the mitochondrial form.

The ion-exchange characteristics and electrophoretic behaviour of the isoenzymes are consistent with reports of two pig cardiac isoenzymes of AK having pI values of 4.7–7.5 and 9.3 (Kubo & Noda, 1974; Itakura *et al.*, 1978), but not with the pI values of 9.3 and 10.1 for bovine enzyme reported by Tomasselli & Noda (1980).

In the present study identical mol.wts. of 21000–22000 have been found for the two forms of AK. This is consistent with values for the pig heart and skeletal-muscle cytoplasmic isoenzyme (Kubo & Noda, 1974) and for the isoenzymes from bovine liver (Markland & Wadkins, 1966) and bovine lens (Klethi, 1968).

In contrast, Tomasselli & Noda (1980) report mol.wts. of 31500 and 21000 for the mitochondrial and cytoplasmic cardiac isoenzymes respectively, and Itakura *et al.* (1978) report mol.wt. 26900 for pig heart mitochondrial AK. The consistent presence of high-molecular-weight species in otherwise homogeneous enzyme preparations implies that AK polymerizes in a manner resistant to dissociation with SDS. The 63000-mol.wt. band is consistent with the monomer–dimer–trimer equilibrium suggested for the rat liver mitochondrial isoenzyme (Criss *et al.*, 1970).

Our pH–stability data confirm that the cardiac isoenzyme with the more acidic isoelectric point is acid-labile (Kubo & Noda, 1974), whereas the isoenzyme with alkaline isoelectric point is acid-stable. That the cardiac acid-labile form is of mitochondrial origin, whereas the cardiac acid-stable form derives from the cytoplasm, agrees with findings from other tissues (Pradan *et al.*, 1974; Kubo & Noda, 1974; Criss, 1970; Chiga & Plaut, 1960).

Tomasselli & Noda (1980) measured temperature-dependence of activity at pH 8.0 for mitochondrial AK and suggested that a conformational change occurs at about 21.5°C, which increases the activation energy for ATP formation at lower temperatures. Our data, at pH 6.9, do not confirm this biphasic response.

Cytoplasmic and mitochondrial AK showed differences in their affinities for AMP, ADP and ATP. The apparent K_m for ATP obtained in the present study for the mitochondrial enzyme agrees with the value of 0.125 mM reported by Font & Gautheron (1980) for pig heart mitochondrial AK. Michaelis constants for cardiac cytoplasmic AK have not previously been reported.

The mitochondrial form had a biphasic Lineweaver–Burk plot for AMP, similar to that reported by Font & Gautheron (1980) for pig heart mitochondrial AK. The relationship is linear for cytoplasmic AK, and the apparent K_m indicates that this isoenzyme has a lesser affinity for AMP.

The relationship between pH and enzyme activity was investigated over a range of pH similar to that occurring within aerobic and ischaemic myocardial cells (Irvine & Dow, 1968; Garlick *et al.*, 1979). The pH-dependence of mitochondrial isoenzyme activity was similar to that reported by Font & Gautheron (1980) for partially purified pig cardiac mitochondrial AK, but different from profiles reported by Tomasselli & Noda (1980) and Itakura *et al.* (1978) for mitochondrial AK from bovine heart and frozen pig heart. The cytoplasmic form showed the higher pH optimum also reported by Noda (1957) for rabbit skeletal muscle, and by Itakura *et al.* (1978) for pig skeletal-muscle AK.

There are indications that cytoplasmic and mitochondrial AK have an integrated function. In aerobic cardiac myocytes the AK substrates are present at concentrations (Neely *et al.*, 1973) which indicate that the cellular reaction is close to its measured equilibrium position (Font & Gautheron, 1980). In ischaemic myocardium, ATP is lost at about 0.4 μmol/min per g wet wt. of tissue. Our data show that AK activity is in large excess over that required to restore ATP from the ADP produced. The metabolic impact of the resulting 10-fold increase in AMP content (Neely *et al.*, 1973) is two-fold. Firstly, AMP inhibits fatty acid–CoA ligase. We find that long-chain fatty acid–CoA ligase is located between the mitochondrial membranes of cardiac tissue, similar to that of the liver enzyme (Alexandre *et al.*, 1969). Cardiac mitochondria have sufficient AK to prevent AMP accumulating under

aerobic conditions. But in oxygen-depleted tissue ADP accumulates between the mitochondrial membranes as the ATP/ADP translocase fails (Pfaff *et al.*, 1965), and will prevent the phosphorylation of AMP. Fatty acids will inhibit liver mitochondrial AK (Sapico *et al.*, 1972), so mitochondrial AK and fatty-acid–CoA ligase, both being loosely attached to the mitochondrial outer membrane, may act in a co-ordinated manner to regulate the activation of fatty acids. This would be energy-conserving by arresting an uneconomical consumption of ATP, for activated fatty acids cannot be further oxidized in anoxic tissue.

Secondly, the accumulating AMP is available to accelerate energy production from carbohydrate metabolism, both by activating phosphofructokinase (Regen *et al.*, 1964; Lorenson & Mansour, 1969) and by activating phosphorylase *b* (Morgan & Parmeggiani, 1964).

Thus, as AK in the two subcellular locations catalyses an increase in the AMP content of hypoxic tissue, the balance between the oxidation of carbohydrate and fatty acids shifts, favouring the carbohydrate pathway and anaerobic ATP production.

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