Biochimica et Biophysica Acta, 612 (1980) 56—66

BBA 68904

PURIFICATION AND CHARACTERIZATION OF ADENYLATE KINASE ISOZYMES FROM RAT MUSCLE AND LIVER

TAKIKO TAMURA *, HIROSHI SHIRAKI and HACHIRO NAKAGAWA **

Division of Protein Metabolism, Institute for Protein Research, Osaka University, 5311 Yamada-Kami, Suita, Osaka, 565 (Japan)

(Received May 30th, 1979)

Key words: Adenylate kinase; Subunit; (Rat muscle, Liver)

Summary

Isozymes of adenylate kinase (ATP:AMP phosphotransferase, EC 2.7.4.3) were purified from skeletal muscle and liver of rats to essentially homogeneous states by acrylamide gel electrophoresis and sodium dodecyl sulfate gel electrophoresis. The isozyme from muscle was purified by acidification to pH 5.0, and column chromatography on phosphocellulose, Sephadex G-75 and Blue Sepharose CL-6B, while that from liver was purified by column chromatography on Blue Sepharose CL-6B, Sephadex G-75 and carboxymethyl cellulose. By these procedures the muscle isozyme was purified about 530-fold in 29% yield, and the liver isozyme about 3600-fold in 27% yield from the respective tissue extracts.

The molecular weights of the muscle and liver isozymes were estimated as about 23 500 and 30 500, respectively, by both sodium dodecyl sulfate gel electrophoresis and molecular sieve chromatography, and no subunit of either isozyme was detected. The isoelectric points of the muscle and liver isozymes were 7.0 and 8.1, respectively. The K_m values of the respective enzymes for ATP and ADP were similar, but the K_m(AMP) of the liver isozyme was about one-fifth of that of the muscle isozyme.

Immunological studies with rabbit antiserum against the rat muscle isozyme showed that the muscle isozyme was abundant in muscle, heart and brain, while the liver isozyme was abundant in liver and kidney.

* Present address: Biochemistry Division, Institute of Immunological Science, Hokkaido University, Kita-15, Nishi-7, Sapporo, Japan 060.

** To whom reprint requests should be sent.

Introduction

It has been suggested that there are at least two adenylate kinase isozymes in a single species of animal. For instance, Khoo and Russell [1] demonstrated immunologically that there are two adenylate kinase isozymes in individual humans and rabbits, and Criss et al. [2] separated four isozymes from rat liver by isoelectric focusing. Both groups showed that one of these isozymes was abundant in muscle, brain and heart (muscle-type isozyme), while another was abundant in liver and kidney (liver-type isozyme). It has also been shown that the muscle-type isozyme is recovered in the supernatant fraction on centrifugation of a muscle homogenate, whereas the liver-type isozyme is localized in mitochondrial intermembrane speces [3,4].

The properties of muscle adenylate kinase from many sources have been extensively studied [5—8], but little is yet known about the properties of the liver-type isozyme [9—11], and various values have been reported for its isoelectric point and molecular weight [9,11—13]. Moreover, the properties of liver and muscle adenylate kinases from a single species have not yet been compared. This paper describes simple and convenient procedures for purification of the liver-type and muscle-type isozymes from rats, and some enzymatic properties of these purified isozymes.

Materials and Methods

Materials

Male Wistar strain rats were used throughout. They were killed by decapitation and their skeletal muscles and livers were removed and stored at —70°C until use.

Chemicals and enzymes were obtained from the following sources: ATP, ADP, dithiothreitol and 2(*N*-morpholino)ethanesulfonic acid (Mes) (Sigma); NADH, NADP, pyruvate kinase, lactate dehydrogenase, hexokinase and glucose-6-phosphate dehydrogenase (Boehringer); AMP (Kyowa Hakko); peroxidase (Nagaze); Sephadex G-25, G-75 and G-150, and Blue Sepharose CL-6B (Pharmacia); carboxymethyl cellulose (CM-52) and phosphocellulose (P-cellulose) (Whatman); ampholine (LKB); complete and incomplete Freund's adjuvant (Difco). Myoglobin, cytochrome *c* and hemoglobin were gifts from Dr. M. Tamura, Institute for Applied Electrics, Hokkaido University. Other chemicals were of reagent grade.

Enzyme assays and protein determination

Adenylate kinase activity in forward reaction (ATP + AMP $\rightleftharpoons$ 2 ADP) was measured at 30°C in reaction mixtures consisting of 50 mM morpholinopropanesulfonic acid buffer (Mops) (pH 7.4), substrates (5 mM ATP and 5 mM AMP for the muscle-type isozyme, and 3 mM ATP and 0.5 mM AMP for the liver-type isozyme), the same concentration of $MgCl_2$ as ATP, 0.3 mM phospho*enol*pyruvate, 0.22 mM NADH, 150 mM KCl, 10 units of pyruvate kinase, 10 units of lactate dehydrogenase and an appropriate amount of adenylate kinase in a volume of 1 ml. The reverse reaction was measured, when necessary, in 1 ml of reaction mixture consisting of 50 mM Mops (pH 7.4), 3 mM ADP, 1.5

mM $MgCl_2$, 100 mM glucose, 0.5 mM NADP, 2.8 units of hexokinase, 2.8 units of glucose-6-phosphate dehydrogenase and adenylate kinase. Adenylate kinase was diluted with 0.1% bovine serum albumin in 50 mM Mops buffer (pH 7.4) and immediately assayed spectrophotometrically by monitoring NADH oxidation or NADP reduction. One unit of enzyme activity was defined as the amount forming 0.5 μmol of ADP per min in the forward reaction or 1 μmol of ATP or AMP per min in the reverse reaction.

Protein was estimated by the method of Lowry et al. [14].

Electrophoresis

Disc electrophoresis was carried out using 7.5% polyacrylamide gel and a buffer system of pH 9.5. Sodium dodecyl sulfate (SDS) gel electrophoresis was performed as described by Weber and Osborne [15] with 10% polyacrylamide gel containing 0.1% SDS. Column isoelectric focusing was carried out by the method of Vesterberg [16].

Immunological procedures

Antiserum against purified rat muscle adenylate kinase was obtained as described previously [17], except that 2 mg of purified muscle adenylate kinase were emulsified with Freund's complete adjuvant. The specificities of the antisera were tested by Ouchterlony's double diffusion technique. The inhibition reaction with anti-muscle adenylate kinase serum was carried out as follows. Reaction mixture containing 20 mM potassium phosphate buffer (pH 7.4), 0.1% bovine serum albumin, 0.02% NaN_3 and an appropriate amount of antiserum or non-immunized serum, and a tissue extract was incubated at 37°C for 30 min, stood overnight at 4°C and then centrifuged at 900 × *g* for 15 min. Then the activity and protein content of the supernatant and precipitate were measured.

Preparation of adenylate kinase from muscle

All procedures were performed in a cold room or in an ice-bath. Fractions I, II and III in Table I were obtained by a procedure similar to that described by Noda et al. [7], except that a buffer of pH 6.7 (Mops) was used instead of a buffer of pH 7.0 for the last washing of P-cellulose adsorbing the enzyme on Buchner funnel. Fraction IV was obtained by Sephadex G-75 column chromatography and gave two bands upon SDS gel electrophresis, and was therefore purified further. The concentrated and desalted Fraction IV was applied to a column of Blue Sepharose CL-6B (1 × 5 cm), washed with 20 mM Mops (pH 7.0) and then the enzyme was eluted with 20 mM Mops (pH 7.0) containing 2 mM ATP and 2 mM AMP, and precipitated from the eluate with 90% saturation of $(NH_4)_2SO_4$ (Fraction V).

Preparation of adenylate kinase from liver

Frozen rat livers were slightly thawed, and homogenized with 3 vols. of 20 mM potassium phosphate buffer (pH 7.0). The homogenate was centrifuged at 105 000 × *g* for 60 min and the supernatant was filtered through nylon cloth and collected (Fraction I). It was applied to a Blue Sepharose column (2.6 × 21 cm) and washed with 20 mM Mops buffer (pH 7.0). A large amount of protein

was not adsorbed on the column. The enzyme was eluted with 2 column volumes of the same buffer containing 2.5 mM ATP and 2.5 mM AMP, and then with 1 column volume of the same buffer containing 1 M NaCl (Fraction II). Fraction II was treated with $(NH_4)_2SO_4$ (30—90% saturation) and dissolved in 20 mM potassium phosphate buffer containing 0.05 mM AMP (Fraction III). Fraction III was applied to a Sephadex G-75 column (1.6 × 60 cm) and the activity was eluted with 20 mM potassium phosphate buffer (pH 7.0) in a small peak after a large peak of inert protein. It was then concentrated with 90% saturation of $(NH_4)_2SO_4$ and dissolved in 20 mM Mops buffer (pH 6.5) (Fraction IV). Fraction IV gave several bands on SDS gel electrophoresis, and was therefore purified further. Desalted Fraction IV was applied to a CM-cellulose column (1.2 × 10 cm), and the column was washed successively with the 20 mM Mops buffer (pH 6.5) and then with the same buffer containing 0.25 M KCl. Then the enzyme was eluted with 0.35 M KCl in the same buffer and concentrated by precipitation with $(NH_4)_2SO_4$ (Fraction V).

Results

Enzyme purification

Table I and II summarize the results of purification precedures for the muscle and liver enzymes, respectively. As shown from these tables, rat muscle adenylate kinase was purified about 1900-fold from the muscle extract in 29% yield and its final specific activity was 1650 units per mg protein, while rat liver enzyme was purified about 3600-fold from the liver extract with an overall yield of 27% to a final specific activity of 1400 units per mg protein.

Homogeneity

The homogeneities of the purified enzymes were examined by disc and SDS polyacrylamide gel electrophoresis. The purified muscle and liver enzymes both gave a single band on disc gel electrophoresis in 7.5% gel with a buffer system of pH 9.5. On SDS gel electrophoresis, the purified liver enzyme gave a faint minor band in addition to the major band.

TABLE I

PURIFICATION OF RAT MUSCLE ADENYLATE KINASE

Starting material, 580 × *g* muscle.

Procedure	Fraction	Volume (ml)	Protein (mg)	Activity		Purification	Yield (%)
				(units/mg)	(units × 10^{-3})		
Extraction	I	1600	25 600	3.56	91.1	(1)	(100)
pH 5.0 filtrate	II	1600	16 300	5.59	91.1	1.6	100
P-Cellulose	III	170	180	248	44.7	70	49.1
Sephadex G-75	IV	36	21.0	1400	29.4	393	32.3
Blue Sepharose CL-6B *	V	21	13.7	1900	26.0	534	28.5

* The Blue Sepharose was as follows: dye binding, 0.5 μmol/ml swollen gel; 1 g of dry powder was equivalent to 20 ml of swollen gel.

TABLE II

PURIFICATION OF RAT LIVER ADENYLATE KINASE

Starting material, 110 × *g* liver.

Procedure	Fraction	Volume (ml)	Protein (mg)	Activity		Purification	Yield (%)
				units/mg	units		
Extraction	I	325	11 400	0.454	5180	(1)	(100)
Blue Sepharose CL-6B *	II	135	105.2	41.5	4370	91.4	84.3
Ammonium sulfate fractionation	III	8.45	85.0	31.4	2670	69.2	51.6
Sephadex G-75	IV	1.98	3.18	481	1530	1060	29.6
CM-Cellulose	V	0.50	0.85	1650	1400	3630	27.0

* The Blue Sepharose was as follows: dye binding, 2 μmol/ml swollen gel; 1 g of dry powder was equivalent to 3.6 ml of swollen gel.

Molecular weight determination

The molecular weights of the native muscle and liver enzymes were determined to be 23 000 and 31 000, respectively, by Sephadex G-150 column chromatography. Their minimum molecular weights were 24 000 and 30 000, respectively, estimated by SDS polyacrylamide gel electrophoresis. Thus, the molecular weight of rat liver adenylate kinase was slightly larger than that of the muscle isozyme and there are no evidence of subunits of either.

Kinetic properties

The optimum concentration of magnesium for muscle isozyme was 3 mM to 8 mM in the presence of 5 mM ATP, while that for liver isozyme was 3 mM to 6 mM in the presence of 3 mM ATP, as shown in Fig. 1. Hence, in enzyme assays equal concentrations of magnesium and ATP were used. The magnesium

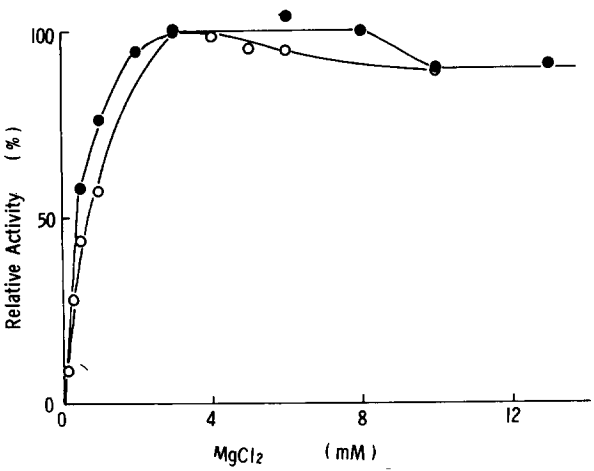

Fig. 1. Relationship between $MgCl_2$ concentration and activity. (•) muscle adenylate kinase; the assay medium contained 5 mM AMP and 5 mM ATP; (○) liver adenylate kinase; the assay medium contained 0.5 mM AMP and 3 mM ATP.

concentration dependencies of these isozymes were similar to that of the enzyme in rabbit muscle [18].

The kinetic constants of the muscle and liver enzymes are given in Table III. The K_m(ATP) and K_m(AMP) values of the muscle enzyme were almost the same and the $K_m(ADP)_f$ value was about one third of the K_m(ADP · Mg). The K_m(AMP) value of the liver enzyme was one third of the K_m(ATP), but the K_m(ADP) and K_m(ADP · Mg) value were nearly the same. The K_m(AMP) value of the liver enzyme was one-fifth of that of the muscle enzyme. AMP at above 0.5 mM inhibited the liver isozyme (Fig. 2(a)), and AMP at above 4 mM appeared to inhibit the muscle isozyme slightly (Fig. 2(b)).

Other properties

The pH-vs. activity curves of the forward reactions of the two isozymes are shown in Fig. 3. The muscle isozyme showed a broad pH optimum of pH 6.0—9.0, like the rabbit enzyme [18], whereas the liver isozyme showed a narrower optimum of pH 6.0—7.5. The activity of the liver isozyme also decreased more steeply than that of the muscle isozyme in acidic regions. The pH values of the muscle and liver isozymes at which the activities were half-maximum were 5.1 and 5.6, respectively. The broad pH optima of the rat muscle and liver isozyme are in contrast with the narrow ones of adenylate kinase from yeast [20], bovine liver mitochondria [10] and porcine heart (possibly mitochondria) [21].

Story reported an inhibitory effect of a physiological concentration of NADH on adenylate kinase from squid mantle [22], and Pradhan et al. reported a stimulatory effect of tricarboxylic acid cycle intermediates. especially, citrate [23]. However, the two isozymes from rat were not affected

TABLE III

KINETIC CONSTANTS OF ADENYLATE KINASE

Assay conditions are given in the text. Kinetic constants were obtained from Lineweaver-Burk plots.

K_m (substrate)	Muscle ($\times 10^3$ M)	Liver ($\times 10^3$ M)
(ATP)	0.56	0.33
(AMP)	0.53	0.10
(ADP · Mg) *	0.30	0.11
$(ADP)_d$	0.092	0.091
V_m (substrate)	Muscle	Liver
	$\times 10^{-3}$ mol · mol per enzyme per min	
(ATP)	45	45
(AMP)	45	49
(ADP · Mg) *	45	49
$(ADP)_f$	45	49

* A K_D value of 0.03 mM for $(ADP)Mg^- \rightleftharpoons (ADP)^{3-} + Mg^{2+}$ [19] was used for determining the concentration of (ADP · Mg) or $(ADP)_f$.

** Molecular weights of 23 500 and 30 500 for muscle and liver adenylate kinase, respectively, were used in the calculation.

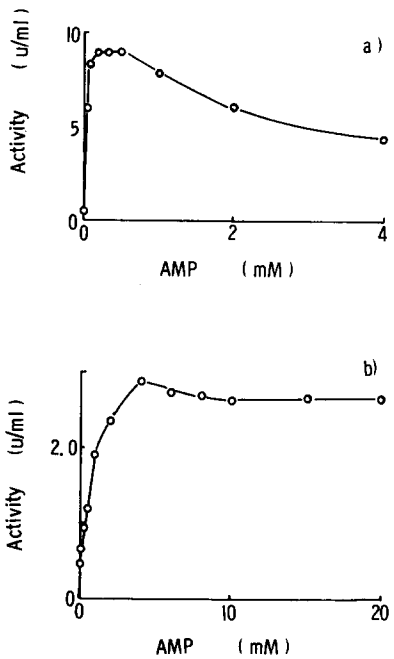

Fig. 2. Effect of AMP concentration on adenylate kinase activity. (a) liver adenylate kinase activity in the presence of 3 mM ATP; (b) muscle adenylate kinase activity in the presence of 5 mM ATP. Other conditions are described in the text.

by 25 μM to 300 μM NADH or by 0.1 μM to 10 mM citrate in an assay system coupled with pyruvate kinase and lactate dehydrogenase.

Isoelectric points

The isoelectric points (p*I* values) of the rat muscle and liver isozymes were

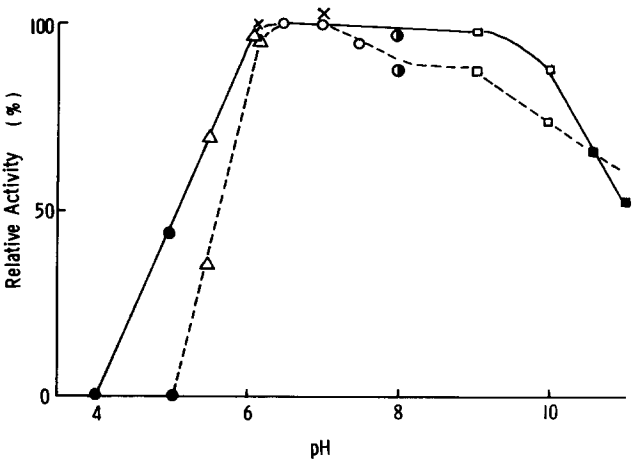

Fig. 3. pH dependency of adenylate kinase. The following buffers (50 mM) were used: ●, acetate; △, Mes; ○, potassium phosphate; X, histidine; ⊗, triethanolamine-HCl; □, lysine-HCl and ■, glycine-NaOH. ———, muscle adenylate kinase; - - - - - -, liver adenylate kinase. other conditions are given in the text.

determined to be 7.0 and 8.1, respectively, by column isoelectric focusing (data not shown). The p*I* values of the isozymes in extracts of rat liver and kidney were also investigated (Figs. 4 a and b). Most of the adenylate kinase activity in both extracts was attributable to the liver-type isozyme, because the p*I* values were 8.1, but a small amount of adenylate kinase with a p*I* value of 7.0, presumably the muscle type isozyme, was also detected in both extracts.

Immunological characteristics

Antiserum to the muscle enzyme formed a sharp single precipitin line against purified muscle adenylate kinase and a less sharp line against a muscle extract in Ouchterlony's double diffusion test (Fig. 5a). Fig. 5b shows the precipitin reaction between antiserum to rat muscle adenylate kinase and extracts of

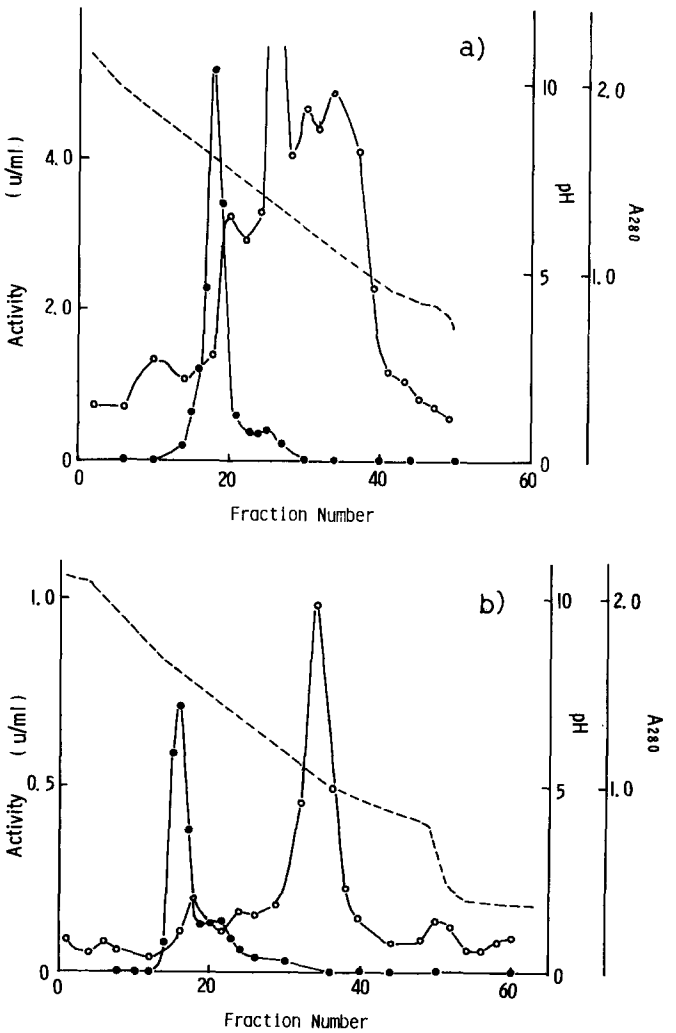

Fig. 4. Column isoelectric focusing patterns of tissue extracts. (a) liver extract, (b) kidney extract; - - - - - -, pH; ●, activity; ○, A_{280}.

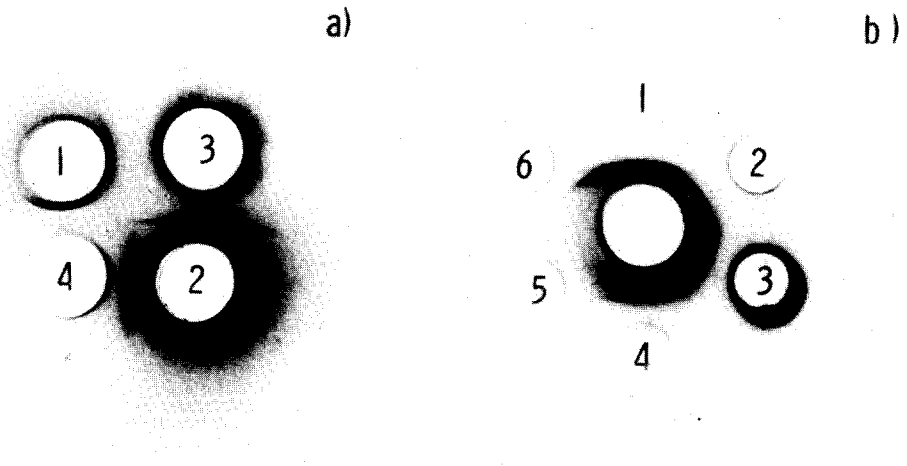

Fig. 5. Ouchterlony's double diffusion analysis. (a) 1, normal serum; 2, anti-muscle adenylate kinase serum; 3, purified muscle adenylate kinase; 4, muscle extract. (b) center well, anti-muscle adenylate kinase serum, 1, muscle extract; 2, brain extract; 3, heart extract; 4, stomach extract; 5, liver extract; 6, kidney extract.

various organs. Precipitin lines were formed between the antiserum and muscle, brain, heart and stomach extracts, but not liver and kidney extracts. The reason why no precipitin lines were formed with the last two extracts, in spite of the existence of small amounts of muscle-type isozyme (Fig. 4a, b and Table IV) may be that the titer of the antiserum and/or the content of muscle-type isozyme was too low.

Tissue distribution of adenylate kinase isozymes

Since AMP inhibited the liver-type isozyme, the activities in various organs were assayed with a concentration of 1 mM AMP, which only inhibited about

TABLE IV

ADENYLATE KINASE ACTIVITIES IN VARIOUS ORGANS AND THEIR INHIBITION BY ANTISERUM AGAINST MUSCLE ADENYLATE KINASE

Organ	Activity * (units/g organ)	Inhibition ** (%)
Muscle	139.4	99
Liver	58.0	8.1
Kidney	46.1	13
Heart	43.4	89
Brain	36.2	92
Stomach	14.1	67
Lung	11.4	33
Small intestine	3.7	10
Spleen	2.6	16

* Activities were measured at 3 mM ATP and 1 mM AMP. Other conditions were as in the ordinary assay.

** Standard deviations were about ±5%.

20% of the activity at the optimum concentration of substrates. As seen in Table IV, the activity was highest in muscle, and decreased in the following order: muscle, liver, kidney, heart, brain.

The inhibition of adenylate kinase by antiserum to the muscle enzyme was also examined to estimate the content of muscle-type isozyme in various organs (Table IV). Most of the adenylate kinase activity in muscle and most of the activity in brain and heart was inhibited by the antiserum. In contrast, little of the activity in liver, kidney, small intestine and spleen was inhibited by the antiserum.

Discussion

Markland et al. [9] first reported a method for purification of adenylate kinase from bovine liver mitochondria, involving 12 steps and giving a preparation of 85—95% purity in about 0.8% yield from the crude homogenate. Subsequently, Criss et al. [11] reported a method for purifying the rat liver enzyme, involving two column chromatographies of Sephadex G-75 and preparative column isoelectric focusing. Our method is superior to these methods in the following respects. (1) Blue Sepharose column chromatography immediately after enzyme extraction resulted in a smaller volume of preparation and a higher specific activity. (2) Our method does not involve isoelectric focusing, which is expensive and gives a variable yield (40—75% in our experiments). (3) Elution of the enzyme from a Sephadex G-75 column with buffer containing ATP, AMP or both resulted in better recovery than elution without these substrates. A similar procedure has been reported for the purification of adenylate kinase from calf liver [24].

We obtained the result that the molecular weight of rat liver adenylate kinase was larger than that of muscle enzyme. Similar results were obtained by others [11,21], except for Markland et al. [9]. Above findings suggest that the liver-type adenylate kinase is slightly larger than the muscle-type enzyme of the same species.

We found that the activity of the liver adenylate kinase was inhibited by AMP (at concentrations above 0.5 mM). The inhibition by AMP to muscle enzyme activity was also reported [25]. It is not known whether these inhibitions by AMP have any physiological signifiance, because the effective concentration of AMP for the inhibition is considerably higher than the physiological concentration in either muscle or liver.

Various p*I* values have been reported for adenylate kinase [5,6,21,26—28]. However, we found that the p*I* value of the purified muscle adenylate kinase from rat skeletal muscle was 7.0 and the isoelectric focusing patterns of liver and kidney extracts had a small peak at pH 7.0, whereas the p*I* value of the purified liver enzyme was 8.1 and the isoelectric focusing patterns of liver and kidney extracts had a large peak of activity at pH 8.1. These findings and the results of our immunological examinations suggest that there are at least two isozymes in rats; namely, the muscle type (p*I* 7.0), which is predominant in muscle, brain and heart, and the liver type (p*I* 8.1), which is predominant in liver and kidney.

It has been presumed that the location of the mucle type isozyme is in

cytosol. Our work also suggested this. Evidence for this is as follows. (1) Most of the adenylate kinase activity in muscle was readily extracted into the soluble fraction, irrespective of the tonicity of the extraction medium, and there was no association of the purified muscle adenylate kinase with myosin or actin in vitro, although it is reported that many muscle enzymes related to glycolysis, glucogenolysis or the purine nucleotide cycle are attached to contractile proteins [17,29—31]. (2) Preliminary experiments by the fluorescent antibody technique showed no special location of adenylate kinase within cells in skeletal muscle. While liver-type adenylate kinase in all tissues is thought to be localized in the mitochondrial intermembrane space [3,4]. Thus the liver-type enzyme exists mainly in mitochondria in liver and kidney, where ATP is actively synthesized, while the muscle type may exist in the cytosol of muscle, brain and heart, where a large amount of ATP is used up. Such localizations of these two isozymes would be closely related to the physiological function of the isozymes. It has been reported that the specificity of adenylate kinase for the AMP site is more rigorous than its specificity for the triphosphate site [32]. Thus the fact that the K_m(AMP) value of the liver enzyme is about one-fifth of that of the muscle enzyme might favor the regeneration of AMP to ADP in the presence of ATP, and consequently favor oxidative phosphorylation in mitochondria. Substantiation of this working hypothesis must await further investigations.

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