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PURIFICATION OF RAT LIVER ADENYLATE KINASE ISOZYME II AND COMPARISON WITH ISOZYME III

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SUMMARY

1. Adenylate kinase (ATP:AMP phosphotransferase, EC 2.7.4.3) isozyme II from rat liver was purified 136-fold to a final specific activity of 60 μmoles of ADP produced per min per mg of protein at 25°. This represented a purification to at least 85% homogeneity. Further purification was hampered by the instability of the enzyme.

2. The final preparation behaved as a single peak in the analytical ultracentrifuge, and gave a single major band and three trace bands on disc gel electrophoresis using sodium dodecul sulfate–urea.

3. The molecular weight, which was estimated to be 46 000 by Sephadex G-100 chromatography and to be 49 000 by analytical ultracentrifugation was similar to the previously published molecular weight (46 000) of the dimer form of rat liver adenylate kinase isozyme III.

4. Both isozymes from rat liver can use ATP and dATP as phosphoryl donors. The K_m values for ATP was 0.39 mM with isozyme II and 0.43 mM with isozyme III. The K_m for dATP was 1.8 mM with isozyme II and 0.83 mM with isozyme III. Both isozymes exhibited about 30 to 35% greater maximal velocity with dATP as a substrate than with ATP.

5. GTP, dGTP, CTP, UTP, and ITP did not serve as substrates for either isozyme.

6. Of several nucleoside monophosphates tested with isozyme II, only 5'-AMP served as a phosphoryl acceptor, with a K_m value of 0.073 mM.

7. In the reverse direction (production of ATP), the K_m for ADP was 0.3 mM.

8. Kinetic comparison of isozymes II and III revealed similar substrate specificities, Michaelis constants, inhibition to AMP, stability in acid, and lability in alkali. However, differences were observed in stability in phosphate buffer at 4°, sensitivity to mercurial reagents, and sensitivity to free fatty acids and detergents.

9. Comparison of physical parameters showed no significant differences between the two isozymes in terms of diffusion coefficients, partial specific volumes, frictional or axial ratios. Whereas isozyme III had the ability to aggregate, isozyme II did not.

Also, there were differences in isoelectric points and immunological cross reactivity with antiserum prepared against isozyme III.

INTRODUCTION

The enzyme adenylate kinase (ATP:AMP-phosphotransferase EC 2.7.4.3) from several sources occurs in tissue-specific forms[1-5] which differ in molecular and catalytic properties[1-7]. Recent findings in our laboratory have established the occurrence of the enzyme in at least 3 isozymic forms of rat liver[8]. The predominant form, isozyme III, which comprises about 77% of the total adenylate kinase activity in rat liver, is decreased in hepatomas[8] and localized in the outer compartment of the mitochondria[9]. On the other hand, isozyme II, which comprises about 18% of the total activity in liver, is unchanged in hepatomas[8] and is localized in the soluble fraction of the cell[9]. Because of the above differences, it was of interest to purify the two isozymes and to compare their physical and kinetic properties.

MATERIALS AND METHODS

Chemicals

$NADP^+$, NADH, ATP, ADP, AMP, pyruvate kinase (ATP:pyruvate phosphotransferase, EC a.7.1.40), and lactate dehydrogenase (L-lactate:NAD^+ oxidoreductase, EC 41.1.27) were obtained from P-L Biochemicals, Milwaukee, Wisc. Hexokinase (ATP:D-hexose 6-phosphotransferase, EC 2.7.1.1), glucose 6-phosphate dehydrogenase (D-glucose 6-phosphate:$NADP^+$ oxidoreductase, EC 1.1.1.49), and other nucleoside mono- and triphosphates were from Sigma, St. Louis, Mo. Phosphoenolpyruvate was purchased from Boehringer-Mannheim, N.Y.: pepsin (EC 3.4.4.1) from Worthington, Freehold, N.J.; horse heart cytochrome *c* from Mann, Orangeburg, N.Y.; human hemoglobin from Pentex, Inc., Kankakee, Ill.; and blue dextran from Pharmacia, Piscataway, N.J. All other chemicals were obtained as described previously[8-12].

Animals

Male CFN rats obtained from Carworth Farms, New York, were maintained on lab chow until they were 200–300 g in body weight.

Enzyme assays

All assays were performed in a 3-ml volume. The rate of oxidation of NADH or reduction of $NADP^+$ at 25° was monitored continuously at 340 nm with a Gilford spectrophotometer. All assays were linear with enzyme concentration. A unit of activity was defined as 1 μmole of product formed per min in the assay. Specific activity was defined as the number of units per mg of protein.

The assay for adenylate kinase in the forward direction (production of ADP) contained 300 mM triethanolamine–HCl buffer (pH 7.5), 400 μM KCl, 8.4 μM $MgSO_4$, 8.1 μM ATP, 0.75 μM AMP, 1.2 μM NADH, 2.1 μM phosphoenolpyruvate, 30 units pyruvate kinase, 30 units lactate dehydrogenase, and rate-limiting amounts of adenylate kinase.

The assay for adenylate kinase in the reverse direction (production of ATP) contained 300 mM triethanolamine–HCl buffer (pH 7.5), 400 μM KCl, 6.4 μM $MgSO_4$, 4.5 μM ADP, 5.1 μM glucose, 1.2 μM NADP, 30 units hexokinase, 25 units glucose 6-phosphate dehydrogenase and rate-limiting amounts of adenylate kinase.

For K_m determinations in the forward direction, the nucleotide concentrations were varied and the $MgSO_4$ to nucleoside triphosphate ratio was maintained at approximately 1:1. For K_m determinations in the reverse direction, the ADP concentration was varied and the $MgSO_4$ to ADP ratio was maintained at approximately 1.4:1.

Analytical procedures

Most analytical procedures have been previously described[9,10]. New procedures are described within the RESULTS section of the present manuscript.

RESULTS

Purification of adenylate kinase II

All operations were performed at 0–4°. A summary of the purification procedure is given in Table I. Most purification steps, namely, the preparation of cytosol, pH fractionation, Sephadex G-75 chromatography, and isoelectrofocusing were performed as previously described[8,10].

Adenylate kinase II was separated from the other isoenzymes at the isoelectrofocusing step. Thus, the enzyme activity prior to electrofocusing (Table I) represents the total activity contributed by all of the adenylate kinase isoenzymes. The activity after the isoelectrofocusing step, represents only isozyme II. We have previously reported that the isoelectric points of isoenzymes II and III were pH 7.0 and 7.6, respectively, when the carrier ampholytes used during electrofocusing had a pH range of 3 to 10. In the present experiments, a pH gradient of 5 to 8 resulted in isoelectric points of pH 7.5 and 8.0, respectively, for isozymes II and III.

TABLE I

PURIFICATION OF ADENYLATE KINASE II*

The enzyme activity prior to electrofocusing represented total adenylate kinase activity contributed by all isoenzyme.

Fraction	*Vol. (ml)*	*Total activity (units)*	*Total protein (mg)*	*Specific activity (units/mg protein)*	*Recovery (%)*	*Purification (-fold)*
Whole homogenate	280	13 500	30 000	0.45	100	—
Cytosol	210	11 340	10 290	1.1	84	2.4
pH 3 supernatant	196	7 762	2 000	3.9	57.4	8.7
Sephadex G-75	96	5 760	384	15.0	42.6	33.4
Electrofocusing	19.6	241	3.3	73.0	18.0	162.0
Sephadex G-75*	26.0	2 560	42.9	60.0	—	136.0

* Electrofocusing fractions obtained over a period of 6–8 months were combined, dialyzed with 30 vol. of 5 mM sodium phosphate containing 1 mM β-mercaptoethanol (pH 7.5) and concentrated before layering on the Sephadex G-75 column.

Rechromatography on Sephadex G-75. Fractions from electrofocusing with the highest adenylate kinase II activity were combined and frozen over a period of 6–8 months. Subsequent purification of isozyme II was accomplished by combining isozyme II from a series of 21 purification runs for adenylate kinase III. The frozen electrofocused fractions were thawed, combined, diluted with 9 vol. of 5 mM sodium phosphate buffer (pH 7.2) containing 1 mM β-mercaptoethanol, and concentrated in an Amicon ultrafiltration cell using a PM-10 membrane. The concentrated preparation (7.1 ml) was layered on a 78.5 cm $\times$ 3.2 cm column of Sephadex G-75 equilibrated with 5 mM phosphate buffer (pH 7.2). The enzyme was eluted with the same buffer and 3-ml fractions were collected and assayed for adenylate kinase activity. Fractions containing most of the enzymatic activity were combined, concentrated and dialyzed with several volumes of buffer.

A summary of the purification scheme is given in Table I. The final specific activity was 60 μmoles of ADP produced per min per mg of protein at 25°. The last Sephadex step did not increase purification, but was necessary to remove ampholytes remaining from electrofocusing and ultrafiltration dialysis.

Properties of adenylate kinase II

Stability. The 136-fold purified isozyme II preparation was stable for about 6 months when frozen, but steadily lost activity when repeatedly thawed and frozen. It lost about 95% of its activity when stored at 4° over a period of two weeks. Further attempts to purify this isozyme were hampered by its extreme lability.

Analytical ultracentrifugation. Only one peak was observed in the analytical ultracentrifuge when adenylate kinase II was centrifuged at 11 mg protein per ml (Fig. 1).

Polyacrylamide gel electrophoresis. Electrophoresis of isozyme II on polyacrylamide gels containing sodium dodecyl sulfate–urea according to the procedure of DUNKER AND RUECKERT[13] revealed one major band and 3 tracer bands.

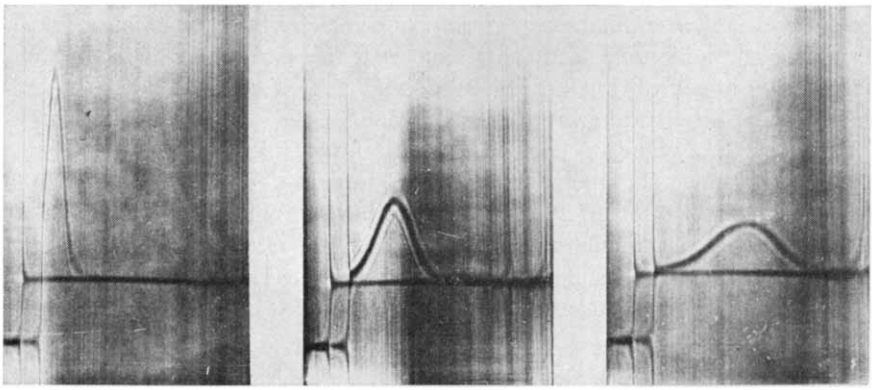

Fig. 1. Sedimentation velocity of adenylate kinase II using a double sector, filled upon centerpiece. The speed was 60 000 rev./min; the direction of sedimentation is from left to right. Photographs were made at 8-min intervals and the 3 selected above were, from left to right, 8, 48, and 96 min after speed was attained. The run was made at 20°; the protein concentration was 11 mg/ml and the solvent was 5 mM sodium phosphate at pH 7.2. The value of $s_{20,w}$ from this determination was calculated to be 2.7.

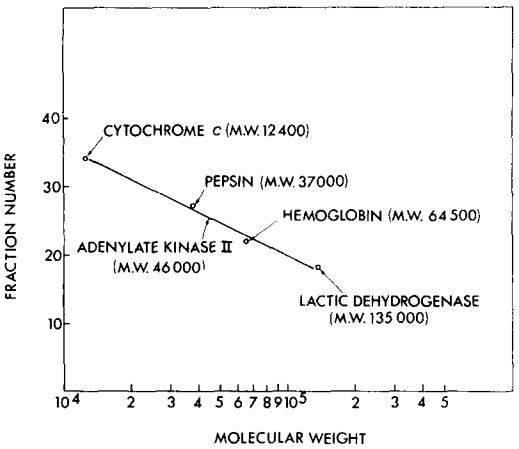

Fig. 2. Plot of elution volume *versus* log molecular weight of the standards for the estimation of the molecular weight of adenylate kinase II. A column (48 cm × 1 cm) of Sephadex G-100 equilibrated with 5 mM sodium phosphate buffer (pH 7.2) containing 1 mM β-mercaptoethanol was calibrated with the molecular weight standards. The same column was then used for the chromatography of a 0.3-ml aliquot of the combined fraction from G-75.

Molecular weight determination with Sephadex G-100. This was performed at 4° as described by ANDREWS[14]. The molecular weight standards were chicken heart lactate dehydrogenase (mol. wt. 135 000)[15], human hemoglobin (mol. wt. 64 500)[16,17], pepsin (mol. wt. 37 000)[18], and horse heart cytochrome *c* (mol. wt. 12 400)[19]. A 0.3-ml aliquot of the combined Sephadex G-75 fraction was layered on a calibrated column (48 cm × 1 cm) of Sephadex G-100 equilibrated with 5 mM sodium phosphate buffer (pH 7.2) containing 1 mM β-mercaptoethanol. The same solution was used to elute 15-drop fractions. From a plot of elution volume *versus* log molecular weight of the standards, the molecular weight of isozyme II was estimated to be about 46 000 (Fig. 2).

Molecular weight determination by analytical ultracentrifugation. A single peak was observed in the analytical ultracentrifuge with the purified preparation containing 11 mg protein per ml (Fig. 1). The Svedberg constant decreased slightly with increasing protein concentration, and extrapolation to zero protein concentration revealed a single $s^0_{20,w}$ value of 3.02 (Fig. 3). In contrast, isozyme III gave two $s^0_{20,w}$ values, 1.32 and 3,52, upon extrapolation to zero protein concentration[10]. From the slope of a plot of σ^2 *vs.* $2t$ (Fig. 4), the diffusion constant of isozyme II at 11 mg protein per ml was calculated to be $5.34 \cdot 10^{-7}$ cm^2/sec. From the $s^0_{20,w}$ and $D_{20,w}$ values at 11 mg/ml, the molecular weight of isozyme II was calculated to be 49 000. This was in good agreement with the molecular weight of 46 000 estimated from G-100 chromatography.

Substrate specificity. We previously reported[10] that isozyme III can utilize ATP, dATP, and dGTP as phosphoryl donors, but have since noted that the activity observed with dGTP was an artifact due to a contaminant in the nucleotide which reacted with the coupling enzymes in the assay. Isozyme III, therefore, is specific for ATP and dATP as phosphoryl donors. The previously published v_{max} values for isozyme III[10] were also in error since the amount of AMP (1 mM) used was inhibitory,

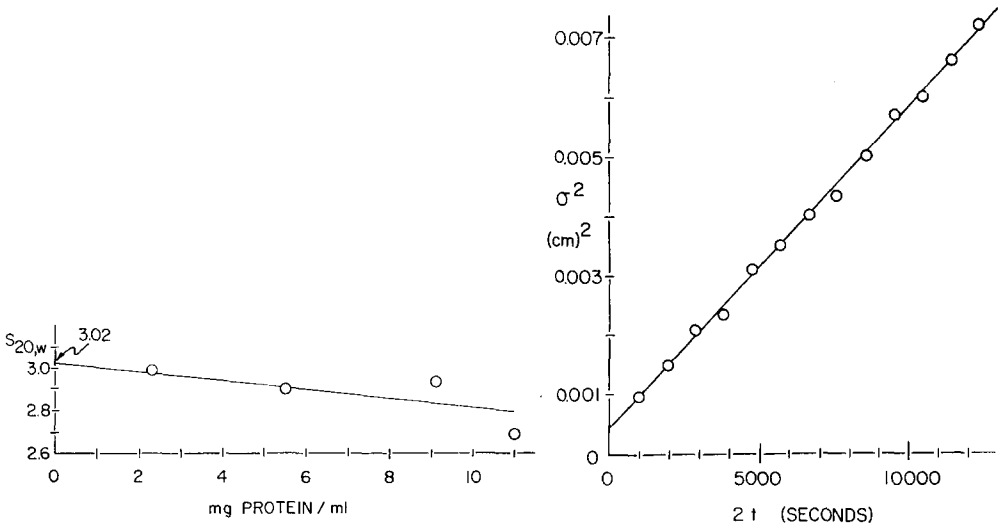

Fig. 3. Plot of $s^\circ_{20,w}$ *vs.* protein concentration of adenylate kinase II preparation.

Fig. 4. Plot of σ^2 *vs.* $2t$ (in sec). From the slope of the plot the diffusion coefficient $D_{20,w}$ of adenylate kinase II at 11 mg protein per ml was calculated to be $5.34 \cdot 10^{-7}$ cm²/sec.

and the $MgSO_4$ to nucleoside triphosphate ratio was not optimal. Under optimal conditions (0.25 mM AMP, 1:1 ratio of $MgSO_4$ to nucleoside triphosphate, and excess nucleoside triphosphate), the percentage activity of isozyme III was 100% with dATP and 70% with ATP; the relative rate of isozyme II reaction was 100% with dATP and 65% with ATP. Neither isozyme exhibited activity with 5 mM dGTP, GTP, CTP, UTP, or ITP. Like isozyme III, isozyme II used only 5'-AMP as a phosphoryl acceptor[10]. The following nucleotides (at 3 mM) did not serve as substrates: 3',5'-cyclic AMP, dAMP, 2'-AMP, 3'-AMP, TMP, CMP, UMP, GMP, dGMP, and IMP.

TABLE II

KINETIC COMPARISON OF LIVER ADENYLATE KINASE ISOZYMES

Characteristics	*Isozyme II*	*Isozyme III*
Subcellular location	Cytoplasm	Mitochondria
Final specific activity	60 units/mg	320 units/mg
Substrates (forward)	Mg^{2+}–ATP, AMP	Mg^{2+}–ATP, AMP
Substrates (backward)	Mg^{2+}–ADP, ADP	Mg^{2+}–ADP, ADP
Rate direction favored	Formation of ADP	Formation of ADP
K_m (ATP) (apparent)	0.39 mM	0.43 mM
K_m (ADP) (apparent)	0.30 mM	0.18 mM
K_m (AMP) (apparent)	0.0735 mM	0.12 mM
K_m (dATP) (apparent)	1.80 mM	0.83 mM
K_i (AMP) (apparent)	≈2.13 mM	≈1.81 mM
Stability at 4° (phosphate buffer)	—	+
Stability in acid	+	+
Stability in alkali	—	—
Sensitivity to mercurials	+	—
Sensitivity to detergents	+	++

TABLE III

STABILITY OF ADENYLATE KINASE ISOZYMES IN ACID AND ALKALI

Treatment included incubation of the enzyme in 5 mM sodium phosphate–1 mM β-mercaptoethanol buffer at the stated pH value for 5 min at 25° and then assay at pH 7.5. Values of adenylate kinase activity are arbitrary units.

	Treatment at pH:				
	2.5	*4.0*	*7.0*	*9.0*	*10.5*
Isozyme II	84	84	86	70	<1
Isozyme III	93	92	94	68	<1

Michaelis constants. The apparent K_m values for the substrates of isozyme II were: ATP, 0.39 mM; dATP, 1.8 mM; AMP, 0.073 mM; and ADP, 0.3 mM. The apparent K_m values of isozyme III for ATP and dATP[10] were redetermined with 0.25 mM AMP and a 1:1 ratio of $MgSO_4$ to nucleoside triphosphate. The corrected values were 0.43 mM for ATP and 0.83 mM for dATP.

Comparison of adenylate kinases II and III

Kinetic comparisons. Numerous kinetic studies have been performed with the two purified liver forms of adenylate kinase. The results of these studies are tabulated in Table II. Adenylate kinase II was located exclusively in the cytoplasm while isozyme III was found in the outer mitochondrial compartment. The substrates were similar for both isozymes. They each catalyzed phosphate transfer between ATP, AMP, ADP, and dATP. The reaction rate ratio (defined as: ATP converted/ATP formed) for isozymes II and III was 2.8 and 3.4, respectively. The apparent Michaelis constants for each substrate were not significantly different between the two isozymic forms. Each isozyme exhibited substrate inhibition by AMP and showed similar apparent K_i values. When stored in 5 mM sodium phosphate buffer containing 1 mM mercaptoethanol at pH 7.2 at 4°, isozyme II lost most of its activity within a few days; under similar conditions, adenylate kinase III lost less than 10% activity in 1 month. Both enzymes are stable at pH 2.5. Both enzymes lost 100% activity within a few minutes at pH 10.5 (Table III). In the presence of 10^{-4} M *p*-chloromercuribenzoate, isozyme II lost 80% activity within 5 min, isozyme III lost less than

TABLE IV

STABILITY OF ADENYLATE KINASE ISOZYMES WITH MERCURIAL REAGENTS

Treatment included incubation of the enzyme in 5 mM sodium phosphate buffer at pH 7.2 for 5 min at 25° in the presence of the mercurial reagents and then assay. Control values are illustrated with only dithiothreitol present. Values of adenylate kinase are arbitrary units.

	Mercurial reagent		
	10^{-4} M p-chloromercuribenzoate	*10^{-4} M hydroxymercuribenzoate*	*10^{-4} M dithiothreitol*
Isozyme II	12	11	64
Isozyme III	62	61	61

TABLE V

STABILITY OF ADENYLATE KINASE ISOZYMES WITH FREE FATTY ACIDS OR DETERGENT

Treatment included incubation of the enzyme in 5 mM sodium phosphate buffer at pH 7.2 for 5 min at 25° in the presence of the fatty acids or detergent and then assay. Values of adenylate kinase are arbitrary units.

	Treatment				
	None	*Myristic acid (50 μM)*	*Palmitic acid (50 μM)*	*Oleic acid (50 μM)*	*Lauryl sulfate (100 μM)*
Isozyme II	78	51	50	53	47
Isozyme III	83	16	14	15	14

10% activity after 4 h (Table IV). Whereas in the presence of 50 μM myristic acid, 50 μM oleic acid, or 100 μM lauryl sulfate, isozyme II lost (irreversibly) about 30% activity at 5 min, while isozyme III lost (irreversibly) about 80% activity at 1 min. Therefore, it would appear that the two liver forms of adenylate kinase are very similar kinetically, but have different subcellular locations and different stability properties.

Physical comparisons. A number of studies designed to determine the physical and structural features of the purified liver adenylate kinases have been made. Table VI tabulates the comparative results. There are no significant differences between isozymes II and III in terms of diffusion coefficient, partial specific volume, frictional or axial ratios. However, molecular weight studies in both the ultracentrifuge and on column chromatography revealed the possibility that adenylate kinase activity could aggregate. Isozyme III was found to exist at molecular weights of 23 000, 46 000, and 68 000. Isozyme II was observed to occur only at 46 000 to 49 000. Thus, it is likely that adenylate kinase III exists in monomer, dimer, and trimer conformations, or at least has the ability to aggregate (molecular weights of greater than 200 000 have frequently been observed on column chromatography for isozyme III). Adenylate kinase II was found only in a possible dimer conformation (assuming a monomer as 23 000). No subunit studies in urea–guanidine · HCl have been performed

TABLE VI

PHYSICAL COMPARISON OF LIVER ADENYLATE KINASE ISOZYMES

Characteristics	*Isozyme II*	*Isozyme III*
Diffusion coefficient	5.7 (10^{-7})	4.8 (10^{-7})
Partial specific volume	0.65	0.74
Frictional ratio	1.3	1.1
Axial ratio	3.8	4.0
Svedberg values	3.02	1.23, 3.52
Molecular weight (centrifugation)	49 000	23 000, 68 000
Molecular weight (chromatography)	46 000	46 000
Isoelectric point	7.5	8.0
Inhibition of anti-adenylate kinase III serum	−	+

TABLE VII

STABILITY OF ADENYLATE KINASE ISOZYMES WITH ANTISERUM PREPARED AGAINST ISOZYME III

Treatment included incubation of the enzyme in 5 mM sodium phosphate buffer at pH 7.2 for 5 min at 25° in the presence of antiserum prepared against adenylate kinase III and then assay. (Control was incubation with 100 μl serum prepared from rabbit inoculated with Freund's adjuvant and saline.) Values of adenylate kinase are arbitrary.

	Antiserum to Rat Liver Adenylate Kinase III				
	Control	*10 μl*	*20 μl*	*50 μl*	*100 μl*
Isozyme II	83	82	84	85	83
Isozyme III	79	<1	<1	<1	<1

with either enzyme. Analysis of amino acids show lysine and glycine among the 4 most abundant amino acids in isozyme II, while they apparently are replaced by alanine and aspartic acid as among the 4 most abundant amino acids in isozyme III. The isoelectric point, as determined by isoelectrofocusing (pH range 5–8), was 7.5 for isozyme II and 8.0 for isozyme III. 10 μl of antiserum prepared by inoculating a rabbit with a total of 12 mg of purified liver adenylate kinase III completely inhibited the activity of isozyme III, but up to 100 μl of the antiserum did not affect the activity of isozyme II (Table VII). Therefore, it would appear that the cytoplasmic and mitochondrial forms of adenylate kinase are distinguishable by differences in ability to aggregate (molecular weights forms), amino acid compositions, isoelectric points, and antibody cross reactivities.

DISCUSSION

Adenylate kinase II could not be brought to the same stage of purity as isozyme III[10] due to the extreme lability of the former. We have thus far not found a suitable reagent to protect isozyme II from continued loss of activity during purification.

Since the estimated molecular weight of isozyme II agreed closely with that of the dimer of isozyme III[10], the question arises whether the former is actually a degradation product of the latter, rather than a distinct species. Previous reports from our laboratory[8–12], however, would indicate that the two are distinct enzymes: Whereas isozyme III is localized in the outer compartment of the mitochondria, isozyme II is in the soluble fraction[9]; II is the predominant form in brain, heart, and muscle, while III predominates in liver and kidney[11]; the ratio of III to II shifts significantly during liver development[12]; and antibody to isozyme III inhibits III but not II (present publication).

We have reported previously[10] that the dimer forms of isozyme III predominates in Sephadex gel columns, whereas only the trimer and monomer were visualized during sedimentation. In contrast, present data indicate that isozyme II exists as only one form during Sephadex chromatography and sedimentation. Therefore, it would appear that there are structural differences between these two isozymes which allow only III to exhibit aggregated froms in very rapid equilibrium.

The two rat liver isozymes differ from other adenylate kinases in their specificity for AMP, ATP, and dATP. Bovine liver mitochondrial[2], rabbit muscle[19], swine

liver[1], and baker's yeast[20] enzymes can use dAMP as a phosphoryl acceptor; ITP, GTP, and GMP serve as substrates for adenylate kinase from various sources[2,20]. Both isozymes from rat liver exhibit a greater maximal velocity with dATP than with ATP. The K_m for ATP, however, was about 1/4 that for dATP.

The results presented here and in previous papers thus far indicate that adenylate kinase isozymes II and III from rat tissues differ in tissue distribution, intracellular localization, response to tissue dedifferentiation and liver development, molecular weight and tendency to aggregate. The two isozymes, however, exhibit similar catalytic and kinetic properties. It has not been determined whether the two enzyme species are truly isozymic in the sense that they have subunits in common, or whether one molecular form might simply be a modified version (*e.g.* acetylated, phosphorylated, *etc.*) of the other molecular form which would alter the former species' ability to aggregate and bind to the mitochondria. Studies designed to resolve the latter problems are currently in progress in our laboratory.

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