Rat Liver Adenosine Triphosphate: Adenosine Monophosphate Phosphotransferase Activity

II. SUBCELLULAR LOCALIZATION OF ADENYLATE KINASE ISOZYMES*

WAYNE E. CRISS

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From the Fels Research Institute, Temple University School of Medicine, Philadelphia, Pennsylvania 19140

SUMMARY

Adenylate kinase activity (ATP:AMP-phosphotransferase, EC 2.7.4.3) was studied in subcellular extracts of rat liver tissue. Essentially all of the activity, over 150 units per g of tissue, wet weight (1.7 units per mg of protein), was released into the cytosol when the tissue was homogenized in 0.025 M sucrose. Isotonic (0.25 M sucrose) homogenization and separation of subcellular fractions by both differential centrifugation and linear sucrose density gradient centrifugation revealed 8 units per g (0.4 units per mg) in the nuclear fraction, 84 units per g (2 units per mg) in the heavy mitochondrial fraction, 22 units per g (0.6 unit per mg) in the light mitochondrial fraction, and 30 units per g (0.5 unit per mg) in the cytosol fraction.

Rat liver adenylate kinase activity was separated into four electrophoretically distinct isozymes. Isozyme I, at 3 units per g of tissue, wet weight, was observed only in the nuclear fractions. Isozyme II was found in the cytosol at 19 units per g. Isozyme III, at 110 units per g, was the predominant adenylate kinase isozyme in the rat liver; it was localized in the outer compartment of the mitochondria. Since adenylate kinase III is easily released from mitochondria, mitochondrial damage can be observed by monitoring this isozyme. Also, because of this release, the true subcellular distribution of rat liver adenylate kinase activity is probably better represented by the corresponding values for each isozyme, not the values obtained by normal subcellular extraction procedures.

We recently reported that rat liver adenylate kinase (ATP:AMP-phosphotransferase, EC 2.7.4.3) was the result of four distinct isozymes (I). The activities of adenylate kinases I, II, and IV remained reasonably constant in liver and in a series of Morris hepatomas; but adenylate kinase III, which was responsive to dietary changes in the liver, decreased almost 10-fold with decreased differentiation. Since the adenine nucleotides are powerful modulators of key regulatory enzymes, the functional significance of these various isozyme forms, which directly catalyze the equilibrium between AMP, ADP, and ATP, must be thoroughly investigated.

Large differences (2) in the level of "soluble" adenylate kinase activity have been reported as soluble (3, 4), mitochondrial (5), or absent (6) in heart and skeletal muscle. Similarly, liver adenylate kinase activity has been located exclusively in the mitochondria (7), partially soluble and partially particulate bound (8, 9), or completely soluble (6). Adenylate kinase is not associated with particulate fractions in yeast (10). Thus, there would appear to be some doubt concerning the relationship of soluble to particulate bound adenylate kinase activity in cells.

We report here the subcellular location of the various adenylate kinase isozymes in rat liver and discuss their possible significance in regulating the adenylate charge of the cell.

EXPERIMENTAL PROCEDURE

Animals—Normal male rats (CFN strain) were purchased from Carworth Farms, Rockland County, New York. The animals were maintained on laboratory chow and were killed when they were between 190 and 240 g.

Tissue Preparation—The animals were decapitated and exsanguinated. The livers were quickly removed and placed in sucrose solutions of indicated concentration. Upon cooling, the livers were blotted, weighed, and homogenized in 10 volumes of sucrose solution of indicated concentration. Homogenization was carried out in ice in a glass homogenizer with motor-driven teflon pestle. Care was taken to eliminate any heating and only four complete passes of the pestle were used. The homogenates were centrifuged for 60 min at 100,000 × g at 2°C to directly obtain nonparticulate bound enzymatic activity.

Subcellular Fractionation—Various subcellular fractions were isolated by two methods. Differential centrifugation was accomplished by centrifuging down the nuclei at 5,000 × g_{min}; large mitochondria at 33,000 × g_{min}; small mitochondria at 300,000 × g_{min}; microsomes at 3,000,000 × g_{min}; and the remaining supernatant fraction was called cytosol. Each fraction had to be kept cold and was washed only twice in the appropriate sucrose solution. Washes were combined to the next postfractional centrifugation. Centrifugation was performed in a...
Sorvall RC2-B automatic refrigerated centrifuge with a SS-34 rotor or in a Beckman L-2 ultracentrifuge with a type 40 rotor.

The second method for separation of organelles was a linear sucrose gradient. A 60-ml gradient of 10 to 60% sucrose was poured into 14- x 39-inch cellulose nitrate tubes. The whole homogenate, 1 to 2 ml, was layered on the top of the gradient tubes. The gradient tubes were placed into a SW28.2 swinging bucket rotor. The rotor was centrifuged for 90 min at 25,000 rpm in a Beckman L-2 ultracentrifuge. After centrifugation, each tube was removed and mounted onto an Instrument Specialties Company model D density gradient fractionator. Fractions, 1 ml each, were collected from the top of the fractionator when a 70% sucrose solution was pumped into the bottom of the gradient tube at a rate of 2 ml per min. The absorbance at 280 nm was continuously recorded during the elution process. All 1-ml fractions were then individually monitored for enzymatic activity.

Mitochondrial Compartmentation Mitochondria were prepared in 0.3 M sucrose and were separated by differential centrifugation. The fraction between 5,000 × gmin and 300,000 × gmin was used for total mitochondria. This fraction was washed twice and electron micrographs of this type of preparation revealed approximately 75% mitochondria. The purified mitochondria were swollen in 0.01 M Tris-phosphate buffer at pH 7.4 for 15 min. This suspension was then diluted with 1 volume of 1.8 M sucrose containing 2 mM ATP and 2 mM MgCl₂. The method was designed to remove the outer mitochondrial membrane but leave the inner membrane intact (11). After 15 min, the mitochondria preparation was recentrifuged at 500,000 × gmin. The supernatant was removed and the mitochondrial pellet was then resuspended in 0.025 M sucrose containing 1 mM phosphate at pH 7.4. This suspension was frozen and thawed twice, and subjected to three 10-sec bursts on a Branson sonifier set at 5. The remaining mitochondrial membranes were centrifuged at 1,000,000 × gmin. The supernatant and resuspended membranes (in same buffer) were assayed for enzymatic activity and prepared for electron microscopy.

Enzymatic Assays—Adenylate kinase activity was measured and the adenylate kinase isozymes were separated as described previously (1). All adenylate kinase activity values reported in this paper were made by assaying in the forward direction (ADP formation). Glucose 6-phosphate dehydrogenase was used as a cytoplasmic marker and was measured by the method of Criss and McKerns (12). Glucose 6-phosphatase was the microsome marker enzyme and it was measured by the method of Harper (13). Lysosomes were located by measurement of acid phosphatase (14). Glutamate dehydrogenase served as a mitochondrial marker enzyme and was assayed by the method of Schmidt (15). Protein determination was by the method of Lowry et al. (16).

Electron Microscopy—Mitochondria were prepared as described above. They were fixed in 3% glutaraldehyde in 0.1 M phosphate buffer at pH 7.2 for 2 hours. They were washed twice and allowed to remain overnight in the phosphate buffer. The mitochondrial preparation was then postfixed in 1% osmium tetroxide in 0.1 M phosphate buffer at pH 7.2 for 1 hour. It was washed in distilled water for 1 hour, postfixed in saturated uranyl acetate for 2 hours, dehydrated in alcohol, and embedded in Epon. Observations were made on a Siemens Electron Microscope 101.

RESULTS AND DISCUSSION

Release of Particulate-bound Adenylate Kinase Activity—Adenylate kinase activity increased in the cytosol fractions as the hypertonicity of the homogenizing media increased (Fig. 1). Adenylate kinase activity, 26 units per g or 0.31 unit per mg, was found in the cytosol when the liver tissue was homogenized in 0.25 M sucrose. However, upon homogenization in 0.025 M sucrose, 160 units per g or 1.71 units per mg was observed in the cytosol fraction. Similar results were obtained with decreasing concentrations of Tris-HCl buffer at pH 7.2 or by homogenizing vigorously at 25° (observations in our laboratory). Variations in the homogenization procedure or extraction conditions may have been the cause of the large differences reported concerning the levels of the soluble versus bound adenylate kinase activity in liver tissue (6-9). The release of the adenylate kinase activity from some particulate fraction during extraction would appear to have been associated with the decrease in the amount of the particulate enzyme. When the liver tissue homogenates were centrifuged at 1,000,000 × g for 60 min, only 0.31 unit/mg of adenylate kinase activity was found in the cytosol fraction.
to be quite specific for this enzyme and not for other proteins, because the observed increases in activity per g of tissue or per mg of extracted protein in the present study were identical.

Subcellular Location of Adenylate Kinase Activity—The origin of the bound adenylate kinase activity was examined by differential centrifugation and by sucrose density gradient centrifugation. Differential centrifugation was carried out with extracts from both 0.25 and 0.025 M sucrose homogenizations (Table I). All washes were performed with the isotonic sucrose, such that only the homogenization procedure was unique. The largest fraction of adenylate kinase activity in rat liver tissue was localized in the mitochondrial fractions (Table I and Fig. 2). The large mitochondria contained over 80 units per g of tissue (2 units per mg of protein) and the small mitochondria had over 20 units per g of tissue (0.6 unit per mg of protein). Eight units per g of tissue (0.4 unit per mg of protein) were found in the nuclear fraction and 30 units per g of tissue (0.5 unit per mg of protein) was observed in the cytosol. Activity in the microsomal fraction was very low. Hypotonic homogenization reduced the mitochondrial adenylate kinase activity to about 8 units per g of tissue (0.2 unit per mg of protein) and increased the cytosol activity to approximately 150 units per g of tissue (2 units per mg of protein). Disappearance of the mitochondrial adenylate kinase activity upon hypotonic homogenization was also observed in the sucrose density gradient studies (Figs. 2 and 3). It would appear the hypotonic homogenization released the mitochondrial-bound adenylate kinase activity.

Submitochondrial Location of Adenylate Kinase Activity—With a combination of procedures previously described (see “Experimental Procedure”), we attempted to localize the mitochondrial adenylate kinase activity. Tissue, 80 units per g, was released from the mitochondria when the outer mitochondrial membrane was ruptured or completely removed from the mitochondria (Fig. 5). Mitochondria with intact double membranes were observed. X 40,000.

Hypotonically prepared rat liver mitochondria. Liver mitochondria were prepared and washed in 0.25 M sucrose. Mitochondria with intact double membranes were observed. X 60,000.

Hypotonically prepared rat liver mitochondria. Liver mitochondria were homogenized in 0.025 M sucrose and washed in 0.25 M sucrose. Mitochondria were observed with gaps in the outer membrane and with the outer membrane missing entirely. X 40,000.

Swollen-shrunk liver mitochondrial preparations. Liver mitochondria were prepared and washed in 0.25 M sucrose. The mitochondria were subjected to a shrink-swell phenomena as described under “Experimental Procedure.” Only single membrane structures were observed, apparently the outer membrane was completely removed. X 40,000.

**Table I**

<table>
<thead>
<tr>
<th>Location</th>
<th>Isotonic homogenation</th>
<th>Hypotonic homogenation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>units/g tissue</td>
<td>units/mg protein</td>
</tr>
<tr>
<td>Nuclei</td>
<td>8.4 ± 4.1</td>
<td>0.42 ± 0.11</td>
</tr>
<tr>
<td>h. mitochondria</td>
<td>83.0 ± 6.2</td>
<td>2.01 ± 0.24</td>
</tr>
<tr>
<td>l. mitochondria</td>
<td>22.3 ± 4.1</td>
<td>0.64 ± 0.16</td>
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<tr>
<td>Microsomes</td>
<td>2.1 ± 0.9</td>
<td>0.10 ± 0.03</td>
</tr>
<tr>
<td>Cytosol</td>
<td>29.8 ± 6.8</td>
<td>0.48 ± 0.17</td>
</tr>
<tr>
<td>Outer mitochondrial compartment</td>
<td>79.8 ± 3.6</td>
<td>0.21 ± 0.41</td>
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<tr>
<td>Matrix of mitochondria</td>
<td>1.8 ± 0.7</td>
<td>0.20 ± 0.07</td>
</tr>
<tr>
<td>Membrane bound in mitochondria</td>
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* 0.25 M sucrose.
* 0.025 M sucrose.
* Activity released by swell-shrink method.
* Activity released from free-thaw and sonic disruption method following activity released by swell-shrink method.
* Activity remaining on mitochondrial membranes after both methods described under Footnotes c and d.

**Table II**

<table>
<thead>
<tr>
<th>Location</th>
<th>Adenylate Kinase activity</th>
<th>Predominant isozyme</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>units/g tissue</td>
<td></td>
</tr>
<tr>
<td>Nuclei</td>
<td>3.2</td>
<td>I + III</td>
</tr>
<tr>
<td>h. mitochondria</td>
<td>&lt;1</td>
<td>III</td>
</tr>
<tr>
<td>l. mitochondria</td>
<td>&lt;1</td>
<td>III</td>
</tr>
<tr>
<td>Microsomes</td>
<td>&lt;1</td>
<td>II</td>
</tr>
<tr>
<td>Cytosol</td>
<td>18.7</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>28.0</td>
<td>114 ± 11</td>
</tr>
<tr>
<td>Hypotonic release</td>
<td>28</td>
<td>156 ± 12</td>
</tr>
</tbody>
</table>

* Activity released by homogenizing in 0.025 M sucrose.
Subcellular Location of Adenylate Kinase Isozymes—Four adenylate kinase isozymes have previously been separated and identified from rat liver tissue and Morris hepatomas (1). With this method, we separated and identified the isozymes within each isolated subcellular or submitochondrial fraction. The subcellular distribution of these four isozymes is given in Table II. Adenylate kinase III was the predominant form found in liver tissue. It was the major isozyme in both heavy and light mitochondria, and was also codominant in the nuclear fraction.

In consideration of the location of adenylate kinase III in the outer compartment of mitochondria, one should be able to determine the percentage of mitochondrial damage during extraction just by determining the amount of isozyme III which is released. Examination of Tables I and II revealed 11 units per g of tissue of isozyme III extracted in the cytosol. This is approximately 10% of the total adenylate kinase III activity in the cell. Calculations from electron micrographs of these mitochondrial preparations indicate about 10 to 13% mitochondrial rupture. Therefore, release of liver adenylate kinase III is a reasonably good index of mitochondrial damage. Similarly, the total adenylate kinase activity reported in the liver cytosol (Table I) is probably too high. Subtracting the amount of adenylate kinase III released from mitochondrial rupture would leave approximately 20 units per g of tissue in the cytosol. This value is in excellent agreement with the total level of adenylate kinase II activity (Table II).

Role of Adenylate Kinase in Regulating Cell Metabolism—Localization of different isozymes of adenylate kinase in both the cytoplasm and the mitochondria could be extremely important in terms of the ability of the liver cell to regulate its energy system, AMP + ADP + ATP. The energy charge of the adenylate system has been defined by Atkinson as the chemical energy charge of the cell and to be equal to one-half the average number of anhydride-bound phosphate groups per adenosine moiety (17, 18). Several enzymes of glycolysis and the citrate acid cycle are inhibited by an adenylate charge of one (high ATP), or are stimulated by an adenylate charge of zero (high AMP), or both; these include phosphofructokinase and pyruvate dehydrogenase (19, 20), citrate synthetase and NAD-specific isocitrate dehydrogenase (21), and pyruvate kinase (22). Correspondingly, several biosynthetic enzymes are inhibited by an adenylate charge of zero (high AMP), or are stimulated by an adenylate charge of one (high ATP), or both. This response was observed with the citrate cleavage enzyme (23), phosphoribosylpyrophosphate synthetase (24), phosphoribosyl ATP synthetase, and aspartokinase (25), and fructose 1,6-diphosphatase (26). Thus the energy charge of the adenylate system provides the cell with fine intracellular regulatory control which is capable of regulating entire metabolic pathways (for excellent reviews see References 18, 27, and 28). Therefore, adenylate kinase, (a) because of its potential role in maintaining a nearly constant adenylate charge, (b) because of its dual location inside and outside of the mitochondrion, (c) because of its existence in multiple isomeric forms, and (d) because of its response to dietary and hormonal stimuli, could be capable of directly regulating the energy flow of the liver cell. The extent of this influence will necessitate further clarification of the various adenylate kinase isozymes and examination of their individual contribution toward regulation of the cell's energy flow. We are pursuing these objectives in our laboratory.

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REFERENCES
Rat Liver Adenosine Triphosphate:Adenosine Monophosphate Phosphotransferase Activity: II. SUBCELLULAR LOCALIZATION OF ADENYLATE KINASE ISOZYMES
Wayne E. Criss


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