An Improved Purification, Crystallization, and Some Properties of Rabbit Muscle 5'-Adenylic Acid Deaminase*

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SUMMARY

A rapid method for the preparation of crystalline 5'-adenylic acid deaminase from rabbit skeletal muscle is presented. The enzyme remains bound to cellulose phosphate under conditions at which apparently no other proteins are bound; thus it was possible to develop, in essence, a one-step method for its purification. The crystalline preparation is homogeneous as indicated by its elution profile, by ultracentrifugation, and by acrylamide gel electrophoresis. The new method results in enzyme that has 5 times the specific activity of the previously reported crystalline preparation and differs in stability and kinetic properties. The enzyme is stable in dilute solution for extended periods at room temperature provided the diluting medium is of high ionic strength and contains 3-mercaptoethanol.

The enzyme follows normal Michaelis-Menten kinetics with respect to adenosine monophosphate concentration when assayed in the presence of KCl, and with respect to potassium concentration. Sodium and potassium, at 0.15 M, function equally efficiently as cation activators.

MATERIALS AND METHODS

Frozen rabbit muscle was obtained from Pel-Freez Biologicals, Inc., Rogers, Arkansas. AMP and cellulose phosphate were obtained from Sigma. The cellulose phosphate was successively washed with 0.5 M KOH, H2O, 0.5 M HCl, H2O, and finally with 5 \times 10^{-6} M EDTA prior to equilibration with extraction buffer. Imidazole was recrystallized from chloroform and petroleum ether. Tetramethylammonium chloride was re-crystallized from absolute ethanol. All other reagents were reagent grade. Distilled water was passed through a mixed bed ion exchange resin before use in making solutions and dilutions.

Protein concentrations were routinely determined by the method of Warburg and Christian (14). Various samples were also determined by the biuret method (15) for direct comparison with Lee’s results (12). Kinetic assays were performed with dilutions in 0.5 M KCl, 0.02 M sodium cacodylate, pH 6.7, and 1 M mercaptoethanol. Then, 10 to 25 \mu l of the appropriately diluted enzyme were added to 1 ml of the buffered AMP solution in a 1-cm quartz cuvette. The kinetic assays for AMP concentrations below 0.2 mM were performed essentially by the method of Kalcak (6) at 30° in 0.1 M potassium succinate, pH 0.5, or in 0.05 M imidazole-HCl, pH 6.5, depending on the experiment. The change in optical density at 295 nm was recorded on a Gilford multiple position absorbance recorder attached to a Beckman model DU spectrophotometer or on a Sargent recorder attached to a Beckman model DB spectrophotometer.
initial slope was determined graphically and converted to micromoles of AMP deaminated per min per mg of protein by dividing by the factor 8.86 and the protein concentration. The factor 8.86 (i.e., 8,860/1,000) was obtained from the difference in molar absorptivity of AMP (14,000) and IMP (5,240) at 265 nm in 0.1 M potassium succinate at pH 6.5.

Assays at concentrations of AMP greater than 0.2 mM were conducted in the same manner except that the increase in optical density was recorded at 265 nm. The difference in molar absorptivity at this wavelength equals 300. Thus, at 265 nm, the micromoles of AMP deaminated per min are equal to the change in optical density per min divided by 0.30.

Sedimentation experiments were carried out with a Spinco model E centrifuge. Calculations of sedimentation rates were done according to the method of Schachman (16).

Disc electrophoresis was performed in polyacrylamide gels according to directions supplied by Canlab Industries. The separating gel contained 5% acrylamide and the sample was applied in 50% glycerol, 0.1 M K₃H₂PO₄, pH 6.9, to the top of the spacer gel.

RESULTS

Purification—One pound of frozen rabbit muscle was cut into small pieces and passed through a meat grinder. To the ground muscle was added 3.3 times its weight of extraction buffer composed of 0.18 M KCl–0.054 M KH₂PO₄–0.035 M K₂HPO₄, pH 6.5. After homogenization for 15 sec at high speed in a large Waring Blender, the slurry was stirred at room temperature for 1 hour. The homogenate was centrifuged at 14,000 × g for 15 min and the precipitate was discarded. The supernatant fraction was poured through two layers of cheese cloth to remove lipid particles. All succeeding steps were performed at room temperature.

To the extract were added 5 g of cellulose phosphate (dry weight), which was previously washed as outlined under “Materials and Methods” and equilibrated with the extraction buffer. This suspension was stirred 10 min or longer after which the cellulose phosphate was allowed to settle. At least 90% of the enzyme was bound to the cellulose phosphate at this stage. The supernatant solution was siphoned off and discarded. The cellulose phosphate slurry was transferred to a sintered glass suction filter where it was washed repeatedly with approximately 2 liters of extraction buffer in 200- to 300-ml portions. During this process the cellulose phosphate pad was not permitted to become dry. The cellulose phosphate was then washed with approximately 1 liter of a solution containing 0.45 M KCl adjusted to pH 7 with 1 M K₂HPO₄, again in 200- to 300-ml portions. This salt solution and all following solutions contained 1 mM mercaptoethanol. When no more protein was eluted in the wash, the cellulose phosphate was transferred to a column (2.5 × 25 cm) and the enzyme was eluted with 1 M KCl, pH 7, or with a linear gradient between 0.45 M KCl and 1.0 M KCl. From all indications it appears that AMP deaminase was the only enzyme held by the cellulose phosphate under these conditions. Gradient elution yielded one symmetrical protein peak coincident with enzyme activity (Fig. 1). The specific activity remained constant within experimental error over the peak. The A₄05: A₄45 ratio for the peak tubes was 1.8 or higher showing little or no contamination by nucleic acids.

The peak tubes were pooled and the enzyme was precipitated by the addition of 0.261 g of solid K₂HPO₄ for each milliliter of solution. The K₂HPO₄ was added slowly with stirring at room temperature, and the suspension was allowed to equilibrate for

![Fig. 1. Elution profile of AMP deaminase from cellulose phosphate. The enzyme was eluted with a 300-ml gradient from 0.45 to 1.0 M KCl containing 1 mM mercaptoethanol at pH 8. The flow rate was 0.45 ml per min and 4.5 ml were collected per tube. ●—●, protein; ○—○, units; ×—×, conductivity. Specific activities were determined at 50 μM AMP.]

![Fig. 2. Photomicrograph of AMP deaminase crystals. X 770](http://www.jbc.org/DownloadedFrom.Http://www.jbc.org/)

By guest on August 28, 2017
harvested by centrifugation, washed twice with 0.45 M KCl, pH 8, at 3°C, and then redissolved in 0.5 M KCl-0.02 M sodium cacodylate, pH 6.7. A typical purification scheme is given in Table I. The enzyme crystallized as hexagonal hipinards during the drop in temperature. The crystals (Fig. 2) were yileds of 10 to 20% and a purification of 240-fold. The specific activity at 50°C. One symmetrical protein peak was observed in addition to a very small amount of high molecular weight material. The high molecular weight contaminant was not seen in the sedimentation patterns of samples prior to crystallization. The ~20, was calculated to be 11.5 S, which is very similar to Lee's preparation where the ~20, was reported to be 12.5 S (17). Samples submitted to disc electrophoresis gave one band in 5% polyacrylamide gel. The enzyme did not move in 7% gels.

The entire procedure was completed within 24 hours with yields of 10 to 20% and a purification of 240-fold. The specific activity at 50 μM AMP was 92, roughly 5 times that reported for the crystalline preparation of Lee (17).

Homogeneity—A sample containing 3.22 mg of protein per ml was run in a Spinco model E analytical ultracentrifuge (Fig. 3). One symmetrical protein peak was observed in addition to a very small amount of high molecular weight material. The high molecular weight contaminant was not seen in the sedimentation patterns of samples prior to crystallization. The ~20, was calculated to be 11.5 S, which is very similar to Lee's preparation where the ~20, was reported to be 12.5 S (17).

Samples submitted to disc electrophoresis gave one band in 5% polyacrylamide gel. The enzyme did not move in 7% gels. In each case there was no evidence for any contaminating material.

**Table I**

**Purification of AMP deaminase**

<table>
<thead>
<tr>
<th>Conditions</th>
<th>Relative activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>H₂O (no mercaptoethanol)</td>
<td>0</td>
</tr>
<tr>
<td>0.5 M KCl (no mercaptoethanol)</td>
<td>0</td>
</tr>
<tr>
<td>10⁻² M mercaptoethanol</td>
<td>0</td>
</tr>
<tr>
<td>0.1 M KCl</td>
<td>41</td>
</tr>
<tr>
<td>0.5 M KCl</td>
<td>95</td>
</tr>
<tr>
<td>0.5 M (CH₃)₂NCl</td>
<td>91</td>
</tr>
<tr>
<td>0.5 M CaCl₂</td>
<td>96</td>
</tr>
<tr>
<td>0.1 M K₂HPO₄, pH 6.5</td>
<td>99</td>
</tr>
<tr>
<td>0.1 M K₂SO₄</td>
<td>94</td>
</tr>
<tr>
<td>0.5 M KCl, pH 6.0</td>
<td>94</td>
</tr>
<tr>
<td>0.5 M KCl, pH 6.7</td>
<td>101</td>
</tr>
<tr>
<td>0.5 M KCl, pH 7.0</td>
<td>10</td>
</tr>
<tr>
<td>0.5 M KCl, pH 7.5</td>
<td>7</td>
</tr>
</tbody>
</table>

**Stability of AMP deaminase**

Unless otherwise indicated, samples contained in addition, 1 mM β-mercaptoethanol and 0.05 M Tris-cacodylate buffer, pH 6.5. Protein concentration was 12 μg per ml. The samples were stored at room temperature (22°C) for 7 days. The activity reported is relative to zero time.

**Table III**

**Relative initial velocities of AMP deaminase with various cations**

<table>
<thead>
<tr>
<th>Ion</th>
<th>Radius of hydrated ion</th>
<th>Ionic radius from crystals data</th>
<th>Relative initial velocity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Li⁺</td>
<td>10.03</td>
<td>0.60</td>
<td>75</td>
</tr>
<tr>
<td>Na⁺</td>
<td>7.90</td>
<td>0.95</td>
<td>99</td>
</tr>
<tr>
<td>K⁺</td>
<td>5.32</td>
<td>1.33</td>
<td>100</td>
</tr>
<tr>
<td>NH₄⁺</td>
<td>5.37</td>
<td>1.48</td>
<td>48</td>
</tr>
<tr>
<td>Rb⁺</td>
<td>5.09</td>
<td>1.48</td>
<td>49</td>
</tr>
<tr>
<td>Cs⁺</td>
<td>5.03</td>
<td>1.60</td>
<td>5</td>
</tr>
<tr>
<td>Ca²⁺</td>
<td>0.99</td>
<td>0.65</td>
<td>0</td>
</tr>
<tr>
<td>Mg²⁺</td>
<td>0.64</td>
<td>0.65</td>
<td>0</td>
</tr>
<tr>
<td>(CH₃)₂N⁺</td>
<td>0.65</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

* Jenny (18) also Nachod and Wood (19).
* Pauling (20).

**Stability**—AMP deaminase purified from skeletal muscle by the above method shows quite different stability properties than reported for previous less active preparations. As noted in Table II, the enzyme rapidly loses activity in the absence of mercaptoethanol or in solutions of low ionic strength. In KCl solutions the enzyme remains more stable as the KCl concentration is increased. In the presence of 1 mM KCl and 1 mM β-mercaptoethanol, the enzyme has remained stable for 20 to 30 days at room temperature at a concentration of 6 μg per ml. MgCl₂ and CaCl₂ at 0.5 M also give excellent stability. Multivalent anions such as PO₄³⁻ and SO₄²⁻ provide good protection; the enzyme remains active in solutions of multivalent ions of lower ionic strength (0.3) than under equivalent conditions with the monovalent salts. The pH of storage is critical. The best range is between 6.0 and 6.7 as measured in Tris-cacodylate buffers. If pH 7 or above the activity rapidly decreases. The enzyme is stored routinely in 1 M KCl, 0.05 M potassium cacodylate, pH 6.5, and 10⁻³ or 10⁻² M mercaptoethanol. Thus far we have been
unable to keep the enzyme stable in crystalline form. Losses in activity of 50% were observed in 1 to 2 weeks.

**Kinetic Properties**—At 50 μM AMP, the deaminase is markedly activated by a monovalent cation. The relative effectiveness of various monovalent cations is given in Table III. K⁺ and Na⁺ are equally efficient, whereas Li⁺, NH₄⁺, and Rb⁺ show 75%, 50%, and 50% activation, respectively. Tetramethylammonium ion is a nonactivating cation. The relationship between K⁺ concentration and the velocity of the reaction is seen in Fig. 4. The Kₐ for potassium activation, calculated from a K⁺/υ against K⁺ plot, is 2 mm.

The relationship between the initial velocity for deamination of AMP and AMP concentration at optimum concentrations of K⁺ is given in Fig. 5. Normal Michaelis-Menten kinetics are obtained yielding a Kₐ of 0.5 to 0.7 mM for AMP.

**DISCUSSION**

The purification and crystallization of AMP deaminase described here has a number of advantages over earlier procedures. It is, in essence, a one-step method easily resulting in crystals within 24 hours. The purified enzyme has 5 times the specific activity reported in the only previous crystallization, and was recovered with a higher yield. The procedure is much milder than that of Lee (12) who used a heat step, ethanol precipitation, ammonium sulfate fractionation, and calcium phosphate gel chromatography. Since Lee had good evidence for a homogeneous preparation, and the sedimentation constant was very similar if not the same as our preparations, we feel that the higher specific activity in our case reflects the milder conditions employed.

More important are the differing kinetic and stability properties of the new crystalline preparation. Our preparations show a requirement for high ionic strength and mercaptoethanol for stability; if either condition is not met, the catalytic activity virtually disappears overnight. At low concentrations of AMP, the enzyme is markedly activated by monovalent cations, K⁺ and Na⁺ preferred. Monovalent cations are not required for activity, however, since it has been shown that the enzyme exhibits the same Vₘₐₓ at high concentrations of AMP (21). A monovalent cation requirement has previously been reported for AMP deaminase from human erythrocytes (22) and brain (23). The brain deaminase is maximally activated by Na⁺ although NH₄⁺, K⁺, and Rb⁺ activate 66%, 47%, and 40% of the optimum, respectively. The human erythrocyte enzyme is activated by K⁺ and NH₄⁺. Na⁺ will activate only in the presence of ATP. The muscle deaminase described here is activated equally efficiently by K⁺ and Na⁺ with Li⁺, NH₄⁺, and Rb⁺ activating less efficiently as noted in Table III. Since it is now known that the muscle enzyme does not exhibit an absolute requirement for univalent cation (21), the univalent cation requirement for other preparations must be re-examined. The univalent cation requirement may be eliminated by high substrate concentration and by ATP, ADP, or other as yet unknown activators.

As noted from a table of 46 monovalent cation-requiring enzymes compiled by Evans (24), the ability of Na⁺ to activate as efficiently as K⁺ is quite rare. In most examples, Na⁺ and Li⁺ give little or no activation or inhibit the enzyme in question, whereas Rb⁺ and NH₄⁺ are usually fairly efficient in replacing the K⁺ requirement. These properties are thought to be a function of the radius of the hydrated ion. For muscle deaminase however, the activation appears to be correlated with the radius of the unhydrated ion; the difference in size between K⁺ and NH₄⁺ or Rb⁺ is critical for optimum activation. A correlation of activation with the radius of the unhydrated ion was previously reported for pyruvate kinase (25).

The mechanism of monovalent cation activation is obscure. In this case the requirement for univalent cations can be eliminated by high concentrations of substrate (21). Similar phenomena have recently been reported by Hunsley and Suelter for yeast pyruvate kinase. In both cases univalent cation serves as an allosteric activator, a function not previously observed with univalent cation.

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REFERENCES

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