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 homogenous 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.

5'‐Adenylic acid deaminase (EC 3.5.4.6, AMP‐aminohydrolase) catalyzes the deamination of adenylic acid to form inosine monophosphate and ammonia. It was first described by Schmidt (1–3) who separated it from adenosine deaminase and designated its specificity for 5'‐AMP. The enzyme, widely distributed in animal tissues (4), is found in highest concentrations in muscle where it is closely associated with myosin (5–7). As yet its biological function is unclear, although it may be important in the regulation of the relative amounts of adenine, hypoxanthine, and guanine nucleotide (8). Levels of AMP deaminase are reportedly lower in mouse dystrophic muscle (9) and in human subjects with hypokaliemic periodic paralysis (10).

The enzyme was previously purified by Kalckar (6) and by Nikiforuk and Colowick (11). They reported preparations with specific activities of 2.4 and 1.13 μmoles of AMP deaminated per min per mg of protein, respectively. Lee (12) described a purification scheme that gave crystalline enzyme with a specific activity of 17.3 μmoles of AMP per min per mg of protein measured at 4.5 × 10⁻⁴ M AMP. Although Lee presented evidence that his preparation was homogenous, the ratio of the optical densities at 280 and 260 nm indicated the presence of a nucleic acid contaminant. Currie and Webster (13) have described the preparation of homogeneous AMP deaminase from rat skeletal muscle by dialysis of actomyosin against phosphate buffer. This procedure was not successful with rabbit muscle actomyosin.

Prior to the investigation of certain aspects of the reaction catalyzed by this enzyme, a new procedure for purification from rabbit muscle was developed. With faster and milder procedures we were able to obtain crystalline enzyme in higher yields and of higher specific activities than reported previously. The resultant enzyme has kinetic properties that differ markedly from those of the enzyme isolated by the earlier procedures.

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 N HCl, H₂O, and finally with 5 × 10⁻⁴ 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 mM mercaptoethanol. Then, 10 to 25 μl 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 Kalckar (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 265 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.
In the subsequent purification process, one pound of frozen rabbit muscle was cut into small pieces and passed through a meat grinder. The ground muscle was mixed with an 8.5-fold excess 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 x 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 fraction 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 M mercaptoethanol. When no more protein was eluted in the wash, the cellulose phosphate was transferred to a column (2.5 x 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 A260/A465 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

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**Figure 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.

**Figure 2.** Photomicrograph of AMP deaminase crystals. × 770
15 min at 0° before centrifugation. The precipitate was next dissolved in a minimal volume of 0.45 M KCl at pH 7 to form a saturated solution at room temperature. This solution was placed in a glass dish of water at 21-24° and allowed to cool slowly in a cold room. The enzyme crystallized as hexagonal bipyramids during the drop in temperature. The crystals (Fig. 2) were harvested by centrifugation, washed twice with 0.45 M KCl, pH 8, at 3°, 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 entire procedure was completed within 24 hours with yields of 10 to 20% and a purification of 240-fold. The specific activity at 50° is increased. In the presence of 1 mM MgCl2 and 1 mM mercaptoethanol, the enzyme has remained stable for 20 to 30 days at room temperature (22°) for 7 days. The activity reported is relative to zero time.

**Table I**

**Purification of AMP deaminase**

<table>
<thead>
<tr>
<th></th>
<th>Total protein</th>
<th>Total units</th>
<th>Activity</th>
<th>Purification</th>
<th>Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>Extract</td>
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<td>6,880</td>
<td>0.38</td>
<td>1 (100)</td>
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</tr>
<tr>
<td>Cellulose phosphate eluation</td>
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<td>2,500</td>
<td>84</td>
<td>212</td>
<td>36</td>
</tr>
<tr>
<td>Crystals</td>
<td>16</td>
<td>1,540</td>
<td>92</td>
<td>240</td>
<td>22</td>
</tr>
</tbody>
</table>

**Table II**

**Stability of AMP deaminase**

Unless otherwise indicated, samples were run in 7% gels. The enzyme did not move in 7% gels. In each case there was no evidence for any contaminating material.

**Table III**

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

Assay mixture was 50 mM AMP-0.05 M imidazole, pH 6.5. All salts were at 0.15 M.

**Fig. 2.** Sedimentation pattern of crystalline AMP deaminase. The enzyme was in 0.5 M KCl, 0.023 M sodium cacodylate, pH 7, and 1 mM mercaptoethanol. The photograph was taken after 28 min at 60,780 rpm. The bar angle was 60°. The protein concentration was 3.22 mg per ml.
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 $K_v$ for potassium activation, calculated from a $K dinosaurs$ against $K_c$ 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 $K_m$ 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 $V_{max}$ 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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