THE ACETATE ACTIVATING ENZYME OF BEEF HEART

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In a recent communication Beinert et al. (1) have described the properties of an acetate activating enzyme system obtained from pig heart muscle. A similar system was first recognized by Nachmansohn and Machado (2) in brain tissue, and later studied by Lipmann and his coworkers in extracts of pigeon liver and yeast (3-5). The AAES catalyzes the over-all reaction

\[ \text{ATP} + \text{CoA} + \text{acetate} \rightleftharpoons \text{AMP} + \text{acetyl CoA} + \text{P-Pi} \]

The present communication deals with the purification and some properties of the enzyme responsible for this reaction in beef heart.

Results

Assay System—The assay system, previously described (1, 6, 7), is based on the conversion of acetyl CoA to citrate in the presence of malic dehydrogenase and condensing enzyme. The oxalacetate necessary for the condensation reaction is generated by the oxidation of malate in the presence of DPN, and the rate of DPN reduction is proportional to the concentration of AAE present, within the limits of the assay conditions (1). The specific activity is defined as micromoles of DPNH formed per mg of protein per 15 minutes. A unit of enzyme activity is defined as that amount which leads to the formation of 1 \( \mu \text{M} \) of DPNH in 15 minutes.

Preparation of Beef Heart Mitochondrial Particles—Fresh beef heart is freed from fat and connective tissue and diced. The subsequent steps are identical with those described previously for beef liver mitochondria (8), except that the acetone step is omitted and the particles are finally sus-

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1 The following abbreviations will be used throughout: AAES (acetate activating enzyme system), ATP (adenosinetriphosphate), AMP (adenosine-5'-phosphate), P-Pi (inorganic pyrophosphate), CoA (coenzyme A), CoASH (reduced CoA), GSH (reduced glutathione), DPN (diphosphopyridine nucleotide), Tris (tris(hydroxy-methyl)aminomethane), \( \Delta F^\circ \) (standard free energy change), \( K_m' \) (Michaelis-Menten constant for substrate under standard assay conditions), \( V \) (velocity at fatty acid concentration = \( \infty \), under standard assay conditions), AAE (acetate activating enzyme).
pended in 0.9 per cent potassium chloride. The suspension is stored frozen in 500 ml. plastic bottles.

Preparation of Extracts The suspensions are frozen and thawed three times and centrifuged after the third thawing. Thawing is accomplished by shaking the bottles in a bath at 38° for about 1 hour. If the suspension is allowed to thaw slowly at 4°, most of the enzyme activity is lost. The time of liberation of AAE from the particles is unpredictable between the first, second, and third thawing, but the maximal yield is always obtained by the third. More than three repetitions of the freezing and thawing process usually result in a lowered yield of enzyme.

After the third thawing the suspended particles are centrifuged in the Spinco preparative centrifuge at 14,000 × g for 10 minutes. This gives a clear supernatant solution. Centrifugation at lower gravitational fields gives a supernatant solution in which fine particulate material is still suspended. These particles seriously interfere with subsequent purification procedures.

Purification of Extract (Table I). Ammonium Sulfate Fractionation—To the extract containing about 5 mg. of protein per ml. are added 21 gm. of ammonium sulfate per 100 ml. and the precipitate is sedimented in the Spinco preparative centrifuge at 14,000 × g. This precipitate is discarded. To the supernatant material are added another 21 gm. of ammonium sulfate per 100 ml. and the precipitate is again sedimented in the Spinco centrifuge. This precipitate is taken up in 0.02 M potassium bicarbonate and the solution, after dilution to give 10 mg. of protein per ml., is further fractionated with saturated ammoniacal ammonium sulfate (pH about 8.9). The fraction obtained at 37 per cent saturation is rejected and the fraction precipitating at 55 per cent saturation is taken up in 0.02 M potassium bicarbonate. The solution is then dialyzed for 6 hours against 0.02 M potassium bicarbonate.

**Table I**

<table>
<thead>
<tr>
<th>Stage No.</th>
<th>Specific activity</th>
<th>Protein</th>
<th>Total units</th>
<th>Per cent recovery of units</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Mitochondrial extract</td>
<td>3.9</td>
<td>9800</td>
<td>38,300</td>
<td>100*</td>
</tr>
<tr>
<td>2. 1st ammonium sulfate fraction</td>
<td>7.2</td>
<td>4470</td>
<td>32,100</td>
<td>84</td>
</tr>
<tr>
<td>3. 2nd &quot; &quot; &quot; pH 8.9</td>
<td>13.0</td>
<td>1710</td>
<td>22,300</td>
<td>61</td>
</tr>
<tr>
<td>4. Alumina Cγ gel supernatant</td>
<td>31.0</td>
<td>420</td>
<td>13,000</td>
<td>34</td>
</tr>
<tr>
<td>5. 3rd ammonium sulfate fraction, pH 8.2</td>
<td>53.0</td>
<td>131</td>
<td>7,000</td>
<td>19</td>
</tr>
</tbody>
</table>

* Arbitrarily set at 100 per cent.
Treatment with Alumina Cγ Gel and Final Fractionation—The 37 to 55 per cent fraction, after dialysis, is diluted with 0.02 M potassium bicarbonate to give a protein concentration of 10 mg. per ml. The solution is mixed with the alumina Cγ gel (freshly prepared) in the proportion of 6 mg. dry weight equivalent of gel per mg. of protein. Both enzyme solution and gel are saturated 10 per cent with respect to ammonium sulfate (pH about 8.9) before mixing. After rapid mixing the suspension is centrifuged and the supernatant solution is treated with ammonium sulfate (42 gm. per 100 ml.). The solution of the precipitate is refractionated at a protein concentration of 10 mg. per ml. with ammoniacal ammonium sulfate (pH about 8.2) between the limits of 40 to 54 per cent saturation.

Purity of Enzyme; Physical Criteria—Enzyme preparations of specific activity 50 to 55 were examined in the Tiselius apparatus and the ultracentrifuge. The preparations were examined in 0.05 M phosphate buffer at pH 7.5 and 6.5 and in 0.1 M Tris containing 0.04 M potassium dihydrogen phosphate at pH 8.5. The mobility of the fastest moving component increased relative to those of the other components as the pH was raised. At the end of the run the major components on both ascending and descending limbs were sampled and it was found, in all three cases, that the fastest moving component (containing about 25 per cent of the total protein) had a specific activity 3 times greater than that of the undifferentiated material recovered from the cell. There was a graded decrease in specific activity down the ascending limb until the specific activity of the undifferentiated material was reached. The components from the descending limb contained little or no enzymatic activity. Material from the fastest moving component would react maximally in an assay system in which “supplementary enzyme” had been replaced with highly purified malic dehydrogenase and condensing enzyme, indicating that the “supplementary enzyme” did not contain a second component necessary for acetate activation. These findings all indicate that the activation of acetate is associated with a single protein component.

Examination of fresh material at specific activity 52 in the ultracentrifuge indicated that the molecular weight of all proteins present lay between 40,000 and 80,000. Similar examination of a sample of the fastest moving component taken from the Tiselius apparatus, and which had lost all activity, indicated a molecular weight in the range of 10,000.

Stability of Enzyme—Crude preparations of AAE obtained from pig heart muscle are extremely stable. These preparations withstand several hours at pH 4.3 in the presence of 20 per cent ammonium sulfate, are stable to dialysis, and can be kept almost indefinitely in the frozen state. By contrast, enzyme preparations at or above specific activity 13 show great instability after dialysis or dilution.
Substrate Specificity—The specificity of the AAE for substances related to acetate was determined by following the disappearance of CoASH in the presence of the substance under test and the enzyme, according to the method of Grunert and Phillips (9) (see Table II). Michaelis constants and maximal velocities for acetate, propionate, and acrylate were obtained by plotting the data according to the method of Lineweaver and Burk (10). Marginal reactions were obtained with samples of glycolate, glyoxalate, β-hydroxybutyrate, D(-)-lactate, and formate.

Reversibility of Reaction—This process was followed by observing the change in optical density at 232 mμ (see Fig. 1), which is a measure of the concentration of acetyl CoA (11). The formation of acyl CoA in the presence of acetate and propionate and its disappearance on addition of P-Pi were demonstrated.

Equilibrium Constant—The equilibrium constant with acetate was determined at pH 7.5, 8.0, and 8.5 in Tris buffer, and identical results were obtained at all three pH values. The constant for propionate was determined at pH 8.0. The equilibrium constant for the acetate activating reaction (Equation 1) was 0.86, and for the analogous propionate activating reaction 1.15. These values were calculated in a manner similar to that described by Mahler, Wakil, and Bock (8). The ΔF° for the reactions

\[
\text{(2)} \quad \text{CH}_3\text{CO-SCoA} + \text{H}_2\text{O} \rightarrow \text{CH}_3\text{COOH} + \text{CoASH}
\]

\[
\text{(3)} \quad \text{CH}_3\text{CH}_2\text{CO-SCoA} + \text{H}_2\text{O} \rightarrow \text{CH}_3\text{CH}_2\text{COOH} + \text{CoASH}
\]

was found to be -11,700 and -11,500 calories, respectively. These figures are uncorrected for the ionization of the acids. They agree closely

<table>
<thead>
<tr>
<th>Substrate Specificity of AAE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Substrate</td>
</tr>
<tr>
<td>----------</td>
</tr>
<tr>
<td>Acetate*</td>
</tr>
<tr>
<td>†</td>
</tr>
<tr>
<td>Propionate*</td>
</tr>
<tr>
<td>Acrylate*</td>
</tr>
</tbody>
</table>

The test system contained ATP, 2 μM; CoASH, 0.2 μM; magnesium chloride, 1 μM; Tris, pH 9.5, 20 μM; borohydride, 0.4 μM; AAE (specific activity 51), 9 γ in the DPN assay and 55 γ in the ΔSH assay. The final volume was 0.2 ml.; the final pH 8.0. The mixture was incubated at 38° for 7 minutes and the reaction stopped by the addition of 1 ml. of saturated sodium chloride. The free sulphydryl was then determined.

* Assayed in the ΔSH system.
† Assayed in the DPN system.
with the figures given by Stadtman (11, 12) and by Stern et al. (7) for the hydrolysis of acetyl CoA.

**Materials and Methods**

*Sources*—ATP was supplied by the Pabst Laboratories and GSH by the Schwarz Laboratories; CoA was prepared by the procedure of Beinert et al. (13). It was converted to the reduced form by treatment with sodium amalgam (13).

**Methods**—The following preparative methods were used: alumina Cγ gel (14), condensing enzyme (15), malic dehydrogenase (16), and "supplementary enzyme" (6).

**Analytical Procedures**—Free sulfhydryl and protein were determined according to Grunert and Phillips (9) and Gornall et al. (17).

**Summary**

Purified preparations of the acetate activating enzyme have been obtained from beef heart mitochondrial particles. Electrophoretic examination shows that the AAE activity is probably associated with a single protein component in these preparations. The enzyme is very unstable under the conditions of electrophoresis, and at the higher purity levels undergoes inactivation after dialysis, although this is not immediately mani-
The equilibrium constants of the acetate activating reaction and of the propionate activating reaction have been determined and found to lie near unity.

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BIBLIOGRAPHY

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