The Purification of Citrate-condensing Enzyme*

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The citrate-condensing enzyme was isolated as a crystalline protein from pig heart by Ochoa et al. (1). These workers reported that pig heart contained considerable quantities of the condensing enzyme. We were surprised when we obtained much higher yields of this enzyme from pig heart tissue with a different extraction procedure (2). This observation led to a purification procedure that yields 30 mg of crystalline condensing enzyme from 100 g of pig heart in 3 to 4 days. Using the new extraction procedure, we have also reinvestigated the condensing enzyme content in a number of tissues.

Sedimentation and diffusion coefficients were determined for this enzyme, and its approximate molecular weight and turn-over number were calculated.

EXPERIMENTAL PROCEDURE

Materials and Methods

The following materials were commercial preparations: CoA (Pabst Laboratories and Sigma Chemical Company), DPN (Pabst Laboratories), DPNH and malate dehydrogenase (C. F. Boehringer and Sohne, Mannheim, Germany), Sephadex G20 (Pharmacia, Uppsala, Sweden), DEAE-cellulose (Brown Company). Acetyl-CoA was prepared by the method of Simon and Shemin (3).

Condensing enzyme was assayed in the three ways described by Ochoa (4): (a) citrate formation assayed colorimetrically (5), (b) OAA1 production measured by DPNH formation in the presence of excess malate dehydrogenase; and (c) acetyl-CoA disappearance as measured by the decrease in absorption at 233 μm.

The citrate formation assay was carried out according to the procedure of Ochoa (4). Routine assays for activities were made with the malate dehydrogenase-coupled system (1). Protein was determined spectrophotometrically by the method of Warburg and Christian (6).

The uncoupled assay followed at 233 μm was performed in cells with a 0.5-cm light path and containing 100 μmole of Tris-HCl buffer, pH 8.0, 0.6 μmole of OAA (freshly prepared and neutralized with solid KHCO3), and 0.22 μmole of acetyl-CoA in a total final volume of 1.3 ml. The reaction was initiated by the addition of enzyme (usually 10 μl). The decrease in absorbance was linear for 4 minutes as long as the rate was less than 0.040 unit per minute. The change in absorbancy at 233 μm is due to both the cleavage of the thiol ester bond and the utilization of OAA. Calculation from values reported in the literature (7, 8) would lead one to the combined molar absorbancy index of 5.9 x 106. When this value was redetermined experimentally by allowing the reaction to go to completion with known concentrations of OAA (determined by use of the malate dehydrogenase assay), the combined molar absorbancy index was found to be 3.4 x 106.

Calcium phosphate gel (12 to 15 mg dry weight per ml) was prepared by the method of Keilin and Hartree (9). Assays for malate dehydrogenase, isocitrate dehydrogenase, aconitase, and fumarase were carried out as described by Ochoa (10), Macoy (11), and Anfinsen (12), respectively.

One unit of enzyme activity is that amount of enzyme that catalyzes the formation of 1 μmole of citrate per minute at 25°.

Specific activity is expressed as units per mg of protein.

All rates are initial velocities and units are expressed at 25°. The temperatures were measured at the end of each reaction by a Tencool No. GR33P insulated thermometer connected to a Wheatstone bridge circuit. The influence of temperature on initial velocities was determined, and the variations in rate due to slight temperature fluctuations were corrected to 25° with the use of this data. Initial velocities varied about 6.7 % per degree when the temperature was approximately 25°.

RESULTS

Extraction—Pig hearts obtained fresh at the slaughter house were packed in ice and carried to the laboratory where they were trimmed of fat and connective tissue, and cut into ¼-inch cubes. The tissue was divided into 100 g portions and weighed into plastic bags, which were then sealed and placed in the deep freeze. Less enzyme can be extracted from fresh tissue than from frozen tissue. No loss in activity occurred in 3 to 6 months of storage of the frozen tissue. Unless otherwise noted, all operations were performed at 0–3°.

Frozen pig heart, 100 g, was placed in a cold Waring Blender containing 400 ml of 0.4 M KCl (pH 7.4) and 50 ml of 0.01 M Tris buffer at -10° (4 volumes of 0.5 M KCl plus 1 volume of absolute ethanol) and homogenized for 10 minutes. The temperature rises to 20° during homogenization, but this does not cause a loss of activity. The homogenate was centrifuged for 15 minutes at 23,000 × g, and the supernatant fluid was dialyzed against 8 liters of cold 0.002 M potassium phosphate (pH 7.4) for 2 hours. The outside fluid was then changed and dialysis continued for another 2 hours. The dialysate was centrifuged at 23,000 × g for 15 minutes, and the precipitate was discarded. This dialysate is stable overnight at 3°.

Ammonium Sulfate Fractionation—Ammonium sulfate, 31.3 g, was added to each 100 ml of dialysate, and the mixture was stirred for 15 minutes. The precipitate was removed by cen-
trifugation at 23,000 × g for 15 minutes; 13.5 g of ammonium sulfate were added to each 100 ml of the supernatant fluid, and this mixture was stirred for 15 minutes. The precipitate, collected by centrifugation, was dissolved in a small amount of cold water.

**TABLE I**

<table>
<thead>
<tr>
<th>Purification of condensing enzyme</th>
<th>Total units* for 100 g</th>
<th>Specific activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ethanolic KCl extract</td>
<td>2350</td>
<td>0.35</td>
</tr>
<tr>
<td>Ammonium sulfate, 50-70%</td>
<td>3170</td>
<td>1.3</td>
</tr>
<tr>
<td>Combined DEAE-cellulose eluate</td>
<td>1460</td>
<td>10.5</td>
</tr>
<tr>
<td>CaPO₄ eluate</td>
<td>1110</td>
<td>25.8</td>
</tr>
<tr>
<td>Supernatant</td>
<td>270</td>
<td>33</td>
</tr>
<tr>
<td>Precipitate</td>
<td>425</td>
<td>33</td>
</tr>
</tbody>
</table>

* Units are expressed in terms of the coupled malate dehydrogenase assay (340 μm).

**TABLE II**

**Extraction of condensing enzyme from pig heart**

Fresh pig hearts were extracted twice with 0.02 M potassium phosphate, pH 7.4, according to the procedure of Ochoa et al. (4). Fresh tissue was also extracted twice with 0.4 M KCl in 20% ethanol (1 part tissue to 10 parts solvent) in a water-cooled Waring Blender for 5 minutes. The range given is for five different samples. All the units are expressed in terms of coupled malate dehydrogenase assay (340 μm) per g of tissue.

<table>
<thead>
<tr>
<th>Solvent</th>
<th>Concentration</th>
<th>Protein</th>
<th>Specific activity</th>
<th>Total units per g</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phosphate, pH 7.4</td>
<td>0.02</td>
<td>20.4</td>
<td>0.073</td>
<td>2.1</td>
</tr>
<tr>
<td>KCl in 20% ethanol</td>
<td>0.4</td>
<td>135</td>
<td>0.28</td>
<td>30-44</td>
</tr>
<tr>
<td>Sucrose</td>
<td>0.25</td>
<td>31*</td>
<td>31*</td>
<td>31*</td>
</tr>
</tbody>
</table>

* The 10% homogenate in 0.25 M sucrose of fresh pig heart tissue was assayed directly in the citrate colorimetric assay.
† An aliquot of the sucrose homogenate was extracted with ethanolic KCl in a Waring Blender and then assayed by the coupled malate dehydrogenase assay.

**TABLE III**

**Distribution of condensing enzyme**

Fresh samples of tissue (each 10 g) were extracted twice in 10 volumes of 0.4 M KCl in 20% ethanol for 5 minutes in a water-cooled Waring Blender. A third extraction was found unnecessary. For each extraction, the homogenate was centrifuged at 20,000 × g for 15 minutes, and the supernatant fluid was assayed by the coupled malate dehydrogenase assay (340 μm). No indication of interfering DPNH oxidase activity in the supernatant fluids was found. The units are expressed in terms of the 340-μm assay. The values given for rabbit tissue are for single extractions of frozen tissue. The pigeon muscle was breast muscle.

<table>
<thead>
<tr>
<th>Animal</th>
<th>Heart</th>
<th>Liver</th>
<th>Kidney</th>
<th>Muscle</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pig</td>
<td>36</td>
<td>3.8</td>
<td>10.8</td>
<td>16.8</td>
</tr>
<tr>
<td>Dog</td>
<td>33</td>
<td>3.1</td>
<td>10.5</td>
<td>16.8</td>
</tr>
<tr>
<td>Rat</td>
<td>39</td>
<td>2.8</td>
<td>6.6</td>
<td>3.1</td>
</tr>
<tr>
<td>Rabbit</td>
<td>23</td>
<td>1.0</td>
<td>6.6</td>
<td>1.2</td>
</tr>
<tr>
<td>Pigeon</td>
<td>31</td>
<td>1.1</td>
<td>40</td>
<td></td>
</tr>
</tbody>
</table>

**DEAE-cellulose Chromatography**—The enzyme solution was placed on a DEAE-cellulose column (4.5 × 20 cm) which had been previously equilibrated with 0.002 M potassium phosphate, pH 7.4. The original extract can be chromatographed without a preliminary ammonium sulfate concentration step if the initial dialysis is thorough. Usually, we have used the ammonium sulfate step. The column was washed with 1 liter of 0.002 M potassium phosphate, pH 7.4, 2 liters of 0.008 M potassium phosphate, pH 7.4, then with 0.018 M potassium phosphate, pH 7.4. The flow rate of effluent from the column was 20 ml per minute, and approximately 500 ml fractions were collected. The enzyme first appeared in the eluate at about the 4th liter of 0.018 M potassium phosphate, pH 7.4, and continued to be eluted until the 10th or 11th liter of effluent had been collected. The later fractions had so little protein that accurate determinations were difficult. Some fractions from the column gave estimated specific activities close to that for pure condensing enzyme. The enzyme can also be purified by a batch procedure in the following way: the salt-free enzyme is added to 35 g of DEAE-cellulose in 1 liter of 0.002 M potassium phosphate, pH 7.4. After filtration, the cellulose was washed with two 1-liter portions of 0.002 M potassium phosphate, pH 7.4, two 1-liter portions of 0.008 M potassium phosphate, pH 7.4, and then with seven 1-liter portions of 0.018 M potassium phosphate, pH 7.4. The cellulose was collected by filtration on a Buchner funnel.

**Comparison of Assays** The specific activities, expressed as micromoles of citrate formed per mg of protein at 25°, show pronounced differences when condensing enzyme is assayed by the three available methods. The highest figure is obtained
with the thiol ester cleavage assay (followed spectrophotometrically at 233 mµ), and if it is assigned a value of 1.0, then the coupled citrate formation assay (followed colorimetrically) is 0.61 and the assay coupled to malate dehydrogenase (DPNH formation followed at 340 mµ) is 0.38. Ochoa (4) has reported a ratio of 0.70 between the last two assays, and our value is 0.62, which is in fair agreement. Because the rate of the reaction coupled to malate dehydrogenase is slowest, perhaps the level of OAA is at too low a steady-state level and below the concentration needed for maximal velocities.

**Extraction**—A comparison of several extraction methods is shown in Table II. The amount extracted by the KCl-ethanol method is in good agreement with the amount found in a sucrose homogenate of the tissue. Using the KCl-ethanol extraction procedure, we found essentially the same relative distribution as Ochoa et al. (1), but with substantially more enzyme in each of the tissues investigated (Table III). Inasmuch as pure condensing enzyme has a specific activity of about 30 and one can find 36 units per g in heart tissue, this would correspond to 1.2 mg of condensing enzyme per g wet weight of heart or 1.1% of the total extracted protein.

**Tests for Other Enzymes**—The following activities were not found in 10 µg of condensing enzyme: fumarase, aconitase, isocitric dehydrogenase. Malate dehydrogenase is the most difficultly removed contamination, but was found to be less than 1 part in 1000 after three recrystallizations.

**Physical Constants**—The sedimentation coefficient (13) of twice recrystallized condensing enzyme was determined in a Spinco model E ultracentrifuge at protein concentrations of 17.3, 12.5, and 10.0 mg per ml. Values were corrected for changes in viscosity and for changes in temperature. The values were 4.2, 4.2, and 5.0 Svedberg units, respectively, in 0.02 m potassium phosphate buffer, pH 7.4, at 20°. The diffusion coefficient (13) of 7.5 x 10^-2 cm² per second (20°) at infinite time, as calculated from Rayleigh fringe patterns, was measured in a Spinco electrophoresis apparatus. Twice recrystallized condensing enzyme was used at a concentration of 3.7 mµg per ml in 0.20 m potassium phosphate buffer, pH 7.4, at 1°. The diffusion was followed for 70 hours. These values give a molecular weight of 56,000, assuming a partial specific volume of 0.70 m³ per g and with a value of 5.0 S for the sedimentation coefficient. Because we do not have a complete study of the effect of protein concentration on the sedimentation and diffusion coefficients, this value is an approximate one. The turnover number based on the thiol ester cleavage assay (233 mµ) is 4,400 moles of citrate per minute per mole of enzyme at 25°. The ratio of absorbancy at 280 mµ to 260 mµ is 1.80 for the enzyme.

Microscopic examination of the crystals prepared with this method showed slender needles very similar in appearance to the crystals obtained by Ochoa et al. (1). A sample of crystalline condensing enzyme, which had been stored at 3° for several years, was kindly furnished by Dr. Ochoa and was found to have a specific activity of 27.

**Discussion**

The high concentration of citrate-condensing enzyme in heart tissue is well correlated to the high QO₂ of this tissue as well as the high concentrations of other proteins, such as cytochrome c, concerned with aerobic oxidations (14). Since the citrate condensing enzyme is probably located in mitochondria of cells, it is not surprising that the relatively mild phosphate extract of Ochoa et al. (1) did not remove more than 5% of the total enzyme. The conditions used at present, ethanolic-KCl, although fairly drastic, have been effective in the extraction of several mitochondrial enzymes. Inasmuch as the same activity is seen in a sucrose homogenate, the ethanolic-KCl extraction seems to be without harmful effect on the enzyme.

Availability of large quantities of pure enzymes has enabled more detailed investigation of mechanisms involved in enzyme-catalyzed reactions. The procedure described here results in the isolation of 30 mg of enzyme rather easily in 3 to 4 days from 100 g of tissue. With the estimated molecular weight of 56,000, 0.5 µµmol of enzyme is obtained from one purification procedure. With this amount, one can look for reactions between enzyme and substrate by conventional means.

**Summary**

Extraction of pig heart with 0.4 m KCl in 20% ethanol showed that the citrate-condensing enzyme content was about 20 times that previously reported. Examination of a number of other tissues also showed larger quantities of citrate-condensing enzyme than earlier reports.

A purification procedure was developed starting with this new extract that yields 30 mg of crystals from 100 g of pig heart. The sedimentation coefficient and diffusion coefficient for this enzyme were determined and the molecular weight is estimated to be 56,000. The turnover number for this enzyme can thus be calculated to be 4,400 moles of citrate per minute per mole of enzyme at 25°.

**Acknowledgments**—We wish to thank C. F. Newton, U. Maitra, and A. N. Stabikha for their help in purifying the enzyme. We also want to thank Dr. N. S. Ling of the Mental Health Research Institute, University of Michigan, for the diffusion coefficient determination and Dr. Dale Osender of this department for assistance with the sedimentation studies.

**References**

The Purification of Citrate-condensing Enzyme
Paul A. Srere and George W. Kosicki