Purification of Rat Heart and Rat Liver Citrate Synthases

PHYSICAL, KINETIC, AND IMMUNOLOGICAL STUDIES*

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SUMMARY

The citrate synthases from rat heart and rat liver have been purified. These homogeneous proteins have a molecular weight of about 1 x 10^5 and are composed of 2 apparently identical subunits. Kinetic constants for acetyl coenzyme A and oxaloacetate and the K_i for ATP are the same for both enzymes. Immunological studies using antibody to the rat heart enzyme also indicate that the enzymes are identical.

Citrate synthase (citrate oxaloacetate lyase (CoA-acetylating), EC 4.1.3.7) has been found in all cells examined for it. The amount of this enzyme in cells is correlated with the ability of the cell to use oxygen. It was first isolated and crystallized from pig heart by Ochoa, Stern, and Schneider (1). A simpler procedure for its isolation was described by Srere and Kosicki (2), and a similar procedure was used to isolate and crystallize it from moth flight muscle and pigeon breast muscle (3). Other preparations of this enzyme from a variety of cells have been described (4), but for the most part these preparations are not homogeneous, nor has data as to the purity of the preparation been presented.

There is interest in knowing the kinetic properties of enzymes from rat tissues so that the extensive literature on metabolism in this animal can be better interpreted at the enzyme level. In addition, there would be of interest to know whether this mitochondrial enzyme is identical in each of the rat tissues. Shepherd and Garland (5) have reported a purification of the rat liver enzyme and estimated its purity at 30%. On the basis of data presented in this paper it is probable that the purity was no greater than 10%.

We report in this paper the purification of rat liver and rat heart citrate synthase. Both preparations appear to be crystalline and are homogeneous by several criteria. We have determined their molecular weights, subunit structure, and kinetic properties. We have also studied the antibody to rat heart citrate synthase and its interaction with a number of citrate synthases. The rat heart and rat liver citrate synthase appear to be identical proteins.

* This research was supported in part by Grant 5R01 AM11313-06 from the United States Public Health Service.

EXPERIMENTAL PROCEDURE

Materials and Methods—The following materials were commercial preparations: DEAE-cellulose (DE-52) from Reeve Angel Company, New York, New York; CM-cellulose from Schleicher and Schuell, Keene, New Hampshire; hydroxylapatite from Clarkson Chemical Company, Williamsport, Maryland; CoA from P-L Biochemicals. Acetyl-CoA was prepared by the method of Simon and Shemin (6).

Citrate synthase activity was determined by measuring the initial rate of the liberation of 1 pmole of CoA per min under those conditions. Specific activity is expressed as units per min per mg of protein.

Acrylamide Gel and Sodium Dodecyl Sulfate Gel Electrophoresis—Acrylamide gel electrophoresis of the enzyme was carried out according to the procedure of Davies (7). Electrophoresis was performed in 7.5% acrylamide gel, with the use of two different electrode buffers: 0.05 M Tris-glycine containing 10 mM citrate, pH 8.3, and 0.35 M Tris-glucose acetic, pH 4.3.

The enzyme sample to be analyzed was layered with an equal volume of 0.2 M sucrose containing the dye bromthymol blue and the electrode buffer. A current of 3 mA per gel was applied for 2 hours at room temperature. The gel was stained in 1% Amido black in 7% acetic acid.

Sodium dodecyl sulfate gel electrophoresis was carried out by the procedure of Laemmli (8). The sample was incubated at 70° for 3 min and then cooled in ice. The sample was layered onto the gel with an equal volume of 90% glycerol containing bromthymol blue. A current of 3 mA per gel was applied for 2 hours at room temperature. The gel was washed with methanol-acetic acid (1:1) solution for 4 hours to remove sodium dodecyl sulfate.

The abbreviations used are: CM-cellulose, carboxymethyl-cellulose; DTNB, 5,5'-dithiobis (2-nitrobenzoate).
Ultracentrifugation Studies—Ultracentrifugation was carried out in a Spinco model E ultracentrifuge. High speed sedimentation equilibrium experiments were performed by the procedure of Yphantis (9) with the use of 12-mm double sector cells with sapphire windows. The protein concentration was between 0.15 and 0.6 mg per ml.

Kinetic Studies—Kinetic studies were carried out in 1.0-cm cuvettes maintained at 28°, and initial velocity was measured using the highest sensitivity on the recorder (full scale was 0.1 A). The DTNB assay method was carefully controlled for changes in rate due to changes in ionic strength and changes in ionic composition. Particularly in experiments using ATP, potassium sulfate was added to the control cuvette to estimate inhibition due to changes in ionic strength and not due specifically to ATP.

Immunological Studies—Antiserum to rat heart citrate synthase was obtained from a rabbit in the following manner. Crystalline rat heart citrate synthase (4.2 mg) was dissolved in 1 ml of 20 mM KPO₄, pH 6.8, and emulsified with an equal volume of complete Freund’s adjuvant. The emulsified sample (1 ml) was injected intramuscularly into the back of a rabbit. The injections were repeated 3 times at weekly intervals. Rabbit serum was tested for antibody to volume of complete Freund’s adjuvant. The emulsified sample was added to the control cuvette to estimate inhibition due to changes in ionic strength and not due specifically to ATP.

Results
Purification of Citrate Synthase from Rat Tissues

Homogenization—Frozen rat livers (670 g) were put in 3 liters of the extraction solution, 40% saturated with ammonium sulfate, 1 mM EDTA, 5 mM citrate in 10 mM potassium phosphate buffer, pH 7.4, and 5 ml of Antifoam-90. Homogenization was performed in a large Waring Blender for four 1-min periods at full speed, cooling the solution between homogenization periods in an ice bath. The homogenate was centrifuged at 13,000 rpm for 30 min at 4° and the precipitate discarded.

Ammonium Sulfate Step—The supernatant solution was brought to 50% of saturation of ammonium sulfate with solid ammonium sulfate (62.7 g per liter of solution). The precipitate was removed by centrifugation as described above. The supernatant solution was brought to 75% saturation of ammonium sulfate (172 g per liter of 50% supernatant solution), stirred for 1 hour, and centrifuged at 13,000 rpm for an hour. The precipitate was dissolved in 800 ml of 20 mM potassium phosphate buffer, pH 6.8, containing 1 mM EDTA. After centrifugation at 13,000 rpm for 15 min, the supernatant solution was dialyzed for 24 hours against 10 liters of 5 mM KPO₄, pH 7.4, containing 1 mM EDTA at 4°. The external solution was changed 4 times.

DEAE-cellulose Chromatography—The dialyzed solution was applied to a DE-52 column (5 x 40 cm) previously equilibrated with 5 mM KPO₄, pH 7.4, and the column was washed with the same buffer. Citrate synthase was removed by this washing. The collected washings were adjusted to pH 6.0 with 0.2 N acetic acid, and a small precipitate was removed by centrifugation.

CM-cellulose Column Chromatography—After centrifugation, the supernatant solution was applied to a CM-cellulose column (5 x 30 cm) previously equilibrated with 5 mM KPO₄, pH 6.0, and, after washing with the same buffer, the column was eluted by a linear gradient of from 0 to 0.2 M KCl in the same buffer. Citrate synthase was eluted at approximately 0.1 M KCl. The eluate was adjusted to pH 6.8 with 0.1 N KOH.

Hydroxylapatite Column Chromatography—The eluate from CM-cellulose was applied to a hydroxylapatite column (2.5 x 30 cm) previously equilibrated with 5 mM KPO₄, pH 6.8, and, after dialysis against 4 liters of 5 mM KPO₄, pH 6.0. After dialysis the solution was centrifuged and the supernatant solution applied to a CM-cellulose column (2.5 x 30 cm). After washing the column, the same gradient method used above was used to elute the enzyme. Citrate synthase fractions were collected and concentrated with solid ammonium sulfate. The precipitate was then dissolved in a small amount of buffer.

First Crystallization—A small amount of solid ammonium sulfate was added to the above enzyme solution with continuous stirring until a slight turbidity appeared. The enzyme solution was stirred by a magnetic stirrer for 3 days at 4°.

Second Crystallization—Crystals of the enzyme were collected by centrifugation and dissolved in 3 ml of 20 mM KPO₄, pH 6.8. The enzyme was recrystallized with additional ammonium sulfate according to the above procedure. Crystalization was repeated once with the same procedure. The pure enzyme could be stored in 20 mM potassium phosphate buffer, pH 6.8, at 4° for 8 months without loss in its activity. An example of the purification procedure is summarized in Table I.

Rat heart citrate synthase was purified in the same way as the rat liver enzyme except that the chromatography step on DEAE-cellulose was deleted. The final specific activity of rat heart citrate synthase was 128, and the over-all yield was 24.3%. Table II shows the purification procedure for rat heart citrate synthase.

In both cases, malate dehydrogenase was difficult to remove, but this was accomplished by repeated recrystallization. The microscopic appearance of the crystals of rat liver and rat heart citrate synthase was that of small fine needles. They were similar in appearance to each other.

Homogeneity, Molecular Weight, and Subunits

The homogeneity of both samples was tested by disc gel electrophoresis. Both enzymes were observed as single protein...
TABLE I
Purification procedure for citrate synthase from rat liver

<table>
<thead>
<tr>
<th>Step</th>
<th>Total activity</th>
<th>Protein</th>
<th>Specific activity</th>
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</thead>
<tbody>
<tr>
<td>Homogenate in 40% (NH₄)₂SO₄</td>
<td>6400</td>
<td>32,000</td>
<td>0.2</td>
</tr>
<tr>
<td>50 to 75% (NH₄)₂SO₄</td>
<td>5500</td>
<td>12,222</td>
<td>0.45</td>
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<tr>
<td>DE-22 cellulose</td>
<td>4048</td>
<td>913</td>
<td>4.4</td>
</tr>
<tr>
<td>First CM-cellulose</td>
<td>2600</td>
<td>110</td>
<td>26</td>
</tr>
<tr>
<td>Hydroxylapatite</td>
<td>2065</td>
<td>31.7</td>
<td>65</td>
</tr>
<tr>
<td>Second CM-cellulose</td>
<td>1114</td>
<td>11.5</td>
<td>96</td>
</tr>
<tr>
<td>First crystallization</td>
<td>635</td>
<td>5.1</td>
<td>124</td>
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<tr>
<td>Second crystallization</td>
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</tr>
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Total activity: 29,000 units

Protein Specific activity:
- 99,031 mg
- 32,000 mg
- 12,222 mg
- 913 mg
- 110 mg
- 31.7 mg
- 11.5 mg
- 5.1 mg

TABLE II
Purification procedure for citrate synthase from rat heart

<table>
<thead>
<tr>
<th>Step</th>
<th>Total activity</th>
<th>Protein</th>
<th>Specific activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Homogenate in 40% (NH₄)₂SO₄</td>
<td>9442</td>
<td>2241</td>
<td>4.2</td>
</tr>
<tr>
<td>1st CM-cellulose</td>
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<td>350</td>
<td>25</td>
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<tr>
<td>Hydroxylapatite</td>
<td>6009</td>
<td>130</td>
<td>40</td>
</tr>
<tr>
<td>2nd CM-cellulose</td>
<td>4065</td>
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<tr>
<td>2nd crystallization</td>
<td>2432</td>
<td>19.0</td>
<td>128</td>
</tr>
<tr>
<td></td>
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<td></td>
<td></td>
</tr>
</tbody>
</table>

Fig. 1. High speed sedimentation equilibrium data of citrate synthase from rat liver. The protein concentration was 0.5 mg per ml in 20 mM KPO₄, pH 6.8, containing 1 mM EDTA. The centrifugation was at 17,980 rpm for 24 hours at an average temperature of 19.0°C. The fringe displacement in micra was measured on a microcomparator. The abscissa represents the square of the distance from the center of rotation.

Fig. 2. Disc gel electrophoresis of citrate synthases treated with sodium dodecyl sulfate. About 25 μg of the dissociated enzyme in SDS were applied to each gel and electrophoresis was carried out 3 ma per tube for 1 hour and 45 min. To check the sensitivity of the method of dissociation, partially purified rat liver citrate synthase (specific activity = 80) was run at the same time and six separate bands were observed.

The results of high speed sedimentation equilibrium studies in 20 mM KPO₄, pH 6.8, containing 1 mM EDTA for both enzymes, show that all the points in the plots of the log of the fringe displacement versus r² (radial distance)², lie on straight lines, indicating the homogeneity of the preparations. The molecular weights of both enzymes were calculated based on the results at protein concentrations of 0.24 mg per ml and 0.35 mg per ml in rat liver, and of 0.17 mg per ml and 0.35 mg per ml in rat heart. For the calculation of molecular weight, the value of 0.74 was used as the partial specific volume (12). The results yield molecular weights of liver and heart citrate synthase of 1.0 x 10⁵. Fig. 1 shows a representative sedimentation equilibrium experiment.

The enzyme solutions (0.3 mg per ml) were dialyzed for 24 hours against 7 M guanidine-HCl and 10 mM 2-mercaptoethanol in 2.0 mM potassium phosphate buffer, pH 6.8. The dialyzed enzymes in 7 M guanidine-HCl were analyzed by the high speed sedimentation equilibrium technique. Assuming the value for partial specific volume as 0.74, the molecular weight of the dissociated enzymes in 7 M guanidine-HCl is approximately 5.8 x 10³ for rat liver enzyme and 5.3 x 10³ for the rat heart enzyme.

Sodium Dodecyl Sulfate

The enzyme solutions in 2% sodium dodecyl sulfate containing 0.1 M 2-mercaptoethanol and 62.5 mM Tris-Cl buffer, pH 6.8, were heated for 3 min at 70°C.

The treated enzymes were applied to sodium dodecyl sulfate gels as described under “Materials and Methods,” and the results are shown in Fig. 2. The sodium dodecyl sulfate-dis-
Kinetic Studies

The apparent Michaelis constant for both substrates was determined. In rat liver citrate synthase, the apparent $K_m$ for acetyl-CoA was 2.8 $\mu$M as determined with oxaloacetate concentrations of 1.25 $\mu$M, 2.5 $\mu$M, and 5.0 $\mu$M. The apparent $K_m$ for oxaloacetate was 3.6 $\mu$M.

Inhibition by ATP

Both citrate synthases from rat liver and rat heart are inhibited by ATP. Plots of the reciprocal initial reaction velocity against ATP concentration at acetyl-CoA concentrations of 10 $\mu$M and 50 $\mu$M and a concentration of 5 $\mu$M oxaloacetate showed that the inhibition was the competitive type with respect to acetyl-CoA, and the $K_i$ values for both enzymes were 0.7 mM (Fig. 5). On the other hand, with a fixed concentration of 10 $\mu$M acetyl-CoA, the same analysis for oxaloacetate (Fig. 6) indicated the inhibition to be noncompetitive for oxaloacetate. The $K_i$ values against oxaloacetate were similar for both enzymes.
RAT LIVER

Fig. 6. Inhibition of ATP on citrate synthase from rat liver (top) and rat heart (bottom). The $K_i$ for ATP was measured by using 10 and 50 $\mu$M acetyl-CoA and 5 $\mu$M oxaloacetate.

RAT HEART

Fig. 7. Double reciprocal plot of initial velocity against oxaloacetate in the absence and the presence of 1 and 4 mM ATP.

Immunological Studies

Antiserum (5 $\mu$l) was placed in the center well of an Ouchterlony plate, and then 5 $\mu$l of the diluted enzyme samples were added to each outer well. Diffusion was allowed to proceed at 4° for 24 hours. On the stained plate, a single precipitin band was visible against both rat heart and rat liver citrate synthase, but no band was visible against guanidine-HCl-dissociated citrate synthases (Fig. 8).

Similar studies against a number of citrate synthases from other species were carried out by means of the same procedure. The pig heart, moth muscle, and Azotobacter enzymes were crystalline preparations, whereas the mango enzyme was a crude preparation. No precipitin reaction occurred between any of these enzymes and the antiserum to rat heart citrate synthase, whereas partially purified citrate synthases from other organs in the rat (kidney (specific activity, 39), brain (specific activity, 30), and spleen (specific activity, 10)) reacted with the antiserum and formed precipitin bands. Precipitin bands were fused to each other at their ends. Moreover, spur formation was observed between rat heart and spleen and between liver and kidney citrate synthase, but not between heart and liver.
Table III shows the formation of precipitin bands on Ouchterlony plate. The positive reaction indicates the presence of antigen-antibody complexes, while the no reaction indicates the absence of such complexes.

<table>
<thead>
<tr>
<th>Positive reaction</th>
<th>No reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat heart citrate synthase</td>
<td>Pig heart citrate synthase</td>
</tr>
<tr>
<td>Liver</td>
<td>Mosquito</td>
</tr>
<tr>
<td>Kidney</td>
<td>Acetobacter</td>
</tr>
<tr>
<td>Brain</td>
<td>Mango</td>
</tr>
<tr>
<td>Spleen</td>
<td>Malate dehydrogenase</td>
</tr>
</tbody>
</table>

* Crystalline enzymes.


discussion

The citrate synthases from rat heart and rat liver appear to be identical proteins. It is somewhat surprising, considering the great differences in metabolic properties of these two tissues, that an enzyme thought to be a key and controlled step in the oxidative function of the cell should be identical in both tissues. Their molecular weights are about $1 \times 10^6$, and they are composed of 2 similar or identical subunits. Their kinetic constants are similar, and the inhibition by ATP shows similar patterns for both. Antibody against rat heart enzyme behaves identically toward the rat liver enzyme. The turnover numbers of both enzymes are about $1.3 \times 10^6$ moles of substrate converted per min per mole of enzyme at 28°C.

The content of citrate synthase in rat liver is 14 units per g. The mitochondria of rat liver is about 20% of the liver cell, and, since the citrate synthase is a mitochondrial enzyme, then there are 70 units per g of mitochondria. This represents $5.8 \times 10^{-6}$ moles per kg of mitochondria. Heart has about 5 times this concentration or about $2.8 \times 10^{-6}$ moles of citrate synthase per kg of heart mitochondria.

It is difficult to compare the kinetic properties that we have measured here at enzyme concentrations of between $5 \times 10^{-5}$ M and $10^{-4}$ M with the behavior at mitochondrial concentrations, 1000 times greater than these. It is possible that the metabolic differences seen in the two tissues are due to the difference in concentrations of the enzymes only. Both substrates, acetyl-CoA and oxaloacetate, have been measured in rat heart and rat liver mitochondria. Oxaloacetate is in the 1 μM range, whereas acetyl-CoA may be as high as 50 μM. If the dissociation constants for oxaloacetate for these enzymes are similar to the one we have measured for the pig heart citrate synthase, 0.7 μM, then the free oxaloacetate in mitochondria is extremely low, and under these conditions the difference in the total amount of citrate synthase in rat liver and rat heart mitochondria could well be a determinant of the free oxaloacetate concentration and an important factor in the regulation of oxidative metabolism of these tissues.

The apparent $K_m$ for oxaloacetate and acetyl-CoA for both these enzymes is in the 5 μM range. These $K_m$ values appear to be independent of each other as was previously reported for the rat liver enzyme (3). This is in contrast to our earlier reported results for pig heart and moth muscle enzyme (3, 13). We have reinvestigated the pig heart enzyme and find that the $K_m$ for acetyl-CoA is 5 μM and is independent of oxaloacetate concentration. The difference between our present results and our previous ones is apparently due to our ability with an expanded scale recorder to measure more accurately initial rates of reaction at these low substrate concentrations.

The differences in $K_m$ values for acetyl-CoA in rat liver enzyme reported here (5 μM) and that reported by Shepherd and Garland (16 μM) (5) are probably due to differences in ionic strength in the various assays. Egggerer, Remberger, and Grünwider (14) have reported that increasing ionic strength inhibits pig heart citrate synthase. Wu and Yang (15) have confirmed these observations, and we have shown that this effect can also be demonstrated in the rat heart and rat liver enzymes (16). These ionic strength effects have been reported to be competitive with acetyl-CoA so that differences in reported apparent $K_m$ values may be due to differences in the ionic strength of the various assay mixtures.

We have reported studies (17) with the use of the antiserum prepared to partially purified pig heart citrate synthase. Although the antiserum had antienzyme activity, no precipitin reaction was obtained between the antiserum and the pure pig heart citrate synthase. However, the antienzyme was active against a number of different vertebrate citrate synthases. We find no interaction between the anti-rat heart citrate synthase and citrate synthase from other animals. There are good precipitin lines formed with partially purified citrate synthases from other rat tissues. It remains to be seen whether the spur formation seen on Ouchterlony plates is indicative of different citrate synthases in these tissues.

Acknowledgments—We would like to thank Dr. H. Itoh and Mrs. B. Böttger for their assistance.

REFERENCES

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