The Citrate Cleavage Enzyme

II. STOICHIOMETRY SUBSTRATE SPECIFICITY AND ITS USE FOR COENZYME A ASSAY*

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The citrate cleavage enzyme catalyzes the following reaction:

\[ \text{Citrate} + \text{ATP} + \text{CoA} \rightarrow \text{Acetyl-CoA} + \text{OAA} + \text{ADP} + \text{Pi} \]

This enzyme is widely distributed in animal tissues and has been partially purified from pigeon liver (1) and extensively purified from chicken liver (2). Because of the complexity of the overall reaction, we attempted at first to fractionate the activity into more than one protein fraction. The results of these earlier studies led to the conclusion that one protein component or tightly bound protein complex was responsible for the catalysis. In further studies of this enzyme we have looked for the formation of possible free intermediates and have assayed for several possible initial enzymic reactions. The results of these earlier papers are presented in this paper along with data on the stoichiometry, substrate specificity, and reversibility of the reaction. In addition, a method for the stoichiometric determination of coenzyme A with this enzyme is described.

EXPERIMENTAL PROCEDURES

Materials

CoA was purchased from Pabel Laboratories and Sigma Chemical Company. A gift of CoA was obtained from C. F. Boehringer and Soehne, Mannheim, Germany. Dr. G. Moffat (University of British Columbia) supplied us with a sample of synthetic CoA. ATP, GTP, UTP, ITP, and ADP were purchased from Pabel Laboratories. Phosphoenolpyruvate was purchased from California Corporation for Biochemical Research. DPNH, malate dehydrogenase, lactate dehydrogenase, and pyruvate kinase were obtained from C. F. Boehringer and Soehne. Adenylate deaminase was a gift of Dr. Guarino. Condensing enzyme assay system (7) was prepared in this laboratory by an unpublished procedure. Citrate cleavage enzyme was prepared as described in earlier papers (1, 2).

Citryl Anhydride, Citryl Hydroxamate, and Citryl CoA—An anhydride of citric acid was prepared with ethyl chloroformate as described for other acids by Wieland and Rueff (3). The hydroxamate made from this anhydride when chromatographed in a butanol-water system, as described by Stadtman and Barker (4), gave a typical hydroxamate spot \((R_f 0.40)\) which is the same as acetyl hydroxamate and also reacted with a nitroprusside spray to give a red spot. The \(R_f\) value of citryl-CoA is 0.34. The material eluted from the spot, having \(R_f\) of 0.24, showed an absorption peak at 232 \(\mu\)m.

RESULTS

Identification of Products of Reaction—When enzyme citrate, ATP, Mg\(^{++}\), and CoA are incubated in the presence of 0.2 \(\mu\)m NH\(_2\)OH, hydroxamate is formed. Isolation and paper chromatography of this hydroxamate \((4)\) show the presence of only one spot \((R_f 0.40)\) which is the same as acetyl hydroxamate and also cochromatographs as a single spot with authentic acetyl hydroxamate. The material eluted from the spot, having \(R_f\) of 0.22, which is where citryl hydroxamate would be expected to appear. If the incubation is carried out in the absence of hydroxylamine, acetyl-CoA as a product of reaction can be identified by its reaction in the condensing enzyme assay system (7).

OAA \(^1\) was identified as a product of the citrate cleavage enzyme by its oxidation of DPNH in the presence of excess malate dehydrogenase. The spectrophotometric assay for citrate cleavage enzyme \((2)\) is dependent upon production of OAA.

The products of ATP breakdown in this reaction were identified as ADP and Pi. The ADP can be identified by its reaction with phosphoenolpyruvate in the presence of DPNH, excess lactate dehydrogenase, and pyruvate kinase. AMP was not detected, as judged by the lack of a material that would react with adenylate deaminase (Table I) \((8)\). Since the enzyme is essentially free from myokinase activity, AMP cannot be a product of the reaction. Formation of Pi was measured by the method of Fiske and SubbaRow \((9)\). Its formation from ATP was found to be dependent upon all factors, citrate, CoA, Mg\(^{++}\), and ethyl chloroformate, then three different mixed anhydrides are possible, and if excess ethyl chloroformate is used, one might expect the mono-, di-, tri-, mixed anhydrides to be formed. When excess ethyl chloroformate was used and the product converted to its hydroxamate, just one hydroxamate was formed, as judged by paper chromatography. Thus the procedure for the formation of the mixed anhydride may, in the case of citric acid, lead to the formation of an internal anhydride of citric acid.

When 0.1 ml of this anhydride is added to a solution of 5 \(\mu\)moles of glutathione or CoA in 1 ml of 0.2 \(\mu\)m NaHCO\(_3\), sulphydryl disappears, as judged by the nitroprusside color reaction \((5)\), and a compound is formed which has an ultraviolet absorption peak at 232 \(\mu\)m, typical of a thioester compound. This preparation \((\text{when CoA is used})\) is referred to as citryl-CoA. Chromatography of citryl-CoA in the acetate-ethanol system of Stadtman \((6)\) showed an ultraviolet absorbing spot \((R_f 0.24)\) which also reacted with a nitroprusside spray to give a red spot. The \(R_f\) values of CoA and acetyl-CoA in this system are 0.52 and 0.34, respectively. The material eluted from the spot, having an \(R_f\) of 0.24, showed an absorption peak at 232 \(\mu\)m.

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1 The abbreviation used is: OAA, oxaloacetate.

2 We would like to thank Mr. Chapman for his cooperation in obtaining rather large quantities of fresh chicken liver.
and enzyme. No inorganic pyrophosphate formation could be detected by the method of Flynn et al. (10).

Stoichiometry—Tables I and II give the results of several experiments which show partial stoichiometry of the reaction. These results confirm our earlier findings with the pigeon liver citrate cleavage enzyme that citrate, ATP, and CoA are used equimolarly to form acetyl-CoA, OAA, ADP, and P\(_i\). This is true both in the presence and absence of hydroxylamine as a trapping agent for acetyl-CoA. The low value obtained for OAA in Table I probably is due to the instability of this compound.

Substrate Specificity—A number of compounds were substituted for citrate in the standard hydroxamate assay for citrate cleavage enzyme. Acetate, malonate, succinate, malate, ascorbate, tricarboxylate, and isocitrate were inactive in the assay. \(\beta\)-Hydroxy-\(\beta\)-methylglutarate at 100 times the usual citrate concentration formed only 4% as much hydroxamate. A slight activity was observed with itaconate, but this is quite likely due to a small contamination of citrate in the itaconate.

The data in Table III show that the enzyme is also rather specific for the ATP. The rate seems to be depressed in the presence of GTP. When Mn\(^{++}\) or Co\(^{++}\) replaces Mg\(^{++}\), only 60 to 80% of the activity is observed. Only 2% of the total can be seen when Zn\(^{++}\) replaces Mg\(^{++}\). Pantetheine could not replace CoA in the enzyme reaction.

\(pH\) Study—In the citrate cleavage assay which is coupled to malate dehydrogenase, there is an optimal activity near pH 7.3 with Tris (Table IV). Phosphate buffers were inhibitory in this assay.

### Table I

<table>
<thead>
<tr>
<th>Stoichiometry</th>
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<tbody>
<tr>
<td>In Experiment 1 the reaction mixture contained 20 (\mu)moles of potassium citrate, 5 (\mu)moles of ATP, 10 (\mu)moles of MgCl(_2), 0.05 (\mu)mole of CoA, 10 (\mu)moles of 2-mercaptoethanol, 200 (\mu)moles of NH(_2)OH (pH 7.4), 60 (\mu)g of enzyme in a total volume of 1 ml. A control tube contained all components except potassium citrate. After incubation for 15 minutes at 37°, 0.1 ml of 50% trichloroacetic acid was added, the protein removed by centrifugation, and aliquots taken for assay. The following analytical methods were used: hydroxamate, Lipmann and Tuttle (11); P(_i), Fiske and SubbaRow (9); ADP as described in text; AMP, Kalckar (8).</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Experiment</th>
<th>(-)Sulf-hydryl</th>
<th>(-)Citrate</th>
<th>(+)Hydroxamate</th>
<th>(+)P(_i)</th>
<th>(+)ADP</th>
<th>(+)OAA</th>
<th>AMP</th>
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<tbody>
<tr>
<td>1</td>
<td>(\mu)moles</td>
<td>(\mu)moles</td>
<td>(\mu)moles</td>
<td>(\mu)moles</td>
<td>(\mu)moles</td>
<td>(\mu)moles</td>
<td>(\mu)moles</td>
</tr>
<tr>
<td>10</td>
<td>0.34</td>
<td>0.32</td>
<td>0.94</td>
<td>0</td>
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<tr>
<td>20</td>
<td>0.3</td>
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<tr>
<td>30</td>
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### Table II

<table>
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<th>Stoichiometry</th>
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<tr>
<td>In Experiment 2 the incubation mixture contained 1.2 (\mu)moles of potassium citrate, 30 (\mu)moles of 2-mercaptoethanol, 30 (\mu)moles of MgCl(_2), 15 (\mu)moles of ATP, 0.15 (\mu)mole of CoA, 300 (\mu)moles of NH(_2)OH (pH 7.4), 3 mg of enzyme in a total volume of 3 ml. A tube without CoA and a zero time tube were used as controls. The reaction was stopped after 2 hours at 37°, with 0.3 ml of 50% trichloroacetic acid. After centrifugation to remove protein, citrate was added, the protein removed by centrifugation, and aliquots taken as above. OAA was determined by the method of Friedemann and Haugen (13).</td>
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</table>

### Table III

<table>
<thead>
<tr>
<th>Nucleotide sensitivity</th>
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<tbody>
<tr>
<td>Assays were performed by following the rate of DPNH oxidation as described previously (2). Each cuvette contained 100 (\mu)moles of Tris pH 7.3, 0.3 (\mu)mole of CoA, 20 (\mu)moles of potassium citrate, 5 (\mu)moles of nucleotide, 10 (\mu)moles of MgCl(_2), 500 units of malate dehydrogenase, 0.15 (\mu)mole of DPNH, and 400 (\mu)g of enzyme in a final volume of 1.0 ml.</td>
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<table>
<thead>
<tr>
<th>Time</th>
<th>(+)OAA</th>
<th>(+)Acetyl-CoA</th>
<th>(+)P(_i)</th>
<th>(+)ADP</th>
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<tbody>
<tr>
<td>min</td>
<td>(\mu)moles</td>
<td>(\mu)moles</td>
<td>(\mu)moles</td>
<td>(\mu)moles</td>
</tr>
<tr>
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<td>0.2</td>
<td>0.3</td>
<td>0.4</td>
<td>0.5</td>
</tr>
<tr>
<td>20</td>
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<td>0.4</td>
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<tr>
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<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
</tr>
</tbody>
</table>

* After initial rates were determined, 5 \(\mu\)moles of ATP were added, and the rate was observed again.

### Table IV

<table>
<thead>
<tr>
<th>pH study</th>
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</thead>
<tbody>
<tr>
<td>Conditions of assay are described in Table III, with the exception that 5 (\mu)moles of ATP were used in all cases and 100 (\mu)moles of the buffer indicated.</td>
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<table>
<thead>
<tr>
<th>Buffer</th>
<th>pH</th>
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<tr>
<td>Tris</td>
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<td>70</td>
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<tr>
<td>7.3</td>
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</tr>
<tr>
<td>7.7</td>
<td>97</td>
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<tr>
<td>8.0</td>
<td>54</td>
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</tr>
<tr>
<td>Phosphate</td>
<td>6.3</td>
<td>5</td>
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<tr>
<td>7.2</td>
<td>41</td>
<td></td>
</tr>
</tbody>
</table>
Reversibility—Some indications of the reversibility of the citrate cleavage enzyme from pigeon liver were reported by us earlier (1). This report now seems to be in error, since repetition of these experiments with the pigeon liver enzyme failed to yield the same results. In addition, we are unable to detect any reversibility of the reaction catalyzed by the highly purified chicken liver enzyme. We cannot find citrate or ATP formation from acetyl-CoA, OAA, ADP, Pi, and enzyme under a variety of conditions, nor can we find the utilization of OAA or Pi, under such conditions. No exchange of Pi into ATP is observed when the components of the reaction (citrate, ATP, CoA, Mg++, and enzyme) are incubated together. Nor can the formation of ATP from acetyl-CoA, OAA, ADP, Pi, and enzyme be observed.

Intermediate Steps—Since no hydroxamate, but acetylhydroxamate can be formed, such intermediates as free citryl-CoA or citryl phosphate seem to be excluded. It has also been observed that 0.2 M hydroxylamine does not affect the rate of OAA formation in the spectrophotometric assay, which also rules out a free carboxyl-activated citrate formation. However, when synthetic citryl-CoA is used in place of citrate, ATP, and CoA in the spectrophotometric assay for citrate cleavage enzyme, a slow reaction is observed. This rate is much less than one would expect if the synthetic material were an intermediate, but still makes it difficult to rule out this compound absolutely as an intermediate.

Neither citrate, ATP, and enzyme, nor CoA, ATP, and enzyme will lead to the production of Pi, nor can ADP formation from these components be demonstrated with the coupled pyruvate kinase-lactate dehydrogenase system. For Pi and ADP formation, citrate, CoA, ATP, and enzyme must be incubated together.

We showed earlier that the cleavage and condensing enzyme activities of chicken and pigeon liver can be completely separated. Indeed, when crystalline condensing enzyme is added to a citrate cleavage reaction, one observes that less hydroxamate is formed. This apparent inhibition may be explained by assuming that the condensing enzyme reforms citrate from OAA and acetyl-CoA and thus competes effectively with hydroxylamine for the acetyl-CoA formed by citrate cleavage.

A Stoichiometric Assay for CoA

A number of methods for CoA determination depend upon the measurement of reaction rate and then a comparison with the rate produced with known CoA samples (14-16). There are several methods now available for the stoichiometric determination of CoA. One can easily convert the CoA to acetoacetyl-CoA and determine the latter with \( \beta \)-keto acyl reductase and DPNH (17, 18). Dr. Bergmeyer has adapted the \( \alpha \)-keto glutarate dehydrogenase assay (16) to give a stoichiometric assay for CoA. Recently Winkl and Hübcher (19) have described a method for CoA determination based on the determination of sorbyl-CoA formed with fatty acid-activating enzyme.

During the course of experimentation with the citrate cleavage enzyme, we noticed that the oxidation of DPNH in the coupled enzyme assay would quickly reach completion when CoA was the limiting substrate. The amount of DPNH oxidized was proportional to the amount of CoA added (Fig. 1). 2-Mercaptoethanol was omitted, since a slow oxidation takes place when this substance (or any other thiol) is present. Thus the method will analyze only reduced CoA. A check on the accuracy of the method was made by determining CoA content of various samples that were assayed in other ways. A sample of CoA assayed by Dr. Bergmeyer with \( \alpha \)-ketoglutarate dehydrogenase as 62% pure was assayed as 59% pure with our method. A sample assayed as 50% pure by the acetylation of sulfanilimide (14) was assayed as 54% pure. A sample of synthetic CoA estimated to be 97% pure was assayed by us as 78% pure, the discrepancy being caused by the presence of oxidized CoA. We have estimated the purity of several CoA samples in our laboratory in several ways: (a) by ion exchange chromatography on Dowex 1 (20); (b) by conversion to aceto-CoA and estimation with condensing enzyme (7); and (c) by the method described here. Good agreement is obtained among these methods.

**DISCUSSION**

The citrate cleavage enzyme catalyzes an ATP-dependent carbon-carbon bond cleavage. In considering an approach to the study of this reaction, it at first seemed probable that several separate reactions were involved. In a similar cleavage of itaconate in rat and guinea pig liver extracts (21), several enzymes seemed to be involved, and itaconyl CoA appeared to be an intermediate. One can thus write a sequence of reactions as follows:

\[
\text{Citrate} \rightarrow \text{ATP, CoA} \rightarrow [\text{citryl-CoA}] \rightarrow \text{acetyl-CoA + OAA}
\]

The first reaction (except for the split products of ATP) is similar to carboxyl activation reactions, and the second reaction is similar to the \( \beta \)-hydroxy-\( \beta \)-methylglutaryl-CoA cleavage reaction (22). Our work on enzyme purification, however, indicated that only one enzyme was responsible for the reaction. In the present work, attempts to trap an activated citrate were unsuccessful, and attempts to demonstrate possible partial reactions such as those catalyzed by CoA-kinase or citrate kinase gave negative results. The slow reaction observed with a synthetic (but not necessarily authentic) citryl-CoA may indicate the oo-

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\( ^* \) Dr. E. Bergmeyer, personal communication.
currence of an enzyme-bound intermediate, or may reflect impurity or the fact that the synthetic citryl-CoA is an isomer of a natural citryl-CoA.

There are few single enzymes known that catalyze reactions of the complexity of the citrate cleavage reaction. The glutamine synthetase reaction (23) has some common features with citrate cleavage in that no free intermediates have been detected. Preliminary kinetic experiments with the citrate cleavage enzyme have indicated that a lag occurs in the coupled reaction, dependent on the order of addition of substrates. This seems to be true with glutamine synthetase as well. The free energy change of the citrate cleavage can be calculated in the following manner:

\[
\begin{align*}
\text{ATP} + \text{H}_2\text{O} &\rightarrow \text{ADP} + \text{Pi} + \text{H}^+ \\
\text{Citrate} + \text{H}^+ + \text{CoA} &\rightarrow \text{acetyl-CoA} + \text{OAA} + \text{H}_2\text{O} \\
\text{Citrate} + \text{ATP} + \text{CoA} &\rightarrow \text{acetyl-CoA} + \text{OAA} + \text{ADP} + \text{Pi} \\
\end{align*}
\]

\[\Delta F' = -7600 \text{ cal/mole (24)}\]
\[\Delta F' = +7600 \text{ cal/mole (7)}\]
\[\Delta F' = 0\]

Since the calculated free energy change is zero, the equilibrium constant should be one. It is therefore surprising that no reversibility could be demonstrated. A similar situation, however, has been found by Colowick et al. (26) in the pyridine nucleotide transhydrogenase of Pseudomonas fluorescens and has been explained by the inhibitory action of a substrate on the reaction.

**SUMMARY**

The enzyme catalyzing an adenosine triphosphate- and coenzyme A-dependent cleavage of citrate has been studied. The stoichiometry of the reaction catalyzed by the purified chicken liver enzyme is in accord with the earlier results with a pigeon liver enzyme.

\[
\text{Citrate} + \text{ATP} + \text{CoA} \rightarrow \text{acetyl-CoA} + \text{oxaloacetate} + \text{ADP} + \text{Pi}
\]

The reaction is highly specific in regard to citrate and adenosine triphosphate. Pantetheine will not replace coenzyme A, but Mg\(^{++}\) could be replaced with Mn\(^{++}\) and Co\(^{++}\). No reversibility of the reaction can be demonstrated. No indication for the formation of free intermediates could be found.

In addition, a stoichiometric assay for coenzyme A with citrate cleavage enzyme is described.

**Acknowledgment**—I am grateful to Mrs. Virginia Johansen for her excellent technical assistance.

**REFERENCES**

The Citrate Cleavage Enzyme: II. STOICHIOMETRY SUBSTRATE SPECIFICITY AND ITS USE FOR COENZYMES A ASSAY
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