Purification and Properties of Acetyl Coenzyme A Synthetase from Bovine Heart Mitochondria*

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In the presence of magnesium ions, acetyl coenzyme A synthetase catalyzes the formation of acetyl coenzyme A from acetate, coenzyme A, and adenosine triphosphate. This reaction proceeds through a two-step mechanism probably involving enzyme-bound acetyl adenylate as an intermediate (1-4).

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\text{Acetate}^{--} + \text{ATP}^{--} + \text{CoA-SH} \rightleftharpoons \text{acetyl-CoA} + \text{AMP}^{--} + \text{PPi}^{--}
\]

Acetyl coenzyme A synthetase has been partially purified from many sources including yeast, bacteria, molds, plants, mammalian organs, and pigeon liver (5-11). Substrate amounts of this enzyme, partially purified by the method of Hele from beef heart mitochondria (10), have been used for the isolation of acetyl adenylate from a reaction mixture containing all reactants except coenzyme A (4). Although the heart preparation provided sufficient adenylate for identification purposes, it was found to contain appreciable activity for the synthesis of butyryl coenzyme A and for the hydrolysis of chemically synthesized acetyl adenylate (4). For further studies concerning the mechanism of acetate activation, large amounts of an acetyl coenzyme A synthetase, essentially free of butyryl coenzyme A synthetase and acetyl adenylate hydrolase, were needed. This objective has been achieved with the isolation of a nearly homogeneous enzyme and the present paper describes both its purification and some of its characteristics.

**EXPERIMENTAL PROCEDURE**

**Enzyme Assays**—The acetate-dependent disappearance of coenzyme A was determined essentially as described by Mahler, Waki, and Bock (12). In a total volume of 0.25 ml, the complete reaction mixture contained 25 μmoles of tris(hydroxymethyl)-aminomethane hydrochloride buffer, pH 8.0, 1 μmole of MgCl\(_2\), 0.8 μmole of dipotassium ATP, 37.5 μmoles of KCl, 0.3 to 0.4 μmole of CoA-SH, 2.5 μmoles of acetate, and as little as 0.0035 mg of enzymatic protein. Both the acetate and the potassium ATP were titrated to pH 8.0 with Tris base. Both complete and control mixtures were incubated for 3 minutes at 37\(^\circ\) and each reaction was terminated by the addition of 0.06 ml of 30% metaphosphoric acid. After centrifugation, a 0.1-ml aliquot of the supernatant liquid was taken for assay in the nitroprrusside reaction as outlined by Grunert and Phillips (13).

Acetyl adenylate disappearance was determined by the hydroxamate reaction of Lipmann and Tuttle (14) under the conditions described by Webster and Campagnari (4). Chemically synthesized acetyl adenylate was formed from acetic anhydride and adenosine monophosphate according to the method of Avison (15). Excess anhydride and acetic acid were removed by extraction with ether at acidic pH (4).

Protein was measured by the biuret method of Gornall, Bardawill, and David with crystalline bovine serum albumin used as the standard (16). One unit of enzyme activity is that quantity which catalyzes the disappearance of 1 μmole of substrate (coenzyme A or acetyl adenylate) per minute under the assay conditions described above; specific activity is expressed in units of enzyme activity per mg of protein.

**Other Methods**—Salt-free hydroxylamine was prepared by the method of Beinert et al. (9). Crystalline pyrophosphatase was kindly provided by Dr. M. Kunitz. Acetyl coenzyme A was isolated by paper chromatography on Whatman No. 3 paper both in an isobutyric acid-NH\(_3\)-water system and in another solvent complex containing ethanol and 0.1 M sodium acetate buffer, pH 4.5 (1:1). The product was identified by its absorption at 232 and 260 m\(\mu\) and by its reactions with -SH reagents after hydrolysis of the thioester bond (17). The alumina Cy gel was prepared according to Willstätter and Kraut (18), kept at a concentration of 20 mg of gel (dry weight) per ml of solution, and used within a period of 4 months. Tubing for dialysis was purchased from the Visking Company and was boiled in two changes of 0.02 M KHCO\(_3\)·0.0005 M Versene to remove impurities.

**Materials**—Nucleotides (ATP, ADP, AMP, GTP, and UTP) and coenzyme A were obtained from the Pabst Laboratories. Glutathione was purchased from the Nutritional Biochemicals Corporation. Pyruvic, succinic, maleic, glycolic, glyoxylic, and γ-aminobutyric acids were purchased from General Biochemicals, Inc. Dr. Warwick Sakami kindly provided us with twice recrystallized L- and DL-lactate and the β-hydroxybutyric acid was a gift of Dr. D. Christensen. Sephadex G 100 was purchased from Pharmacia in Uppsala, Sweden, and the triethylaminomethyl cellulose (TEAE) was obtained from the Brown Company, Berlin, New Hampshire.

Ammonium sulfate used to precipitate highly purified enzyme was recrystallized at alkaline pH in the presence of 0.0005 M Versene. Other chemicals were the commercially available reagent grade.
RESULTS

Purification of Acetyl Coenzyme A Synthetase—Beef heart mitochondria were isolated and broken by three freezing and thawing steps essentially as described by Hele (10). All subsequent procedures were carried out at 0 to 4°C. After the third thawing the mitochondrial suspension was centrifuged at 20,000 × g for 15 minutes, the precipitate was discarded, and the remaining solution (mitochondrial supernatant, Table I) was treated with 21 g of solid ammonium sulfate per 100 ml. The resulting precipitate was discarded and an additional 23 g of ammonium sulfate per 100 ml of original solution were then added. The precipitate containing most of the enzyme was collected by centrifugation.

The ammonium sulfate precipitate was dissolved in sufficient 0.02 M KHCO₃-0.0005 M Versene to give a protein concentration of 8 mg per ml. Then saturated liquid ammonium sulfate (brought to pH 8.5 with NH₄OH) equal to 5% of the volume was added followed by 20% of the original volume as alumina C₄ gel. The gel was removed by centrifugation at 5000 × g for 5 minutes and discarded, and the enzyme was precipitated by the addition of 42 g of solid ammonium sulfate per 100 ml of supernatant solution. This precipitate was dissolved and diluted with 0.02 M KHCO₃-0.0005 M Versene to a protein concentration of 10 mg per ml and a saturated ammonium sulfate solution, pH 8.5, was added. The precipitate collected between 0.35 and 0.38 saturation was used for further purification (second ammonium sulfate precipitate, Table I).

Protein

Of protein were observed in the elution curve. The second peak of protein contained the enzymatic activity and its descending limb comprised the highly purified enzyme employed for definitive studies (second Sephadex, Table I).

Enzyme Stability and Purity—The best fractions of acetyl-CoA synthetase (specific activity, 12 to 13.3) lost activity slowly if kept at 3°C. When stored as a frozen ammonium sulfate precipitate, the enzyme was quite stable but excess salt usually had to be removed by dialysis or with Sephadex G-100 before full activity was noted. With prolonged dialysis, the preparation usually lost 15 to 20% of its activity; repeated freezing and thawing also caused a substantial loss.

In contrast to cruder material, the best preparations showed only one peak of protein when chromatographed on either TEAE or Sephadex G-100 and the various fractions of protein throughout this peak had essentially a constant specific activity for acetate activation (Figs. 1 and 2). During centrifugation in the Spinco model E analytical ultracentrifuge, nearly all the protein appeared in a single peak which had a sedimentation constant of 4.4 S (Fig. 3). Under identical conditions, cruder preparations of acetyl-CoA synthetase showed both heavier and lighter components which blended with the major peak of protein.

The enzyme was found to be active with acetate, acrylate, and propionate. Butyrate had about 1% of the activity found with acetate. That this was due to a contaminant was shown by the absence of this activity in the latter fractions from the first TEAE column. The best preparations of acetyl-CoA synthetase contained no detectable acetyl adenylate hydrolase, acetyl-CoA deacetylase, ATPase, or pyrophosphatase. Little or no nucleic acid was present (0.25 to 0.5%) as calculated from the ultraviolet absorption ratio between 280 and 260 nm (19). The enzyme showed only the usual peak at 280 nm.

Characteristics in CoA Disappearance Assay The assay procedure for the acetate-dependent disappearance of CoA-SH gave reproducible results. At 37°C, product formation was linear with time up to 4 minutes, after which it diminished particularly when very low concentrations of enzyme were used. When a 3-minute incubation period was used the acetate-dependent disappearance of CoA-SH was proportional to enzyme concentration, and this time interval was therefore selected. The pH curve showed a broad peak with an optimum near pH 8.0 as previously described (4).

Michaelis constants were calculated for the various substrates,
Acetyl Coenzyme A Synthetase

Vol. 238, No. 5

70 80 so 100 110

Fig. 1 (above). Chromatography of acetyl-CoA synthetase over a column (23 x 1 cm) of TEAE-cellulose showing a nearly constant ratio of units to protein concentration. Gradient elution between 0.02 M KHC03-0.0005 M Versene and 1.4 M KHC03-0.0005 M Versene (120 ml in both the elution chamber and the reservoir); 74% of the added protein and 72% of the units were recovered. Average specific activity of the eluted protein was 12.6 units per mg.

Fig. 2 (below). Chromatography of acetyl-CoA synthetase over a column (23 x 1 cm) of Sephadex G-100 showing a nearly constant ratio of units to protein concentration. Protein was eluted with 0.02 M KHC03-0.0005 M Versene; 101% of the added protein and 98% of the units were recovered. The average specific activity of the eluted protein was 11.8 units per mg.

i.e. acetate, propionate, acrylate, ATP, MgCl2, and coenzyme A from the data shown in Figs. 4 to 9. There was inhibition of rate at high concentrations of most substrates. Acrylate and propionate had apparent Km values approximately 14-fold greater than that of acetate (1.1 x 10^-3 M as compared to 7.9 x 10^-4 M). The previously reported product inhibition with high concentrations of pyrophosphate was confirmed (4) and inhibition was noted with adenosine monophosphate (50% inhibition at a concentration of 0.012 M). However, the amount of pyrophosphate formed during the assay procedure was not sufficient to produce significant product inhibition and, as might be expected, pyrophosphatase had no stimulatory effect. No inhibition was noted with either adenosine diphosphate or inorganic phosphate.

Several organic acids had no detectable activity (less than 0.5% that obtained with acetate) when substituted for acetate in the CoA-SH disappearance assay. These included formic, L- and DL-lactic, pyruvic, β-hydroxybutyric, succinic, malic, glycolic, glyoxylic, benzoic, salicylic, and γ-aminobutyric acids. Glycine, alanine, and β-alanine likewise were not substrates at a concentration of 0.024 M. Adenosine triphosphate was the only nucleotide which was active; no effect was obtained with adenosine diphosphate and monophosphate or guanosine and cytidine triphosphates. When glutathione was substituted for CoA-SH, no activity was detected.

While the CoA-SH assay was being studied, it was noted that further stimulation of enzymatic activity was obtained when chloride salts of potassium and ammonium were added to the routine incubation mixture; lithium and sodium chloride were inhibitory under identical conditions. Since 0.123 M cation (mostly Tris) was already present, these experiments were repeated at a lower cation concentration (0.023 M) while the pH was maintained near 8.0. Similar results were obtained (Fig. 10) suggesting that the enzyme has an absolute requirement for specific monoivalent cation.

There was inhibition of enzymatic activity obtained with high concentrations of certain anions, chloride being the least effective in this regard (Fig. 11). This was of practical significance in that certain ammonium sulfate fractions yielded inordinately low specific activities which improved after most of the salt was removed by dialysis or treatment with Sephadex G-100. The inhibitory effect of anion was noted only when a stimulatory cation was employed, e.g. potassium or ammonium.

The product of the synthetase reaction was isolated by ascending paper chromatography in two solvent systems as described in “Experimental Procedure.” The acetyl-CoA spots were identified by ultraviolet absorption and also by a nitroprusside reaction after exposure to either dilute alkali or hydroxylamine as out-

FIG. 3. Appearance of purified acetyl-CoA synthetase in the Spinco model E analytical ultracentrifuge. The enzyme (specific activity, 12.1) was dialyzed for 4 hours against hourly changes of 0.02 M KHC03-0.0005 M Versene. The protein concentration was 5.3 mg per ml and centrifugation was continued for 168 minutes after maximal speed was obtained at 59,805 r.p.m. with a rotor temperature of 6.7°. The above photographs were taken 16, 48, 88, 112, 144, and 160 minutes after the rotor reached maximal speed. The bar angel was 50° for the first plate and 40° for the remaining plates.
Figs. 4 to 9. The effect of increasing concentrations of individual substrates is shown on the rate of acetyl-CoA formation. Enzyme with a specific activity of 9.0 to 9.6 units per mg was employed and the assay procedure is described in the text. Enzyme concentrations were adjusted so that no more than 65% of the rate-limited substrate disappeared during the 3-minute incubation at 37°. Apparent Michaelis constants were as follows: acetate, \(7.9 \times 10^{-4}\) M; propionate, \(1.1 \times 10^{-2}\) M; acrylate, \(1.1 \times 10^{-2}\) M; ATP, \(1.8 \times 10^{-3}\) M; MgCl_2, \(1.4 \times 10^{-2}\) M; coenzyme A, \(1.4 \times 10^{-3}\) M.

Purification of Acetyl Adenylate Hydrolase—The greenish colored fractions obtained by chromatography of crude preparations of acetyl-CoA synthetase on TEAE contained a high level of acetyl adenylate hydrolase. Furthermore, acetyl-CoA
Fig. 10. The effect of increasing cation concentrations on the rate of acetyl-CoA formation. Enzyme with a specific activity of 9.0 units per mg and the chloride salts of the stated cations were used; the assay procedure is described in the text. The basic incubation mixtures contained 0.023 M cation without the stated additions.

Fig. 11. The effect of increasing anion concentrations on the rate of acetyl-CoA formation. Enzyme with a specific activity of 9 units per mg and the potassium salts of the stated anions were used; the assay procedure is described in the text. The basic incubation mixtures contained 0.123 M anion without the stated additions.

Table II
Purification of acetyl adenylate hydrolase

<table>
<thead>
<tr>
<th>Purification Procedure</th>
<th>Specific Activity</th>
<th>Purification Factor</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mitochondrial supernatant</td>
<td>0.03-0.07</td>
<td>2-3</td>
</tr>
<tr>
<td>Second (NH₄)SO₄ precipitate</td>
<td>0.17-0.33</td>
<td>5</td>
</tr>
<tr>
<td>First TEAE</td>
<td>0.35</td>
<td>7</td>
</tr>
<tr>
<td>Heat and second TEAE</td>
<td>0.75</td>
<td>15</td>
</tr>
</tbody>
</table>

The purification procedure is described in the text. Enzyme assays were performed by hydroxamate disappearance as outlined by Webster and Campagnari (4).

DISCUSSION

Purification of acetyl-CoA synthetase from beef heart mitochondria was carried out in order to obtain an enzyme suitable for further studies on the mechanism of this reaction. Preparations with a specific activity of 13.0 to 13.7 appeared to be quite homogeneous as judged by column chromatography and analysis in the ultracentrifuge. Assuming that the molecular weight of this protein is approximately 70,000 (4.4 S), a turnover rate of 910 moles of acetyl-CoA per mole of enzyme per minute can be calculated. If one makes the further assumption that the specific activities of the crude mitochondrial supernatant are correct, it can be estimated that acetyl-CoA synthetase represents about 1% of the protein released from the mitochondria by the freezing and thawing technique.

The present fractionation yielded a 7- to 8-fold purification above the best enzyme previously prepared from beef heart mitochondria (10). Furthermore, it resulted in the removal of both butyryl-CoA synthetase and acetyl adenylate hydrolase, both of which contaminate cruder preparations (4). The marginal activities which Hele described for glycolate, glyoxy-late, β-hydroxypyruvate, D(-)-lactate, and formate were eliminated by further purification and the apparent Michaelis constants for several substrates were found to differ also. In this regard, it was observed that, as compared to acetate, both propionate and acetate were quite poor substrates.

The previously reported stimulatory effect of potassium and ammonium ions was confirmed as well as the inhibitory properties of sodium and lithium (21). It appears likely that there is an absolute requirement for specific monovalent cation, but this cannot be stated with certainty because a small quantity of cation was used to titrate the various acid substrates to the pH required for the reaction (Fig. 10). Although a slight (20%) increase in the apparent Michaelis constant for acetate was observed when potassium chloride was omitted from the reaction mixture, this does not yet prove that monovalent cation is required for the binding of acetate to the enzyme.

In studying the mechanism of the over-all reaction, it was found that acetyl adenylate hydrolase did not decrease the rate at which acetyl-CoA was formed from acetate, ATP, and coenzyme A. Since acetyl adenylate is suspected of being an intermediate in this reaction, the above observation is consistent with the proposed formation of the adenylate on the enzyme surface (2, 4). An alternative explanation is that the hydrolase could not destroy the acetyl adenylate if the latter were present in catalytic quantities.

The purified acetyl-CoA synthetase has just been used to isolate enzyme-bound acetyl adenylate directly. ¹ Preliminary experiments indicate that the molecular weight of this enzyme as

¹ L. T. Webster, Jr., unpublished observations (1963).
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F. Campagnari and L. T. Webster, Jr.

1633

determined by its maximal binding of acetyl adenylate is in the same range as that expected from the sedimentation constant (4.4 S) herein reported. Further experiments concerning the role of acetyl adenylate in this reaction are now in progress.

SUMMARY

Acetyl coenzyme A synthetase has been purified over 100-fold from a crude mitochondrial preparation of bovine heart. At 37°C, the best preparation catalyzed the formation of 13.7 μmoles of acetyl coenzyme A per minute per mg of protein. It migrated as a single protein peak (4.4 S) in the analytical ultracentrifuge and also appeared quite homogeneous as judged by column chromatography. The enzyme appears suitable for quantitative studies concerning the mechanism of acetyl coenzyme A formation from acetate, adenosine triphosphate, and free coenzyme A. Several of its properties are described.

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