Studies of the Acetyl Coenzyme A Synthetase Reaction

II. CRYSTALLINE ACETYL COENZYME A SYNTHETASE*

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Acetyl coenzyme A synthetase, which catalyzes the biosynthesis of acetyl coenzyme A from acetate, adenosine triphosphate, and coenzyme A, has previously been obtained in highly purified form from bovine heart mitochondria (1). The enzyme appeared to be fairly homogeneous because only a single protein peak was detected in two chromatographic systems and the pattern observed in the analytical ultracentrifuge showed only minor contamination. However, the preparative procedure was lengthy and difficult to reproduce.

Now, crystalline acetyl-CoA synthetase has been obtained which shows a specific activity over twice that found previously. This report describes the improved fractionation procedure and certain properties of the purified enzyme including its molecular weight.

EXPERIMENTAL PROCEDURE

Methods—Activity of acetyl-CoA synthetase was measured in an acetate-dependent reaction by the disappearance of free coenzyme A as determined by the nitroprusside reaction of Grunert and Phillips (2). The assay procedure is described in detail by Campagnari and Webster (1), but concentrations of reaction components found optimal for enzymatic activity were revised. In a total volume of 0.25 ml, complete incubation mixtures contained 25 μmoles of Tris-HCl buffer, pH 8.0, 0.75 μmole of MgCl₂, 37.5 μmoles of KCl, 0.6 μmole of dipotassium ATP, 0.35 μmole of coenzyme A, 0.75 μmole of potassium acetate, and as little as 0.001 mg of enzyme. Acetyl-CoA disappeared linearly with increasing time and enzyme concentrations. Protein concentrations were determined by the biuret method of Gornall, Bardawill, and David (3); crystalline bovine plasma albumin was used as the standard. One unit of enzyme activity catalyzes the disappearance of 1 μmole of CoA—SH per min at 37° under the assay conditions employed, and specific activities are expressed as units of activity per mg of protein.

The second Sephadex fraction of acetyl-CoA synthetase (Table I) was assayed for three potential enzymatic contaminants. Butyryl-CoA synthetase activity was measured as described by Webster, Gerowin, and Rakita (4). For ATPase and inorganic pyrophosphatase, complete reaction mixtures contained 50 μmoles of Tris-HCl buffer, pH 8.0, 1.5 μmoles of MgCl₂, 2.4 μmoles of substrate (potassium ATP or pyrophosphate), and 1.11 mg of enzyme in a total volume of 0.5 ml. After incubation (5 min, 37°) complete and control (-Mg²⁺) reactions were terminated with 0.12 ml of 25% trichloroacetic acid. Inorganic phosphate in an aliquot of the deproteinized supernatant solution was determined by the method of Fiske and SubbaRow (5). Activities are expressed in terms of inorganic phosphate liberated (ATPase) or inorganic pyrophosphate hydrolyzed (pyrophosphatase).

For experiments in which acetyl-¹⁴C-adenylate was formed as the product of a partial reaction, the preparation of carrier acetyl adenylate and the isolation of isotopic product by chromatography on Dowex 1 (Cl⁻) have been described (6). Specific experimental conditions are given under Fig. 4.

Certain procedures were employed throughout the fractionation procedure. The dissolved protein was usually kept in an environment of 0.5 mM EDTA-3 mM mercaptoethanol at 0-4°. When solid ammonium sulfate was added to an enzymatic solution, the resulting mixture was titrated to pH 8.0 (paper), with m ammonium hydroxide. Each time the enzyme was precipitated by ammonium sulfate, the recovered precipitate was re-centrifuged in order to remove excess liquid ammonium sulfate (high concentrations of this salt inhibit the activity of acetyl-CoA synthetase).

Triethylaminoethyl cellulose and Sephadex G-100 used for column chromatography were washed three times in 4 volumes of 0.1 M KOH-m KCl-0.18 M KHCO₃ at a protein concentration of approximately 12 mg per ml. The enzyme was rapidly dialyzed against a continuous wash of the above solvent for 3

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hours at 4° and studies were done immediately afterward. Molecular weights were obtained from Raleigh interference patterns photographed in a Spinco model F analytical ultracentrifuge during short column sedimentation equilibrium experiments performed according to methods reviewed by Schachman (8). Initial protein concentrations were determined by counting the number of half-fringes across a boundary formed in a synthetic boundary cell at low speed. Molecular weight experiments were done in an epoxy double sector cell equipped with sapphire windows and column heights varied from 3.07 to 3.35 mm. The position of the zero order fringe at equilibrium was identified by the white light fringe; it was also located by periodic slit photography of the sample during its approach to equilibrium. Protein concentrations at the meniscus, a detailed tabular representation of the natural logarithm of the protein concentration versus the square of the distance from the axis of rotation, calculations of molecular weights at the meniscus and base, and computations of weight average and Z-average molecular weights were obtained from the observed data by a computer program modified from that of Schachman (8). Manual calculations were also made in some cases.

Materials—Adenosine triphosphate and coenzyme A were products of P-L Biochemicals. Sephadex G-100 was obtained from Pharmacia, and triethylaminoethyl cellulose was purchased from the Brown Company. Crystallized bovine plasma albumin was obtained from Armour Pharmaceutical Company. FC 43, (C4HJ3N, heptacosfluorotributylamine, was supplied by Minnesota Mining and Manufacturing Company. Other chemicals were the commercially available reagent grade.

Results

Purification of Acetyl-CoA Synthetase—The mitochondrial protein obtained from processing 10 Kg of ground bovine myocardium (4.0 kg) was processed as described in the text. For enzymatic assay, a complete reaction mixture contained 25 μmoles of tris(hydroxymethyl)aminomethane-hydrochloride buffer, pH 8.0, 0.75 μmole of MgCl2, 0.6 μmole of dipotassium ATP, 37.5 μmoles of KCl, 0.75 μmole of acetate, 0.35 μmole of CoA—SH, and enzyme in a total volume of 0.25 ml. The control (without acetate) and complete mixtures were incubated at 37° for 3 min, and the reaction was terminated with 0.06 ml of 30% metaphosphoric acid. After centrifugation, 0.1-ml aliquots of the reaction mixtures were analyzed by the nitroprusside reaction of Grunert and Phillips (2). The amount of enzyme was adjusted to give a difference of 0.1 to 0.2 optical density unit between the control and experimental samples.

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Specific activity</th>
<th>Protein</th>
<th>Activity</th>
<th>Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mitochondrial supernatant</td>
<td>0.55</td>
<td>14,500</td>
<td>7980</td>
<td>100</td>
</tr>
<tr>
<td>First (NH4)2SO4 precipitate</td>
<td>0.99</td>
<td>7.700</td>
<td>6690</td>
<td>87</td>
</tr>
<tr>
<td>Gel (NH4)2SO4 precipitate</td>
<td>1.6</td>
<td>4.100</td>
<td>6140</td>
<td>81</td>
</tr>
<tr>
<td>Second (NH4)2SO4 precipitate</td>
<td>2.8</td>
<td>1.700</td>
<td>4760</td>
<td>60</td>
</tr>
<tr>
<td>First Sephadex fraction</td>
<td>3.1</td>
<td>0.810</td>
<td>4280</td>
<td>54</td>
</tr>
<tr>
<td>Triethylaminoethyl cellulose</td>
<td>24.0</td>
<td>0.125</td>
<td>3000</td>
<td>38</td>
</tr>
<tr>
<td>Second Sephadex fraction</td>
<td>35.4</td>
<td>0.020</td>
<td>710</td>
<td>9</td>
</tr>
</tbody>
</table>

The colorless supernatant solution was stored at O-4° and after dialysis against 100 volumes of 0.02 M KCl-0.5 mM EDTA-3 mM mercaptoethanol buffer to give a protein concentration of 50 mg per ml. The solution was dialyzed against 100 volumes of the same solvent for 1 hour at 4° after which any insoluble material was removed by centrifugation. This preparation was pipetted onto a column (35 × 2.1 cm) of Sephadex G-100 which had been equilibrated overnight with the bicarbonate-KCl solution. The protein was eluted with the same solution at a flow rate of not less than 0.5 ml per min and fractions of 6 to 8 ml were collected. Most of the enzymatic activity appeared slightly after the protein peak both before and overlapping a reddish pigment.

A total recovery of approximately 800 to 900 mg of protein (specific activity, 4.8 to 5.6) was obtained by combining the most active fractions (“First Sephadex fraction,” Table I). The solution was diluted with 0.02 M KHCO3-0.5 mM EDTA-3 mM mercaptoethanol to a concentration of 6 mg of protein per ml, and was allowed to drip on a column (37 × 2.2 cm) of triethylaminoethyl cellulose at a flow rate of 15 to 20 ml per hour; the column was equilibrated with 0.02 M KHCO3-0.5 mM EDTA prior to use. Gradient elution was started with 220 ml of 0.02 M KHCO3-0.05 M KCl-0.5 mM EDTA-3 mM mercaptoethanol in the mixing bottle and 220 ml of 0.02 M KHCO3-1.4 M KCl-0.5 mM EDTA-3 mM mercaptoethanol in the reservoir; the flow rate was 15 to 20 ml per hour. The enzyme was eluted in fractions of 5 to 7 ml shortly after and overlapping a greenish pigment which was readily apparent during the chromatographic procedure. Fractions having a specific activity of greater than 19.5 units per mg of protein were pooled (Table I, triethylaminoethyl cellulose), and the protein was precipitated by addition of 47 g of ammonium sulfate per 100 ml of solution. After collection by centrifugation (15 min at 27,000 × g) the precipitate was reconstituted to remove excess ammonium sulfate. The protein was dissolved in a minimal volume of 0.02 M KHCO3-0.05 M KCl-0.5 mM EDTA-3 mM mercaptoethanol and dialyzed against the same solution for 1 hour.

About 100 to 130 mg of the dialyzed protein were placed on a column (40 × 1.2 cm) of Sephadex G-100 which had been equilibrated with 0.02 M KHCO3-0.18 M KCl-0.5 mM EDTA; elution was carried out with the same solution at a flow rate of 0.5 ml per min. Either a broad peak or two peaks of protein were eluted. Enzyme of essentially constant specific activity was located in the descending limb either of the broad peak or of the second protein peak when two peaks were present. Ammonium sulfate was added, 47 g per 100 ml of the pooled second Sephadex fractions (Table I, second Sephadex fraction), and the precipitate was collected by centrifugation at 27,000 × g for 15 min. The supernatant solution was decanted, and the precipitate was packed by another centrifugation before it was stored at −30°.

For crystallization, the precipitate was suspended at a concentration of 10 to 20 mg of protein per ml in 0.55 saturated (0°) ammonium sulfate solution adjusted to pH 8.0 with NH4OH. The mixture was stirred for 10 min after which the undissolved protein was removed by centrifugation at 27,000 × g for 15 min. The colorless supernatant solution was stored at 0-4° and after...
a period of up to 4 days a chalky white material settled out either in crystalline or amorphous form. The enzyme was crystallized with greater consistency if seed crystals were added to the supernatant solution before storage. Occasionally, crystalline material was obtained by a second method. An ammonium sulfate precipitate of material from the second Sephadex step was dissolved in 0.02 M KHCO₃-0.5 mM EDTA at a protein concentration of 30 to 50 mg per ml, and solid ammonium sulfate was slowly added until a faint cloudiness appeared; seed crystals were added, and the preparation was stored at 0° and checked daily for the appearance of crystals.

Crystals were easily detected by the presence of a schlieren pattern when the contents of the tube were swirled. Under the light microscope the crystals had the appearance of hexagonal plates which easily became fractured or dissolved (Fig. 1). Both the crystalline and amorphous material reacted with biuret

**Fig. 1.** Crystalline acetyl-CoA synthetase suspended in 2.8 M ammonium sulfate. The magnification factor is 180.

**Fig. 2.** The effect of increasing concentrations of acetate, ATP, and CoA on the rate of acetyl-CoA formation. The assay procedure is described in the text. Concentrations of enzyme (specific activity ~24 units per mg) were adjusted so that no more than 66% of the rate-limiting substrate disappeared during the 3-min incubation period.
reagent and had specific activities essentially unchanged from that of the second Sephadex preparation (approximately 35.4 units per mg of protein). No further increase in specific activity was found when the protein was recrystallized.

**Stability and Characteristics of Enzyme**—The second ammonium sulfate precipitate (specific activity, 2.4 to 3.3) lost less than 20% of its activity after storage for 6 weeks at -30°C. Highly purified enzyme (second Sephadex or crystalline material) lost appreciable activity within a few days when stored in the same manner or in solution at 4°C. The enzyme was stabilized in solution by addition of equal quantities of crystalline bovine plasma albumin. Purified acetyl-CoA synthetase (second Sephadex fraction) had a specific activity for butyryl-CoA synthetase of about 0.1 unit per mg; specific activities for ATPase and inorganic pyrophosphatase were less than 0.01 unit per mg.

When the highly purified enzyme was rechromatographed on short columns of Sephadex G-100 or triethylaminoethyl cellulose immediately following its isolation, a single sharp peak of protein was observed in which the ratio of enzymatic activity to protein concentration was essentially constant (1). However, if there was a delay of several days before rechromatography, the single protein peak eluted from Sephadex showed some spreading toward the solvent front. Enzymatic activity appeared predominantly in the descending limb of the protein elution curve and total units recovered were substantially decreased as compared to the starting material. Changes in solubility characteristics also were correlated with the age of the purified preparation and in increased specific activity of the soluble protein remaining. At times, the specific activity of older preparations could be improved by preincubation with or dialysis against 3 mM mercaptoethanol. In the analytical ultracentrifuge, older or extensively dialyzed second Sephadex fractions appeared to contain a heavier material as shown by skewing of the main peak toward the base of the cell.

Certain kinetic parameters of acetyl-CoA synthetase prepared by the new method were found to differ from those described previously (1). The new assay conditions, described under "Experimental Procedures," resulted in specific activity of the soluble protein remaining approximately 1.5-fold those obtained by the old procedure. Apparent $K_m$ values were 8 X 10^-4 M for acetate, 9 X 10^-3 M for ATP, and 4 X 10^-4 M for coenzyme A (Fig. 2). The constants for ATP and coenzyme A were somewhat lower than those found before.

**Determinations of Molecular Weight**—The freshly purified protein migrated as a single peak and had an $s_{20, 	ext{w}}$ of 3.5 when

![Graph of sedimentation equilibrium data obtained from an experiment with acetyl-CoA synthetase.](image)
studied at a protein concentration of approximately 4 mg per ml in the analytical ultracentrifuge. A plot of the results of one of the sedimentation equilibrium studies is shown in Fig. 3. The molecular weight at the meniscus of the cell was calculated from the observed data to be 30,570 whereas that at the bottom was 55,740. The calculated weight average molecular weight for this experiment was 35,790 and the z-average molecular weight was 71,000. More than one molecular species was present as shown by the z-average molecular weight and the deviation from linearity of the graph plotted in Fig. 3 (9). The molecular weight data are consistent with the s20,w of 3.5 described above. A second experiment done 5 days later on the same preparation, at a concentration of 6.12 mg of protein per ml, gave a calculated weight average molecular weight of 38,790, but there was some protein precipitated at the bottom of the cell. In this experiment, calculated molecular weights ranged from 26,230 at the meniscus to 50,060 at the bottom of the cell. Other sedimentation equilibrium studies of protein solutions maintained at a lower salt concentration (0.02 M KHCO3-0.08 M KCl instead of 0.02 M KHCO3-0.15 M KCl) or of aged preparations of enzyme revealed appreciably higher weight average molecular weights and more heterogeneity throughout the cell.

The molecular weight of acetyl-CoA synthetase was estimated also from experiments in which the enzyme was saturated with acetyl adenylate (6). The millimicromoles of adenylate saturating a given number of milligrams of homogeneous enzyme was calculated from a reciprocal plot of acetyl adenylate yields at equilibrium as a function of increasing concentrations of acetate (Fig. 4). Molecular weights obtained by this method in four experiments ranged from 31,000 to 34,000 if one assumes a 1:1 stoichiometry between enzyme and maximal yields of acetyl adenylate.

**DISCUSSION**

Acetyl-CoA synthetase from bovine heart mitochondria is one of the best characterized enzymes catalyzing a biosynthetic reaction in which adenosine triphosphate is cleaved to adenosine monophosphate and pyrophosphate with the intermediate formation of an enzyme-bound acyladenylate (6). It is the second such enzyme that has been crystallized, the first having been the firefly luciferase of Green and McElroy (10).

The present acetyl-CoA synthetase preparation is quite homogeneous by several criteria. Although crystallization per se is not a good measure of enzyme purity, it was noted that no increase in specific activity occurred when acetyl-CoA synthetase was recrystallized. The purified enzyme contained only traces of butyryl-CoA synthetase, ATPase, and pyrophosphatase activities which were appreciable in cruder preparations. Acetyl-CoA synthetase has previously been shown to chromatograph as a single peak in two systems used for its purification (1).

The freshly purified enzyme probably has a molecular weight near 32,000. This estimate is based upon both sedimentation equilibrium studies (Fig. 3) and the maximum yield of acetyl adenylate obtained at equilibrium for a given quantity of enzyme (Fig. 4). When plotted as in Fig. 3 the sedimentation equilibrium data theoretically should give a straight line relationship if just one molecular species is present (9). Actually a curved plot was obtained; whether this finding was caused by a contaminant or contaminants or by aggregation of acetyl-CoA synthetase cannot be stated with certainty although other data strongly indicate that the latter mechanism is operative.

When the maximum yield of enzyme-bound acetyl adenylate at equilibrium was obtained as a function of the quantity of fresh enzyme in the reaction mixture, molecular weights of 31,000 to 34,000 were calculated which support the molecular weights determined by physical methods. This finding also provides more evidence that the adenylate is indeed enzyme-bound (6) and that there is one active binding site per enzyme molecule. If one assumes a molecular weight of 32,000, the turnover number of acetyl-CoA synthetase (specific activity, 35.4 units per mg) is 1.13 X 10^6 moles of acetyl-CoA formed per mole of enzyme at 37°.

A molecular weight of 32,000 for acetyl-CoA synthetase does not agree with the rough estimates made previously (1). Nearly homogeneous enzyme was reported to have a specific activity of 13.3 units per mg and an s20, w of 4.4; the latter finding was thought to be consistent with a molecular weight in the range of 60,000 to 80,000 as were later results calculated from maximal acetyl adenylate binding to the enzyme (6). The most obvious explanation for this discrepancy is that an aggregated form (dimer?) of acetyl-CoA synthetase was studied previously in which only one enzymatic site was active. Several other observations support such a hypothesis. The use of high concentrations of salt in the latter stages of the current fractionation procedure was the principal modification from our previous purification technique. This was associated with a change in optimal assay conditions (lower apparent Km values for ATP and CoA) and a maximal specific activity of 35.4 for the present preparation instead of the 13.3 units per mg of protein previously reported (1). Higher molecular weights (ranging from 40,000 at the meniscus to 73,000 at the base of the cell) were found in two sedimentation equilibrium experiments in which freshly purified enzyme was studied at lower concentrations of salt (0.02 M KII CO3-0.08 M KCl instead of 0.02 M KII CO3-0.18 M KCl).

Other experiments suggest that acetyl-CoA synthetase tends to aggregate and that the lower molecular weight species has the higher specific activity. Multiple short column sedimentation equilibrium studies of purified aged enzyme, having specific activities amounting to 50 to 70% that of freshly purified enzyme, gave results quite different from those cited above. Calculated molecular weights at the meniscus varied widely for the aged preparations (58,000 to 104,000) but were invariably higher than the weight average molecular weight of 35,200 recorded for fresh enzyme; material in aged preparations usually sedimented during an equilibrium experiment, making it impossible to determine the molecular weight at the bottom of the cell. The presence of more than one molecular species in theaged preparations was also apparent from the non-Gaussian shape of the sedimenting peak which was skewed toward the base of the analytical cell. The diminished solubility and increased specific activity of soluble protein remaining after removal of insoluble material indicate that the aggregated forms of the protein were not as active as the lower molecular weight species. This is substantiated by the rechromatography of purified aged enzyme on Sephadex G-100; fractions with the greatest specific activities always were eluted after that containing the highest concentration of protein.

**SUMMARY**

Crystalline acetyl coenzyme A synthetase has been obtained from a preparation of bovine heart mitochondria. Only traces of adenosine triphosphatase, pyrophosphatase, and butyryl

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coenzyme A synthetase activities which were present in cruder preparations were detectable in the purified enzyme. Short column sedimentation equilibrium studies of fresh enzyme gave a weight average molecular weight of 35,200; the preparation contained more than one molecular species and several correlated observations suggest that the enzyme exists in both monomer and aggregated forms. From four experiments in which the maximal yield of enzyme-bound acetyl adenylate under equilibrium conditions was determined for a given quantity of enzyme, molecular weights ranging from 31,000 to 34,000 were calculated with the assumption of a 1:1 stoichiometry between acetyl adenylate and enzyme.

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