Purification and Characteristics of a Butyryl Coenzyme A Synthetase from Bovine Heart Mitochondria

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During the purification of acetyl coenzyme A synthetase from bovine heart mitochondria it was noted that cruder preparations catalyzed appreciable butyrate-dependent disappearance of coenzyme A; activity toward butyrate disappeared from the acetate enzyme during advanced stages of the fractionation procedure (1). The present report describes the purification of a butyryl-CoA synthetase from bovine heart mitochondria. It was found that this enzyme has a different spectrum of substrate specificity than that described either for the acetate enzyme isolated from this source or for the intermediate fatty acyl-CoA synthetases of liver and kidney mitochondria (2, 3). Other distinguishing features of the butyrate enzyme of heart muscle are described also.

EXPERIMENTAL PROCEDURE

Enzyme Assays—Butyryl-CoA synthetase was assayed by determining the butyrate-dependent disappearance of CoA according to the method of Mahler, Wakil, and Book (2). The complete reaction mixture contained 31.2 μmoles of Tris-hydrochloride buffer, pH 8.0, 3.75 μmoles of MgCl₂, 2.8 μmoles of sodium ATP, pH 8.0, 4.2 μmoles of sodium butyrate, pH 8.0, 0.6 to 0.8 μmole of CoA, and 0.001 to 1.5 mg of enzyme in a final volume of 0.25 ml. After incubation at 37° for 23 minutes, the experimental and control (minus butyrate) reactions were terminated with 0.06 ml of 30% metaphosphoric acid. Precipitated protein was removed by centrifugation. The sulfhydryl concentration of the supernatant solutions was then assayed by the nitroprusside method of Grunert and Phillips (4). Acetyl-CoA synthetase activity was determined by the acetate-dependent disappearance of CoA under conditions outlined by Campagnari and Webster (1).

Protein concentrations were measured by the biuret method of Gornall, Bardawill, and David (5) with crystalline bovine serum albumin as the standard. One unit of thiokinase is defined as the amount that catalyzes the fatty acid-dependent disappearance of 1 μmole of CoA per minute under the conditions described. Specific activities are expressed in units of enzyme activity per mg of protein.

Other Methods—Pyrophosphate was measured as orthophosphate after enzymatic hydrolysis with crystalline pyrophosphatase (6). Butyryl-CoA was isolated by chromatography on DEAE-cellulose in a manner similar to that described for acetyl-¹⁴C-CoA (7). Butyryl coenzyme A was identified further by its absorption at 232 and 260 nm and by its reaction with —SH reagents before and after hydrolysis of the thioester bond (8).

Alumina Cy was prepared according to Willstätter and Kraut (9) and was kept at 4° at a concentration of 15 to 20 mg of gel, dry weight, per ml of solution. Just prior to use it was equilibrated in 0.0005 M EDTA-mercaptoethanol (1:5000, v/v), and adjusted to pH 8.0. Dialysis tubing was boiled in three changes of 0.02 M KHCO₃-0.0005 M EDTA and dried prior to use.

Materials—The materials used in this study were obtained from the following sources: nucleotides and coenzyme A, Pabst Laboratories; glutathione and L-cysteine, Nutritional Biochemicals Corporation; butyric, valeric, caproic, and crotonic acids, Eastman Organic Chemicals; isobutyryl acid, The Matheson Company, Inc.; propionic acid and recrystallized ammonium sulfate, General Biochemicals, Inc.; sodium butyrate-¹⁴C, Isotopes Specialties Company, Inc.; Sophadex G-100, Pharmacia, Uppsala, Sweden; triethylaminomethyl cellulose, Brown Company, Berlin, New Hampshire. The last two products were prepared for use by washing three times with 0.1 M KOH and once with 0.1 M HCl, and then were brought to constant pH by repeated washing with 0.02 M KHCO₃-1 M KCl. Before use both polymers were equilibrated overnight with the buffer to be used in the fractionation procedure. DL-β-Hydroxybutyric and acetoacetic acids were gifts from Dr. J. Stern, and crystalline pyrophosphatase was a gift of Dr. M. Kunitz.

RESULTS

Purification of Butyryl Coenzyme A Synthetase—Steps of the purification of butyryl-CoA synthetase are summarized in Table I. All steps were carried out at 0–4°, and whenever solid ammonium sulfate was added, the pH was adjusted to approximately 8.0 with 1 M ammonium hydroxide. When the enzyme was precipitated with ammonium sulfate and collected by centrifugation, the supernatant was decanted and the precipitate was recentrifuged to remove excess ammonium sulfate.

Ice-chilled fresh beef hearts were defatted and freed from most of their connective tissue. The left ventricular myocardium was processed to a medium pulp in a meat grinder, and 1,000 g of this material were homogenized with 2,800 ml of 0.13 M KCl in a 1-gallon Waring Blender (15 to 20 seconds at high speed and 20 seconds at low speed). The homogenate was centrifuged at 1,200 × g for 10 minutes in an International PR-2 refrigerated centrifuge, and the crude supernatant solution was passed through several layers of cheesecloth. The supernatant was then centrifuged in a Lourdes LRA refrigerated
beef myocardium were combined, dissolved, and diluted to a
v/v.
resulting crude mitochondrial supernatant (Table I) was clear
and had a protein concentration of 2.6 to 5.4 mg per ml; mer-
sulfate was added until the solution became faintly cloudy
precipitate collected by centrifugation at 20,000 x g for 10
minutes was discarded. The supernatant solution was treated
with solid ammonium sulfate (final concentration of 45.5 g/100
ml) and suspended by brief homogenization at low speed in a
Waring Blendor. The resulting mixture was frozen at -20".

To the crude mitochondrial supernatants obtained from 10
kg of ground beef myocardium, solid recrystallized ammonium
sulfate was added until the solution became faintly cloudy
about 22 g of salt per 100 ml of enzyme solution). The small
precipitate collected by centrifugation at 20,000 x g for 10
minutes was discarded. The supernatant solution was treated
with solid ammonium sulfate (final concentration of 45.5 g/100
ml of original solution), and the precipitate, which was collected
by centrifugation (10 minutes at 20,000 x g), was stored at
-20°.

The ammonium sulfate precipitates obtained from 20 kg of
beef myocardium were combined, dissolved, and diluted to a
protein concentration of 8 mg per ml with a solution of 0.02 M
potassium bicarbonate; mercaptoethanol was added to a final
concentration of 1:5,000, v/v. In a typical preparation 5% of
the enzyme volume was then added as saturated ammonium
sulfate, pH 8.3, followed by 15% of the enzyme volume as alu-
mia Cg gel. The gel suspension was added slowly with rapid
stirring, which was continued for 5 minutes after the addition
had been completed. The gel was collected by centrifugation at
6,000 x g for 5 minutes and discarded. Recrystallized am-
onium sulfate (45.5 g) was then added to 100 ml of gel super-
natant solution, and the enzyme was sedimented at 20,000 x g
for 10 minutes (Table I, gel fraction). The concentration of gel
was varied depending upon the age of the gel so that 55 to 60% of
the protein was recovered in the final ammonium sulfate
precipitate.

The precipitate was dissolved and diluted to a concentration
of 8 mg of protein per ml with 0.02 M KHCO3-0.0005 M Versene-
mercaptoethanol (1:5,000, v/v). Saturated liquid ammonium
sulfate (67 ml, pH 8.3, 0°) was added to 100 ml of solution.
After removal of the precipitate by centrifugation, the super-
natant was brought to 0.67 saturation by addition of 18 g of
solid ammonium sulfate per 100 ml of solution. The precipi-
tate obtained by centrifugation at 20,000 x g for 10 minutes
was taken up in a minimal volume (protein concentration about
50 mg per ml) and dialyzed against 100 volumes of 0.02 M potas-
sium bicarbonate-mercaptoethanol (1:5,000, v/v) for 1 hour
(Table I, second ammonium sulfate fraction).

The dialyzed protein (1.8 to 2.5 g) was pipetted onto two
columns (70 x 2.2 cm) of Sephadex G-100 which had been washed
at 4° overnight with 0.02 M KHCO3. The columns were eluted
with the same solution at a flow rate of approximately 0.5 ml per
minute, and 6- to 8-ml fractions were collected. The protein

### Table I

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Protein</th>
<th>Specific activity</th>
<th>Units</th>
<th>Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mitochondrial supernatant</td>
<td>17.000</td>
<td>0.036</td>
<td>613</td>
<td>100</td>
</tr>
<tr>
<td>First (NH₄)SO₄ precipitate</td>
<td>7.700</td>
<td>0.070</td>
<td>539</td>
<td>88</td>
</tr>
<tr>
<td>Alumina C₇</td>
<td>4.300</td>
<td>0.125</td>
<td>538</td>
<td>88</td>
</tr>
<tr>
<td>Second (NH₄)SO₄ precipitate</td>
<td>1.570</td>
<td>0.26</td>
<td>486</td>
<td>79</td>
</tr>
<tr>
<td>First Sephadex</td>
<td>0.780</td>
<td>0.50</td>
<td>300</td>
<td>61</td>
</tr>
<tr>
<td>Second Sephadex</td>
<td>0.300</td>
<td>0.94</td>
<td>282</td>
<td>46</td>
</tr>
<tr>
<td>TEAE-cellulose</td>
<td>0.003</td>
<td>3.3</td>
<td>10</td>
<td>1.6</td>
</tr>
</tbody>
</table>

**FIG. 1.** Chromatography of butyryl- and acetyl-CoA synthetases on TEAE-cellulose. Protein (184 mg; 175 units of butyryl-CoA synthetase activity and 675 units of acetyl-CoA synthetase activity) from a second Sephadex fraction was chromatographed on TEAE-cellulose. Assay conditions are described in the text.

**FIG. 2.** The effect of pH on butyrate-dependent disappearance of coenzyme A. Assays were performed as described in the text with Tris-HCl buffers.
which appeared near the solvent front had little enzymatic activity, and fractions containing the highest enzymatic activity were eluted from the column near the peak of protein concentration and before a reddish material. Fractions with the highest specific activity were pooled, and the protein (700 to 1000 mg from both columns) was recovered by precipitation with 45.5 g of cold ammonium sulfate per 100 ml of solution followed by centrifugation at 4°C. The precipitate was taken up in a minimal volume of 0.02 M KHCO₃-0.0005 M EDTA (protein concentration, about 50 mg per ml) and dialyzed for 1 hour against 100 volumes of the same buffer at 0°C (Table I, first Sephadex). The Sephadex procedure was repeated with another column (70 × 2.2 cm) and the combined active fractions were diluted to a protein concentration of 5 mg per ml (Table I, second Sephadex).

This solution was introduced at a rate of 10 to 12 ml per hour into a column (40 × 2.1 cm) of DEAE-cellulose that had previously equilibrated with 0.02 M KHCO₃-0.0005 M EDTA. Gradient elution was employed with 200 ml of 0.02 M KHCO₃-0.0005 M EDTA in the mixing chamber and an equal volume of 1.4 M KHCO₃-0.0005 M EDTA in the reservoir. The flow rate was about 15 ml per hour, and fraction volume was 3 to 4 ml.

The chromatographic separation of butyryl- and acetyl-CoA synthetase activities on DEAE-cellulose is shown in Fig. 1. Enzyme showing activity with butyrate was eluted shortly before and overlapping with both acetyl-CoA synthetase and a colored material. Certain fractions had specific activities with butyrate as high as 3.4 pmoles per minute per mg of protein (Table I) and were essentially free of activity toward acetate. These fractions lost appreciable activity within 24 hours at 4°C. Stability was substantially better if the enzyme was stored as a frozen ammonium sulfate precipitate at -20°C; i.e., over 50% of the original activity with butyrate was recovered after 1 month. The enzyme was not destroyed by dialysis for 1 to 2 hours against 0.02 M KHCO₃-0.05 M KCl-mercaptoethanol (1:5000, v/v). Some preparations which had lost considerable activity could be partially reactivated by incubation at 0°C for 2 hours with fresh mercaptoethanol (1:5000, v/v).

The enzyme isolated by chromatography on DEAE-cellulose did not appear to be homogeneous; the ratio between units and protein concentration was not constant even in those fractions...
2.5 units per mg and did not contain appreciable pyrophosphatase.

The enzyme had a specific activity of 3.0 pmoles of CoA with or without (control) 16.8 pmoles of pH 8.0, 11.2 pmoles of sodium ATP, pH 8.0, 15 pmoles of MgCl₂, and 3.0 pmoles of CoA with or without (control) 18.8 pmoles of sodium butyrate, pH 8.0. The enzyme was characterized in the text. Incubations and determinations of CoA disappearance of butyryl-CoA formed in the 2-minute assay period (Table II).

<table>
<thead>
<tr>
<th>Substrate preincubated with enzyme</th>
<th>CMB concentration</th>
<th>Butyryl-CoA formed</th>
<th>Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>0</td>
<td>1.06</td>
<td>0</td>
</tr>
<tr>
<td>None</td>
<td>1 x 10⁻⁶</td>
<td>0.91</td>
<td>14</td>
</tr>
<tr>
<td>None</td>
<td>1 x 10⁻⁵</td>
<td>0.68</td>
<td>36</td>
</tr>
<tr>
<td>None</td>
<td>1 x 10⁻⁴</td>
<td>0.21</td>
<td>80</td>
</tr>
<tr>
<td>ATP</td>
<td>1 x 10⁻⁴</td>
<td>0.87</td>
<td>45</td>
</tr>
<tr>
<td>Butyrate</td>
<td>1 x 10⁻⁴</td>
<td>0.55</td>
<td>48</td>
</tr>
</tbody>
</table>

Activity was decreased over 50% by preincubation with 10⁻⁴ M p-chloromercuribenzoate (Table III). The enzyme was partially protected from this reagent by either butyrate or ATP; butyrate was the more effective.

**Product Identification and Substrate Specificity**—The identification of butyryl-CoA as a product of the enzymatic reaction was established by the following studies. When butyrate-¹⁴C was incubated with ATP, MgCl₂, free CoA, and enzyme under standard assay conditions, a radioactive product formed which was not extracted by ether under mildly acidic conditions (pH 2.0). This product was brought to pH 6.0, mixed with 2 amoles of authentic butyryl-CoA, and chromatographed on a column (11 x 1.2 cm) of DEAE-cellulose at room temperature. After approximately 85 ml of 0.05 M KCl-0.005 M HCl had passed through the column, the bulk of radioactivity was eluted in fractions which absorbed strongly at 260 nm. These fractions, containing both radioactive material and authentic butyryl-CoA, were combined, desalted, concentrated, and spotted on Whatman No. 1 paper. After chromatography in a descending solvent system (ethyl alcohol-0.5 M ammonium acetate buffer, pH 4.0, 5:2), radioautography showed coincidence between most of the radioactivity and the ultraviolet absorption of the nonradioactive butyryl-CoA. Butyryl coenzyme A was identified on the paper by its reaction with —SH reagents before and after hydrolysis of the thioester bond by alkali and neutral hydroxylamine (8). When the material was eluted from the

**FIG. 7. Substrate specificity of acetyl (Ac. CoA S.) and butyryl (Bu. CoA S.) coenzyme A synthetases from bovine heart mitochondria.** Assay conditions for the butyrate enzyme are described in the text. Fatty acid concentrations were 0.0016 M. The assay procedure for acetyl CoA synthetase was that described by Cagnagnari and Webster (1). Fatty acid concentrations were 0.01 M. Activities are expressed as percentage of that obtained with acetyl coenzyme A synthetase and with butyrate for butyryl coenzyme A synthetase.
paper in H₂O₂, its absorption at 232 με decreased after alkalimina-
tion as would be expected.

A balance study of an incubation mixture containing added
pyrophosphatase showed that 2 μmoles of phosphate formed
per μmole of CoA disappearance, which suggests pyrophosphate
as a product. Phosphate production in the absence of added
pyrophosphatase was negligible.

Straight chain acids having 3, 5, or 6 carboxyl were less active
as substrates than butyrate at a concentration of 0.0168 M
(Fig. 7). Formate, acetate, heptanoate, and octanoate gave
negative results. Of other acid analogues tested, isobutyrate
and crotonate had about 20% of the activity found with butyrate.
Insignificant activity was found with 0.0168 M pyruvate,
malate, acrylate, fumarate, succinate, α-hydroxybutyrate,
α-ketobutyrate, α-aminobutyrate, γ-hydroxybutyrate, aceto-
acetate, dl-β-methylbutyrate, γ-aminobutyrate, benzoate, phenyl
acetate, L-phenylalanine, L-tyrosine, and L-tryptophan.

Guanosine triphosphate did not replace ATP, and both
inosine and cytidine triphosphates possessed slight activities.
Neither cysteine nor glutathione substituted for reduced CoA
as the acceptor.

**DISCUSSION**

Studies presented in this communication indicate that heart
mitochondria contain a butyryl-CoA synthetase which is not
identical with acetyl-CoA synthetase. The acetyl-CoA syn-
hetase of bovine heart mitochondria, which recently has been
obtained in crystalline form, displays less than 2% of its acetate
activity toward butyrate.¹ A clear separation of the two ac-
tivities was achieved only by chromatography on TEAE-cellu-
lose (Fig. 1).

The butyryl-CoA synthetase appeared to differ from acetyl-
CoA synthetase of heart in parameters other than substrate
specificity. For example, the former is not differentially affected
by monovalent cations, whereas the acetate enzyme is stimulated
by ammonium and potassium and inhibited by sodium and
lithium salts (11). Also, as will be shown in a subsequent re-
port, the subcutaneous administration of acetate is accompanied
by an increased level of mitochondrial acetyl-CoA synthetase
activity, whereas butyryl-CoA synthetase activity remains
unchanged. The converse is observed when butyrate is in-
jected. Such physiological experiments lend further support
to the concept of separate acetyl- and butyryl-CoA synthetases.

Although the acetyl- and butyryl-CoA synthetases of heart
both show activity with high concentrations of propionate, the
question remains open as to whether there is a sepa.
rate propionate enzyme as well.

Differences were found between the beef heart butyryl-CoA
synthetase and the intermediate chain length fatty acid-activat-
ing enzyme purified from liver mitochondria of the same species
by Mahler, Wakil, and Bock (2). In contrast to the heart
enzyme (Fig. 7), the liver preparation showed peak activity
with heptanoate and significant activity with straight chain
acids of up to 12 carbon atoms. Certain aromatic acids and
amino acids also served as substrates for the hepatic enzyme,
whereas these compounds were inactive with the enzyme from
heart. Heart acetyl and butyryl-CoA synthetases both differ
from the intermediate fatty acid enzyme of liver in that the
latter was quite sensitive to product inhibition with pyrophos-
phate and was appreciably stimulated by added pyrophosphatase
(12).

The two cardiac enzymes were not readily separated by the
salt and gel procedures and were closely related in chromato-
graphic behavior. This casts some doubt on the occurrence of
a single hepatic intermediate fatty acyl-CoA synthetase with
broad substrate specificity. This has been proposed on the
basis of the constancy of the ratio of substrate activities through-
out a relatively minor degree of enzyme purification (2). It is
doubtful that the butyrate enzyme obtained here after 10-fold
purification was homogeneous because a constant ratio did not
exist between the units of activity and the protein concentra-
tions (Fig. 1). In view of these findings it seems reasonable to
speculate that there are a variety of mitochondrial short and
intermediate chain fatty acyl-CoA synthetases within a single
tissue and that there are certain genetic differences in these
enzymes from tissue to tissue. Studies concerning some of the
possibilities will be presented subsequently.

**SUMMARY**

A butyryl coenzyme A synthetase has been purified approxi-
imately 10-fold from a crude extract of bovine heart mitochon-
dria. The enzyme displays peak activity toward butyrate and
acceptable activity with propionate, valerate, and caproate.
The butyrate enzyme is distinguished from the acetyl coenzyme
A synthetase of beef heart by its substrate specificity and lack of
differential response to monovalent cations. Its substrate
specificity is for fatty acids of shorter chain length and is less
broad than that of the intermediate fatty acid-activating en-
zyme of beef liver; butyryl coenzyme A synthetase from heart
is also less sensitive to inhibition by pyrophosphate than the
hepatic enzyme.

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Carter and Mr. Robert Ray for their technical assistance.

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