Enzymic Synthesis and Metabolism of Malonyl Coenzyme A and Glutaryl Coenzyme A*

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During the course of studies on the prevalence of enzymic Coenzyme A (CoA) transfer reactions (1--4) in animal tissues and yeast, evidence was obtained for the occurrence of the following new reactions of this type:

Acetoacetyl-CoA + malonate $\rightleftharpoons$ malonyl-CoA + acetoacetate (1)

Acetoacetyl-CoA + glutarate $\rightleftharpoons$ glutaryl-CoA + acetoacetate (2)

Acetoacetyl-CoA + R(CH2)COOH = R(CH2)CO-S-CoA + acetate (3)

This paper describes the experiments which led to the identification of these reactions and which demonstrated that they are catalyzed by the previously described enzyme, succinyl-$\beta$-ketoacetyl-CoA transferase (4). The conversion of both synthetic and enzymically prepared malonyl-CoA to acetyl-CoA in pigeon liver is also demonstrated.

EXPERIMENTAL PROCEDURE

Methods and Preparations

Test System for Enzymic CoA Transfer—The principle used in testing for enzymic CoA transfer was to determine whether the addition of carboxylic acids accelerated the disappearance of acetyl-CoA in the presence of various enzyme preparations as would be predicted from the general reaction (Reaction 3), in which R = H or COOH. Stimulation of acetyl-CoA disappearance by carboxylic acid would result if the equilibrium of Reaction 3 were significantly toward R(CH2)CO-S-CoA or if the latter disappeared in secondary reactions. In practice, acetyl-CoA was incubated with enzyme fractions with and without added carboxylic acids under the conditions described in Table I. At the end of the incubation period, 0.1 volume of 30% metaphosphoric acid was added, the suspension neutralized carefully with 2 N KOH, and centrifuged. A sample of the clear supernatant solution was used for enzymic determination of acetyl-CoA (5).

Substrates — Acetyl - CoA, succinyl - CoA, and glutaryl - CoA were prepared from the corresponding anhydrides (6), and acetoacetyl-CoA from diketene (7). $\beta$, $\beta$-Dioxoacylglutaric, $\alpha$-oxoacylglutaric, and $\beta$-methylglutaric acids were purchased from Sapon Laboratories, New York; methylmalonic and dimethylmalonic acids from K and K Laboratories, New York; methylsuccinic acid. Reaction of malonyl dichloride with hydroxylamine, and

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Effect of mono- and dicarboxylic acids on acetyl-CoA metabolism

The test system contained (in μmoles): Tris buffer pH 7.6 (100), MgCl₂ (8), mono- or dicarboxylic acid, K salt (100), acetyl-CoA (0.7) and enzyme. Volume 1.0 ml. Incubation, 20 minutes at 37°C. The enzymes used were dialyzed yeast extract (4.0 mg of protein), dog heart 0.35 to 0.65 saturated (NH₄)₂SO₄ fraction (10.8 mg of protein), dog skeletal muscle 0.35 to 0.65 saturated (NH₄)₂SO₄ fraction (10.8 mg of protein), and a pig heart fraction (3.7 mg of protein) prepared by salt fractionation, gel absorption, and elution.

Results

Stimulation of Acetyl-CoA Disappearance—As shown in Table 1, acetyl-CoA itself was metabolized by enzyme fractions from yeast, dog heart and skeletal muscle, and pig heart. This disappearance resulted almost exclusively from deacetylation (direct or indirect), as it was accompanied by roughly equivalent sulphhydril (i.e. CoA-SH) release. In yeast, acetyl-CoA disappearance was increased by succinate, glutarate, propionate, and butyrate, but not by malonate. In heart and muscle, all three dicarboxylic acids increased acetyl-CoA disappearance. In the case of dog muscle, the occurrence of the overall Reaction 3 was further confirmed by converting residual thioesters to their corresponding dicarboxylic acids (Reaction 7) in analogous manner to the succinyl-β-ketoacyl-CoA transferase of pig heart (4).

Acetyl-CoA + succinate → succinyl-CoA + acetate (4)

Acetyl-CoA + propionate → propionyl-CoA + acetate (5)

Further evidence for Reaction 4 was obtained by coupling the reverse Reaction 4 with endogenous citrate-condensing enzyme and measuring citrate synthesis from oxalacetate (90 μmoles), succinyl-CoA (1 μmole), and acetate (100 μmoles) in the presence of Mg²⁺ (8 μmoles), GSH (20 μmoles), KPO₄ buffer pH 7.5 (100 μmoles), and a yeast fraction (6.1 mg of protein) precipitated by 40% ethanol at −10°C. Oxalacetate alone gave 0.05 μmole of citrate; oxalacetate + acetate, 0.13 μmole; oxalacetate + succinyl-CoA, 0.06 μmole; and oxalacetate + succinyl-CoA + acetate, 0.50 μmole. However, propionyl-CoA did not lead to extra citrate synthesis when substituted for succinyl-CoA in the above system. Tests for CoA transfer from acetocetyl-CoA to malonate (Reaction 1) and to succinate by direct optical assay (4) were negative in various yeast fractions, indicating the absence of succinyl-β-ketoacyl-CoA transferase.

CoA Transferase in Tissue Extracts—Experiments similar to the above failed to show any activation of acetate by succinyl-CoA, i.e. the reverse Reaction 4, by heart and muscle enzyme fractions. For this reason, a second mechanism which can explain the stimulation of acetyl-CoA disappearance by dicarboxylic acids was explored. This mechanism would result in the synthesis of acetoacetyl-CoA according to Reaction 6. The acetoacetyl-CoA could then transfer its CoA moiety to the dicarboxylic acid (Reaction 7) in analogous manner to the succinyl-β-ketoacyl-CoA transferase of pig heart (4).

2 Acetyl-CoA ⇌ acetoacetyl-CoA + CoA-SH (6)

Acetocetyl-CoA + (CH₃)₂C=O + acetoacetate (7)

COOH

COOH

COOH

Sum: 2 Acetyl-CoA + (CH₃)₂C=O + acetoacetate + CoA-SH

COOH

COOH

This reaction sequence predicts that stimulation of acetyl-CoA disappearance by dicarboxylic acid should (a) result in concomitant acetocetate formation and (b) be abolished by iodoacetamide which inactivates thiolase (7, 19) but not the succinyl-β-ketoacyl-CoA transferase of pig heart (4).

Additions

Yeast Dog heart Dog muscle Pig heart

Acetyl-CoA 0.31 0.26 0.19 0.02

Acetyl-CoA + malonate 0.30 0.47 0.35 0.21

Acetyl-CoA + succinate 0.56 0.68 0.59 0.44

Acetyl-CoA + glutarate 0.49 0.33 0.23

Acetyl-CoA + propionate 0.58 0.33

Acetyl-CoA + butyrate 0.35 0.30 0.08

Table II

Effect of dicarboxylic acids on acetocetate synthesis from acetyl-CoA

Conditions as in Table I. Enzyme fractions (10 mg of protein) from dog skeletal muscle and heart as in Table I.

Additions

Dog muscle Dog heart

Acetyl-CoA 0.24 0.26

Acetyl-CoA + malonate 0.42 0.47

Acetyl-CoA + succinate 0.50 0.68

Acetyl-CoA + glutarate 0.39 0.33

* Ac-CoA = acetyl-CoA.

† AcAc = acetocetic acid.

‡ Assays as acetocetate by Walker method.
Table III

Inhibition by iodoacetamide of dicarboxylic acid stimulation of acetyl-CoA metabolism

Conditions as in Table I. The pH 7.4 gel eluate fraction of dog skeletal muscle (2.5 mg of protein) was used. The enzyme fraction was incubated with $10^{-4}$ M iodoacetamide for 30 minutes at 0°. Glutathione was then added to $10^{-2}$ M concentration and the fraction dialyzed for 4 hours against three changes of 0.02 M KPO$_4$ buffer pH 7.5. Values are μmoles of acetyl-CoA disappearing.

<table>
<thead>
<tr>
<th></th>
<th>Untreated enzyme</th>
<th>Iodoacetamide-treated enzyme</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetyl-CoA</td>
<td>0.00</td>
<td>0.07</td>
</tr>
<tr>
<td>Acetyl-CoA + malonate</td>
<td>0.17</td>
<td>0.07</td>
</tr>
<tr>
<td>Acetyl-CoA + succinate</td>
<td>0.64</td>
<td>0.05</td>
</tr>
</tbody>
</table>

Relative rates of CoA transfer from acetoacetyl-CoA to dicarboxylic acids

The reaction mixture contained (in μmoles): Tris buffer pH 8.15 (150), dipotassium malonate or succinate (60), acetoacetyl-CoA (0.3), and dog muscle enzyme fraction as indicated. Volume 1.5 ml. Direct optical assay of CoA transferase (4). Specific activity equals $-\Delta A_{340}$ per minute per mg of protein.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Malonate</th>
<th>Succinate</th>
</tr>
</thead>
<tbody>
<tr>
<td>35-65 AmSO$_4$</td>
<td>0.28</td>
<td>12.2</td>
</tr>
<tr>
<td>40-67 Acetone</td>
<td>0.44</td>
<td>18.0</td>
</tr>
<tr>
<td>7.4 Gel eluate</td>
<td>0.78</td>
<td>38.6</td>
</tr>
<tr>
<td>CoA transferase*</td>
<td>75.0</td>
<td>3750.0</td>
</tr>
</tbody>
</table>

* Practically pure enzyme from pig heart (4). See legend to Fig. 2.

Fig. 1. Optical assay of CoA transferase in muscle. The complete reaction mixture is given in Table IV. pH 7.4 eluate fraction (3.2 mg of protein) of dog muscle was used. Silica cuvettes, $d = 0.5$ cm. Volume 1.50 ml. Temp. 22°. At zero time the reaction was started by addition of enzyme and dicarboxylic acid to the other components as follows: O--O, no dicarboxylic acid added; △--△, glutarate; ⌞--⌟, malonate; ▲--▲, succinate.

Fig. 2. Specificity of pig heart CoA transferase. Conditions as in Fig. 1. Practically pure CoA transferase, original specific activity 1000 (4) was used. It had been stored at −20° for 5½ years and now had a specific activity of 200. The amounts of enzyme used in each experiment are shown on the figure. Curves: O--O, acetoacetyl-CoA without or with added glutarate; ⌞--⌟, acetoacetyl-CoA without or with added succinate; ▲--▲, succinate added.
Table V
Conversion of "enzymic" malonyl-CoA to acetyl-CoA by pigeon liver

<table>
<thead>
<tr>
<th>Additions</th>
<th>Citrate formation</th>
<th>Experiment 1</th>
<th>Experiment 2</th>
<th>Experiment 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oxaloacetate</td>
<td>0</td>
<td>0.04</td>
<td>0.03</td>
<td></td>
</tr>
<tr>
<td>Oxaloacetate + malonyl-CoA</td>
<td>0.30</td>
<td>0.27</td>
<td>0.24</td>
<td></td>
</tr>
<tr>
<td>Oxaloacetate + malonyl-CoA</td>
<td>0.07</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Oxaloacetate + succinyl-CoA</td>
<td>0.72</td>
<td>0.72</td>
<td>0.82</td>
<td></td>
</tr>
</tbody>
</table>

Conversion, %: 32 29 26

* Tested after alkaline hydrolysis.

Transfer to both malonate and succinate in pig heart and dog muscle. The dog muscle enzyme either differs from the pig heart enzyme in having significant action with glutarate or else a discrete CoA transferase specific for glutarate may occur in dog muscle and heart, and perhaps pig heart (cf. Table I and Fig. 1). The following compounds were inactive as CoA acceptors from acetooacetyl-CoA when tested with the pig heart CoA transferase (24 μg) or the dog muscle Eluate fraction (cf. Fig. 1): 2-methylmalonate, methylsuccinate, 2,2-dimethylmalonate, α- or β-methylglutarate, α-α- or β-β-dimethylglutarate.

Preparation of Malonyl-CoA from Acetooacetyl-CoA—The reaction mixture, volume 6.0 ml, contained: Tris buffer pH 7.0, 1000 μmoles; dipotassium malonate, 200 μmoles; acetoacetyl-CoA, 20.1 μmoles; and iodooacetamide-treated 40 to 57% acetone fraction of dog muscle extract, 81 mg of protein. The mixture was incubated at 37° and samples were removed at intervals for enzymic determination of residual acetooacetyl-CoA with crystalline dehydrogenase (14). After 35 minutes the mixture was heated to 100° for 1 minute, cooled rapidly on ice, and the precipitate removed by centrifugation. Analysis of the supernatant solution showed that 1.0 μmole of acetooacetyl-CoA and 14.4 μmoles of thioester (as determined with hydroxylamine) remained; also, 1.2 μmoles of acetyl-CoA had been formed. Acetooacetyl-CoA reacts with hydroxylamine, but the resulting hydroxamic acid gives a negligible color with Fe+++ The accumulated thioester on chromatography in ethanol-acetate solvent pH 4.7 (18) gave an Rf of 0.42. The Rf values for synthetic malonyl-CoA, acetyl-CoA, succinyl-CoA, glutaryl-CoA, and acetooacetyl-CoA under the same conditions were 0.46, 0.56, 0.54, 0.55, and 0.60, respectively. Thus malonyl-CoA can be separated from these thioesters. The hydroxamic acid of the accumulated thioester on chromatography in water-saturated butanol (17) had an Rf of 0.32. The Rf values of synthetic malonononohydroxamic, acetohydroxamic, and glutaromono hydroxamic acids were 0.32, 0.49, 0.38, and 0.42, respectively. In this experiment then, the yield of malonyl-CoA (13.2 × 100 + 19.1) was 69%.

Table VI
Conversion of synthetic malonyl-CoA to acetyl-CoA in pigeon liver

<table>
<thead>
<tr>
<th>Additions</th>
<th>Citrate formation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Experiment 1</td>
</tr>
<tr>
<td>Oxaloacetate</td>
<td>0.04</td>
</tr>
<tr>
<td>Oxaloacetate + malonyl-CoA</td>
<td>0.24</td>
</tr>
<tr>
<td>Oxaloacetate + malonate + CoA</td>
<td>0.07</td>
</tr>
<tr>
<td>Thioester added</td>
<td>0.76</td>
</tr>
</tbody>
</table>

Conversion, %: 22 19 23

Preparation of Malonyl-CoA from Succinyl-CoA—The fact that CoA transferase catalyzes an exchange of succinate with succinyl-CoA (20) and also of acetooacetate with acetooacetyl-CoA (1) has led to the formulation (20, 21) of a CoA-enzyme intermediate:

\[ \text{Succinyl-CoA} + \text{E} \rightleftharpoons \text{CoA-enzyme} + \text{succinate} \] (9)

These results suggest that the enzyme would also catalyze the reversible synthesis of malonyl-CoA from succinyl-CoA:

\[ \text{Succinyl-CoA} + \text{malonate} \rightleftharpoons \text{malonyl-CoA} + \text{succinate} \] (10)

To test for this reaction, Tris buffer pH 7.0, 500 μmoles; dipotassium malonate, 200 μmoles; succinyl-CoA, 6.5 μmoles (20 μmoles hydroxamic acid) and 40 to 57% acetone fraction of dog muscle extract, 40 mg of protein, in a volume of 3.0 ml were incubated for 30 minutes at 22°. The mixture was heated at 100° for 1 minute to hydrolyze any residual succinyl-CoA (malonyl-CoA is stable), although a control experiment indicated that in the absence of malonate all the succinyl-CoA disappeared under the experimental conditions. After rapid cooling, the protein was removed by centrifugation. Hydroxylamine assay of the supernatant solution showed 2.5 μmoles of thioester were present. The identity of the thioester as malonyl-CoA was indicated by its enzymic conversion to citrate (i.e. acetyl-CoA) in the presence of oxaloacetate and pigeon liver enzyme fraction (Table V, Experiment 3). Thus, 0.32 μmole of thioester gave 0.21 μmole of citrate. Substitution of 2.0 μmoles of succinyl-CoA for the thioester yielded no citrate in this system. This experiment clearly demonstrates that CoA transferase can catalyze the exchange of CoA between succinate and malonate and lends further support to the formulation of a CoA-enzyme intermediate in the reaction.

Enzymic Conversion of Malonyl-CoA to Acetyl-CoA—The possible conversion of malonyl-CoA to acetyl-CoA by pigeon liver was tested by coupling with endogenous citrate-condensing enzyme and measuring citrate synthesis from malonyl-CoA and oxaloacetate. Both enzymic and synthetic malonyl-CoA were determined to be free of acetyl-CoA by direct enzymic assay (5), and both were converted to citrate (i.e. acetyl-CoA) by a pigeon liver enzyme fraction (Tables V and VI). This conversion did not occur after alkaline hydrolysis of enzymic malonyl-CoA. Since there were variable amounts of malonate in the thioester solution, and about 50% of the thioester was decarboxylated under

1 J. R. Stern, and A. del Campillo, unpublished experiments.
the experimental conditions, it was further shown that malonate plus CoA-SH did not replace malonyl-CoA (Table VI). These experiments rule out an indirect synthesis of citrate via decarboxylation of malonate to acetate followed by possible conversion of the latter to acetyl-CoA. This decarboxylation does not involve reversal of the acetyl-CoA carboxylase reaction (22, 23) since this enzyme fraction did not fix any C4O2 in the presence of acetyl-CoA, propionyl-CoA, or butyryl-CoA (24) and hence was devoid of propionyl-CoA carboxylase (25). This enzymic conversion of malonyl-CoA to acetyl-CoA was also observed in the 0.3 to 0.6 saturated (NH4)2SO4 fraction of dog skeletal muscle in which the conversion was 15 to 18%. The decarboxylation of malonyl-CoA to acetyl-CoA has been independently noted by Wakil (26) in pigeon liver and by Lynen and Kessel (27) in yeast.

**DISCUSSION**

These experiments demonstrate the synthesis of malonyl-CoA and of glutaryl-CoA by enzymic transfer of CoA from acetoacetyl-CoA (Reactions 1 and 2) and establish that the synthesis is catalyzed by the enzyme succinyl-β-ketoacyl-CoA transferase. Thus another biosynthetic route to malonyl-CoA may be added to the following known mechanisms: (a) activation of malonate to malonyl-CoA via an ATP-, CoA-dependent reaction, first described in *Pseudomonas* (16) and later in rat kidney (28) and pig heart (29); (b) carboxylation of acetyl-CoA to malonyl-CoA by various tissues (22-24); (c) oxidation of malonyl semialdehyde-CoA to malonyl-CoA in *Clostridium kluyveri* (30). This route can operate in peripheral tissues but not in liver which is devoid of CoA transferase. Malonyl-CoA has been identified as an intermediate in fatty acid synthesis (22, 29). The CoA transferase reaction by exchanging CoA between succinyl-CoA and malonate and between acetoacetyl-CoA and malonate provides a link in peripheral tissues between the citric acid cycle and fatty acid synthetase. The fatty acid oxidation cycle and fatty acid synthesis on the other hand, and the fatty acid oxidation cycle and fatty acid synthesis on the other.

In the presence of α-Ketoglutarate + DPN+ + CoA ⇔ succinyl-CoA + DPNH + H+ + CO2 (11)

Succinyl-CoA + GDP + P_i ⇒ succinate + GTP + CoA (12)

Succinyl-CoA + malonate ⇒ malonyl-CoA + succinate (13)

Malonate or glutarate, CoA transferase could replace the requirement for the P enzyme (Reaction 12) in the coupled oxidation of α-ketoglutarate (Reaction 11) to succinate. However, it is not clear in what way malonate may arise in peripheral tissues, since no complete enzymic pathway for biosynthesis of malonate other than by carboxylation, e.g. from β-alanine or propionate (31), is known.

The decarboxylation of malonyl-CoA to acetyl-CoA by a reaction other than reversal of the carboxylation of acetyl-CoA provides a means of oxidizing malonyl-CoA via the citric acid cycle and account for the conversion of malonate carbon to CO2 in the intact mouse (32) and to acetocacetate in rat liver slices (28). The significance of this reaction is obscure, since malonyl-CoA has not been shown to be an intermediate in degradation processes. The reactivity of glutarate with CoA transferase provides a means whereby glutarate, generated in the metabolism of lysine (33) and of tryptophan (34), can be converted to glutaryl-CoA by exchange with acetoacetyl-CoA, succinyl-CoA, or malonyl-CoA. This conversion is probably the first step in the oxidation of glutarate to acetate (35, 36) by a pathway of which the enzymic steps have been partly elucidated (37). Animal tissues, including liver, also possess a second enzyme, the specific glutarate-activating enzyme (38), which catalyzes the conversion of glutarate to glutaryl-CoA in the presence of CoA and ATP.

**SUMMARY**

1. A general method for testing for enzymic transfer of coenzyme A (CoA) from acetyl-coenzyme A to a carboxylic acid is described.

2. Yeast was shown to possess the enzyme, acetyl-succinyl-CoA transferase, which catalyzes the reverse reaction, acetyl-CoA + succinate ⇒ succinyl-CoA + acetate.

3. Dog muscle and heart extracts were shown to catalyze the following CoA transfer reactions: (a) acetoacetyl-CoA + malonate ⇒ malonyl-CoA + acetocacetate, and (b) acetoacetyl-CoA + glutarate ⇒ glutaryl-CoA + acetoacetate, as well as (c) acetoacetyl-CoA + succinate ⇒ succinyl-CoA + acetoacetate. It was further demonstrated that the highly purified succinyl-β-ketoacyl-CoA transferase of pig heart which catalyzes reaction (c) also catalyzes reaction (a) but not reaction (b).

4. Malonyl-CoA has been prepared synthetically with malonyl dichloride and CoA, and also enzymically from either acetoacetyl-CoA or succinyl-CoA via CoA transfer to malonate.

5. Both the synthetic and the enzymic preparations of malonyl-CoA are converted to acetyl-CoA by extracts of pigeon liver and of dog skeletal muscle. This reaction does not involve reversal of the acetyl-CoA carboxylation reaction and is presumably a direct decarboxylation.

**REFERENCES**

Metabolism of Malonyl Coenzyme A and Glutaryl Coenzyme A

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