A ROLE OF ACETOACETYL-CoA SYNTHETASE IN ACETOACETATE UTILIZATION BY RAT LIVER CELL FRACTIONS

Joseph R. Stern

Department of Pharmacology, School of Medicine
Case Western Reserve University
Cleveland, Ohio 44106

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Summary: The enzyme acetoacetyl-CoA synthetase which catalyzes the synthesis of acetoacetyl-CoA from acetoacetate, CoA and ATP is shown to be present in mitochondrial and cytoplasmic fractions of rat liver. It was decreased in both specific activity and amount after starvation for 48 hours. It is proposed that the synthetase normally functions in the re-utilization of some of the acetoacetate produced within the mitochondrion as well as that reaching the cytoplasm, and that acetoacetate can function as an acetyl carrier between mitochondria and cytoplasm.

The capacity of liver to utilize acetoacetate by direct conversion to acetoacetyl-CoA by means of reaction 1, catalyzed by the enzyme acetoacetyl-CoA synthetase, was first demonstrated in pigeon liver by Stern and Ochoa (1).

(1) Acetoacetate + CoA + ATP $\rightleftharpoons$ acetoacetyl-CoA + (AMP + PP)

Acetoacetyl-CoA synthetase has since been shown to be present in pig kidney and yeast (2) pigeon brain (3), dog heart and skeletal muscle, rabbit and sheep liver, and Rodospirillum rubrum (4). The failure to observe acetoacetate oxidation in rat liver slices (5) and mitochondria (6) has been interpreted as evidence for the absence of acetoacetyl-CoA synthetase in rat liver (7, 8). This paper demonstrates that acetoacetyl-CoA synthetase is present in both the mitochondria and the cytoplasm of rat liver and that its activity in both fractions is significantly reduced after starvation.

METHODS

The livers of 3 male chow-fed or 48 hr. starved rats (Sprague-

Dawley strain) were diced, then suspended in 0.3 M sucrose, 5mM morpholinopropane sulfonic acid (MOPS) and 2 mM EDTA, pH 7.2, (3.2 ml. per g. wet wt.) and homogenized in a mechanically driven all-glass homogenizer at low speed. Cell debris and nuclear material were removed by centrifuging at 425 g for 15 min, and the supernatant fluid was re-centrifuged at 9,000 g for 10 min. The mitochondria were then washed twice in 0.3 M sucrose-MOPS and reisolated by centrifuging at 9,000 g for 10 min. They were finally suspended in this medium to give a protein concentration of 40-50 mg. per ml. The mitochondrial suspension was subjected to 3 cycles of rapid freeze-thawing, then rapidly frozen and stored overnight at -80° before being thawed (in a 25° water bath) for use. This procedure resulted in a 3-fold increase in the rate of acetoacetate synthesis (9) and a 2-fold increase in acetoacetyl-CoA synthetase activity.

The 9,000 g supernatant fluid was spun at 100,000 g for 1 hr. to obtain the 100,000 g supernatant fluid representing the cytoplasmic fraction.

Acetoacetate synthesis from acetyl-CoA was measured by the catalytic assay system of Stern et al (10). Acetoacetate was determined by the method of Walker (11). The amount of glutathione in the sample ($<$1mM) did not interfere with color development. Incubation was for 30 min. at 37° and the amount of CoA-SH added (0.56 μmole) gave maximum rates. As with ox liver (10) acetoacetate synthesis in rat liver was markedly decreased (by 40-50%) when either Mg^{++} or glutathione was omitted from the assay system.

Acetoacetyl-CoA synthesis from acetoacetate, ATP and CoA was assayed by coupling Reaction 1 with thiolase and citrate synthetase (1,2) and measuring citrate formation. The assay system contained: potassium phosphate buffer pH 7.5, 100 μmoles; $MgCl_2$, 8 μmoles; ATP, 10 μmoles;

CoA, 0.5 μmole; glutathione 20 μmoles; potassium acetoacetate, 50 μmoles; potassium oxalacetate, 20 μmoles, crystalline citrate synthase 5 μg, and cell fraction. Final volume was 2.0 ml. After incubation for 30 minutes at 38°, proteins were precipitated with trichloracetic acid and citrate determined on the supernatant. In each case, a control without acetoacetate was used to correct for endogenous citrate formation (presumably from endogenous acetate since acetyl-CoA synthetase was present). The latter amounted to 10-35% of the value with acetoacetate.

Specific activity is defined as nanomoles of acetoacetate (or acetoacetyl-CoA) formed per hour per mg protein.

RESULTS

Both the mitochondrial and cytoplasmic fractions of rat liver formed citrate (i.e., acetoacetyl-CoA) from acetoacetate, CoA and ATP (Table 1). Although not shown, both ATP and CoA, as well as acetoacetate, were required for maximal synthesis of acetoacetyl-CoA. The mitochondrial fraction had the highest specific activity for acetoacetyl-CoA synthetase but 70% of the total units was present in the cytoplasmic fraction.

The rate of acetoacetate synthesis from acetyl-CoA was also measured in these fractions. It is seen (Table 1) that the specific activity of the acetoacetate synthesizing system was 33-fold greater in the mitochondrial than the cytoplasmic fraction and that only 11.8% of the total units was found in the cytoplasm. This agrees well with a value of 11% previously reported (12). In a future publication it will be shown that the acetoacetate-synthesizing activity found in the cytoplasm can be accounted for completely by the contamination of the cytoplasmic fraction with mitochondrial protein, as a result of rupture of some mitochondria during the resolution procedure (13). Thus, the acetoacetate synthesizing enzyme system is exclusively

Table 1

Synthesis of Acetoacetyl-CoA and of Acetoacetate by Liver Fractions from Fed Rats

Fraction	Protein	Acetoacetyl-CoA Synthesis		Acetoacetate Synthesis	
		Sp. Activity*	Units	Sp. Activity	Units
	mg per g. wet wt.		per g. liver		per g. liver
Mitochondrial	19.4	89.3	1,733	3310	64,200
Cytoplasmic	81.0	51.1	4,140	108	8,750
Total Units			5,873		72,950
Units in cytoplasm (%)			70.4		11.8

* Nanomoles per hour per mg. protein

mitochondrial in location and can be used as a measure of contamination of the cytoplasmic fraction.

On the other hand, the acetoacetyl-CoA synthetase must be present in both mitochondria and cytoplasm, since the higher specific activity of the enzyme in the mitochondria (as well as the method of preparation) precludes any significant contamination of the mitochondrial fraction by the cytoplasmic fraction, and the proportion of total enzyme found in the cytoplasm is much too high to be accounted for by mitochondrial contamination which, as shown above, was 11.8%. After correction, there is about 1.5 times more synthetase present in the cytoplasm (59%) than in the mitochondria (41%). It should be noted that the total activity of the acetoacetyl-CoA synthetase in the mitochondrion was 8.1% of that of acetoacetate synthesizing enzyme system.

The effect of starving the rat for 48 hours on the acetoacetyl-CoA synthetase and the acetoacetate synthesizing system is shown in Table 2. The specific activity of the synthetase in mitochondrial and cytoplasmic fractions from "starved" liver fell to 23 and 25% of the values for the same fraction from fed rats. The total units of enzyme in "starved" liver

Table 2

Synthesis of Acetoacetyl-CoA and of Acetoacetate by Liver Fractions from 48 hr Starved Rats

Fraction	Protein	Acetoacetyl-CoA Synthesis		Acetoacetate Synthesis	
		Sp. Activity	Units	Sp. Activity	Units
	mg per g. wet wt.		per g. liver		per g. liver
Mitochondrial	31.6	20.3	641	2590	81,900
Cytoplasmic	80.0	12.8	1,024	104	8,320
Total Units			1,665		90,220
Units in cytoplasm (%)			61.6		9.2

was only 28% of that in the fractions from "fed" liver. In contrast, the specific activity of the acetoacetate synthesizing system in the mitochondrial fraction from starved rats was 78% of that in the same fraction from fed rats, while the total amount of enzyme units in the "starved" liver was somewhat greater than in "fed" liver. This agrees with earlier conclusions (9, 12, 14) that the activity of the liver acetoacetate synthesizing system measured *in vitro* does not increase on starvation and consequent ketonemia.

DISCUSSION

These experiments demonstrate that the enzyme acetoacetyl-CoA synthetase that catalyzes Reaction 1 is present in both the mitochondrial and cytoplasmic fractions of rat liver. Hence the mitochondrion is capable of re-utilizing some of the acetoacetate normally produced within it by conversion to acetoacetyl-CoA. Likewise the cytoplasm can re-utilize some of the acetoacetate normally entering it from the mitochondrion before the acetoacetate exits from the cell into the hepatic venous circulation. Thus acetoacetate formed in the mitochondrion could function as a carrier of acetyl units ("active" acetate) from mitochondrion to cytoplasm, in a manner analogous to citrate which is produced in the mitochondrion and enters the

cytoplasm to be cleaved by the citrate cleavage enzyme to acetyl-CoA and oxalacetate (15). Acetoacetate, coupled to cytoplasmic acetoacetyl-CoA synthetase would be a more efficient carrier of "active" acetate than citrate, since, as a result of cytoplasmic thiolase activity (12) twice as much acetyl-CoA would be formed per mole of ATP utilized than results from cleavage of citrate to acetyl-CoA.

The large decrease in the capacity of the liver of starved rats to convert acetoacetate to acetoacetyl-CoA adds a new parameter to enzymic factors influencing ketogenesis. Thus a decreased rate of re-utilization within the hepatic cell of acetoacetate normally (or abnormally) produced may contribute significantly to ketonemia. In vitro the rate of liver acetoacetate synthesis was 73 μmole/hr/g. wet wt. at 38°, while that of acetoacetate activation to acetoacetyl-CoA was 5.9 μmole/hr/g. wet wt (Table 1). The data of Krebs et al (16) showed that in the perfused liver of fed rats total ketone body formation proceeded at a rate of 4.9 μmole/g wet wt. in absence of substrate and increased to 50 with added fatty acid. With liver from starved rats the rates were 30 and 120 respectively. Unless the activity of acetoacetyl-CoA synthetase can be significantly greater in vivo than appears from these in vitro measurements (assuming no enzyme inactivation or unknown activators), it is clear from these calculations that the synthetase can be of quantitative importance in regulating ketogenesis in the rat only when the rate of ketogenesis is low or intermediate.

The possibility that acetoacetyl-CoA synthetase may function in the reverse direction to effect an AMP- and PP-dependent deacylation of aceto-acetyl-CoA to acetoacetate coupled to substrate level phosphorylation is most unlikely since the activity of this enzyme was decreased in starvation which increases ketogenesis.

Parallel experiments have been performed using Wistar strain rats. The results were very similar, except that the fall in acetoacetyl-CoA synthetase after fasting was not so great.

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