Effects of Starvation and Development on Mitochondrial Acetoacetyl-Coenzyme A Thiolase of Rat Liver

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(Received 23 April 1976)

The activity of the putative ketogenic β -oxoacyl-CoA thiolase from mitochondria of rat liver increases with starvation, during neonatal life, and after the injection of glucagon. These changes are associated with alteration in ketonaemia. The changes in activities of this species of thiolase are not associated with significant alterations in the apparent affinity (K_m) for the ketogenic substrate, acetyl-CoA. These results support a role for thiolase in the regulation of ketogenesis.

Hepatic acetoacetyl-CoA thiolase (EC 2.3.1.9) (thiolase) from various species is generally considered to be present in three different forms and in two locations, i.e. two are mitochondrial and one cytosolic (Clinkenbeard *et al.*, 1973; Middleton, 1972). The cytoplasmic thiolase apparently participates in cholesterogenesis, whereas the mitochondrial species function in β -oxidation and ketogenesis (Clinkenbeard *et al.*, 1973, 1975*a,b*; Middleton, 1973*a,b*). The mitochondrial thiolases, both of matrix origin (Middleton, 1973*a*; Chapman *et al.*, 1973; Clinkenbeard *et al.*, 1975*a*), have been separated by phosphocellulose chromatography into two types with either a long- or a short-chain β -oxoacyl-CoA specificity (Huth *et al.*, 1974; Middleton, 1973*a,b*). Huth *et al.* (1973, 1974) have implicated the mitochondrial thiolase in the regulation of ketogenesis by demonstrating changes in the activity and kinetic properties of the short-chain-specific species as a function of the ketotic state of the animal. In view of these findings, a comparison between the activities and kinetic characteristics of the mitochondrial-matrix thiolase species and the nutritional or hormonal status of the rat was studied with regard to the regulation of hepatic ketogenesis. A preliminary account of this work has appeared (Reed *et al.*, 1975).

Experimental

Materials

Acetoacetyl-CoA was prepared as described by Simon & Shemin (1953) by using diketene. All other reagents, CoA, potassium phosphate, Hepes* buffer, Triton X-100, glycerol, 3-hydroxyacyl-CoA dehydrogenase (EC 1.1.1.35), acetyl-CoA, NADH, glucagon

and insulin were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.).

Animals

Laboratory-bred Wistar rats were used in measuring the effects of various physiological changes on blood ketone bodies and hepatic thiolase activities. Neonatal animals were studied at 3, 7 and 14 days of age in the fed state. Adults of both sexes weighing approx. 200g were killed in the fed state or after 48–72h of starvation. In the adults, injections of glucagon (0.1mg/kg) were given in 0.9% NaCl intraperitoneally 30min before death. The blood ketone-body values (Table 1) were determined in another group of equivalent animals, and the data for neonatal rats have been published previously (Ozand *et al.*, 1975).

Assay of ketone bodies

The blood ketone bodies, β -hydroxybutyrate and acetoacetate, were assayed enzymically (Ozand *et al.*, 1975). Total ketone bodies (β -hydroxybutyrate + acetoacetate) produced from [1- 14 C]octanoic acid were measured as the Denigés salt (Bates *et al.*, 1968) after conversion of β -hydroxybutyrate into acetoacetate as described by McGarry & Foster (1969).

Enzyme resolution and assay

In the cleavage direction, thiolase was assayed by monitoring the decrease in A_{303} with a Gilford 2400 spectrophotometer as described by Clinkenbeard *et al.* (1973), except that potassium phosphate (50mM) was added to all assays for maximum enzyme activation (cf. Middleton, 1973*a*). In the condensation direction (acetoacetyl-CoA synthesis),

* Abbreviation: Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid.

thiolase was measured by the method of Lynen *et al.* (1952), with the loss of NADH being followed fluorimetrically with a Turner fluorimeter (model 111) connected to a Heath model 20B recorder. The final reaction volume was 0.25 ml and contained 20 μ M-NADH, 100 mM-Tris/HCl, pH 8.0, 500 μ M-acetyl-CoA, 50 mM-potassium phosphate and 2 units of β -hydroxyacyl-CoA dehydrogenase (1 unit is μ mol/min under standard assay conditions).

In all of the groups of animals (Table 1), liver tissue was removed, immediately chilled on ice in buffer, homogenized, and centrifuged at 0–4°C to obtain the mitochondria, as described below. Preparations of hepatic mitochondrial matrix were obtained by a modification of the procedure of Middleton (1973a). Glycerol was added to 25% (v/v) and included in all subsequent operations at this concentration, except in the actual enzyme assay. The mitochondria were prepared by homogenizing minced liver in 0.25 M-sucrose/5 mM-Hepes, pH 7.4 (adjusted with KOH), with a motor-driven glass/Teflon homogenizer. The homogenate was centrifuged at 600g for 15 min. The supernatant from this step was centrifuged at 9000g for 15 min. The sedimented mitochondria were gently resuspended by using a 'cold-finger', adjusted to one-half the volume of the original homogenate, and centrifuged again at 9000g for 15 min. The mitochondrial pellet was resuspended in homogenization medium to a final protein concentration of approx. 70 mg/ml, as assayed by the method of Murphy & Kies (1960). This method agrees with the biuret method in the concentration range 60–80 mg/ml. The addition of 10% (v/v) Triton X-100 at 2 μ l/mg of mitochondrial protein and incubation at 0°C for 5 min before centrifugation at 105000g for 30 min was as described by Middleton (1973a).

This procedure yielded a preparation of mitochondrial matrix virtually free of cytoplasmic contamination (<1%) as judged by the assay of lactate

dehydrogenase (EC 1.1.1.27). The matrix release was greater than 90% as indicated by the recovery of the matrix marker, malate dehydrogenase (EC 1.1.1.37), as well as of the thiolase (results not shown). Lactate dehydrogenase and malate dehydrogenase were assayed as cited in Clinkenbeard *et al.* (1975a).

The amount of mitochondrial matrix equivalent to thiolase activity of 30 units was layered on a phosphocellulose column (12 mm $\times$ 250 mm) previously equilibrated with 20 mM-potassium phosphate, pH 6.6, containing 25% (v/v) glycerol. The protein content did not exceed 4 mg per column. Thiolase activity was eluted with a linear potassium phosphate gradient (20–500 mM), pH 6.6, containing 25% glycerol. The differences in resolution between peaks of Fig. 1(a), as compared with Fig. 1(b), were due to variability in the flow rate used with the columns. That is, Figs. 1(a) and 1(b) are not exactly superimposable owing, apparently, to the fact that the column flowed at a lower rate when the chromatography was performed in order to assay the eluting fractions in the synthetic direction. This lower flow rate apparently separated the ketogenic thiolase 2a and 2b peaks, from each other to a greater extent than had been seen in the earlier work when the eluting fractions were assayed for the thiolytic cleavage of acetoacetyl-CoA.

Results

Blood ketone bodies and experimental rationale

In contrast with the fed adult rat, the fed newborn and the starved adult with or without glucagon injections had significantly higher values of β -hydroxybutyrate and acetoacetate in blood (Table 1). These different conditions of ketonaemia allow comparative studies of the activities and kinetic properties of the species of mitochondrial thiolase present in the matrix to be performed.

Table 1. *Values of blood ketone bodies in fed and variously ketonaemic rats*

The blood ketone bodies, β -hydroxybutyrate and acetoacetate, were determined on 100 μ l samples of blood withdrawn after decapitation of rats, and the samples were deproteinized with HClO₄. The concentrations per ml of blood were determined by the automated fluorimetric methods described by Ozand *et al.* (1975). The results are expressed as means $\pm$ S.E.M.

	No. of animals	Hydroxybutyrate (nmol/ml)	Acetoacetate (nmol/ml)
Adult fed	19	103.0 $\pm$ 9.1	15.8 $\pm$ 4.8
Adult starved	137	823.5 $\pm$ 44.4	235.7 $\pm$ 11.4
Adult starved (48 h) + glucagon	26	1646.5 $\pm$ 63.0	432.7 $\pm$ 66.0
2 Weeks fed*	20	1130.0 $\pm$ 112.0	60.0 $\pm$ 3.5
2–3 Days fed	10	610.0 $\pm$ 150.0	190.0 $\pm$ 50.0

* Pooled

Chromatography of matrix thiolase

The chromatography of the mitochondrial matrix on phosphocellulose was assayed by monitoring both the cleavage and synthesis of acetoacetyl-CoA. The elution profiles obtained appear in Figs. 1(a) and 1(b). For enzyme activity in the direction of cleavage, there are two major peaks, the first (1), eluted at approx. 120mM-potassium phosphate, represents the long-chain 3-oxoacyl-CoA-specific activity, whereas the second (2a+2b), eluted at and above approx. 260mM, represents the short-chain-specific thiolase [cf. Huth *et al.* (1974) and Middleton (1973a)].

When the eluted fractions were assayed for acetoacetyl-CoA-synthetic activity, two major peaks are

Fig. 1. Phosphocellulose chromatography of 3-oxoacyl-CoA thiolase activity from rat liver mitochondria

(a) Typical profile (fed adult rat) of thiolase activity assayed in the thiolytic cleavage direction is shown when a matrix sample is chromatographed on phosphocellulose and subjected to potassium phosphate gradient elution. The qualitative appearance of this profile was virtually identical in over 30 chromatograms. (b) The same type of gradient elution and fraction volume collection shown in (a) (with a fed adult) was performed and assayed in the direction of acetoacetyl-CoA synthesis. The form of the profile shown was observed in eight different chromatograms assayed in the direction of acetoacetyl-CoA formation. ----, Potassium phosphate gradient.

also observed. However, the first thiolase activity occurs at a gradient concentration corresponding, in Fig. 1(a), to the 'peak-2a' thiolase and the second to 'peak-2b' thiolase. The apparent absence (from Fig. 1b) of a peak of thiolase activity corresponding to the 'peak-1' enzyme of Fig. 1(a) is considered to be due to the fact that all acetoacetyl-CoA-synthetic (or 'ketogenic') activities should reside in the short-chain thiolase. Identical conditions of column volume, units of enzyme applied, gradient volume and tube volumes were used in all experiments, and recoveries of enzyme activity were within the range 92–108%.

To compare the distribution of thiolase activities in the various states of ketonaemia, the enzyme activities eluted from columns and within a Gaussian distribution of each peak were examined in both the synthesis and cleavage directions (cf. Figs. 1a and 1b). The total activity (30 units) applied to each column was identical and the activities comprising each peak were compared in different ketotic states. Measuring only in the cleavage direction, the increase in the activity of 'peaks-2a+2b' thiolase was associated with a decrease in peak 1 during hyperketotic states (see Table 2). 'Peaks-2a+2b' thiolase is presumably short-chain-specific and involved in ketogenesis and the terminal step of β -oxidation, whereas peak 1 represents the relative non-specific class of thiolases associated with the β -oxidation of long-chain β -oxoacyl-CoA fatty acids (Middleton, 1973a; Huth *et al.*, 1974). For ease of comparison the thiolase activities have been expressed in terms of activity/g wet wt. of liver and not according to the units applied to the column. On infrequent occasions, the amount of thiolase eluted between peaks 1 and 2 represented not more than 18% of the total units/g wet wt. This thiolase did not possess acetoacetyl-CoA-synthetic activity, since it was eluted below approx. 260mM-potassium phosphate (cf. Fig. 1b), and was therefore not included in the estimate of total ketogenic thiolase. Because of this and because some activity was excluded from the calculations if it occurred in tubes outside a Gaussian-shaped activity peak, the sum of activities in the peaks is not always a constant value.

In contrast with the fed adult, there is an increase in total cleavage activity in starved adults as well as in fed neonatal rats, both of which are hyperketonaemic (Table 2). The changes in 'peak-1' thiolase are minimal, with only a 2-fold increase noted in 14-day-old animals. However, the increases in 'peak-2' enzyme ranged from 2.5- to 4-fold (Table 2). The administration of glucagon increased the activity in peaks 2a+2b and decreased it in the peak 1, in both fed and starved animals (Table 2). Although not studied in detail, it was observed that isolated mitochondria from rats either starved or injected with glucagon produce ketone bodies from [1- 14 C]octanoic acid in approximately the same order of magnitude

Table 2. Activity of mitochondrial 3-oxoacyl-CoA thiolase from neonatal, fed and starved adult rats and after glucagon administration to fed and starved adult rats (assayed in the direction of acetoacetyl-CoA cleavage)

Thiolase activity in peaks 1 and 2a+2b were evaluated by assaying it in each tube comprising the particular peak and summing the values. The activities in each peak were then converted into units/g wet wt. of liver by appropriate calculations by using total matrix thiolase activity/g wet wt. of liver. The increase in total activity/g wet wt. of liver was essentially equal to the sum of the two main peaks 1+2a+2b. *P* values are indicated in parentheses for statistical comparison with the value for the fed adult. Where appropriate, values are means $\pm$ S.E.M.

State	No. of expts.	Activity (μ mol/min per g wet wt.)	
		Peak 1	Peak 2a+2b
Adult rats			
Fed	10	4.8 $\pm$ 1.1	1.9 $\pm$ 1.26
Starved 48 h	3	5.0 $\pm$ 3.81	4.7 $\pm$ 0.26 (0.005 > <i>P</i> > 0.001)
Starved 72 h	3	6.0 $\pm$ 1.56	6.0 $\pm$ 1.56 (<i>P</i> < 0.001)
Neonatal rats			
3 days	3	3.8 $\pm$ 0.87	7.2 $\pm$ 3.8
7 days	1	7.0	9.9
14 days	2	11.1*	4.7*
Adult fed+glucagon	2	3.1†	5.9†
Adult 48 h-starved+glucagon	2	3.6†	4.1†

* Average of two values.

† Killed 30 min after injection.

and direction as the changes in 'peaks-2a+2b' thiolase activity (Table 2). For example, in two experiments utilizing just two animals each, liver mitochondria from fed adult rats produced total ketone bodies (i.e. β -hydroxybutyrate+acetoacetate) at a rate of 3.6 nmol/min per mg. However, when fed adult rats were injected with glucagon (0.1 mg/kg) 30 min before death, their hepatic mitochondria yielded ketone bodies at a rate of 7.0 nmol/min per mg, i.e. an increase of approx. 2-fold. In another two experiments with two animals each, hepatic mitochondria from 48h-starved young adult rats (5–6 weeks of age) injected with glucagon only increased their output of ketone bodies 1.6-fold. Thus there appears to be a positive relationship between the increased 'peak-2a+2b' thiolase activity and hyperketonaemia. It may be noted here that in another experiment utilizing two rats injected with insulin (15 μ g/kg) 30 min before death, there was an increase of 2.6-fold in 'peak-2a+2b' thiolase compared with the average activity in 27 control rats (i.e. from 1.4 μ mol/min per g wet wt. in control to 3.6 μ mol/min per g; the average of two insulin-treated rats).

Kinetic considerations of matrix thiolase species

The kinetic data were derived from activities in the fraction of peaks 1 or 2a+2b with the highest specific activity (Figs. 2 and 3 and Table 3).

For 'peak-1' thiolase, the Michaelis–Menten plots of the cleavage assay were linear at low values of CoA

Table 3. Values for the apparent K_m of the putative ketogenic 3-oxoacyl-CoA thiolase when assayed in the direction of acetoacetyl-CoA synthesis from acetyl-CoA

Apparent K_m values were calculated from plots of $1/v$ versus $1/\text{acetyl-CoA}$ for thiolase obtained from the fraction in peak 2b with the highest specific activity. The thiolase assay used was in the direction of acetoacetyl-CoA synthesis from acetyl-CoA.

State	K_m (app.) (synthesis) (mM)
Adult fed	0.42
Adult fed+glucagon	0.32
Adult 48 h-starved	0.40
Adult 48 h-starved+glucagon	0.35
Neonatal fed (12–14 days)	0.39

and acetoacetyl-CoA. Substrate inhibition was observed, however, when the concentrations of either of these co-substrates was increased about 10 μ M (not shown). For 'peak-2b' thiolase, the double-reciprocal plots of thiolase (cleavage activity) in 48h-starved animals were linear with respect to CoA and consistent with the observation by Middleton (1973a) and Huth *et al.* (1974) (Fig. 2). The substrate inhibition by acetoacetyl-CoA of this 'peak-2b' thiolase activity was a variable function of the CoA concentration and occurred in all experimental animals. Since no single concentration of CoA tested gave plots that were suitable in every case to calculate an apparent K_m , the kinetics in the direction of acetoacetyl-CoA synthesis were investigated. This condensation assay has the advantages of utilizing a single substrate

Fig. 2. Typical double-reciprocal plots of thiolase cleavage activity for both co-substrates of the 'peak-2b' thiolase. This enzyme species was obtained by phosphocellulose chromatography of total mitochondrial thiolase activity (from a starved adult rat). Samples from those tubes exhibiting maximal acetoacetyl-CoA-cleavage activity were assayed at various concentrations of CoA (a) and acetoacetyl-CoA (b) to obtain Lineweaver-Burk plots. The appearance of the plots (for both co-substrates) was found to be the same from fed and starved adult rats. That is, substrate inhibition of the 'peak-2b' thiolase was observed for acetoacetyl-CoA, but not for CoA in fed and starved adult rats.

and of exhibiting no acetyl-CoA inhibition (Fig. 3). The apparent K_m was determined by using the fraction of 'peak-2b' enzyme with the highest specific activity, and the results are shown in Table 3. These data show that there was no substantial change in apparent K_m . The increases noted in 'peaks-2a+2b' thiolase activity in these experimental states were not associated with a particular change in affinity of thiolase for its ketogenic substrate, acetyl-CoA.

Discussion

The results presented in this report suggest that a positive correlation exists between thiolase activity of the liver mitochondrial matrix and elevated blood

Fig. 3. Typical double-reciprocal plot of 'peak-2b' thiolase activity when assayed for the synthesis of acetoacetyl-CoA.

Selected tubes from the phosphocellulose chromatography of mitochondrial thiolase were assayed by measuring acetoacetyl-CoA cleavage to locate the tubes (in various experiments) with the highest activity. After location of the most active fraction, samples were removed from the tubes and assayed in the formation of acetoacetyl-CoA (i.e. synthesis) at various concentrations of acetyl-CoA. The plot shown is for neonatal animals (12-14 days of age). The K_m for this experiment was $0.39 \times 10^{-3} M$ and the V_{max} was $0.86 \mu\text{mol/min per g wet wt}$.

ketone-body concentrations. Specifically, the activities of the thiolase eluted at higher concentrations of potassium phosphate ('peak-2a+2b' thiolase, Fig. 1a) are increased during states of physiological hyperketonaemia in neonatal animals, as well as during the ketonaemia induced by simple starvation of adults (Table 2). Although it was true that 'peak-2a+2b' thiolase activities always increased when blood ketone bodies were increased, there was not a linear correlation. This is not particularly surprising, since the concentration of blood ketone bodies is influenced by a variety of additional factors, such as catabolic metabolism and effector modulation (such as alanine concentration, Ozand *et al.*, 1976). 'Peak-1' thiolase activity, which is eluted at a lower concentration of potassium phosphate and has been shown by Middleton (1973a) and Huth *et al.* (1974) to be long-chain 3-oxoacyl-CoA-specific and presumably β -oxidative in nature, exhibits minimal changes and responds in a less obvious manner to these conditions of hyperketonaemia.

The administration of glucagon produces a rise in blood ketones (Table 1) and in the short-chain-specific thiolase activities (Table 2). These increments in

enzyme activities may have various effects on ketone-body production, because acetoacetyl-CoA thiolase catalyses the reaction in both the forward and the reverse direction. In the condensation direction this short-chain-specific thiolase is the first enzyme of the ketogenic pathway, whereas in the cleavage direction it is the terminal reaction of the β -oxidation of even-numbered-chain-length fatty acids. Accordingly, the increase in ketogenic thiolase activity in neonatal life, with starvation or after glucagon administration, may well interact with other, as yet incompletely defined, ketogenic factors (e.g. increased rates of fatty acid oxidation, lower values of free CoA and the presence of elevated malate concentrations) to increase the production of ketone bodies. The administration of an anti-ketogenic agent, i.e. insulin, increased the thiolase activity of peaks 2a+2b, as mentioned above. Since one of the species of acetoacetyl-CoA-specific thiolases (perhaps peak 2a) may catalyse the thermodynamically favourable reaction of acetoacetyl-CoA cleavage (Hartmann & Lynen, 1961), an increase in the activity of this species would elicit a decrease in the concentration of the first intermediate in ketone-body synthesis. Thus the potential for production of ketone bodies would be diminished. The increases in thiolase of peaks 2a+2b were not associated with any dramatic change in the affinity of the enzyme for acetyl-CoA, i.e. no large change in apparent K_m was observed.

In summary, the data demonstrate increases in the activity of mitochondrial ketogenic acetoacetyl-CoA thiolase of rat liver in conjunction with increased blood ketone-body concentrations. These increases in activity are not accompanied by substantial changes in apparent K_m for acetyl-CoA in the synthesis of acetoacetyl-CoA. Although these results support a role for ketogenic thiolase in the regulation of ketone-body production, the changes noted are probably not sufficient, in themselves, to account totally for the regulation of hepatic ketone-body formation.

We acknowledge the excellent technical assistance of Mr. Frank R. Mills and Mr. Peter J. Baab. We also thank Mr. Jeffrey Chircus for his participation in this study. These studies were supported in part by NIH grants AM 18127, HD 03959 and HD 06291 and by the John A. Hartford Foundation grant no. 74266.

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