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SUBMITOCHONDRIAL LOCALIZATION AND PARTIAL PURIFICATION OF THE SUCCINYLCoA: 3-HYDROXY-3-METHYLGLUTARATE COENZYME A TRANSFERASE FROM RAT LIVER

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The presence and the localization of the enzyme catalyzing the transfer of a coenzyme A molecule from succinyl-CoA to 3-hydroxy-3-methylglutarate has been established in rat liver mitochondria. The enzyme was found mainly in the mitochondrial matrix but some activity was also found in the inner membrane fraction. The enzyme has been purified about 100-fold from sonically-disrupted mitochondria by high-speed centrifugation, DEAE-cellulose chromatography, $(NH_4)_2SO_4$ precipitation and Sephadex G-100 filtration. The enzymatic activity was recovered in the final step as a single peak. The coenzyme A transferase appears to have a molecular weight of 42 000, the highest activity at pH 8.5 and an energy of activation of 13 kcal/mol. Mercaptoethanol increases the activity and improves its stability. The enzyme is different from the succinylCoA: 3-oxoacids coenzyme A transferase and is active also on malonylCoA. The apparent K_m values obtained for succinylCoA, malnylCoA and 3-hydroxy-3-methylglutarate were $2.2 \cdot 10^{-4}$ M, $3.7 \cdot 10^{-4}$ M and $1.7 \cdot 10^{-3}$ M, respectively. Acetoacetate, which is the final product of the mitochondrial metabolism of hydroxy-methylglutarylCoA, showed an inhibitory effect on the enzyme activity with a K_i of 0.5 mM. The physiological role of the enzyme is discussed.

Introduction

It is well known that the liver of a number of mammals contains an active 3-hydroxy-3-methylglutarylCoA hydrolase which converts 3-hydroxy-3-methylglutarylCoA into free 3-hydroxy-3-methylglutaric acid (HMG) [1–3].

Conflicting data have been reported on the physiological role of HMG. Bloch et al. [4] and Rabinowitz and Gurin [5] found that HMG is converted into cholesterol and therefore could be considered as a cholesterologenic compound, while Dituri et al. [6] showed that HMG is not incorporated into cholesterol precursor squalene. On the other hand HMG shows a hypocholesterolemic effect when administered to rats or rabbits maintained on an atherogenic diet [7–9].

It is now established that HMG can be converted into ketone bodies, via its coenzyme A ester HMG-CoA in kidney and liver [3,10,11]. The activation of HMG to HMGCoA was first proposed to be catalyzed in liver by an ATP-dependent kinase, but we have provide evidence that the transformation of HMG into HMGCoA depends in liver on a coenzyme A transferase reaction, in which succinylCoA acts as a coenzyme A donor [3,11].

In this paper a procedure for a partial purification of the succinylCoA : HMG coemzyme A transferase from rat liver mitochondria is reported.

Part of this work has been presented at the 13th FEBS Meeting, Jerusalem, 1980.

Abbreviations: HMG, 3-hydroxy-3-methylglutarate; EGTA, ethylenglycol-bis(aminoethyl)tetraacetate.

Materials and Methods

Wistar albino rats (both male and female, approx. 300 g body weight) fasted overnight, were used.

Preparation of mitochondrial fractions. Liver mitochondria were isolated according to Chappell and Hansford [12] in 0.25 M sucrose/10 mM Tris-HCl (pH 7.3). They were washed twice and resuspended in 50 mM Tris-HCl (pH 9.0)/2 mM mercaptoethanol and exposed to ultrasonic vibrations three times for 20 s at 0°C (Branson Sonic Co. Instrument, 15 KHz, 70 W).

Submitochondrial fractions were prepared and tested for their purity essentially according to Schnaitman and Greenawalt [13]. The mitoplasts obtained after the digitonin treatment of mitochondria were resuspended in 30 mM Tris-HCl (pH 9.0)/1 mM mercaptoethanol and disrupted by ultrasonic vibrations. The resulting suspension was centrifuged 15 min at 20 000 × *g* and the pellet was discarded. The supernatant was centrifuged again 1 h at 100 000 × *g*; the latter pellet was resuspended in the same buffer used for sonication and considered as inner membrane fraction while the supernatant was considered as soluble mitochondrial matrix.

Monoamino oxidase activity was determined according to Schnaitman et al. [14]; adenylate kinase activity was followed by the enzymatic method described by Bergmeyer [15]; succinate dehydrogenase activity was determined spectrophotometrically according to King [16] and malate dehydrogenase activity was tested according to Dupourque and Kun [17].

SuccinylCoA : HMG coenzyme A transferase assay. Neither the direct measurement of HMGCoA formed nor the amount of succinylCoA consumed were considered to be suitable parameters for the measurement of the enzymatic activity. The crude mitochondrial homogenate contains in fact high activities of HMGCoA lyase, HMGCoA hydrolase and succinylCoA hydrolase which make the direct determination of either HMGCoA or succinylCoA unreliable, at least in the first steps of the purification. The enzyme activity was followed instead by measuring the amount of ketone bodies formed in the presence of an excess of HMGCoA lyase which should convert most of the HMGCoA formed into acetoacetate, which in turn could be reduced to 3-hydroxybutyrate.

The standard assay mixture contained 0.2 M Tris-HCl (pH 8.5)/5 mM $MgCl_2$/2 mM reduced glutathione freshly prepared/10 mM HMG (potassium salt)/2 mM succinylCoA. HMGCoA lyase preparation obtained from acetone-dried powders of ox liver mitochondria by the method of Stegink and Coon [18] was added to the incubation medium (0.3 mg/1.5 ml assay mixture). The amount of coenzyme A transferase preparation was added at an amount ranging between 2 and 0.2 mg of protein according to the fraction assayed. The incubations were carried out at 30°C, started by addition of succinylCoA and stopped after 10 min by addition of 0.3 ml of cold 25% (w/v) $HClO_4$. The pellets were removed by centrifugation and both acetoacetate and 3-hydroxybutyrate were measured enzymatically in aliquots of the neutralized supernatants [19]. Two blank incubations were carried out in parallel in which either HMG or succinylCoA was omitted.

In the experiments where either acetoacetylCoA was tested as possible coenzyme A donor or acetoacetate as possible coenzyme A acceptor the transferase activity was followed by measuring the acetoacetyl-CoA formed or disappeared by both the enzymatic [25] and spectrophotometric methods [26].

Estimation of molecular weight. The molecular weight of the purified transferase was determined by Sephadex G-100 chromatography using chymotrypsigen, pepsin, ovalbumin and bovine serum albumin as standards.

Protein assay. In the early stages of enzyme purification, protein content was detemined by the method of Lowry et al. [20]; in later stages, with low protein concentration, the Bio-Rad assay kit was used [21]. The 280 nm absorbance was used to monitor the eluates from chromatography columns.

Chemicals. The HMGCoA used for checking the activity of the HMGCoA lyase preparation was synthesized following the method of Low et al. [22], succinylCoA was synthesized as described by Wieland and Rueff [23], [3-^{14}C]HMG was purchased from New England Nuclear, Boston; HMG from Fluka, Buchs; Coenzyme A from Boehringer-Mannheim; DEAE-cellulose (23 SS) from Serva, Heidelberg and Sephadex G-100 from Pharmacia, Uppsala. All other reagents were analytical grade.

Results

Submitochondrial localization of the succinyl-CoA : hydroxymethylglutarate CoA transferase. Table I shows the coenzyme A transferase activity in the various submitochondrial fractions. The soluble intramitochondrial fraction exhibited the highest activity but a certain amount was also found in the inner mitochondrial membranes, whereas the outer membranes and the intermembrane fractions were devoid of any activity. The enzyme responsible for the inner membrane was partially extractable by treatment at high ionic strength (data not shown).

Enzyme purification. The stages of purification of the succinylCoA : HMG coenzyme A transferase activity from rat liver mitochondria are summarized in Table II.

All procedures were carried out at 0–4°C. The suspension of sonicated mitochondria pooled from six rat livers and containing about 50 mg protein/ml was centrifuged 10 min at 12 000 rev./min, the pellet was discarded and the supernatant was centrifuged 1 h at 100 000 × *g*. The latter supernatant contained most of the activity, however a low activity, which was disregarded for the enzyme purification procedure, was found in the high-speed pellet. The supernatant was adjusted at pH 9.0 with Tris-base and applied to a DEAE-cellulose 23 SS column (2.0 × 7.0 cm) previously equilibrated with 50 mM Tris-HCl (pH 9.0), containing 1 mM mercaptoethanol. The column was then washed with the same buffer and eluted with a linear gradient (0–0.45 M) of NaCl.

The peak of activity was found at a 0.15 M NaCl concentration. Subsequent elution of the column with 0.8 M NaCl did not induce any further release of the enzyme. The fractions which contained enzyme activity were pooled and $(NH_4)_2SO_4$ was added to 35% saturation. The suspension was stirred for 30 min and centrifuged 20 min at 20 000 × *g*. The supernatant was brought to 60% saturation with $(NH_4)_2SO_4$ and the precipitate obtained after centrifugation (20 min at 20 000 × *g*) was dissolved in 50 mM Tris-HCl (pH 9.0)/1 mM mercaptoethanol and applied to a Sephadex G-100 column (1.8 × 75 cm). Proteins were eluted at a rate of 2 ml/h and fractions of 2.5 ml were collected. Fig. 1 shows the chromatographic profile obtained. The enzyme was eluted as a single peak which exhibited a specific activity about 100-fold higher than the sonicated mitochondrial suspension. The fractions containing the highest activity were pooled and concentrated by ultrafiltration through a Diaflo UM-10 membrane. The resulting preparation was used for all other determinations. The acetoacetylCoA thiolase activity present in our preparations was negligible eliminating the possible interference of this enzyme on acetoacetate formation.

Enzyme stability. Reduced sulphydryl groups seem to be a stability requirement for the enzyme at any step of the purification. Addition of bovine serum albumin at a concentration of 3 mg/ml only slightly increased the enzyme stability, whereas glycerol added at a final concentration of 10% proved to be a much better stabilizer. Enzyme preparations with glycerol and mercaptoethanol retained 80% of their activity after 2 weeks of storage at –20°C. Decreasing the pH to values lower than 6.0 caused an irreversible loss of the enzyme activity.

Properties of the enzyme. Under our experimental conditions the enzyme activity was proportional to the amount of enzyme preparation added and linear

TABLE I

SUBMITOCHONDRIAL LOCALIZATION OF THE SUCCINYLCoA : 3-HYDROXY-3-METHYLGLUTARATE COENZYME A TRANSFERASE

The activity of the coenzyme A transferase and that of marker enzymes are expressed as nmol · min^{-1} · mg $protein^{-1}$.

Fraction	Proteins (mg)	Coenzyme A transferase	Monoamine oxidase	Adnylate kinase	Succinate dehydrogenase	Malate dehydrogenase
Outer membranes	37	0.0	162	157	92	275
Intermembranes fraction	62	0.0	13	1 214	35	630
Inner membranes	196	0.18	7	109	405	780
Matrix	328	0.63	4	0	76	1 960

TABLE II

PURIFICATION OF THE COENZYME A TRANSFERASE FROM RAT LIVER MITOCHONDRIA

Fraction	Total proteins (mg)	Specific activity (nmol/ketone bodies/min per mg protein)	Total activity (nmol/ketone bodies/min)	Yield (%)
Sonically disrupted mitochondria	1 115	0.21	234	100
100 000 × supernatant	532	0.80	426	182
DEAE-cellulose eluate	118	2.2	260	111
(0–35%) $(NH_4)_2SO_4$ supernatant	83	2.9	241	103
(35–60%) $(NH_4)_2SO_4$	55	4.1	225	94
Sephadex G-100 eluate	3.6	19.4	70	30

with time up to 15 min.

A number of substrates were tested as coenzyme A donors, the results reported in Table III show that the partially purified enzyme catalyzes the transfer of coenzyme A to HMG also from malonylCoA although to a lower rate than that obtained from succinylCoA. In contrast no HMGCoA was produced when acetylCoA, acetoacetylCoA or ATP plus CoA were used as substrates, and butyrylCoA gave only negligible amounts of HMGCoA. Acetoacetate cannot substitute for HMG as coenzyme A acceptor ruling out the possibility that this enzyme is the same succinylCoA : 3-oxoacids CoA transferase found in heart and muscles [27,28].

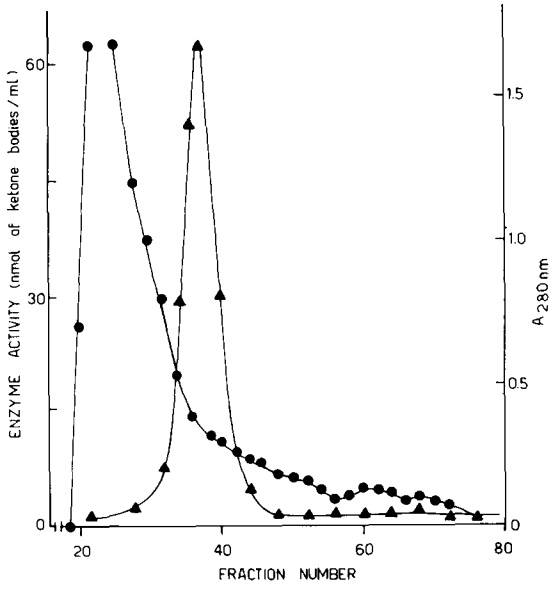

Fig. 1. Elution profile of coenzyme A transferase from Sephadex G-100. 1-ml fraction aliquots were tested for enzyme activity (▲——▲) and absorbance at 280 nm (●——●).

The apparent K_m values of the partially purified enzyme were determined for each substrate, namely succinylCoA, malonylCoA and HMG at the saturating levels of the other substrate. Under our experimental conditions the K_m values obtained were $2.2 \cdot 10^{-4}$ M for succinylCoA, $3.7 \cdot 10^{-4}$ M for malonylCoA and $1.7 \cdot 10^{-3}$ M for HMG, respectively.

The enzyme showed the highest activity at pH 8.5

TABLE III

SUBSTRATE SPECIFICITY OF THE PARTIALLY PURIFIED COENZYME A TRANSFERASE

All substrates were added at the final concentration of 2 mM. SuccinylCoA, malonylCoA, butirylCoA, acetoacetylCoA and acetylCoA were tested as coenzyme donors to HMG. Acetoacetate was tested as coenzyme A acceptor from succinylCoA; in this case the enzyme activity was followed by measuring the acetoacetylCoA formed.

Substrate	Enzyme activity ($nmol \cdot min^{-1} \cdot mg\ protein^{-1}$)
SuccinylCoA	19.4
MalonylCoA	12.7
ButurylCoA	1.0
AcetoacetylCoA	0.0
AcetylCoA	0.0
ATP + CoA	0.0
Acetoacetate	0.0

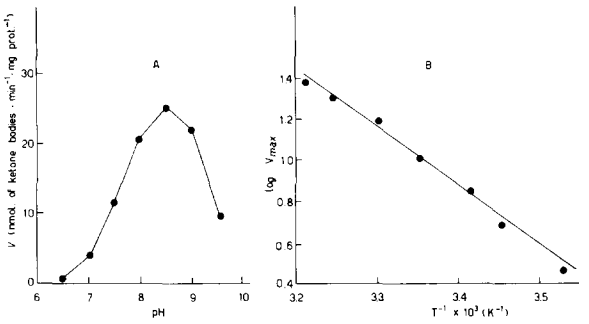

Fig. 2. Effects of pH (A) or temperature (B) on the coenzyme A transferase activity.

(Fig. 2A) and an activation energy, obtained by the Arrhenius plot, of 13 Kcal/mol (Fig. 2B). For these experiments the HMGCoA lyase preparation was added to our assay mixture in such a large excess that its change of activity at different pH and temperature

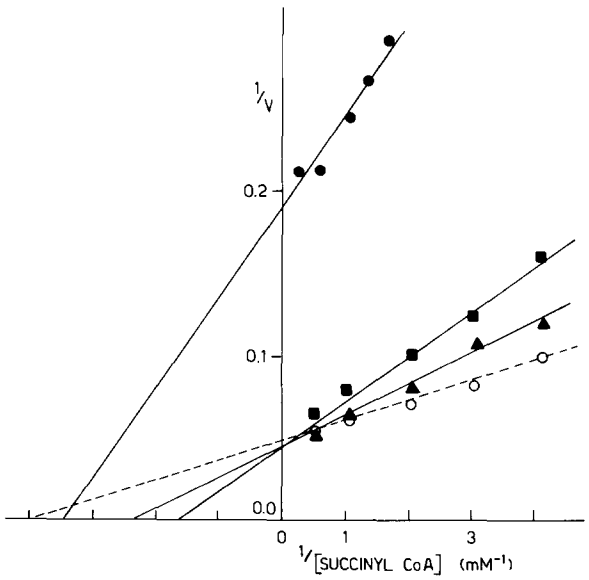

Fig. 3. Lineweaver-Burk plots of the transferase activity in the presence of different inhibitors. Incubations were carried out with: no inhibitor (o------o), 1.5 mM acetoacetate (•———•), 3 mM succinate (▪———▪) and 3 mM malonate (▲———▲). Enzyme activity (v) is expressed as nmol of acetoacetate · min^{-1} · mg $protein^{-1}$. In the experiments where acetoacetate was added the enzyme was followed by measuring the amount of [^{14}C]acetoacetate formed from [3-^{14}C]HMG according to the method reported [24].

had no influence on the determination of succinyl-CoA : HMG CoA transferase activity.

An apparent molecular weight of 42000 was found by Sephadex G-100 filtration.

The enzyme was inhibited by succinate, malonate and acetoacetate as shown in Fig. 3. These results indicate that the previously reported inhibition shown by these compounds in intact mitochondria [3] is due to direct effects on the coenzyme A transferase. The inhibition exhibited by succinate and malonate is of competitive type, whereas that of acetoacetate seems to be non-competitive. A $K_i = 1.5 \cdot 10^{-3}$ M for succinate, $K_i = 2.1 \cdot 10^{-3}$ M for malonate and $K_i = 0.5 \cdot 10^{-3}$ M for acetoacetate were found.

Conclusions

This study provides clear evidence of the existence of the succinylCoA : HMG coenzyme A transferase in rat liver mitochondria and confirms our previous results and hypothesis [3,11]. It is perhaps premature to define the physiological relevance of this enzyme although the K_m values for succinylCoA and HMG are similar to those found for the succinylCoA : 3-oxoacids coenzyme A transferase of the skeletal muscle [28]. The apparent enhancement of the total enzyme activity shown by the high speed supernatant (Table II) indicates the presence of an inhibitor associated with the membranes. This inhibitor may cause an underestimation of the enzyme activity, particularly of that found in the membrane fraction. Crude preparations of the membrane-bound enzyme show properties similar to those of the soluble fraction, indicating that they are probably involved in the same catalytic reactions.

Acetoacetate is a product of the mitochondrial HMGCoA metabolism and its feed-back inhibitory effect on the succinylCoA : HMG coenzyme A transferase might indicate that the enzyme is involved in ketogenesis. The fact that mitochondria obtained from livers of rats in ketosis show a lower coenzyme A transferase activity [3] supports this hypothesis. The enzyme might also be involved in the regulation of intracellular levels of HMG, the metabolic fate of which is still to be completely clarified. However, it has been reported that HMG inhibits the cholesterol synthesis [7–9], and therefore HMG might

be a link between ketogenesis and cholesterolgenesis. Another physiological role which is conceivable for the enzyme is that of regulating the succinylCoA concentration when the tricarboxylic acid cycle function is impaired.

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