MULTIPLE MITOCHONDRIAL FORMS OF ACETOACETYL-CoA THIOLASE IN RAT LIVER: POSSIBLE REGULATORY ROLE IN KETOGENESIS.

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SUMMARY: Two mitochondrial forms of acetoacetyl-CoA thiolases (A and B) have been obtained from rat liver. A kinetic analysis of thiolytic activities revealed for both enzymes marked inhibition by the substrate acetoacetyl-CoA and negative cooperativity with respect to CoA. In the reverse reaction (condensation) different kinetic properties were evident: The affinity of enzyme A for acetyl-CoA is lowered by acetoacetyl-CoA. A possible allosteric control of enzyme A by acetoacetyl-CoA is deduced from negative cooperativity with respect to binding of acetyl-CoA, depending on the concentrations of acetoacetyl-CoA. On the contrary, enzyme B shows simple Michaelis-Menten kinetics with acetyl-CoA that are not altered by acetoacetyl-CoA. A possible regulatory role of acetoacetyl-CoA thiolase A in ketogenesis is deduced from the above data.

In metabolic states with increased rates of ketogenesis nearly all the acetoacetate is formed from the acetyl-CoA pool (1,2). In view of an insufficient correlation between acetyl-CoA levels and ketone body production in vivo (3) and in vitro (4), in addition to already known control points of ketogenesis a control between acetyl-CoA and acetoacetate has also been proposed. The close relation between [^{14}C]carbonyl/[^{14}C]carboxyl ratios of acetoacetate formed from [1-^{14}C]fatty acids and the rates of ketone body production in vivo and in vitro (1,2) locates this control point at the level acetyl-CoA/acetoacetyl-CoA. In order to learn more about the mechanism of control at

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this level, various mitochondrial thiolases specific for acetoacetyl-CoA and long-chain ß-ketoacyl-CoA were separated into individual fractions. Preliminary results on the catalytic and regulatory properties of two mitochondrial acetoacetyl-CoA thiolases are reported in this communication.

MATERIALS AND METHODS

Male Albino rats of the Sprague-Dawley strain, fed on rat pellets (Altromin GmbH, Lage/Lippe, Germany) and weighing 150-220 g were used for the studies. The heavy mitochondrial fraction of rat liver were isolated in 0.25 M sucrose according to de Duve et al. (5). The acetone dry powder of the heavy mitochondrial fraction was prepared according to Dahlen et al. (6). Acetoacetyl-CoA, trans-2,3-enoyl-CoA and [1-^{14}C] acetyl-CoA were prepared as described by Huth et al. (2).

In the course of purification, ß-ketoacyl-CoA thiolase activities were assayed according to Seubert et al. (7). The assay system for acetoacetyl-CoA thiolase contained in 1 ml: 100 mM Tris-HCl buffer, pH 8.9; 5 mM $MgSO_4$; 0.5 mg serum albumin; 25 µM acetoacetyl-CoA; 0.25 mM CoA. The following extinction coefficients of ß-ketoacyl-CoA compounds are valid for the above experimental conditions: C_4 = 12.98 [$cm^2 \cdot \mu Mol^{-1}$]; C_8 = 8.85 [$cm^2 \cdot \mu Mol^{-1}$] (7).

The affinity of acetoacetyl-CoA thiolase for CoA and acetoacetyl-CoA in the thiolytic reaction was assayed in the isotope assay according to Huth et al. (2). Reaction mixtures contained in 0.5 ml: 100 mM Tris-HCl buffer, pH 8.1; 48 nmoles D,L[4-^{14}C] aspartate (sp act 2.11 mCi/mmole); 1 mM α-oxoglutarate; 0.36 U aspartate amino transferase; 0.14 U citrate synthase; CoA and acetoacetyl-CoA as indicated in the figures. Incubation period 1 min.

The affinity towards acetyl-CoA in the condensation reaction was measured in the isotope exchange assay according to Huth et al. (2). Reaction mixtures contained in 1 ml: 100 mM Tris-

HCl buffer, pH 8.1; acetoacetyl-CoA and [1-^{14}C] acetyl-CoA (sp act 4.32 µCi/µMol) as indicated in the figures.

RESULTS

In Fig. 1 the separation of mitochondrial thiolases into indi-

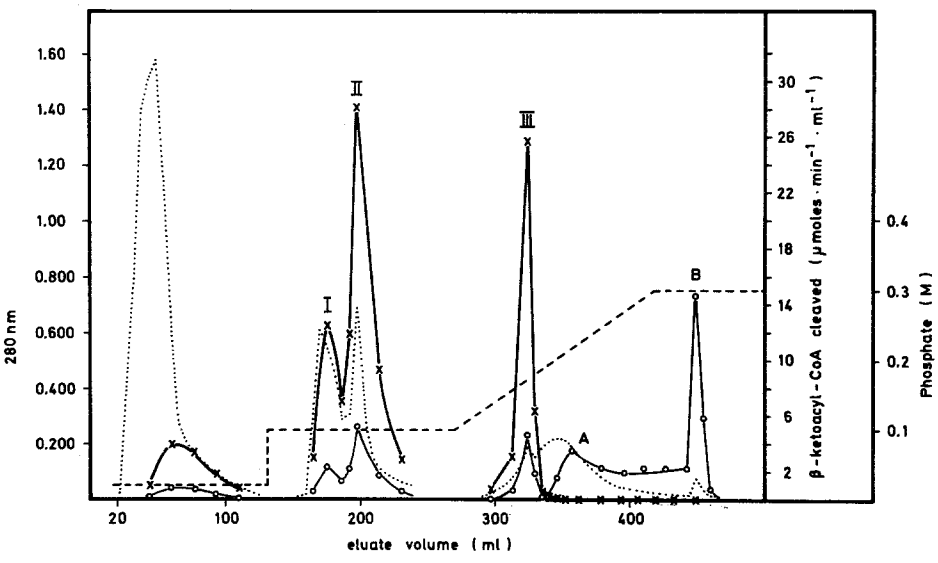

Fig. 1. Resolution of ketoacyl-CoA thiolases by chromatography on cellulose phosphate.

The extract of 12 g acetone dry powder of heavy mitochondria from rat liver (sp act 0.48 U/mg) was fractionated with solid ammonium sulfate between 70 and 85 % saturation. The precipitate was adjusted after dialysis against 20 mM potassium phosphate buffer, pH 7.4, containing 1 mM EDTA and 1 mM ß-mercaptoethanol to pH 6.6. 133 mg protein were applied to a 1.5 x 30 cm cellulose phosphate column equilibrated with 0.02 M potassium phosphate buffer pH 6.6. The column was washed with potassium phosphate buffer, pH 6.6, of increasing concentrations as illustrated in the figure. •••• Absorbance at 280 nm, o——o Distribution of activity assayed with acetoacetyl-CoA, x——x Distribution of activity assayed with ß-ketooctanoyl-CoA, ---- Concentration of potassium phosphate buffer pH 6.6.

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vidual fractions by chromatography on cellulose phosphate is illustrated. Three fractions (I, II, III) show activities with both acetoacetyl-CoA (open circles) and long-chain ß-ketoacyl-CoA (daggers). Two additional fractions A (sp act 26.6 U/mg)

and B (sp act 56.5 U/mg), eluted at high phosphate concentrations, show restriction to acetoacetyl-CoA. The purification effects for A and B are about 200-fold and 480-fold, respectively, when compared with crude extracts of liver. Separation of mitochondrial thiolases into 4 different components has also been reported by Middleton (8).

In view of a possible regulation of ketogenesis at the level of acetyl-CoA/acetoacetyl-CoA deduced from former investigations (2) preference was given to a study of the catalytic and regulatory properties of enzymes A and B (Fig. 1), because of their pronounced specificity for acetoacetyl-CoA. In the thiolytic direction both enzymes show high affinities to acetoacetyl-CoA with the following K_m-values: Enzyme A = 6 μM; enzyme B = 4 μM. At higher concentration of acetoacetyl-CoA (enzyme A 15 μM; enzyme B 10 μM) marked inhibition is apparent (Fig. 2a). With respect to binding of CoA a deviation from a simple Michaelis-Menten kinetic is obvious (Fig. 2b). Interference of the binding of CoA with acetoacetyl-CoA as the cause of this biphasic relationship (Fig. 2b) is evident from Fig. 3. Thus a divergence from a hyperbolic relationship (Fig. 3a, curve I, acetoacetyl-CoA 5 μM, $K_{m\ CoA}$= 22 μM) is only visible at concentrations of acetoacetyl-CoA > 5 μM (Fig. 3a, curves II and III). In accordance with Levitzky and Koshland (9) the reciprocal plots (Fig. 3a) and the Hill plots (Fig. 3b) of curves II and III show a deviation from a straight line relationship. From the acetoacetyl-CoA dependent variability of the n-values (Fig. 3b), changes between negative and positive cooperativity (9), induced by the second ligand acetoacetyl-CoA, have to be deduced. With respect to the direction of condensation, a regulatory

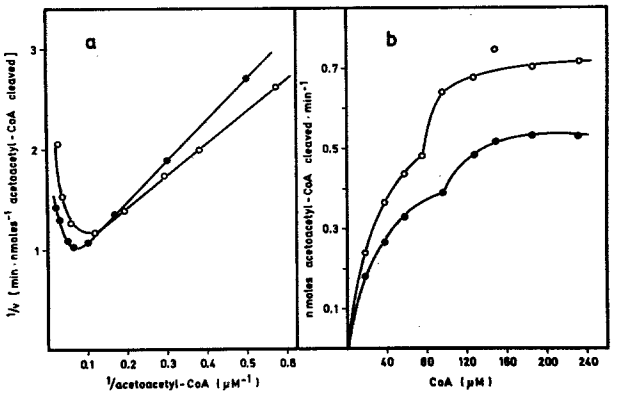

Fig. 2. Relation between thiolytic activities of acetoacetyl-CoA thiolase A (●——●) and B (o——o) and substrate concentrations.

1 munit of enzyme A (sp act 11.5 U/mg) and enzyme B (sp act 56.4 U/mg). (a) Reciprocal velocities as function of reciprocal acetoacetyl-CoA concentrations. Concentration of CoA: 1oo μM. (b) Velocities as function of CoA concentration (acetoacetyl-CoA: 15 μM and 1o μM for enzyme A and B, respectively).

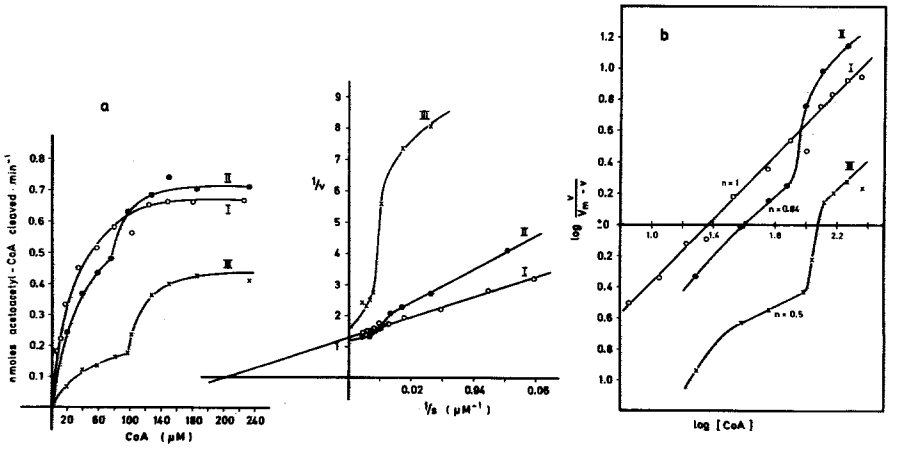

Fig. 3. Relation between thiolytic activities of acetoacetyl-CoA thiolase B and coenzyme A at various fixed aceto-acetyl-CoA concentrations.

1 munit enzyme (sp act 56.4 U/mg), acetoacetyl-CoA concentrations: o——o 5 μM, o——o 1o μM, x——x 3o μM, (a) Velocities as function of CoA concentration and plot of reciprocal data, (b) Hill plot of data from (a).

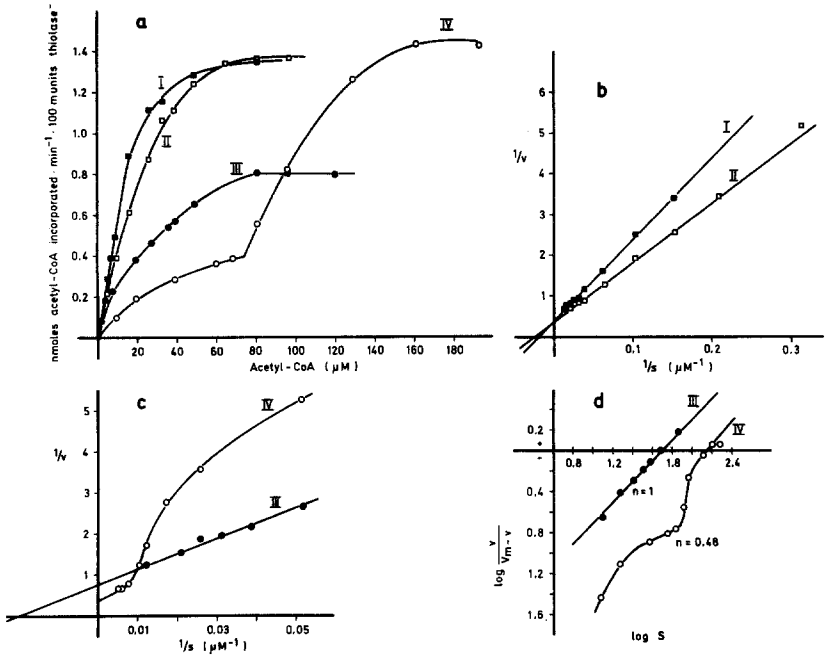

Fig. 4. Relation between acetyl-CoA concentration and activities of acetoacetyl-CoA thiolase A and B in the direction of condensation at various acetoacetyl-CoA concentrations.

60 munit of enzyme A (sp act 26.6 U/mg) and B (sp act 56.5 U/mg); o——o enzyme A, 15 μM acetoacetyl-CoA; ●——● enzyme A, 5 μM acetoacetyl-CoA; □——□ enzyme B, 10 μM acetoacetyl-CoA; ■——■ enzyme B, 5 μM acetoacetyl-CoA; (a) Velocities as a function of acetyl-CoA concentration, (b) Lineweaver-Burk plot for data of enzyme B, (c) Lineweaver Burk plot for data of enzyme A, (d) Hill plot from data of enzyme A.

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role of acetoacetyl-CoA in the conversion of acetyl-CoA to acetoacetyl-CoA is only evident with enzyme A (Fig. 4). While enzyme B shows almost unchanged affinities for acetyl-CoA (K_m = 49-42 μM) at different acetoacetyl-CoA concentrations (Fig. 4 a, b, curves I and II) enzyme A shows negative cooperativity with respect to binding of acetyl-CoA at acetoacetyl-CoA concentrations of 15 μM (Fig. 4 a,c,d, curve IV) and simple Michaelis-Menten kinetics at 5 μM acetoacetyl-CoA (curve III) with an apparent K_m for acetyl-CoA of 50 μM. For a calculation of the

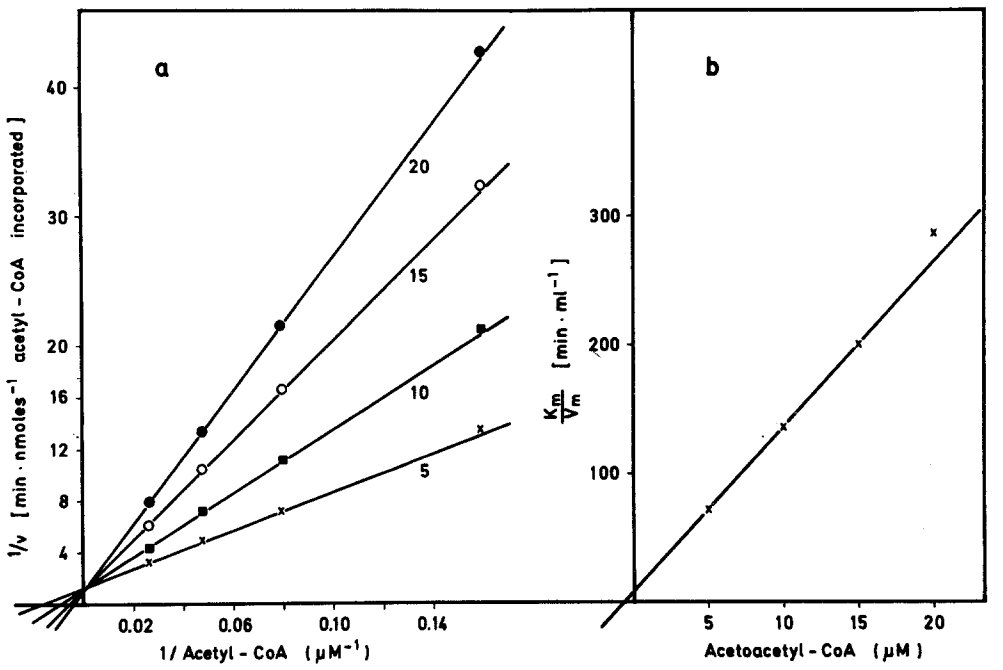

Fig. 5. Extrapolation of the affinity of the acetoacetyl-CoA thiolase A to acetyl-CoA in absence of acetoacetyl-CoA.

Enzyme: 42 munit (sp act 26.6 U/mg), (a) Lineweaver-Burk plots at different concentrations of acetoacetyl-CoA (μM) as indicated, in a concentration range of 6-4o μM acetyl-CoA. Apparent K_m-values for acetyl-CoA at the given acetoacetyl-CoA concentrations: 55 μM, 1o5 μM, 154 μM and 222 μM. (b) Replot of slopes vs. the different fixed acetoacetyl-CoA concentrations.

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real affinity of enzyme A for acetyl-CoA, the ratios K_m/V_{max} for different apparent K_m-values, obtained at various fixed concentrations of acetoacetyl-CoA in the range between 6 to 4o μM acetyl-CoA (Fig. 5a), were plotted against acetoacetyl-CoA concentrations (Fig. 5b). From the intercept of this straight line with the ordinate (Fig. 5 b) a K_m of 6.2 μM for acetyl-CoA was extrapolated. Whether the regulatory properties of enzyme A, evident at higher acetyl-CoA concentrations (Fig. 4 a,c,d, curve IV) are the result of variations of the intrinsic binding constants, catalytic constants or the number of binding sites as deduced by Teipel and Koshland (1o) from theoretical models,

will be the subject of further investigations.

DISCUSSION

According to Williamson (see discussion in 11) the mitochondrial concentration of acetoacetyl-CoA may range from 4 to 50 μM, dependent on an uniform distribution within the cell and an exclusive location in the mitochondria, respectively. Whatever the real mitochondrial acetoacetyl-CoA concentration is, the above range would be sufficient to exert a negative feedback on acetoacetyl-CoA synthesis by enzyme A (K_i = 1,4 μM). These regulatory properties of acetoacetyl-CoA thiolase A and its extremely high affinity towards acetyl-CoA (K_m = 6.2 μM) favor this enzyme to be the regulator of ketogenesis at the level acetyl-CoA/acetoacetyl-CoA as deduced from former studies (1,2). Negative and positive cooperativity observed at higher concentrations of acetoacetyl-CoA (Fig. 4) may represent an additional regulatory property allowing exceeding rates of ketogenesis at pathological situations (high levels of acetyl-CoA and acetoacetyl-CoA). Its physiological significance, however, still has to be analyzed. Whether acetoacetyl-CoA thiolase B takes part in the oxidative degradation of fatty acids or represents a b-form of interconvertable enzymes involved in ketogenesis, is subject of current investigations in our laboratory.

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