Some Properties of 3-Hydroxy-3-methylglutaryl-Coenzyme A Synthase from Ox Liver

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Mitochondrial 3-hydroxy-3-methylglutaryl-CoA synthase (EC 4.1.3.5) was purified from ox liver, and obtained essentially free from 3-oxoacyl-CoA thiolases. The kinetic behaviour, like that of the synthases from chicken liver and yeast, is compatible with a reaction pathway involving condensation of an acetyl-enzyme with acetoacetyl-CoA. The K_m for acetoacetyl-CoA, less than $1\ \mu\text{M}$ at pH 7.8, may possibly be low enough to permit rapid ketogenesis under physiological conditions without the need for a binary complex between the synthase and oxoacyl-CoA thiolase.

Although HMG-CoA synthase has often been assumed to be the rate-limiting enzyme of ketogenesis in mammalian liver very little is known about the enzyme from this tissue. Two distinct types of HMG-CoA synthase, mitochondrial and cytoplasmic, have been purified from chicken liver (Clinkenbeard *et al.*, 1975b; Reed *et al.*, 1975); the former is supposed to be involved in the synthesis of acetoacetate and the latter in that of mevalonate (Clinkenbeard *et al.*, 1975a). The HMG-CoA synthase of yeast has also been extensively studied (Middleton, 1972; Middleton & Tubbs, 1972, 1974).

Kinetic and chemical experiments with the yeast and chicken liver synthases have suggested that the formation of 3-hydroxy-3-methylglutaryl-CoA proceeds by the stepwise mechanism in Scheme 1.

This scheme involves binding of acetyl-CoA (step 1); acetylation (step 2) of the enzyme as a thioester (Middleton & Tubbs, 1974; Miziorko *et al.*, 1975); binding of acetoacetyl-CoA (step 3); reaction (step 4) of the acetyl-enzyme with acetoacetyl-CoA to yield a 1,5-dithioester of 3-hydroxy-3-methylglutarate (Miziorko *et al.*, 1976); and finally hydrolysis (step 5) of this to liberate 3-hydroxy-3-methylglutaryl-CoA and enzyme. The kinetics of the synthase are of interest in view of the extremely low acetoacetyl-CoA concentrations that may exist *in vivo* as a consequence of the very unfavourable equilibrium of the acetoacetyl-CoA thiolase reaction (Wakil, 1963). It has been suggested (Greville & Tubbs, 1968) that a thiolase-synthase binary complex, with direct 'channelling' of acetoacetyl-CoA between the two enzymes, may exist. We report here some kinetic properties of the HMG-CoA synthase of ox liver mitochondria; these are in agreement with the reaction pathway in Scheme 1 and confirm a very high affinity for acetoacetyl-CoA.

Abbreviation used: HMG-CoA synthase, 3-hydroxy-3-methylglutaryl-CoA synthase (EC 4.1.3.5).

Methods

Enzyme purification

The synthase was usually prepared from frozen whole ox liver, but the same purification procedure was also effective starting with frozen mitochondria. The liver was homogenized with 3 vol. of cold deionized water, centrifuged at 15000g for 20 min, and the supernatant fractionated at 0–5°C with $(\text{NH}_4)_2\text{SO}_4$. The protein that was precipitated between 225 and 355 g of $(\text{NH}_4)_2\text{SO}_4$ /litre was dissolved in 10 mM-potassium phosphate (pH 7.5) containing 0.5 mM-dithiothreitol, and the solution was desalted by passage through a large column of Sephadex G-25 equilibrated with this buffer. Glycerol was then added to 25% (v/v), and together with the dithiothreitol was present in all potassium phosphate buffers used in later steps, which were carried out at pH 7.5 and (except for the ultrafiltration) at room temperature. The solution was passed through a column of DEAE-cellulose equilibrated with the above buffer containing 25% (v/v) glycerol. The synthase was slightly retarded by the column, and emerged after most of the haemoglobin. The active fractions were applied to a column of cellulose phosphate equilibrated as above, and a linear gradient of phosphate (10–150 mM) was applied. The synthase was normally eluted at about 70 mM-phosphate, but with some batches of cellulose phosphate a higher concentration was required.

The fractions containing HMG-CoA synthase were pooled and concentrated at 4°C by ultrafiltration through an Amicon Diaflo PM10 membrane (Amicon, Lexington, MA 02173, U.S.A.). Further purification was achieved by passage through two columns (see below) of Sephadex G-150 equilibrated with 50 mM-phosphate containing 0.5 mM-dithiothreitol and 15% glycerol. The enzyme was stored at –20°C after addition of glycerol to 30% (v/v).

Scheme 1. Formation of 3-hydroxy-3-methylglutaryl-CoA by a stepwise mechanism

Abbreviations: E, enzyme; AcCoA, acetyl-CoA; AcAcCoA, acetoacetyl-CoA; HMGCoA, hydroxymethylglutaryl-CoA.

This procedure is quite similar to that for the purification of the chicken liver mitochondrial HMG-CoA synthase (Reed *et al.*, 1975). The main consideration was the separation of the synthase from the several types of 3-oxoacyl-CoA thiolase (Middleton, 1973). The cytoplasmic and mitochondrial thiolases specific for acetoacetyl-CoA were removed by the DEAE-cellulose and cellulose phosphate columns respectively. The mitochondrial general-specificity thiolase exists in at least two forms (Middleton, 1974), of which one is like the synthase in its elution behaviour from cellulose phosphate but emerges rather earlier from Sephadex G-150. The apparent molecular weights of this thiolase and of the synthase, deduced from their gel-filtration behaviour, were about 125000 and 88000 respectively, and synthase free of thiolase activity was obtained by passing 1.5 ml of enzyme concentrated as above through two columns of Sephadex G-150, each 1 m long and 2.5 cm in diameter, mounted in series.

HMG-CoA synthase assay

The assay system contained 100 mM-Tris, 10 mM-MgCl₂ and 50 mM-KCl; the pH was adjusted with HCl to the desired values at 30°C. Acetoacetyl-CoA and enzyme were added, the blank rate was observed, and the reaction started with acetyl-CoA. Disappearance of acetoacetyl-CoA was followed at 303 nm. The specific activity of the purified enzyme with 100 μM-acetyl-CoA and 5 μM-acetoacetyl-CoA at pH 7.8 was about 3 μmol of 3-hydroxy-3-methylglutaryl-CoA synthesized/min per mg of protein (Fig. 1a).

Substrates

Acetyl-CoA and acetoacetyl-CoA were prepared by treating CoA with acetic anhydride or diketene; acetoacetyl-CoA was purified by chromatography on DEAE-cellulose at pH 2.7 (Middleton, 1972). The absorption coefficients at 303 nm for acetoacetyl-CoA utilization are 11×10^3 and 21.6×10^3 litre·mol⁻¹·cm⁻¹ at pH 7.8 and 8.7 respectively, under the conditions used for the kinetic experiments.

Results and Discussion

Fig. 1(a) shows, in double-reciprocal form, the effects of varying the substrate concentrations on the reaction rate at pH 7.8. The results show that the K_m values for acetoacetyl-CoA are exceedingly low (below 1 μM), and that at high concentrations (over 10 μM) this substrate is inhibitory; similar behaviour is found with the chicken liver enzymes (Clinkenbeard *et al.*, 1975b; Reed *et al.*, 1975). The lines representing different acetyl-CoA concentrations are apparently parallel, indicative of Ping Pong (Cleland, 1963) behaviour; this suggests that the first product (presumably CoA) leaves the enzyme before the second substrate (acetoacetyl-CoA) binds. The data in Fig. 1(a) also show that the K_m value for acetyl-CoA is very high (over 100 μM), even when the concentration of acetoacetyl-CoA is low; again the chicken enzymes are similar in behaviour (Clinkenbeard *et al.*, 1975b; Reed *et al.*, 1975).

The determination of the K_m for acetoacetyl-CoA at pH 7.8 is difficult, since it is very small compared with experimentally convenient concentrations. The slopes in Fig. 1(a) (essentially zero) are equal to the ratio $K_m^{\text{acetoacetyl-CoA}}/V_{\text{max}}$; similarly, if the v^{-1} values are plotted against $[\text{acetyl-CoA}]^{-1}$ the resulting slope is $K_m^{\text{acetyl-CoA}}/V_{\text{max}}$. Comparison of these ratios showed that at pH 7.8 $K_m^{\text{acetoacetyl-CoA}}$ is less than one five-hundredth of $K_m^{\text{acetyl-CoA}}$.

At pH 8.7 the K_m values for acetoacetyl-CoA are considerably higher than at pH 7.8, so that the observed rates are not independent of this substrate's concentration (Fig. 1b); at this pH the K_m for acetoacetyl-CoA is only about one-hundredth that for acetyl-CoA. The fact that the lines in Fig. 1(b) appear parallel again suggests Ping Pong kinetic behaviour. As discussed by Middleton (1974) and Tsopanakis & Herries (1975), the use of mixtures in each of which the concentration of the second substrate is in a fixed ratio to that of the first can reveal the presence of a modified enzyme intermediate (in the present case probably acetyl-enzyme). If such an intermediate occurs the double-reciprocal plots will be linear, whereas they will be parabolic if the intermediate is kinetically insignificant; this method avoids the difficulty of deciding whether plots such as those in

Fig. 1. Effect of different substrate concentrations on 3-hydroxy-3-methylglutaryl-CoA synthase activity: (a) at pH 7.8, (b) at pH 8.7. The assay method is described in the text.

Fig. 2. 3-Hydroxy-3-methylglutaryl-CoA synthase activity as a function of the acetoacetyl-CoA in substrate mixtures in which the molar ratios (n) of acetyl-CoA/acetoacetyl-CoA were as shown: (a) at pH 7.7, (b) at pH 8.7.

Figs. 1(a) and 1(b) are truly parallel or gradually converging. The results of such mixture experiments at pH 7.8 and 8.7 are shown in Figs. 2(a) and 2(b). It seems that at pH 7.8 the proposed acetyl-enzyme does exist to a significant extent, but the curves in Fig. 2(b) show that it is very transient at pH 8.7. Presumably this difference is due to an effect of pH on the relative rates of steps 2 and 4 (acetylation and

condensation) in Scheme 1. The results in Fig. 2(a) correspond to Case III of Middleton (1974), in which only one substrate is inhibitory, and the intercept shows that the inhibition by acetoacetyl-CoA has a K_i value of about $12 \mu\text{M}$.

The results suggest that the mechanism of the HMG-CoA synthase from ox liver mitochondria is similar to that of the yeast (Middleton, 1972;

Middleton & Tubbs, 1972) and chicken liver enzymes (Clinkenbeard *et al.*, 1975a,b; Reed *et al.*, 1975), and may be represented by Scheme 1.

As with those other enzymes, the ox liver synthase can be separated from the 3-oxoacyl-CoA thiolases. A thiolase-synthase complex may exist, which could overcome the difficulty of achieving rapid ketogenesis in spite of the presumed extremely low acetoacetyl-CoA concentration *in vivo*, but if so it is readily disrupted. The very low K_m for acetoacetyl-CoA (less than $1\mu\text{M}$ at pH 7.8, and probably still lower at neutral pH) shown by the synthase and the lack of inhibition by acetyl-CoA are in accord with the physiological situation, and may render a binary enzyme complex unnecessary. The properties of the synthase and of the thiolase reaction (Middleton, 1974; Huth *et al.*, 1975) are such that the rate of formation of 3-hydroxy-3-methylglutaryl-CoA, and hence of acetoacetate, should vary as a sensitive function of the acetylation state of the mitochondrial CoA; this is indeed observed (Greville & Tubbs, 1968; Bremer & Aas, 1969). Free CoA strongly inhibits the yeast HMG-CoA synthase (Middleton, 1972), and the amount of free CoA in liver is decreased by starvation, diabetes or a fatty diet, conditions which are accompanied by rapid acetoacetate synthesis. Reed *et al.* (1975) did not observe inhibition of the chicken liver mitochondrial synthase by CoA, but were using a high acetoacetyl-CoA concentration ($50\mu\text{M}$). Inhibition of HMG-CoA synthase (and also of thiolase; Huth *et al.*, 1975) by free CoA may be metabolically important in mammalian liver, but

remains to be studied by using the enzyme uncontaminated by thiolase.

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