THE EXISTENCE OF KETOACYL-CoA THIOLASES OF DIFFERING PROPERTIES AND INTRACELLULAR LOCALIZATION IN OX LIVER

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Summary. Ox liver contains at least four different ketoacyl-CoA thiolases separable by ion exchange chromatography and isoelectric focusing. The cytoplasm contains an acetoacetyl-CoA-specific thiolase with a pI of 5.2 accounting for 14% of the thiolase activity of liver extracts. Mitochondria contain another acetoacetyl-CoA-specific thiolase making up 46% of the liver thiolase activity, which has a pI of 8.0 and a requirement for K^+ with a Ka of 2.3 mM. Ox liver mitochondria also contain two ketoacyl-CoA thiolases of more general specificity towards ketoacyl-CoA substrates and with pIs of 5.7 and 6.7. Together these two thiolases comprise about 40% of the thiolase activity of liver extracts with acetoacetyl-CoA as substrate.

Ketoacyl-CoA thiolase activity is widely distributed among mammalian tissues (1) and two types of mammalian thiolase have been purified: acetoacetyl-CoA thiolase (EC 2.3.1.9) from pig heart (2) and a general ketoacyl-CoA thiolase (EC 2.3.1.16) from ox liver (3). This paper describes the enzymic properties and intracellular location of various ketoacyl-CoA thiolases of ox liver.

MATERIALS AND METHODS

Liver extracts were prepared by blending the twice frozen-thawed tissue at 0° with 4 volumes of 50 mM Na phosphate pH 7.2 at maximum power using a Polytron overhead blender (Kinematica GmbH, Lucerne, Switzerland). Triton X-100 was then added to 0.5% (v/v) final concentration and the homogenate was centrifuged at 100,000 g for 30 min.

Mitochondria and cytoplasmic fractions of fresh ox liver were

prepared in 0.3 M sucrose by conventional differential centrifugation. Mitochondrial extracts were prepared as above.

Ketoacyl-CoA thiolase activity was determined at 30° by following the decrease in extinction at 303 nm in an assay system containing 100 mM tris-HCl (pH 8.2), 25 mM $MgCl_2$, 50 mM KCl, 10 μM 3-ketoacyl-CoA, and 50 μM CoA in a total of 2.0 ml. K^+ activation was determined by substituting an equal concentration of NaCl for the KCl. The rates of ketoacyl-CoA hydrolysis in the absence of added CoA were <0.1% of the measured thiolase rates. Under these assay conditions the apparent millimolar extinction coefficients of ketoacyl-CoA compounds were: C4[1], 20; C6, 17.9; and C10, 15.

Ketoacyl-CoA substrates, with the exception of the C4 compound (prepared from diketene and CoA (4)), were synthesised by the method of Vagelos and Alberts (5). These substrates were prepared in Na^+ media and were assayed by the method of Decker (6).

Isoelectric focusing was performed in a LKB-8101 column at 15° using Ampholine pH 3-10 in a 20-60% (v/v) glycerol gradient.

RESULTS

Ox liver extracts prepared as above were assayed with C4- and C6-CoA substrates. With the C4 substrate the tissue activity was 39.7 μmol/min/g. wet weight and the specific activity was 0.25 μmol/min/mg soluble protein. In addition the activity with the C4 (but not the C6) substrate was stimulated 1.7-fold by K^+. With the C6 substrate the tissue activity was 75.6 μmol/min/g. wet weight and the specific activity was 0.48 μmol/min/mg. The thiolase activities in cytoplasm and mitochondria after subcellular fractionation are shown in Table 1. The properties of the enzymes in the two fractions

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[1] Abbreviations: C4, acetoacetyl; C6, 3-oxohexanoyl; C10, 3-oxodecanoyl.

Table 1

Ketoacyl-CoA thiolase activities of ox liver fractions.

Fraction	Specific activity[1] with different ketoacyl-CoA substrates		Ratio[2] $\frac{C6\text{-CoA rate}}{C4\text{-CoA rate}}$	Ratio $\frac{C4\text{-CoA rate with } K^+}{C4\text{-CoA rate with } Na^+}$
	C4-CoA	C6-CoA		
Cytoplasm	72.5	48.7	0.67	1.1
Mitochondria	307	632	2.06	1.62

1. nmol/min/mg, measured under standard assay conditions.
2. In the presence of 50 mM K^+.

appear to differ, that in the cytoplasm having a lower C6-CoA/C4-CoA activity ratio and a lower K^+ activation than the mitochondrial thiolase(s). This suggests that activity seen in the cytoplasm represents a cytoplasmic thiolase and is not due to mitochondrial leakage.

Separation of thiolases by isoelectric focusing. This technique was applied to cytoplasm and to mitochondrial extracts. Fig. 1a shows that mitochondrial thiolases were separated into three major peaks with pIs of 5.7, 6.7 and 7.9. Only the thiolase with pI of 7.9 is activated by K^+. All these thiolases used C4-CoA as a substrate but C6-CoA was a substrate only for the peaks at pH 5.7 and 6.7. The relative specificity (C6-CoA rate/C4-CoA rate) of these enzymes was about 5:1 from pH 5.2 - 6.5 but fell at higher pH values due to the presence of C4-CoA specific thiolase. As shown below, purification on cellulose phosphate gave complete separation from the latter and shows that these two thiolases of pIs 5.7 and 6.7 have identical specificities. Thus ox liver mitochondria contain a thiolase of pI 7.9, specific for C4-CoA and activated by K^+, and two ketoacyl-CoA thiolases with pIs of 5.7 and 6.7 of more general specificity.

Cytoplasmic thiolase (Fig. 1b) gave one peak of C4-CoA activity specific for C4-CoA only and with a pI of 5.2. Contamination by mito-

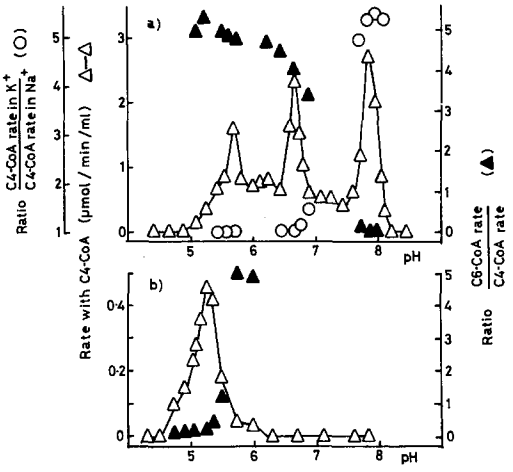

Fig. 1. Isoelectric focusing of ketoacyl-CoA thiolases from a) 100,000 g supernatant of mitochondria and b) cytoplasm.

chondrial thiolase was shown by the presence of a small amount of general specificity enzyme focusing at pH 5.7. The cytoplasmic C4-CoA-specific thiolase, apart from its low pI, differed from the mitochondrial enzyme of similar specificity by the lack of any K^+ activation.

Although isoelectric focusing gives good recovery (>90%) of mitochondrial thiolases, the cytoplasmic thiolase regularly loses 35-40% of its activity during the separation. Therefore, the following ion exchange separation techniques have been developed.

Separation and purification of individual ox liver thiolases by ion exchange chromatography. The cytoplasmic C4-CoA-specific thiolase can be separated and purified on DEAE-cellulose. Ox liver cytoplasm (800 mg soluble protein), equilibrated with 100 mM tris-HCl pH 8.2, containing 20% (v/v) glycerol and 0.5 mM dithiothreitol, was applied to a 6 cm x 3 cm DEAE-cellulose column equilibrated with the same buffer. A linear gradient of tris-HCl pH 8.2 was then applied. Fig. 2a shows that two peaks of activity were observed. Peak I, a minor unretarded peak, had a C6-CoA/C4-CoA rate ratio of 3.7:1 whilst peak II, eluted by

the gradient, was absolutely specific for C4-CoA and was not stimulated by K^+. Peak II thiolase is therefore identical to the enzyme of pI 5.2. This technique gave complete recovery of thiolase activity and purified the cytoplasmic enzyme to a specific activity of 0.75 µmol/min/mg. The technique was applied to an ox liver extract as shown in Fig. 2b. Peak II, representing 14% of the whole tissue thiolase activity with C4-CoA was identified as the cytoplasmic enzyme by its elution behaviour, its absolute specificity for C4-CoA and its lack of stimulation by K^+. The unretarded peak I resembled a mitochondrial extract in its specificity (C6-CoA rate/C4 CoA rate = 2.2) and its K^+ stimulation of 1.6-fold.

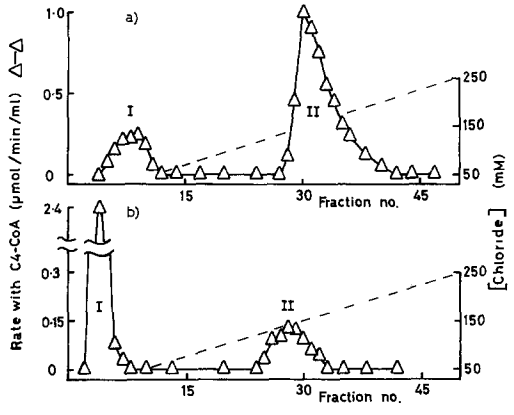

Fig. 2. Isolation of cytoplasmic acetoacetyl-CoA thiolase on DEAE-cellulose at pH 8.2 from a) cytoplasm and b) 100,000 g supernatant of liver homogenate.

The mitochondrial thiolases can be isolated from peak I. Such material (700 mg protein), after equilibration with 20 mM Na phosphate pH 6.6 containing 20% (v/v) glycerol was applied to a 6 cm x 3 cm cellulose phosphate column. Fig. 3 shows that elution with a linear gradient of phosphate at pH 6.6 gave two separate peaks on assay with C4-CoA. Peak I, comprising all the activity with C6-CoA, on isoelectric focusing gave two peaks at pH 5.7 and 6.7 plus a small peak at 6.3. The substrate specificities were identical (table 2). Peak I therefore contained the

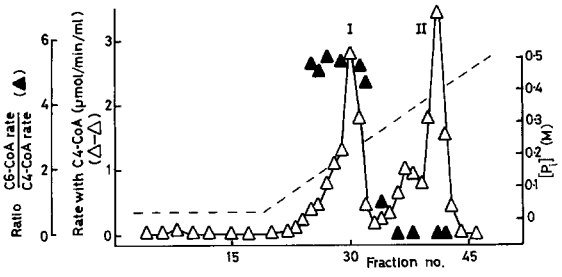

Fig. 3. Separation of ketoacyl-CoA thiolases from peak I Fig. 2b on cellulose phosphate at pH 6.6.

Table 2

Properties of ketoacyl-CoA thiolases isolated from ox liver.

pI	Relative rates[1] with different ketoacyl-CoA substrates	Effect of K^+	Ka^1 for K^+	app. Km^1 for C4-CoA	app. Km^1 for CoA	Location in liver
	C4 : C6 : C10	C4-CoA rate with K^+/rate with Na^+	(mM)	(μM)	(μM)	
5.2	1 : <.01 : <.01	1	-	20	7[2]	Cytoplasm
5.7	1 : 5 : 4.5	1	-	10	10	Mitochondria
6.7	1 : 4.9 : 4.5	1	-	10	10	Mitochondria
8.0	1 : <.01 : <.01	5.5	2.3	10[3]	29	Mitochondria

1. Measured under standard assay conditions, see text for details.
2. Shows marked substrate inhibition above 50 μM CoA.
3. Shows marked substrate inhibition above 50 μM C4-CoA.

mitochondrial general specificity thiolases. Peak II, which showed a small shoulder, was throughout absolutely specific for C4-CoA and was stimulated by K^+. Isoelectric focusing of the most active fractions of peak II gave a pI of 8.0 confirming that this was the mitochondrial C4-CoA specific thiolase.

Cellulose phosphate chromatography of these enzymes gave complete

recovery of activity and purified the general specificity thiolase 17-fold to a specific activity (with C6-CoA) of 7.9 μmol/min/mg. The C4-CoA-specific enzyme was purified to a specific activity of 12.4 μmol/min/mg giving a 53-fold purification over the liver extract activity.

These ion exchange separation techniques show that the observed ox liver thiolase activity with C4-CoA is made up as follows: cytoplasmic C4-CoA-specific thiolase, 14%; mitochondrial K^+-stimulated C4-CoA-specific thiolase, 46%; and the mitochondrial general specificity thiolases, 40%.

DISCUSSION

The properties of the purified ox liver thiolases are summarized in Table 2 together with apparent Km values for CoA and C4-CoA measured under standard assay conditions. The Ka for K^+ activation of the mitochondrial C4-CoA-specific thiolase is independent of Mg^{2+}. This activation is found in the C4-CoA-specific thiolases of other tissues (7) including pig heart and is being further investigated.

The thiolases with pIs of 5.7 and 6.7 are clearly identical in specificity and kinetic constants and they may be part of a family of isoenzymes. If this is so then the thiolase purified by Seubert et al (3), which resembles them in specificity and cell location, must be one of the isoenzymes.

The occurrence in ox liver (and in other mammalian and avian tissues (7)) of several ketoacyl-CoA thiolases, all of which use acetoacetyl-CoA, means that the use of this substrate, particularly when the K^+ concentration is undefined, cannot give an accurate measurement of the acetoacetyl-CoA-specific thiolase.

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