Acetyl-Coenzyme A Hydrolase, an Artifact?

THE CONVERSION OF ACETYL-COENZYME A INTO ACETATE BY THE COMBINED ACTION OF CARNITINE ACETYLTRANSFERASE AND ACETYLCARNITINE HYDROLASE

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1. The nature of the acetyl-CoA hydrolase (EC 3.1.2.1) reaction in rat and sheep liver homogenates was investigated. 2. The activity determined in an incubated system was 5.10 and 3.28 nmol/min per mg of protein for rat and sheep liver homogenate respectively. This activity was not affected by the addition of L-carnitine, but was decreased by the addition of D-carnitine. 3. No acetyl-CoA hydrolase activity could be detected in rat or sheep liver homogenates first treated with Sephadex G-25. This treatment decreased the carnitine concentrations of the homogenates to about one-twentieth. Subsequent addition of L-carnitine, but not D-carnitine, restored the apparent acetyl-CoA hydrolase activity. 4. Sephadex treatment did not affect acetyl-carnitine hydrolase activity of the homogenates, which was 5.8 and 8.1 nmol/min per mg of protein respectively for rat and sheep liver. 5. Direct spectrophotometric assay of acetyl-CoA hydrolase, based on the reaction of CoA released with 5,5'-dithiobis-(2-nitrobenzoic acid), clearly demonstrated that after Sephadex treatment no activity could be measured. 6. Carnitine acetyltransferase (EC 2.3.1.7) activity measured in the same assay system in response to added L-carnitine was very low in normal rat liver homogenates, owing to the apparent high acetyl-CoA hydrolase activity, but was increased markedly after Sephadex treatment. The V_{max} for this enzyme in rat liver homogenates was increased from 3.4 to 14.8 nmol/min per mg of protein whereas the K_m for L-carnitine was decreased from 936 to 32 μ M after Sephadex treatment. 7. Acetyl-CoA hydrolase activity could be demonstrated in disrupted rat liver mitochondria but not in separated outer or inner mitochondrial membrane fractions. Activity could be demonstrated after recombination of outer and inner mitochondrial membrane fractions. The outer mitochondrial membrane fraction showed acetylcarnitine hydrolase activity and the inner mitochondrial membrane fraction showed carnitine acetyltransferase activity. 8. The results presented here demonstrate that acetyl-CoA hydrolase activity in rat and sheep liver is an artifact and the activity is due to the combined activity of carnitine acetyltransferase and acetylcarnitine hydrolase.

The initial report describing the activity of an acetyl-CoA hydrolase in a pig heart preparation only implied the presence of such an enzyme (Gergely *et al.*, 1952), and on the basis of that report the enzyme was classified and numbered. The enzyme has been reported to be widely distributed in rat and sheep tissues (Knowles *et al.*, 1974) and in bovine tissues (Quraishi & Cook, 1972). In each case a high activity was reported in the liver with the activity predominantly localized in the mitochondria. Murthy & Steiner (1973) have claimed that, in rat-liver, this enzyme is associated with the inner mitochondrial membrane. It is difficult to conceive a physiological function of such an enzyme in the inner mitochondrial membrane since the well-characterized enzyme, carnitine acetyltransferase, is localized in this membrane (Edwards

et al., 1974). This latter enzyme has a K_m for acetyl-CoA of approx. 0.04 mM (Fritz *et al.*, 1963; Chase, 1967) whereas the K_m for acetyl-CoA of the acetyl-CoA hydrolase is approx. 0.7 mM (Knowles *et al.*, 1974), and the concentration of acetyl-CoA in rat liver or sheep liver is in the order of 0.04–0.08 mM (Allred & Guy, 1969; Snoswell & Henderson, 1970).

In our investigations on the acetylcarnitine hydrolase of rat and sheep liver (see the preceding paper, Costa & Snoswell, 1975) we observed that acetyl-CoA hydrolase was completely inhibited by low concentrations of bromoacetyl-L-carnitine (Costa & Snoswell, 1974), which is a potent inhibitor of carnitine acetyltransferase (Chase & Tubbs, 1969). This suggested that acetyl-CoA hydrolase activity might be due to the combined

action of carnitine acetyltransferase and acetyl-carnitine hydrolase. In the present work we have examined the nature of the acetyl-CoA hydrolase reaction and have found that in both sheep and rat liver the activity is due to a combination of carnitine acetyltransferase present in the inner mitochondrial membrane and acetylcarnitine hydrolase present in the outer mitochondrial membrane.

Experimental

Methods

Animals. The rats used were hooded Wistar females (150–200g) which had been fed on a pelleted rat diet (Charlicks, Adelaide, S. Austral., Australia).

The sheep used were 9-month-old Merino wethers which had been fed on a diet of lucerne-chaff.

The rats and the sheep were killed by severing the necks and samples of liver were collected into 0.25M-sucrose–23mM-potassium phosphate, pH 7.4.

Tissue preparation and homogenates. Homogenates (20%, w/v) were prepared in 0.025M-sucrose–2.3mM-potassium phosphate (pH 7.4)–0.1% Triton X-100 solution with a Potter–Elvehjem homogenizer. These homogenates were then centrifuged at 8000g for 3 min and the supernatants used for assay. In addition, 2ml fractions of supernatant were passed down a column (22.5cm × 1.5cm) of Sephadex G-25 (coarse) in 25mM-potassium phosphate, pH 7.4. The protein peak and total acid-soluble carnitine peak were monitored. Fractions at the beginning of the protein peak were taken for enzyme assay.

Subcellular fractions. Mitochondria were prepared from rat liver by the method described by Snoswell & Koundakjian (1972). The mitochondria were suspended in 0.44M-sucrose–10mM-triethanolamine (pH 7.6)–2mM-EDTA as described by Brdiczka *et al.* (1969). The method of Hoppel & Tomec (1972) was used to separate the submitochondrial fractions which were resuspended in 0.25M-sucrose–23mM-potassium phosphate, pH 7.4. The integrity of each fraction was established by using the marker enzymes as described in the preceding paper (Costa & Snoswell, 1975).

Enzyme assays. All assays were determined at 37°C by using the instrumentation described in the preceding paper (Costa & Snoswell, 1975).

Carnitine acetyltransferase. This was assayed in homogenates from sheep and rat liver both before and after treatment with Sephadex G-25, and in submitochondrial fractions from rat liver. The reaction was monitored spectrophotometrically at 412nm as described in the preceding paper (Costa & Snoswell, 1975). The kinetics of carnitine acetyltransferase before and after G-25 Sephadex treatment were investigated over the range of 2μM–4.5mM-L-carnitine. The data from these experiments

were fitted to Lineweaver–Burk plots by statistically weighting $1/v$ by the method described by Wilkinson (1961).

Acetylcarnitine hydrolase. This was assayed in homogenates from sheep and rat liver both before and after treatment with Sephadex G-25 and also in submitochondrial fractions derived from rat liver. The activity was determined by the method described in the preceding paper (Costa & Snoswell, 1975).

Acetyl-CoA hydrolase. This was assayed in homogenates from sheep and rat liver both before and after treatment with Sephadex G-25 and also in submitochondrial fractions derived from rat liver. The activity was assayed spectrophotometrically at 412nm. The assay contained 400mM-Tris-HCl (pH 8.2), 100μM-5,5'-dithiobis-(2-nitrobenzoic acid) and 100μM-acetyl-CoA and enzyme preparation in a total volume of 1.0ml. The 5,5'-dithiobis-(2-nitrobenzoic acid) was added first and the rate allowed to become linear before the addition of acetyl-CoA.

Also the activity was assayed in an incubated system similar to that described by Knowles *et al.* (1974). L- or D-Carnitine (3.3mM) was added to this system. The reaction was started by adding 4.0mM-acetyl-CoA.

Metabolite assays. Total acid-soluble carnitine was measured by the method of Pearson & Tubbs (1964) and free carnitine by the method of Marquis & Fritz (1964).

Acetyl-CoA and acetyl-CoA plus free CoA were measured by the kinetic method of Allred & Guy (1969); free CoA was determined by difference. In these kinetic determinations a standard curve was prepared on each occasion by using a CoA standard solution, the concentration of which was determined with phosphotransacetylase (EC 2.3.1.8) by the method of Michal & Bergmeyer (1963). However, as this method only assays reduced CoA, the standards were preincubated for 10min at 25°C with 2μl of 0.2M-dithiothreitol to ensure that all the CoA was in a reduced form.

Acetate was determined by the kinetic method of Knowles *et al.* (1974).

Protein was determined by the biuret method.

Chemicals

L-Carnitine and D-carnitine hydrochloride were generously supplied by Otsuka Pharmaceuticals, Osaka, Japan. CoA and acetyl-CoA were obtained from Calbiochem, Los Angeles, Calif., U.S.A., and enzymes from C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany. Acetyl-CoA was also prepared from free CoA and redistilled acetic anhydride by the method of Stadtman (1957).

Results

The results in Table 1 indicate that, after the passage of homogenates of both rat and sheep

Table 1. *Effect of stereoisomers of carnitine on acetyl-CoA hydrolase activity in normal and Sephadex G-25-treated homogenates of rat and sheep liver*

Homogenates from rat and sheep liver were prepared and assayed as described in the text. The values are means $\pm$ S.E.M. of three experiments. N.D., Not detectable.

Additions	Acetyl-CoA hydrolase activity in rat liver (nmol/min per mg of protein)		Acetyl-CoA hydrolase activity in sheep liver (nmol/min per mg of protein)	
	Normal	Sephadex G-25-treated	Normal	Sephadex G-25-treated
None	5.10 $\pm$ 0.98	N.D.	3.28 $\pm$ 0.28	N.D.
+L-Carnitine	4.87 $\pm$ 0.87	5.98 $\pm$ 1.26	3.27 $\pm$ 0.14	11.56 $\pm$ 1.26
+D-Carnitine	3.23 $\pm$ 1.00	N.D.	2.39 $\pm$ 0.15	N.D.

Fig. 1. *Spectrophotometric assay of acetyl-CoA hydrolase and carnitine acetyltransferase in normal and Sephadex-treated homogenates from rat liver*

Recording of E_{412} is shown; a downward deflexion represents an increase in absorbance. The reaction was started by adding 5,5'-dithiobis-(2-nitrobenzoic acid) and the recording shown was obtained after this initial reaction became linear. Homogenates were prepared and assayed as described in the text. Acetyl-CoA (0.1 mM) and L- or D-carnitine (3.3 mM) were added as indicated. (a) and (b) Normal homogenate. (c) Homogenate that had first been passed down a Sephadex G-25 column (1.5 cm $\times$ 22.5 cm) equilibrated with potassium phosphate (pH 7.4). The initial tubes of peak protein concentration were used.

liver down columns of Sephadex G-25, no acetyl-CoA hydrolase activity could be detected, whereas appreciable activity could be measured in untreated homogenates from both species. The total acid-soluble carnitine concentration in the homogenates initially was 40 and 24 nmol/ml for rat and sheep liver respectively, and this was decreased to approximately one-twentieth of these values by the Sephadex treatment (not shown in Table 1).

Addition of L-carnitine to untreated homogenates of either rat or sheep liver had no effect on the acetyl-CoA hydrolase activity but resulted in appreciable apparent acetyl-CoA hydrolase activity in the Sephadex-treated homogenates (Table 1). In fact the activity in the Sephadex-treated sheep liver homogenate supplemented with L-carnitine was considerably higher than activity in the untreated homogenate. The reason for this is not clear.

Addition of D-carnitine significantly ($P < 0.01$) decreased the activity of acetyl-CoA hydrolase in untreated homogenates of sheep liver and decreased the activity in rat liver homogenate but failed to result in any activity in the Sephadex-treated extracts, in marked contrast with the addition of L-carnitine (Table 1).

It should be emphasized that the acetylcarnitine hydrolase activity determined at the same pH, 8.2, as the acetyl-CoA hydrolase activity, was 5.8 and 8.1 nmol/min per mg of protein respectively for the rat and sheep liver homogenate. This activity remained after the extracts were treated with Sephadex. Also carnitine acetyltransferase activity was present after Sephadex treatment and was 14.8 and 22.2 nmol/min per mg respectively for the rat and sheep liver homogenate.

All the acetyl-CoA hydrolase activities shown in Table 1 were determined on the basis of acetate

and free CoA released in an incubated system; in each case there was a stoichiometric balance between acetyl-CoA utilized and acetate and free CoA formed. An alternative direct spectrophotometric assay of this enzyme activity, based on the development of a yellow complex absorbing at 412 nm formed by reaction of the free CoA released with 5,5'-dithiobis-(2-nitrobenzoic acid), is often used in the assay of this enzyme and demonstrates even more clearly some of the features shown in Table 1.

The results shown in Fig. 1 clearly show that there is no acetyl-CoA hydrolase activity in rat liver homogenates treated with Sephadex G-25 (compare Fig. 1c with 1a). Fig. 1(b) shows a much more dramatic inhibition of added D-carnitine on acetyl-CoA hydrolase activity with normal homogenates than is shown in Table 1, where the incubated assay system was used. In the spectrophotometric assay system the ratio of added D-carnitine to endogenous L-carnitine is considerably greater than in the incubated system.

The results shown in Fig. 1(a) indicate that in rat liver homogenates the activity of carnitine acetyltransferase appears to be very low owing to the apparent high rate of the acetyl-CoA hydrolase reaction which is deducted from the rate in the presence of L-carnitine. However, prior treatment of the rat liver homogenate with Sephadex abolished the apparent acetyl-CoA hydrolase activity and a substantial carnitine acetyltransferase activity was then observed (Fig. 1c). This effect of Sephadex treatment on carnitine acetyltransferase activity is illustrated in Fig. 2 which shows Lineweaver-Burk plots of computer-fitted experimental data. $V_{\max}$ was increased from 3.4 to

Fig. 2. Lineweaver-Burk plot for the carnitine acetyltransferase reaction in rat liver homogenate and for homogenate treated with Sephadex G-25

The Sephadex-treated homogenate prepared as in Fig. 1 was used. Carnitine acetyltransferase activity was determined as in Fig. 1. Acetyl-CoA concentration was 0.1 mM and L-carnitine concentration varied between 2 μ M and 4.5 mM. Experimental data were weighted for accuracy by the statistical method of Wilkinson (1961) by using an appropriate computer program. ●, Normal; ▲, Sephadex G-25-treated.

14.8 nmol/min per mg of protein after Sephadex treatment and the K_m for L-carnitine was substantially decreased from 936 to 32 μ M.

The results presented in Fig. 3 show the measurement of acetyl-CoA hydrolase activity in rat liver mitochondria. No acetyl-CoA hydrolase activity was detected in other subcellular fractions. Appreciable activity was measured in disrupted whole mitochondria (Fig. 3a); however, no activity could be detected in either the inner mitochondrial membrane

Fig. 3. Acetyl-CoA hydrolase activity in rat liver mitochondria and in inner and outer mitochondrial membrane fractions

Acetyl-CoA hydrolase was assayed as described in Fig. 1. Acetyl-CoA (0.1 mM) was added as indicated. (a) Disrupted rat liver mitochondria; (b) inner mitochondrial membrane fraction; (c) outer mitochondrial membrane fraction; (d) recombined inner and outer membrane fractions (1:1 ratio).

fraction (Fig. 3b) or the outer mitochondrial membrane fraction (Fig. 3c), but on recombination of these two membrane fractions acetyl-CoA hydrolase activity could again be detected (Fig. 3d). The inner mitochondrial membrane fraction showed appreciable carnitine acetyltransferase activity, whereas the outer membrane fraction showed acetylcarnitine hydrolase activity.

Discussion

The results presented here clearly demonstrate that in both rat and sheep liver the measurement of acetyl-CoA hydrolase activity depends on the presence of endogenous carnitine. When this was substantially removed by passage through a Sephadex G-25 column, no acetyl-CoA hydrolase activity was observed. Also addition of D-carnitine, a competitive inhibitor of carnitine acetyltransferase (Fritz & Schultz, 1965), decreased acetyl-CoA hydrolase activity in both rat and sheep liver homogenates, particularly in the spectrophotometric assay with normal rat liver homogenate where the ratio of D-carnitine to endogenous L-carnitine was greater than in the incubated enzyme assay system. On the other hand, when L-carnitine was added to homogenates from which endogenous L-carnitine was previously removed, then the apparent hydrolase activity reappeared. Finally, it was demonstrated that, although acetyl-CoA hydrolase activity could be measured in whole rat liver mitochondria, neither the outer membrane, which contained acetylcarnitine hydrolase (see preceding paper, Costa & Snoswell, 1975), nor the inner membrane, which contained carnitine acetyltransferase, showed any acetyl-CoA hydrolase. However, on combination of these two membrane fractions acetyl-CoA hydrolase activity could again be measured. These results then clearly show that, in sheep and rat liver, acetyl-CoA hydrolase is an artifact and the activity measured is due to the combined action of two enzymes, carnitine acetyltransferase present in the inner mitochondrial membrane and acetylcarnitine hydrolase present in the outer mitochondrial membrane. The sum of these two reactions does lead to an overall acetyl-CoA hydrolase reaction as indicated below:

This finding has a number of important implications. First, it explains the anomalous situation referred to in the introduction of having two enzymes, namely carnitine acetyltransferase and acetyl-CoA hydro-

lase, present in the inner mitochondrial membrane both using acetyl-CoA. Second, it provides a system whereby acetyl groups may be removed from the mitochondria as acetate in times of increased 'acetyl pressure'.

The other important finding arising from these studies is that use of the spectrophotometric method for the assay of carnitine acetyltransferase based on the reaction of CoA liberated with 5,5'-dithiobis-(2-nitrobenzoic acid) at 412nm, after adding L-carnitine, underestimates the activity of the enzyme. The initial rate of acetyl-CoA breakdown is attributed to acetyl-CoA hydrolase, but in fact is due to carnitine acetyltransferase reacting with endogenous carnitine. This is particularly so with rat liver. It has been assumed in the past that in this type of assay the small amount of endogenous carnitine present would be insufficient to allow any appreciable measurement of carnitine acetyltransferase. However, the results presented here indicate that the K_m of the rat liver enzyme for L-carnitine, namely $32\mu\text{M}$, is considerably less than $120\mu\text{M}$ reported for pigeon breast-muscle enzyme (Chase, 1967) or 0.31mM for pig heart enzyme (Fritz *et al.*, 1963). Thus previous reports of low carnitine acetyltransferase in rat liver from this (Snoswell & Henderson, 1970) and other laboratories (Barker *et al.*, 1968; Solberg *et al.*, 1972) appear to be underestimates. This also raises some doubts about various activities of carnitine acetyltransferase and short-chain transferases obtained with this assay procedure after various treatments, e.g. clofibrate treatment of rats (Solberg *et al.*, 1972). Slight variations in the concentrations of endogenous carnitine in the liver under various conditions could lead to considerable differences in carnitine acetyltransferase activity.

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