Carnitine Palmitoyltransferase Activities (EC 2.3.1.-) of Rat Liver Mitochondria

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1. A continuously recording and sensitive fluorimetric assay is described for carnitine palmitoyltransferase. This assay has been applied to whole or disintegrated mitochondria and to soluble protein fractions. 2. When rat liver mitochondria had been disintegrated by ultrasound, the specific activity of carnitine palmitoyltransferase was 15-20m-units/mg of protein. Only one-fifth of this activity was assayable (with added substrates) before mitochondrial disintegration. 3. It is concluded that there are two carnitine palmitoyltransferase activities in rat liver mitochondria, of which one (type I) is relatively superficial in location and catalyses an acyl-group transfer between added CoA and carnitine, whereas the other (type II) is less superficial and catalyses an acyl-group transfer in unbroken mitochondria between added carnitine and intramitochondrial CoA. The existence of two distinct carnitine palmitoyltransferases was predicted by Fritz & Yue (1963). 4. In unbroken mitochondria, type I transferase is accessible to the inhibitor 2-bromostearoyl-CoA whereas the type II transferase is inaccessible. 5. A major part of the total carnitine palmitoyltransferase activity of rat liver mitochondria is membrane-bound and of type II. 6. These observations, when considered in conjunction with the penetration of mitochondria by CoASH or carnitine, indicate that the type II transferase is attached to the inner mitochondrial membrane.

A number of schemes have been proposed to explain the involvement of L-carnitine in mitochondrial fatty acid oxidation (Fritz & Yue, 1963; Bremer, 1962; Bode & Klingenberg, 1965; Chappell & Crofts, 1965; van den Bergh, 1967; Yates & Garland, 1966; Garland & Yates, 1967; Allmann, Galzigna, McCaman & Green, 1966). A central but hypothetical feature of the majority of these schemes is the presence of carnitine palmitoyltransferase activities on either side of a barrier that is impermeable to CoA and its acyl esters but not to carnitine and its acyl esters. Scheme 1, which is essentially the scheme of Fritz & Yue (1963), shows an outer compartment that contains an acyl-CoA synthetase activity separated by a barrier from an inner compartment that contains intramitochondrial CoA and the enzymes of fatty acid oxidation. It was proposed (Fritz & Yue, 1963) that each compartment contained a carnitine palmitoyltransferase (transferases I and II) which catalysed the following reactions.

In this paper we present experimental evidence in favour of the proposal that there are two mitochondrial carnitine palmitoyltransferases with different locations. Preliminary reports of this work have been published (Yates & Garland, 1966; Garland & Yates, 1967).

MATERIALS AND METHODS

Rat liver mitochondria were prepared from Wistar rats, of either sex as described previously (Garland, Shepherd & Yates, 1965). Mitochondria were prepared in a similar manner from rat kidney, and from ox heart by the method of Sanadi & Fluharty (1963). Palmitoyl-CoA was prepared by the method of Seubert (1960), palmitoyl-DL-carnitine by the method of Bremer (1962) and 2-oxo-glutarate dehydrogenase (EC 1.2.4.2) as described by

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Scheme 1. Fritz & Yue (1963) type of scheme for the disposition of mitochondrial carnitine palmitoyltransferase. For details see the text. I and II are two differently located carnitine palmitoyltransferases.

Sanadi, Littlefield & Bock (1952). 2-Bromostearoyl-CoA was a kind gift from Dr P. K. Tubbs and Dr J. F. A. Chase. Bovine plasma albumin (Armour Pharmaceutical Co., Eastbourne, U.K., fraction V) was freed of fatty acids by the procedure of Goodman (1958).

Continuous fluorimetric assay for carnitine palmitoyltransferase. CoA released from palmitoyl-CoA in a carnitine-dependent manner (reaction 1) was assayed by coupling with a fluorimetric assay (Garland *et al.* 1965) by using oxoglutarate dehydrogenase (reaction 3):

This assay was performed at 25°C in a final volume of 2.1 ml, containing KCl (160 μmol), tris-chloride buffer, pH 7.2 (40 μmol), EDTA (2 μmol), rotenone (0.8 μg), KCN (2.0 μmol), defatted bovine plasma albumin (10 mg), NAD⁺ (1 μmol), tris-oxoglutarate, pH 7.2 (2 μmol), 0.1 unit of oxoglutarate dehydrogenase, and sufficient of the sample under study to contain 0.1–5.0 m-units of carnitine palmitoyltransferase. The fluorescence of NADH was measured with a modified Eppendorf fluorimeter (Garland *et al.* 1965) and a base-line was recorded for 1 min before the addition of palmitoyl-CoA (40 nmol). The fluorescence was recorded for a further 1 min, during which time any rate of NAD⁺ reduction due to carnitine-independent CoA release (e.g. palmitoyl-CoA hydrolysis) was measured. Finally, DL-carnitine (4 μmol) was added to initiate reaction 1. The presence of rotenone and KCN prevented any change of NADH fluorescence due to oxidoreductions of the mitochondrial respiratory chain. Calibration of the fluorimeter was made by addition of spectrophotometrically standardized NADH to the assay system lacking only palmitoyl-CoA.

Discontinuous assay for carnitine palmitoyltransferase. Reaction 1 was assayed in the reverse direction by measuring palmitoyl-CoA formed from palmitoylcarnitine and CoA. The assay medium at 25°C contained, in a final volume of 10.5 ml; KCl (800 μmol), tris-chloride buffer, pH 7.2 (200 μmol), EDTA (10 μmol), rotenone (10 μg), KCN (10 μmol), defatted bovine plasma albumin (50 mg) and amounts of CoA and mitochondrial protein as

described in the legend to Fig. 3. Then 1 min after addition of mitochondria to the assay mixture, the reaction was initiated by adding palmitoyl-DL-carnitine (800 nmol). Samples (1.0 ml) were removed at appropriate intervals before and after the addition of palmitoyl-DL-carnitine (see Fig. 2), quenched by mixing with 1.0 ml of 10% (w/v) HClO₄ at 0°C, and assayed for palmitoyl-CoA by the fluorimetric modification (Garland *et al.* 1965) of the method of Tubbs & Garland (1964).

Ultrasonic disintegration of mitochondria was effected at 0°C with 60 W MSE ultrasonic power unit (Measuring and Scientific Equipment Ltd., London S.W.1, U.K.) operated at full power for 1 min. This procedure was performed on 4.0 ml of mitochondrial suspension containing KCl (240 μmol), tris-chloride buffer, pH 7.2 (60 μmol), EDTA (3 μmol), sucrose (200 mmol) and mitochondrial protein (80 mg). Separation of ultrasonically disintegrated mitochondria into membrane (pellet) and soluble (supernatant) fractions was made at 0°C by centrifugation at 50 000 rev./min (144 000g) in the 10 × 10 ml angle rotor of an MSE 50 centrifuge for 45 min.

RESULTS

Fluorimetric assay for carnitine palmitoyltransferase. The high sensitivity of fluorescence measurements of NADH and the low K_m (<10⁻⁷ M; Massey, 1960) of oxoglutarate dehydrogenase for CoA provide favourable conditions for accurate determination of initial velocities in the coupled assay system. Under otherwise constant conditions there was a linear relationship between the carnitine palmitoyltransferase activity and mitochondrial protein concentration (0.025–0.25 mg of mitochondrial protein/ml), as shown in Fig. 1. The presence of a relatively high concentration of bovine plasma albumin in the assay medium was used to maintain the total protein concentration approximately constant. Palmitoyl-CoA binds strongly to bovine plasma albumin (Tubbs & Garland, 1964), and the

Fig. 1. Relationship between the carnitine palmitoyltransferase activity and amount of mitochondrial protein. The continuous fluorimetric assay was used for measuring reaction (1) in the forward direction. Details are given in the Materials and Methods section.

true concentration of palmitoyl-CoA in the assay medium would have been much lower than the analytical content. One effect of the presence of bovine plasma albumin was to lower the rate of carnitine-independent CoA release from palmitoyl-CoA. A similar observation was made by Bremer & Norum (1967).

The latency of carnitine palmitoyltransferase: continuous assay. If latency is defined in the present context as being the behaviour of a mitochondrial enzyme such that the enzyme is inactive towards its added substrate(s) unless the mitochondria are first disrupted, then it is readily predicted from scheme 1 that transferase I would not be latent whereas transferase II would be. The observed transferase activity of unbroken mitochondria towards added palmitoyl-CoA and carnitine would be partially latent to a degree that depended on the relative absolute activities of transferases I and II. This predicted behaviour has been unfailingly observed by us with mitochondria from rat liver, and typical assays are shown in Fig. 2. Other features that emerge from Fig. 2 are (i) that palmitoyl-CoA hydrolase activity (EC 3.1.2.2, equated with the carnitine-independent rate of CoA release from palmitoyl-CoA) is also partially latent; (ii) the palmitoyl-CoA deacylase activity is absent from the membrane fraction; and (iii) 85% of the total transferase activity is membrane-bound whereas the remainder is solubilized, a distribution similar to that reported by Bremer, Norum & Farstad (1967). The solubilized enzyme is unstable on storage as the soluble fraction at 0°C, and 90% of the activity was lost in 8 h.

Studies were also made with mitochondria from

Fig. 2. Carnitine palmitoyltransferase activities of rat liver mitochondria before and after ultrasonic disintegration. The recorder tracings (a)–(d) are from the continuous fluorimetric transferase assay carried out as described in the Materials and Methods section. Palmitoyl-CoA was added at P and DL-carnitine at C. In all cases 0.25 mg of mitochondrial protein, or the soluble or membrane fraction derived therefrom, was used. The designations for each tracing and the respective activities (nmol/min per mg of mitochondrial protein) of palmitoyl-CoA deacylase (the rate of NAD⁺ reduction between additions P and C) and of carnitine palmitoyltransferase (the extra rate after C) were: (a) unbroken mitochondria, 1.6, 4.0; (b) ultrasonically disintegrated mitochondria, 4.0, 16.0; (c) soluble fraction, 4.0, 3.8; (d) membrane fraction, 0, 13.6. All activities refer to the starting amount of mitochondrial protein from which the fraction was prepared.

other sources. With rat kidney mitochondria, the carnitine palmitoyltransferase activity towards added palmitoyl-CoA and carnitine was increased from 0.003 to 0.007 units/mg of protein after ultrasonic disintegration, and with ox heart mitochondria the corresponding increase was from 0.001 to 0.008 units/mg of protein.

Latency of carnitine palmitoyltransferase: discontinuous assay in the reverse direction. Rat liver mitochondria contain approximately 2.0 nmol of CoA/mg of protein, and over 60% of this can be acylated by the addition of palmitoylcarnitine (Garland *et al.* 1965). On the basis of scheme 1 the acylation of intramitochondrial CoA by added palmitoylcarnitine should involve only transferase II, whereas the acylation of added CoA should involve only transferase I in unbroken mitochondria. The velocities of these reactions were determined in the experiments shown in Fig. 3, which show that the intramitochondrial CoA was acylated at a rate of 10 nmol/min per mg of protein. This reaction was rapidly completed owing to the limitation imposed by the amount of intramitochondrial CoA. When CoA was added to the suspension of unbroken mitochondria the time-course was biphasic, there being a rapid initial

phase corresponding to the acylation of intra-mitochondrial CoA and a slower protracted phase corresponding to the acylation of added CoA. The acylation of added CoA was accelerated fivefold by prior ultrasonic disintegration of the mitochondria. Some 90% of the transferase activity of the disintegrated mitochondria was membrane-bound.

Studies with DL-2-bromostearoyl-CoA. Tubbs & Chase (1967) have reported that DL-2-bromostearoyl-CoA is a potent inhibitor of carnitine palmitoyltransferase, provided that carnitine is also present. There is no reason to suppose that the permeability of mitochondria to bromostearoyl-CoA would differ from that for palmitoyl-CoA, and it can therefore be predicted from scheme 1 that transferase I would be inhibited by added bromostearoyl-CoA and carnitine whereas transferase II would be insensitive unless the mitochondria were first broken. The design and results of an experiment that tests this prediction are shown in Table 1, which shows that the non-latent transferase activity (equated with transferase I) was inhibited by the addition of bromostearoyl-CoA whereas the latent transferase (equated with transferase II) is unaffected unless the mitochondria are broken

before exposure to the inhibitor. Analogous observations were first made by Tubbs & Chase (1967), who reported that bromostearoyl-CoA inhibited the carnitine-dependent mitochondrial oxidation of palmitoyl-CoA but not the oxidation of palmitoylcarnitine.

DISCUSSION

The above experimental results are consistent not only with two types of transferase in each individual mitochondrion but also with two types of mitochondria each with one or the other of the transferases. Functionally, the end result would be the same for the experiments described here. A third possibility is that all of the transferase activity is normally latent, and that the emergence of non-latent activity is due to mitochondrial damage. The implication of this third possibility is that extramitochondrial palmitoyl-CoA could not be oxidized *in vivo*.

The behaviour of mitochondrial carnitine acetyltransferase is similar to that of carnitine palmitoyltransferase in that its activity towards added substrates is greatly increased by disruption of the mitochondria (Garland & Yates, 1967; Barker, Fincham & Hardwick, 1968).

Alternative evidence for the presence of two carnitine palmitoyltransferases comes from a quantitative correlation between the non-latent activity and the activity solubilized on ultrasonic disintegration followed by centrifugation. For both the forward and reverse assays (Figs. 2 and 3) the solubilized and membrane-bound activities corresponded respectively to the non-latent and latent activities. A similar correspondence occurred with the effects of bromostearoyl-CoA; the loss of activity when the mitochondria were first temporarily exposed to the inhibitor before ultrasonic disintegration was clearly greatest in the soluble fraction (Table 1.) Allmann *et al.* (1966) have claimed that the carnitine oleoyltransferase of ox heart mitochondria is completely non-latent, in contrast to our results with palmitoyl-CoA, which is presumably a substrate for the same enzyme. The failure of Allmann *et al.* (1966) to observe latency was probably due to the fact that their transferase assay system contained 2.2mM-oleoyl-CoA in the absence of added bovine plasma albumin. A further curiosity of the transferase assay medium of Allmann *et al.* (1966) was the presence of 6.5% (v/v) ethanol.

Although the partial latency of carnitine palmitoyltransferase is an essential feature of Scheme 1, such behaviour by itself does not directly identify the functionally defined permeability barrier of that scheme with one or the other of the mitochondrial membranes; either would be equally

Fig. 3. Carnitine palmitoyltransferase activities of rat liver mitochondria before and after ultrasonic disintegration. The discontinuous assay was used to measure palmitoyl-CoA formation from added palmitoylcarnitine. The assay system contained 7.0mg of mitochondrial protein or the fraction derived therefrom. When added, the amount of CoA was 200nmol. The reaction was started at zero time by the addition of 800nmol of palmitoyl-DL-carnitine. Other experimental details are described in the Materials and Methods section. Symbols: ●, unbroken mitochondria with added CoA; ○, unbroken mitochondria without added CoA; ▲, ultrasonically disintegrated mitochondria; △, membrane fraction; □, soluble fraction.

Table 1. *Effects of 2-bromostearoyl-CoA on carnitine palmitoyltransferase activity*

Intact mitochondria (60mg of protein) were suspended in a final volume of 5.0ml containing sucrose (250 μ mol), KCl (320 μ mol), tris-chloride buffer, pH 7.2 (80 μ mol), EDTA (4 μ mol), DL-carnitine (5 μ mol) and 2-bromostearoyl-CoA (5nmol) at 25°C for 5min. A 20 μ l sample (fraction A, intact mitochondria) was withdrawn for measurement of the transferase activity (continuous assay) and the remaining mitochondria were sedimented at 0°C and 10000g in the 8 $\times$ 50ml head of an MSE 18 centrifuge, 10ml tubes and adaptors being used. The supernatant was discarded and the pellet resuspended in 5ml of a medium at 0°C containing KCl (400 μ mol), tris-chloride buffer, pH 7.2 (100 μ mol), EDTA (5 μ mol) and defatted bovine plasma albumin (50mg). The mitochondria were again sedimented and resuspended as described above, but with the omission of bovine plasma albumin. A 20 μ l sample (fraction B, washed mitochondria) was removed for palmitoyltransferase assay. The remaining mitochondria were then ultrasonically disintegrated (fraction C) and separated into a soluble fraction (D) and a membrane fraction (E) as described in the Materials and Methods section. A parallel experiment was run but with the omission of 2-bromostearoyl-CoA. Thus for each fraction there were two types, those derived from mitochondria treated with 2-bromostearoyl-CoA and those that were not. In addition, samples of fraction E were reassayed in the presence of 2-bromostearoyl-CoA (final concn. 1 μ M), added simultaneously with carnitine and followed after 1 min by palmitoyl-CoA. This changed order of additions in the assay exposed the membrane fraction to the inhibitory combination of bromostearoyl-CoA and carnitine before the assay was initiated. 2-Oxoglutarate dehydrogenase was not inhibited under these conditions.

Initial concn. of 2-bromostearoyl- CoA in incubation medium (μ M)	Fraction ...	Carnitine palmitoyltransferase activity (m-units/mg of original mitochondrial protein) in fraction					
		A (intact)	B (washed)	C (disintegrated)	D (soluble)	E (membrane)	E (reassay with 1 μ M- 2-bromostearoyl- CoA)
1		0.4	3.0	13.0	0.7	9.8	3.1
0		7.7	7.7	14.0	3.5	14.4	2.7

suitable. However, when these results are considered in conjunction with the mitochondrial permeable-space measurements for CoA (Yates & Garland, 1966) and acetyl-CoA (if that is representative of acyl-CoA in general: Haddock, Yates & Garland, 1970) it is clear that the behaviour of the latent (type II) transferase is more readily explicable if the enzyme is located on the inner rather than the outer membrane. There are fewer constraints on the position of the non-latent (type I) transferase; it could be associated in intact mitochondria with the outer membrane, the outer aspect of the inner membrane, or the intermembrane space. The solubilization of the type I transferase by ultrasonic disintegration does not exclude any of these possibilities. The permeable-space measurements indicate that all of these situations are accessible to added carnitine, CoA and their acyl esters.

A further point that arises from the permeable-space experiments (Yates & Garland, 1966) concerns the apparent failure of carnitine to penetrate the sucrose-inaccessible space that contains intramitochondrial CoA. Despite this, intramitochondrial palmitoyl-CoA can acylate added carnitine (Bremer, 1963) and added palmitoylcarnitine can acylate intramitochondrial CoA (Garland *et al.* 1965). It is therefore possible that the latent and

membrane-bound transferase II catalyses a vectorial reaction:

where 'in' and 'out' refer to the opposite aspects of the inner mitochondrial membrane.

None of these considerations provide a satisfactory explanation for the observation that carnitine can stimulate the oxidation of long-chain fatty acids under conditions where the initial activation step appears to occur intramitochondrially (Fritz & Yue, 1963; Chappell & Crofts, 1965; van den Bergh, 1967; Garland & Yates, 1967).

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