The effect of malonyl-CoA on overt and latent carnitine acyltransferase activities in rat liver and adipocyte mitochondria

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(Received 20 September 1982/Accepted 10 November 1982)

1. Carnitine palmitoyltransferase and carnitine octanoyltransferase activities were measured in mitochondria at various acyl-CoA concentrations before and after sonication, thus permitting assessment of both overt and latent activities. 2. Overt carnitine palmitoyltransferase in liver and adipocyte mitochondria and overt carnitine octanoyltransferase in liver mitochondria were inhibited by malonyl-CoA. None of the latent activities were affected by this metabolite. 3. 5,5'-Dithiobis-(2-nitrobenzoic acid) stimulated latent hepatic carnitine palmitoyltransferase at low [palmitoyl-CoA]. 4. Starvation (24 h) decreased overt carnitine palmitoyltransferase activity in adipocyte mitochondria, but did not alter the sensitivity of this activity to malonyl-CoA.

There is considerable evidence that mitochondria from mammalian tissues have a bimodal distribution of CPT (Fritz & Yue, 1963; Yates & Garland, 1970; West *et al.*, 1971; Hoppel & Tomec, 1972; Harano *et al.*, 1972; Brosnan *et al.*, 1973; Harper & Saggerson, 1975; Saggerson & Carpenter, 1981*b*). Available evidence suggests that, in intact mitochondria, that part of the activity which is overt (CPT₁) is associated with the outer aspect of the inner membrane (Hoppel & Tomec, 1972; Brosnan *et al.*, 1973). On the other hand, a second fraction (CPT₂), which is latent in intact mitochondria, is considered to be firmly bound to the inner aspect of the inner membrane. McGarry *et al.* (1978) have shown that malonyl-CoA is a potent inhibitor of rat liver CPT. Inhibition of CPT by this metabolite is also seen in other rat tissues (Saggerson & Carpenter, 1981*b*, 1982*a,b*; Saggerson, 1982). McGarry *et al.* (1978) have concluded that CPT₁, but not CPT₂, is susceptible to inhibition by malonyl-CoA. This is based on the following evidence. Firstly, whereas malonyl-CoA can totally inhibit palmitate oxidation by intact mitochondria (in the presence of ATP, CoA and carnitine), malonyl-CoA only inhibits approximately half of the total CPT activity in membranes obtained from ruptured mitochondria (McGarry *et al.*, 1978). Secondly, malonyl-CoA inhibits approximately half of the CPT activity measured in intact mitochondria by an isotope-

exchange assay which is supposed to measure both CPT₁ + CPT₂ activities (McGarry *et al.*, 1978). In addition, near to 100% of the overt CPT activity is malonyl-CoA-suppressible in intact mitochondria from fed rats (Saggerson & Carpenter, 1981*a*). However, it has not been directly demonstrated that latent CPT activity is malonyl-CoA-insensitive. Since sonication is reported not to affect the susceptibility of malonyl-CoA-sensitive CPT to malonyl-CoA (see Table III of McGarry *et al.*, 1978), it should be possible to investigate the susceptibility of CPT₂ to malonyl-CoA simply by assaying before and after sonication of mitochondria and obtaining, by difference, a measure of CPT₂ activity. The degree of inhibition of CPT₁ by malonyl-CoA is dependent on the palmitoyl-CoA concentration (Saggerson, 1982; Saggerson & Carpenter, 1981*c*, 1982*a*; Saggerson *et al.*, 1982). Malonyl-CoA increases the [palmitoyl-CoA] that gives half-maximal activity, but has no effect at saturating concentrations of this substrate (Saggerson & Carpenter, 1981*c*; Saggerson *et al.*, 1982). We have therefore investigated the effect of malonyl-CoA on CPT₂ activity at several palmitoyl-CoA concentrations. Although there is little reason to propose an interaction between malonyl-CoA and CPT₂ within the cell, the apparent insensitivity of CPT₂ to this regulator is of interest. It would suggest either that CPT₁ and CPT₂ could be different proteins or that CPT₂ does not interact with some regulatory component in the membrane which confers malonyl-CoA-sensitivity on CPT₁.

This paper also includes some studies made with white-adipocyte mitochondria. These were per-

Abbreviations used: COT, carnitine acyltransferase activity with octanoyl-CoA; CPT, carnitine palmitoyltransferase (EC 2.3.1.21); CPT₁ and CPT₂, the overt and latent forms respectively of CPT; Nbs₂, 5,5'-dithiobis-(2-nitrobenzoic acid).

formed for two reasons. Firstly, when assayed at a single [palmitoyl-CoA] ($40\mu\text{M}$), CPT₁ activity in adipocyte mitochondria was found to be extremely low, although these mitochondria contained a relatively high activity of latent CPT (Saggerson & Carpenter, 1981b). We have therefore investigated the substrate-dependence of adipocyte CPT over a range of [palmitoyl-CoA]. Secondly, the CPT₁ in this lipogenic tissue, as in the liver, was sensitive to malonyl-CoA (Saggerson & Carpenter, 1981b). Starvation considerably decreases the sensitivity of rat liver CPT₁ to malonyl-CoA (Saggerson & Carpenter, 1981a,c; Bremer, 1981; Saggerson *et al.*, 1982; Robinson & Zammit, 1982). There is evidence that the somewhat limited fatty acid oxidation thought to occur in white adipose tissue is increased or decreased in conditions that respectively decrease or increase fatty acid synthesis (and hence, presumably, malonyl-CoA formation) (Wertheimer & Shapiro, 1948; Flatt & Ball, 1964; Flatt, 1970). Also, it has been suggested that, in starvation, adipose tissue has a decreased ability to restrain the oxidation of endogenous fatty acid (Flatt, 1970). We have therefore investigated the effect of starvation on the malonyl-CoA-sensitivity of adipocyte CPT₁.

Materials and methods

Animals

These were male Sprague-Dawley rats bred in the Animal Colony at University College London and maintained on GR3-EK cube diet (E. Dixon and Sons, Ware, Herts., U.K.). Animals were killed at approx. 6 weeks (160–180 g body wt.). Starvation was commenced by removal of food at 10:00 h 1 day before they were killed.

Chemicals

These were obtained and treated as described by Saggerson *et al.* (1982). Collagenase (crude) was from International Enzymes (Windsor, Berks., U.K.)

Experimental methods

Liver mitochondria were isolated as described by Saggerson (1982) and finally suspended in ice-cold 0.3 M-sucrose containing 10 mM-Tris/HCl buffer (pH 7.4) and 1 mM-EGTA (4 ml/original g of liver).

Adipocytes were isolated by disaggregation of the epididymal fat-pads of eight to ten rats by using collagenase (Rodbell, 1964) and were then broken by swirling for 45 s in a glass tube on a vortex mixer (Martin & Denton, 1970) with 25 ml of ice-cold 0.25 M-sucrose containing 10 mM-Tris/HCl (pH 7.4), fatty acid-poor albumin (10 mg/ml) and 1 mM-EGTA. Centrifugation conditions for the isolation of adipocyte mitochondria were the same as those used for liver (Saggerson, 1982) and previously for adipocyte mitochondria (Saggerson *et al.*, 1980).

Finally, the preparations of adipocyte mitochondria were suspended in 1.5 ml of ice-cold 0.3 M-sucrose containing 10 mM-Tris/HCl buffer (pH 7.4) and 1 mM-EGTA.

The protein contents of these mitochondrial preparations, which were determined by the method of Lowry *et al.* (1951), were: liver 4.08 ± 0.16 (16), adipocyte (fed) 2.30 ± 0.20 (5), and adipocyte (starved) 2.73 ± 0.20 (4) mg/ml (means $\pm$ S.E.M. for the indicated numbers of preparations).

CPT assays were performed within 1 h of the isolation of mitochondria. For assay of CPT₁ (Saggerson & Carpenter, 1982b; Saggerson *et al.*, 1982), 50 μl of intact mitochondria were pre-incubated at 25°C for 2 min in 1.0 ml containing 220 mM-sucrose, 40 mM-KCl, 10 mM-Tris/HCl (pH 7.4), 1 mM-EGTA, fatty acid-poor albumin (1.3 mg/ml), 0.5 mM-dithiothreitol and the indicated concentrations of palmitoyl-CoA and malonyl-CoA. Respiratory inhibitors were found to be unnecessary (Saggerson & Carpenter, 1982b). The reaction was initiated by addition of 20 μl containing 0.4 μmol of L-carnitine and 1 μCi of DL-[methyl-³H]carnitine. After 4 min the reaction was terminated, and the palmitoyl[³H]carnitine was extracted into butanol and counted for radioactivity as described by Saggerson *et al.* (1982). Total CPT activity was measured similarly in 25 μl of mitochondria which had been sonicated (MSE Sonicator at full power, 8 μm peak-to-peak) for 2 min at 2°C. CPT₂ activity was calculated by subtraction of CPT₁ from total CPT.

Overt COT and total COT activities were measured similarly to CPT₁ and total CPT respectively, except that octanoyl-CoA was substituted for palmitoyl-CoA. Latent COT activity was calculated by subtraction of overt COT from total COT.

Assays were linear with time and mitochondrial protein concentration.

Results and discussion

Hepatic carnitine palmitoyltransferase

Fig. 1(b) demonstrates the effects of malonyl-CoA and Nbs₂ on CPT₁ activity. As shown previously (Saggerson & Carpenter, 1981c; Saggerson *et al.*, 1982), malonyl-CoA ($10\mu\text{M}$) inhibited CPT₁ at low but not at high [palmitoyl-CoA]. As also shown previously (Saggerson & Carpenter, 1982b), Nbs₂ considerably increased CPT₁ activity at lower [palmitoyl-CoA]. The values for CPT₁ shown in Fig. 1(b) were then subtracted from values for total CPT measured in sonicated mitochondria to give the values for CPT₂ shown in Fig. 1(a). With the assay conditions employed, the relationship between CPT₂ activity and [palmitoyl-CoA] was quite different from that seen with CPT₁. In the

Fig. 1. Effects of [palmitoyl-CoA], Nbs₂ and malonyl-CoA on CPT activities in liver mitochondria from fed rats. L-Carnitine was present throughout at 400 μM. For full details see the text. (a) CPT₂ activity; (b) CPT₁ activity. The values are means ± S.E.M. for four experiments in every case. O, No further additions; ●, with 10 μM-malonyl-CoA; □, with 100 μM-Nbs₂.

absence of malonyl-CoA or Nbs₂, CPT₂ activity was maximal at approx. 40 μM-palmitoyl-CoA; higher concentrations of substrate were inhibitory. As a consequence, the activity ratio CPT₁/CPT₂ varied considerably with the [palmitoyl-CoA], i.e. from 0.62 ± 0.05 at 10 μM- to 4.86 ± 0.81 at 100 μM-

palmitoyl-CoA. Malonyl-CoA (10 μM) had no appreciable effect on CPT₂ at any tested [palmitoyl-CoA]. The suggestion of McGarry *et al.* (1978) that only CPT₁ is malonyl-CoA-sensitive therefore appears to be substantiated. Nbs₂, however, did affect CPT₂ activity, moving the activity-versus-[palmitoyl-CoA] curve to the left, resulting in appreciable activation at low [palmitoyl-CoA]. This finding is relevant to studies in which CPT activity is measured by an Nbs₂-linked assay rather than by the radiochemical method used here. We do suggest that these findings be treated with caution because of the obvious problems inherent in comparing enzyme activities in differently treated preparations (sonicated and non-sonicated).

CPT activity released from rat liver mitochondria on treatment with Tween-20 (McGarry *et al.*, 1978), or 'outer' CPT solubilized from bovine liver mitochondria (Edwards & Edwards, 1981), is almost insensitive to malonyl-CoA. Similarly, addition of Triton X-100 to rat heart mitochondria greatly decreases the sensitivity of CPT to malonyl-CoA (Saggerson, 1982). It is concluded from these previous studies that membrane-bound CPT₁ is far more sensitive to malonyl-CoA than the enzyme in a 'solubilized' form. Therefore it was considered possible that the sonication procedure used here might cause extensive detachment of CPT from the mitochondrial membrane. If so, the apparent insensitivity of CPT₂ to malonyl-CoA (Fig. 1a) could be an artefact. Accordingly, the following experiment was performed with four separate liver mitochondria preparations (values are means ± S.E.M.) in which CPT assays were performed at 40 μM-palmitoyl-CoA. Freshly sonicated mitochondria were centrifuged at 100 000 *g*_{av.} for 60 min; $86 \pm 1\%$ of the CPT activity was sedimented by this procedure. Malonyl-CoA (10 μM) inhibited total CPT in the 100 000 *g* pellet by $31 \pm 6\%$. The small fraction ($14 \pm 1\%$) of the CPT activity in the 100 000 *g* supernatant was inhibited by $16 \pm 3\%$ by 10 μM-malonyl-CoA. It is concluded that the sonicated procedure did not result in extensive liberation from the membrane of malonyl-CoA-insensitive CPT.

Hepatic carnitine octanoyltransferase

It has been proposed that mammalian tissues contain a medium-chain carnitine acyltransferase activity that is distinct from both carnitine palmitoyl- and acetyl-transferases (Solberg, 1971, 1972; Markwell *et al.*, 1976). This activity is generally referred to as carnitine octanoyltransferase (COT), is found in rat liver mitochondria, peroxisomes and microsomal fractions (Markwell *et al.*, 1977) and has subsequently been observed in at least 11 rat tissues (Choi *et al.*, 1977; Saggerson & Carpenter, 1981b). The acyl-chain-length specificity of this proposed

enzyme is likely to overlap with those of carnitine acetyltransferase and CPT. Clarke & Bieber (1981a) proposed that bovine heart COT is attributable to these latter activities and is unlikely to be a distinct enzyme, although that possibility was not totally discounted. It is still unresolved whether liver contains a distinct COT enzyme. However, rat liver overt COT and CPT₁ activities differ in several respects, i.e. response to $[K^+]$, $[Mg^{2+}]$, the effect of Nbs₂, sensitivity to malonyl-CoA and kinetics with respect to [acyl-CoA] (Saggerson & Carpenter, 1981a,c, 1982b; Saggerson, 1982). Since both overt and latent COT activity is observed in rat liver mitochondria (Saggerson, 1982), it was decided to compare the effects of malonyl-CoA on these activities. As shown previously (Saggerson & Carpenter, 1981c), unlike CPT₁ (see Fig. 1a), the relationship between overt COT activity and [acyl-CoA] is hyperbolic rather than sigmoidal (Fig. 2a). Also, although malonyl-CoA is an inhibitor of COT

activity (Saggerson & Carpenter, 1981a,c, 1982b), the effect is not of a competitive type with respect to [octanoyl-CoA] (Fig. 2a, and Saggerson & Carpenter, 1981c). Fig. 2(b) shows that the latent COT activity in these mitochondria was not inhibited by malonyl-CoA ($10\mu M$). The affinity of the latent COT activity for octanoyl-CoA was clearly less than that of the overt activity. Unlike CPT₁, overt COT activity in rat liver mitochondria is inhibited by Nbs₂ (Saggerson & Carpenter, 1982b). The same was found with latent COT activity; $100\mu M$ -Nbs₂ decreased both overt and latent COT activities by approx. 25% (three experiments; results not shown).

Fig. 2. Effect of [octanoyl-CoA] and malonyl-CoA on COT activities in liver mitochondria from fed rats. L-Carnitine was present throughout at $400\mu M$. For full details see the text. (a) Overt COT activity; (b) latent COT activity. The values are means $\pm$ S.E.M. for four experiments. O, No further additions; ●, with $10\mu M$ -malonyl-CoA.

Fig. 3. Effect of [palmitoyl-CoA] on CPT activities in adipocyte mitochondria from fed (O) and 24 h-starved (●) rats.

(a) CPT₁ activity; (b) CPT₂ activity. For full details see the text. The values are means $\pm$ S.E.M. for four experiments in each case. * indicates $P < 0.05$ for comparison of CPT₁ activities in the fed and starved states (Student's t test). Hill plots for CPT₁ data (derived from Fig. 3a) were good-fit straight lines ($r = 0.998$ and 0.999 for the fed and starved conditions respectively), yielding values for the Hill coefficient of 2.21 (fed) and 3.57 (starved) and for the $s_{0.5}$ for palmitoyl-CoA of $30\mu M$ (fed) and $28\mu M$ (starved).

Adipocyte carnitine palmitoyltransferase

Fig. 3 demonstrates the dependence of adipocyte CPT₁ and CPT₂ activities on [palmitoyl-CoA]. In the fed state the shapes of the curves were similar to those for CPT₁ and CPT₂ in liver mitochondria from fed animals (Fig. 1). However, adipocyte CPT₁ specific activity was only approx. 20% of that found in liver, whereas adipocyte CPT₂ activity was approximately twice the hepatic activity. Adipocyte CPT activities at 40 μ M-palmitoyl-CoA were comparable with those reported by Saggerson & Carpenter (1981b), using the same radiochemical assay. As in liver mitochondria, the [palmitoyl-CoA] influenced the activity ratio CPT₁/CPT₂, but in adipocytes (fed state) this varied from 0.06 ± 0.02 (10 μ M) to 0.28 ± 0.05 (100 μ M-palmitoyl-CoA).

We have now assayed CPT activities in mitochondria from both white and brown adipose tissue under identical assay conditions. A very clear distinction can be seen, in that a far larger proportion of the total activity is overt in brown fat (Saggerson & Carpenter, 1981a). In addition, the maximum specific activity of CPT₁ in white-fat mitochondria is only approximately one-fifteenth of that in brown fat.

As shown previously (Harper & Saggerson, 1975), starvation resulted in a small decrease in the specific activity of adipocyte CPT₁ (Fig. 3a). This may be contrasted with the increase in hepatic CPT₁ activity that is observed on starvation (Harper & Saggerson, 1975; Bremer, 1981; Saggerson *et al.*, 1982). In addition, starvation increased the Hill coefficient, but had little effect on the $s_{0.5}$ for palmitoyl-CoA (Fig. 3a). The adipocyte CPT₁ activities and the CPT₁/CPT₂ activity ratios reported here are considerably lower than those reported by Harper & Saggerson (1975). However, in that earlier study, CPT was assayed in a system (Harano *et al.*, 1972) which is hypo-osmotic compared with that used here (Saggerson, 1982) and may have caused some swelling and rupture of the mitochondria. In addition, the assay of Harano *et al.* (1972) is an Nbs₂-linked assay, and it is now recognized that Nbs₂ has quite marked effects on CPT activities [see Fig. 1, and also Saggerson & Carpenter (1982b) and Saggerson (1982)]. The absolute and relative activities of adipocyte and liver CPT₁ and CPT₂ reported here may be correlated with measurements of palmitoyl-group oxidation made by Harper & Saggerson (1975). The rate of palmitoylcarnitine oxidation in white-adipocyte mitochondria was shown to be comparable with or greater than that in liver mitochondria. However, whereas liver mitochondria oxidized palmitoyl-CoA + carnitine at approx. 50% of the rate seen with palmitoylcarnitine, adipocyte mitochondria oxidized palmitoylcarnitine at more than 10 times the rate seen with palmitoyl-CoA + carnitine (Harper &

Saggerson, 1975). This is an important difference between these two tissues and can now be more clearly seen to be due to the extremely low CPT₁ activity in the white adipocyte (Fig. 3a).

Unlike the hepatic enzyme (Saggerson & Carpenter, 1981a,c; Bremer, 1981; Saggerson *et al.*, 1982; Robinson & Zammit, 1982), the sensitivity to malonyl-CoA of adipocyte CPT₁ was unaffected by starvation (Fig. 4). In the liver it has been suggested that adaptive changes in malonyl-CoA-sensitivity serve to amplify the response of CPT₁ activity to alterations in [malonyl-CoA] (Robinson & Zammit, 1982). This would not appear to be the case in adipose tissue, nor in muscle, where it has been shown that malonyl-CoA inhibition of palmitate oxidation is unaffected by starvation (Veerkamp & Van Moerkerk, 1982). Clearly, adipocyte CPT₁ is sensitive to low concentrations of malonyl-CoA (Fig. 4, and Saggerson & Carpenter, 1981b). There are no reported measurements of malonyl-CoA concentrations in adipose tissue at present. It has yet to be established whether, in this tissue, malonyl-CoA helps to vary the rate of β -oxidation inversely to the rate of fatty acid synthesis, as has been proposed for liver (McGarry & Foster, 1980). Unlike liver, factors influencing the rate of fatty acid esterification may be particularly important in determining the flux of fatty acids into β -oxidation in adipose tissue (Harper & Saggerson, 1976).

As with liver mitochondria (Fig. 1), the malonyl-CoA-sensitivity of adipocyte CPT₂ was also investigated. At 20 μ M-palmitoyl-CoA, malonyl-CoA (10 μ M) only decreased CPT₂ by $6 \pm 3\%$ and by $6 \pm 4\%$ in the fed and starved states respectively

Fig. 4. Effect of malonyl-CoA on CPT₁ activity in adipocyte mitochondria from fed (○) and 24 h-starved (●) rats

Assays were performed with 40 μ M-palmitoyl-CoA and 400 μ M-L-carnitine. For further details see the text. The values are means $\pm$ S.E.M. for five fed and four starved sets of animals. Activities in the absence of malonyl-CoA were 0.84 ± 0.04 (fed) and 0.69 ± 0.03 (starved) nmol/min per mg of protein.

(means of five and four experiments), i.e. the effect on adipocyte CPT₂ was negligible.

General conclusions

It is not known whether CPT₁ and CPT₂ are products of a single or of separate genes. CPT₁ is more readily solubilized than CPT₂ (Hoppel & Tomec, 1972; Bergstrom & Reitz, 1980) and there have been several reports demonstrating both kinetic (Chase & Tubbs, 1972; Edwards, 1977; Scholte *et al.*, 1979) and immunochemical (Brosnan *et al.*, 1973; Kopec & Fritz, 1973) differences between them. The present study demonstrates that CPT₁ and CPT₂ activities in liver and adipocyte differ in their response to [palmitoyl-CoA] and in inhibition by malonyl-CoA. On the other hand, Bergstrom & Reitz (1980) have provided evidence that these two activities are essentially identical with regard to kinetic properties and molecular weight once they have been removed from the mitochondrial membrane. It is therefore possible that differences in properties result from association with different membrane environments. It is known that membrane faces are different on the outer and inner sides of the mitochondrial inner membrane (Racker, 1970) and that the kinetic properties of purified ox heart CPT are influenced by environment (Clarke & Bieber, 1981b).

Purified 'outer' CPT from ox liver is a monomeric protein (mol.wt. 59000) (Edwards, 1977). It is not yet known whether the enzyme from rat tissues is similar. The sigmoidal kinetics with respect to palmitoyl-CoA seen with CPT₁ *in situ* in mitochondria from several rat tissues (Figs. 1 and 3; Saggerson & Carpenter, 1981c, 1982a,b; Saggerson *et al.*, 1982) are not of the type generally expected for a simple monomeric enzyme. It remains to be established which components in the outer side of the inner membrane are responsible for conferring both of this property and of sensitivity to malonyl-CoA.

The same questions apply when the relationship between overt and latent COT is considered. The physiological role of COT in hepatic mitochondrial metabolism is unclear, although COT activities could play a role in mitochondrial utilization of medium-chain fatty acids if these are released by peroxisomes as their carnitine esters (Mannaerts & Debeer, 1981).

This work was supported by a project grant from the Medical Research Council.

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