Increased sensitivity of carnitine palmitoyltransferase I activity to malonyl-CoA inhibition after preincubation of intact rat liver mitochondria with micromolar concentrations of malonyl-CoA *in vitro*

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Carnitine palmitoyltransferase I in rat liver mitochondria preincubated with malonyl-CoA was more sensitive to inhibition by malonyl-CoA than was the enzyme in mitochondria preincubated in the absence of malonyl-CoA. For carnitine palmitoyltransferase I in mitochondria from starved animals this increase also resulted in the enzyme becoming significantly more sensitive than that in mitochondria assayed immediately after their isolation. Concentrations of malonyl-CoA that induced half the maximal degree of sensitization observed were 1–3 μM .

A positive, quantitative, relationship exists between the sensitivity of CPT I to malonyl-CoA inhibition in isolated rat liver mitochondria and the concentration of malonyl-CoA in the livers from which the mitochondria are isolated (Robinson & Zammit, 1982). One of the mechanisms suggested to account for this relationship was the continued tight binding of malonyl-CoA (proportional to its concentration in the liver *in vivo*) to CPT I during preparation of the mitochondria at a site which imparted sensitivity to CPT I (Robinson & Zammit, 1982). In the present study the effect of prior exposure of isolated mitochondria to malonyl-CoA *in vitro* on the sensitivity of CPT I to malonyl-CoA inhibition in subsequent assays has been investigated. The results show that preincubation of liver mitochondria with micromolar concentrations of malonyl-CoA increases significantly the sensitivity of CPT I to malonyl-CoA inhibition.

Materials and methods

Sources of rats and chemicals were as described previously (Zammit, 1981; Robinson & Zammit, 1982). Rat liver fatty acid synthase was purified as described by Zammit (1981).

Preparation and incubation of mitochondria

Mitochondria were isolated and washed as described by Robinson & Zammit (1982). The final mitochondrial pellet was resuspended in 10 ml of

extraction medium (250 mM-sucrose/5 mM-Tris/HCl/1 mM-EGTA, pH 7.4 at 0–2°C). One 3 ml portion of the mitochondrial suspension was used to assay CPT I activity immediately ('I' activity) either in the absence or in the presence of different concentrations of malonyl-CoA. Two other 3 ml portions of the mitochondrial suspension were added separately to 90 ml of a medium which contained 120 mM-KCl, 5 mM-Tris/HCl (pH 7.4 at 0–2°C), 1 mM-EGTA, 0.5 mM-glutathione (reduced), dialysed fatty acid-free albumin (10 mg/ml) and a known concentration of malonyl-CoA. The resulting mitochondrial suspensions were incubated for 10 min at 0°C and were then centrifuged at 9000 g for 5 min. The supernatants were decanted and the sides of the tubes dried and rinsed with fresh KCl medium without resuspending the pellets. The mitochondria were resuspended in 5 ml of KCl medium (from which malonyl-CoA was omitted), and the resulting suspensions were layered on top of a mixture (2 ml) of dibutyl phthalate and dinonyl phthalate (2:1, v/v), which had been layered on a 2 ml cushion of sucrose medium (see above) in 10 ml polycarbonate tubes. The tubes were centrifuged for 60 s at 12000 g in an MSE PrepSpin 50 centrifuge. The mitochondria migrated through the oil layer and formed pellets in the sucrose medium at the bottom of the tubes. The supernatant KCl medium was removed and the sides of the tubes and the surface of the oil layers were washed with fresh KCl medium. The oil and sucrose layers were decanted, the mitochondrial pellets were resuspended in 4.5 ml of fresh sucrose medium, and the activity of CPT I in the resulting mitochondrial suspensions was assayed in

Abbreviation used: CPT I, carnitine palmitoyltransferase I (EC 2.3.1.21).

the absence of malonyl-CoA ('control' activity) and at three different malonyl-CoA concentrations.

Respiratory control ratios (routinely found to be >6.0) were measured with a Clark-type oxygen electrode as described by Robinson & Zammit (1982), with 5 mM-glutamate and 5 mM-L-malate as substrates.

Assay of CPT I activity

CPT I activity was assayed at 37°C as described previously (Robinson & Zammit, 1982), except that the concentration of palmitoyl-CoA was 80 μ M and that 1 mM-glutathione (reduced) replaced 0.5 mM-dithiothreitol. A higher concentration of palmitoyl-CoA was used in order to increase the malonyl-CoA concentration required to inhibit the enzyme (competitively) and thus minimize the effects of any possible carry-over of malonyl-CoA from the preincubation medium to the assay medium (see below). Routinely, the reactions were stopped by addition of acid after 2 min, and [14 C]acylcarnitine formation was measured as described previously (Robinson & Zammit, 1982).

Assay of malonyl-CoA

This was performed as described by Ziegenhorn & Lynen (1974).

Expression of results

CPT I activity in mitochondria which were not preincubated (I) or which were preincubated in the presence (M+) or absence (M-) of malonyl-CoA was measured in the absence of malonyl-CoA ('control' activity) and in the presence of three different concentrations of malonyl-CoA. In Figs. 1-3 the activities obtained in the presence of malonyl-CoA in the assay medium are expressed as a percentage of the 'control' activity of the same preparation of mitochondria. Absolute values for 'control' activities of the mitochondria exposed to various conditions are given in the legends to the Figures.

Results and discussion

Fed virgin and starved late-pregnant rats were chosen for the present experiments because previous studies had shown that the sensitivity of CPT I activity in mitochondria from livers of these animals differed markedly (Robinson & Zammit, 1982). It was considered desirable not to use mitochondria in which CPT I activity is very sensitive to malonyl-CoA inhibition (e.g. from fed late-lactating or fed late-pregnant animals), to minimize the danger of interference from contamination of the assay medium by malonyl-CoA carried over from the preincubation mixture (see below). The experiments were designed to ascertain whether preincubation of

Fig. 1. Effect of preincubation of liver mitochondria from fed virgin female rats in the presence (●) or absence (○) of 65 μ M-malonyl-CoA on the subsequent inhibition of CPT I activity by increasing concentrations of malonyl-CoA in the assay medium

Results for CPT I activity in mitochondria that were not preincubated are denoted by (Δ). The 'control' activities were 0.98 ± 0.07 , 0.81 ± 0.05 and 0.72 ± 0.07 nmol/min per mg of mitochondrial protein at 37°C for 'I', 'M-' and 'M+' mitochondria respectively. Values are means $\pm$ S.E.M. for determinations on six separate mitochondrial preparations.

mitochondria with malonyl-CoA resulted in the sensitization of CPT I to malonyl-CoA inhibition and whether any such sensitization could be retained after separation of the mitochondria from the malonyl-CoA-containing incubation medium. In the course of preliminary experiments it was observed that the sensitivity of CPT I activity in mitochondria from fed virgin animals was somewhat decreased after preincubation in the absence of added malonyl-CoA (Fig. 1). This phenomenon was not observed for the enzyme in mitochondria from starved pregnant animals (which were originally much less sensitive) (Fig. 2). These observations suggested that, during preincubation and subsequent separation of the mitochondria, a factor that imparted the high degree of sensitivity shown by mitochondria from the fed animals was lost, with

Fig. 2. Effect of preincubation of liver mitochondria from starved 19-day-pregnant rats in the presence (●) or absence (○) of 65 μM-malonyl-CoA on the subsequent inhibition of CPT I activity by increasing concentrations of malonyl-CoA in the assay medium

Results for CPT I activity in mitochondria that were not preincubated are denoted by (Δ). The 'control' activities were 1.25 ± 0.14 (4), 0.91 ± 0.05 (9) and 0.91 ± 0.06 (8) nmol/min per mg of mitochondrial protein at 37°C for 'I', 'M-' and 'M+' mitochondria respectively. Values are means $\pm$ S.E.M. for the numbers of determinations (shown in parentheses) performed on separate mitochondrial preparations.

consequent desensitization, whereas in the mitochondria from the starved animals the content of this putative factor was low initially and little or no further loss occurred during processing of the mitochondria. As discussed below, it is suggested that this putative factor could be malonyl-CoA.

In the experiments in which preincubation of mitochondria with malonyl-CoA was performed, it was necessary to effect as efficient a separation of the mitochondria from the malonyl-CoA-containing incubation medium as possible, to prevent contamination of the assay medium with malonyl-CoA. Since successive washes of the mitochondria tended to result in loss of CPT I sensitivity (see above), it was decided to separate the mitochondria by centrifugation through oil (this procedure neces-

Fig. 3. Effect of increasing concentrations of malonyl-CoA in the preincubation medium on the subsequent activity of CPT I in mitochondria from starved 19-day-pregnant rats measured at 1.2 μM-malonyl-CoA in the assay medium (expressed as a percentage of 'control' activity)

Values are means $\pm$ S.E.M. for three to nine determinations on separate mitochondrial preparations. The 'control' activities were 0.91 ± 0.05 (9), 0.73 ± 0.05 (3), 1.10 ± 0.01 (4), 1.15 ± 0.04 (5) and 0.91 ± 0.06 (8) nmol/min per mg of protein at 37°C for mitochondria incubated in the presence of 0, 3.9 μM-, 15.6 μM-, 31.2 μM- and 65 μM-malonyl-CoA respectively.

sitated the use of a KCl-based iso-osmotic medium). That significant carry-over of malonyl-CoA from the preincubation medium into the assay medium did not occur was evident from the observation that the 'control' activities of 'M-' and 'M+' mitochondria were not very different. Mitochondria were preincubated with relatively large volumes of malonyl-CoA-containing KCl medium to prevent significant destruction of malonyl-CoA by the malonyl-CoA-deacylating activity of liver mitochondria (McGarry & Foster, 1981) and maintenance of a near-constant (known) malonyl-CoA concentration throughout the period of preincubation. To ascertain that no significant changes in malonyl-CoA concentration occurred during preincubation at 0°C, the malonyl-CoA concentration in the medium before and (in the supernatant) after incubation and separation of the

mitochondria was measured. No changes in malonyl-CoA concentration occurred during incubation even at the lowest concentrations used (results not shown).

Incubation of mitochondria from livers of fed virgin rats (Fig. 1) or starved pregnant animals (Fig. 2) with $65\mu\text{M}$ -malonyl-CoA resulted in a marked increase in the sensitivity of CPT I activity to malonyl-CoA compared with that shown by the enzyme preincubated concurrently in the absence of malonyl-CoA. In mitochondria from the fed animals this increase was primarily the result of the prevention of loss in sensitivity incurred during preincubation (see above), whereas in the starved animals (in which no loss in sensitivity occurred during preincubation; see Fig. 2) incubation with malonyl-CoA resulted in a marked increase in sensitivity of the enzyme compared with that found for 'I' activity of the same preparation of mitochondria. Therefore, only in mitochondria from starved animals was the induced sensitivity greater than that found in 'I' mitochondria. This difference in sensitization characteristics of the mitochondria from the two types of animals also suggested the involvement of a factor that was initially present in mitochondria from fed animals, but was absent (or present in much smaller quantities) in those from starved pregnant rats. Although the sensitivity of CPT I in mitochondria from starved pregnant rats was markedly increased after incubation with a maximally effective concentration of malonyl-CoA (see below), it did not reach the extent of the sensitivity found for mitochondria from fed virgin rats. Several characteristics of the system *in vitro* used (e.g. incubation of mitochondria with malonyl-CoA at 0°C and in dilute suspension) could account for this residual difference.

There was a small (<20%) decrease in the 'control' activity of CPT I between 'I' and 'M-' or 'M+' mitochondria. This decrease occurred independently of the changes in inhibitory characteristics. The possibility that it could have been caused by the decreased coupling of the mitochondrial respiration was excluded by the observation that the respiratory control ratios for 'I', 'M+' and 'M-' mitochondria were the same and that oxygen consumption of the three types of mitochondria was negligible in the absence of added ADP (results not shown).

In a second series of experiments the dependence of the malonyl-CoA-induced sensitization of CPT I activity on the concentration of malonyl-CoA in the

preincubation medium was studied using mitochondria from starved pregnant rats [in which the degree of sensitization, measured at $1.2\mu\text{M}$ -malonyl-CoA in the assay medium, was larger (Fig. 2) and, consequently, more accurately determined]. Preincubation of mitochondria with a concentration of malonyl-CoA as low as $6\mu\text{M}$ induced a near-maximal sensitization of the enzyme (Fig. 3). The concentration range of malonyl-CoA at which half-maximal sensitization was induced was $1\text{--}3\mu\text{M}$. These concentrations of malonyl-CoA are within the physiological range of concentrations previously found to occur in freeze-clamped liver samples of rats in different physiological conditions ($0.4\text{--}6.8\text{nmol/g}$; Zammit, 1981). It is suggested that the malonyl-CoA-induced sensitization of CPT I to malonyl-CoA inhibition observed in the present study could be the mechanism whereby the quantitative relationship between the sensitivity of the enzyme *in vitro* and the malonyl-CoA concentration *in vivo* (Robinson & Zammit, 1982) is mediated. Furthermore, it is suggested that, since the increase in sensitivity was observed at micromolar and physiological concentrations of malonyl-CoA, the sensitivity of CPT I to malonyl-CoA inhibition *in vivo* is also affected by the cytosolic concentration of malonyl-CoA in the liver. Malonyl-CoA-induced malonyl-CoA inhibition of CPT I could provide a potent amplification mechanism for the regulation of acylcarnitine synthesis by small changes in malonyl-CoA concentration. These suggestions, if valid, would imply that malonyl-CoA binds to CPT I at a site other than the site at which it competes with long-chain acyl-CoA (see, e.g., Saggerson & Carpenter, 1981) and that the malonyl-CoA-induced sensitization is possibly mediated through an allosteric mechanism.

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