Changes in the ability of malonyl-CoA to inhibit carnitine palmitoyltransferase I activity and to bind to rat liver mitochondria during incubation *in vitro*

Differences in binding at 0°C and 37°C with a fixed concentration of malonyl-CoA

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1. Time courses for inhibition of carnitine palmitoyltransferase (CPT) I activity in, and [^{14}C]malonyl-CoA binding to, liver mitochondria from fed or 48 h-starved rats were obtained at 37°C by using identical incubation conditions and a fixed concentration of malonyl-CoA (3.5 μM), which represents the middle of the physiological range observed previously [Zammit (1981) *Biochem. J.* **198**, 75–83]. 2. Incubation of mitochondria in the absence of malonyl-CoA resulted in a time-dependent decrease in the ability of the metabolite instantaneously to inhibit CPT I and to bind to the mitochondria. Both degree of inhibition and binding were restored in parallel over a period of 6–8 min on subsequent addition of malonyl-CoA to the incubation medium. However, the increased inhibition of CPT I activity on addition of mitochondria directly to malonyl-CoA-containing medium was not accompanied by an increase in the amount of [^{14}C]malonyl-CoA bound to mitochondria at 37°C. 3. Time courses for binding of [^{14}C]malonyl-CoA performed at 0°C were different from those obtained at 37°C. There was little loss of ability of [^{14}C]malonyl-CoA to bind to mitochondria on incubation in the absence of the metabolite, but there was a time-dependent increase in binding on addition of mitochondria to malonyl-CoA-containing medium. 4. It is suggested that these temperature-dependent differences between the time courses obtained may be due to the occurrence of different changes at 37°C and at 0°C in the relative contributions of different components (with different affinities) to the binding observed at 3.5 μM -malonyl-CoA. Evidence for multi-component binding was obtained in the form of strongly curvilinear Scatchard plots for instantaneous (5 s) binding of malonyl-CoA to mitochondria. Such multi-component binding would be expected from previous results on the different affinities of CPT I for malonyl-CoA with respect to inhibition [Zammit (1984) *Biochem. J.* **218**, 379–386]. 5. Mitochondria obtained from starved rats showed qualitatively the same time courses as those described above, with notable quantitative differences with respect both to the absolute extents of CPT I inhibition and [^{14}C]malonyl-CoA binding achieved as well as to the time taken to attain them.

Previous work from this laboratory established the time-dependence of sensitivity to inhibition by malonyl-CoA of overt CPT activity in isolated rat liver mitochondria (Zammit, 1984). This time-dependent increase in sensitivity of the enzyme was primarily due to a malonyl-CoA-induced

transition of the enzyme from a form with low affinity to one with high affinity for the metabolite. It was suggested that the reverse transition, i.e. desensitization of the enzyme, could also occur at physiological temperatures *in vitro*, and that if sensitization and desensitization also occur *in vivo* and are freely reversible they would provide a mechanism whereby the effective sensitivity of the enzyme to malonyl-CoA *in vivo* would be determined by the relative amounts of the low- and

Abbreviations used: CPT I, carnitine palmitoyltransferase I (EC 2.3.1.21); $I_{0.5}$, concentration required to give 50% inhibition.

high-affinity forms. These, in turn, would reflect the concentration of malonyl-CoA in the liver. In the present study we have set out to demonstrate the process of desensitization *in vitro* and its reversibility by subsequent incubation of mitochondria with malonyl-CoA.

If at any one chosen concentration of malonyl-CoA an increase in the degree of inhibition occurs with time of incubation of the mitochondria in the metabolite-containing medium, then it is possible that time-dependent changes in the extent of binding of malonyl-CoA to the mitochondria could also be observed. We have investigated the time-course of binding of [^{14}C]malonyl-CoA to rat liver mitochondria at 37°C, using the same concentration of malonyl-CoA (3.5 μM) used for the activity studies such that a direct comparison was possible. In addition, we studied binding at 0°C in order to test our previous suggestion (Zammit, 1984) that the complete loss of sensitivity to malonyl-CoA in isolated mitochondria could be prevented by a very slow rate of loss of malonyl-CoA binding which may exist at the low temperatures employed during the preparation of the mitochondria. The results are discussed in terms of the mechanism of desensitization of CPT I *in vitro* and its malonyl-CoA-induced reversal, as well as of the caution required in performing (and interpreting the data obtained from) malonyl-CoA-binding experiments on rat liver mitochondria.

Materials and methods

Animals

Female Wistar rats (160–200 g) were maintained as described previously (Zammit, 1983a,b). Starved rats were deprived of food for 48 h before being killed, but had continuous access to water.

Chemicals

[2- ^{14}C]Malonyl-CoA and [^3H]sucrose were obtained from Amersham International (Amersham, Bucks., U.K.). All other chemicals and biochemicals were obtained from sources described previously (Zammit, 1984).

Preparation of mitochondria

Rat liver mitochondria were prepared essentially as described previously (Zammit, 1980), except that mitochondria were washed once in a sucrose-free medium containing 150 mM-KCl, 5 mM-Tris/HCl and 1 mM-EGTA, pH 7.4, at 0°C before being sedimented and resuspended at a concentration of approx. 50 mg of protein/ml in the same medium. Stock mitochondrial suspensions were kept on ice in this medium; experiments were performed within 1 h of final resuspension of the mitochondria.

Assay of CPT I activity

This was performed at 37°C essentially as described previously (Zammit, 1984), except that the medium contained no sucrose. The composition of the assay medium (2.0 ml) was 150 mM-KCl, 5 mM-Tris/HCl, 1 mM-EGTA, 10 mg of albumin/ml, 1 μg of antimycin A/ml, 2 μg of rotenone/ml and 1 mM-dithiothreitol. The final pH was 7.4 at 37°C. Reactions were started either by addition of mitochondria (2–3 mg of mitochondrial protein) or by addition of a prewarmed mixture of palmitoyl-CoA–albumin and L-carnitine. The final concentration of palmitoyl-CoA was 80 μM and that of L-carnitine was 0.4 mM (containing 0.4 μCi of [^{14}C]carnitine). In experiments started by addition of mitochondria, palmitoyl-CoA and carnitine were already included in the assay medium; in those started by addition of substrates mitochondria were incubated for various times in the assay medium in the presence or absence of 3.5 μM -malonyl-CoA before initiation of the reactions which were terminated after 15 s by addition of 0.3 ml of 6 M-HCl. The [^{14}C]acylcarnitine formed was quantified as described previously (Robinson & Zammit, 1982).

Measurement of [^{14}C]malonyl-CoA binding to mitochondria

Binding experiments were performed in a medium identical with that described for CPT I assays (above), except that the final volume was 1.0 ml and 0.45 μCi of [^3H]sucrose (sp. radioactivity 9.8 mCi/ μmol) was also included (this represented a concentration of 50 μM -sucrose; identical sucrose-space values were obtained with 2.5 mM- and 5 mM-sucrose). The mixture was contained in 1.5 ml Eppendorf capped plastic tubes. When the instantaneous binding (5 s) of [^{14}C]malonyl-CoA to mitochondria was measured, malonyl-CoA (3.5 μM in 10 μl ; 58 Ci/mol) was added to the medium containing approx. 2 mg of mitochondrial protein. If no incubation of mitochondria in the absence of malonyl-CoA was required, a portion (50 μl) of mitochondrial suspension was added directly to the [^{14}C]malonyl-CoA-containing medium. The mixture was mixed for 5 s and centrifuged (see below). In experiments in which instantaneous binding was to be studied after a period of incubation of the mitochondria in the absence of malonyl-CoA, the medium containing the mitochondria was incubated (at 0°C or 37°C) for the required period of time, followed by addition of malonyl-CoA, mixing and centrifugation as above. In experiments in which the effect on binding of different times of incubation of mitochondria with malonyl-CoA was to be studied, the medium containing mitochondria and [^{14}C]-

malonyl-CoA was incubated for the required time before centrifugation. Separation of the mitochondria from the medium was achieved by centrifugation at 12000g for 90s in Eppendorf 5412 centrifuges, the rotors of which were kept ice-cold before use. The supernatant was immediately removed and retained. The mitochondrial pellet and the sides of the tube were freed as much as possible from adhering medium. The pellets were resuspended in 0.3 ml of ice-cold 6% (v/v) HClO₄, followed by centrifugation. Samples of the original supernatant and of that of the acidified pellets were taken and measured for their contents of ¹⁴C and ³H radioactivity to obtain a measure of specific binding of malonyl-CoA to the rat liver mitochondria.

Results and discussion

Measurement of CPT I activity and [¹⁴C]-malonyl-CoA binding to mitochondria were performed in identical media (except that 50 μM-[³H]-sucrose was present in the binding experiments; this minimal concentration of the sugar was thought to be unlikely to alter malonyl-CoA interactions to any significant extent). The respective time courses for the effects of mitochondrial incubation in the absence or presence of malonyl-CoA were obtained in the absence of substrates (except, of course, that activity measurements necessitated the addition of the substrates at the end of the appropriate incubation periods to measure the amount of product formation over a 15s period). As explained previously (Zammit, 1984), 15s was the shortest reproducible assay time that could be used and that resulted in the production of easily measurable amounts of [¹⁴C]acyl-carnitine. The importance of obtaining as near an instantaneous measurement of enzyme activity (or binding) as possible was emphasized by previous results (Zammit, 1984). For [¹⁴C]malonyl-CoA binding experiments [in which the restraints imposed by the enzyme assay system (above) were not present], we obtained an assessment of near-instantaneous binding by mixing mitochondria with [¹⁴C]malonyl-CoA for 5s before centrifugation. The term 'instantaneous' is used below to denote the shortest possible mixing time before centrifugation. For both CPT I assay and [¹⁴C]-malonyl-CoA-binding experiments the same concentration of malonyl-CoA (3.5 μM) was used, so that for both sets of experiments performed at 37°C the time courses of the changes in sensitivity and binding observed were directly comparable. The concentration of malonyl-CoA used was chosen for several reasons: (i) it represents the middle of the range of concentrations measured previously in freeze-clamped liver samples from animals in a

variety of physiological conditions (Zammit, 1981); (ii) it is intermediate between the I_{0.5} of low- (>10 μM) and high- (<0.3 μM) affinity forms of CPT I (see Zammit, 1984); (iii) it represents an approx. 10-fold excess over the lower of these two I_{0.5} values, such that after complete sensitization (6–8 min) with this concentration of malonyl-CoA the observed inhibition would be essentially maximal (see Zammit, 1984), although we appreciated that, under the conditions used in the present study to assay CPT I activity, the absolute values of the respective I_{0.5} values may have differed from those determined in a sucrose/KCl medium (Zammit, 1984). However, in view of (i) above, it was considered that 3.5 μM represented the most 'physiological' concentration of malonyl-CoA that we could use.

Time-dependence of CPT I inhibition by malonyl-CoA

For each time point illustrated in Fig. 1 the activity of CPT I was measured in the presence and absence ('control') of 3.5 μM-malonyl-CoA. This enabled us to determine the sensitivity of CPT I to this concentration of malonyl-CoA (under the assay conditions used) even at those time points for which complete thermal equilibration could not be assumed, e.g. 30 and 60s after addition of mitochondrial suspension (at 0°C) to the assay medium (at 37°C). We had to resort to such a method, since changes in sensitivity occurred even during this time period, and, although the exact temperature could not be ascertained, it was reasonable to assume that the rate of thermal equilibration was reproducible for a fixed volume of mitochondrial suspension at 0°C added to the incubation medium at 37°C. The control activity increased by 10–20% during the first 90s of incubation, and thereafter remained constant for all time periods studied (results not shown). The use of a sucrose-free medium in the final wash during mitochondrial isolation and for the CPT I assay medium resulted in control activities which were approx. 6-fold higher than those obtained previously (Zammit, 1984), but the relative activities for mitochondria from fed and starved animals as well as the ratios for CPT activity in whole and sonicated mitochondria were unchanged (results not shown). The activation of the enzyme in rat liver mitochondria by K⁺ was also reported by Saggerson (1982).

CPT I desensitization and its reversal

The activity of CPT I was inhibited by approx. 50% in the presence of 3.5 μM-malonyl-CoA in mitochondria obtained from fed rats when the reactions were started by addition of mitochondrial suspension (Fig. 1). If the mitochondria were incubated at 37°C in assay medium in the

Fig. 1. Time course of changes in the inhibition of CPT I activity by $3.5 \mu\text{M}$ -malonyl-CoA in rat liver mitochondria

Mitochondria from fed (closed symbols) or starved rats (open symbols) were preincubated at 37°C in the absence of malonyl-CoA ($\blacksquare$, $\square$) followed by measurement of activity in the presence or absence of malonyl-CoA. The results with the inhibitor present are depicted as percentages of the activity measured without the inhibitor. After 8 min of preincubation, samples of mitochondria were further incubated with malonyl-CoA for various lengths of time before measurement of activity ($\bullet$, $\circ$). Other samples of mitochondria were preincubated in the presence of malonyl-CoA from the beginning ($\blacktriangle$, $\triangle$). CPT I assays were performed over 15 s as described in the Materials and methods section. Values are averages of three determinations ($\pm$ S.E.M.) on separate mitochondrial preparations. Where S.E.M. bars are not shown, values are averages of two experiments. Control activities after 2 min were 2.0 ± 0.4 and 3.4 ± 0.8 (nmol/15 s per mg of mitochondrial protein at 37°C) for mitochondria from fed and starved rats respectively ($n = 3$).

absence of malonyl-CoA before addition of substrates and malonyl-CoA, there was a progressive decrease in sensitivity of CPT I to inhibition such that after 2 min the enzyme was only inhibited by $7 \pm 5\%$. Thereafter there was a several-fold decrease in inhibition of the enzyme by $3.5 \mu\text{M}$ -malonyl-CoA after 2 min incubation. Therefore (up to 18 min) the degree of inhibition remained within this range. These observations verified our previous suggestion that the reverse process to the sensitization observed *in vitro* (Zammit, 1984) could be produced by incubation of mitochondria at 37°C in the absence of malonyl-CoA.

Addition of mitochondria directly to a malonyl-CoA-containing medium at zero time resulted in

an increase in sensitivity. For mitochondria from fed animals inhibition increased from $50.0 \pm 6.6\%$ to $70.0 \pm 3.0\%$ of control activity within 2 min and was constant thereafter up to 8 min. (As mentioned above, this degree of inhibition is likely to represent the maximal amount of the enzyme that was inhibitable by malonyl-CoA.) Addition of $3.5 \mu\text{M}$ -malonyl-CoA to mitochondria that had been incubated at 37°C in the absence of malonyl-CoA for 8 min resulted in complete reversal of the desensitization (see above). The re-sensitization process took approx. 6–8 min to complete, and by the end of this period the sensitivity of the enzyme was similar to that achieved after addition of mitochondria directly to malonyl-CoA-containing medium at zero time (Fig. 1).

The desensitization–resensitization cycle was qualitatively similar for CPT I in mitochondria from 48 h-starved rats, although it showed several important quantitative differences. (i) The initial sensitivity of CPT I was very much lower than in mitochondria from fed rats (inhibition = $14.1 \pm 3.7\%$ and $50.0 \pm 6.6\%$ for mitochondria from starved and fed rats respectively). This was a much larger difference than that found in our previous study; this may have been due to the fact that the difference in sensitivity of the enzyme in the two types of mitochondria was decreased after incubation of the mitochondria for any length of time before addition of malonyl-CoA and substrates during the time-dependent desensitization described above and of which we were not aware at the time of our previous experiments. Nevertheless, CPT I in ‘starved’ mitochondria appeared to be desensitized more completely (Fig. 1). The difference in sensitivity of CPT I to inhibition by $3.5 \mu\text{M}$ -malonyl-CoA in the two types of mitochondria was completely abolished only after 14–18 min of incubation of the mitochondria in the absence of malonyl-CoA. (ii) The rate of sensitization of CPT I in mitochondria from fed or starved rats was markedly different when mitochondria were added directly to the malonyl-CoA-containing medium (Fig. 1). However, sensitization after 8 min of incubation of mitochondria in the absence of malonyl-CoA occurred at the same rate for CPT I in both types of mitochondria. (iii) The maximal inhibition attained after completion of sensitization was significantly larger for CPT I in mitochondria from fed rats (70%) than in those from 48 h-starved animals (55%). This difference was not dependent on the rate at which the maximal inhibition was attained (see above), and was the same (about 16%) irrespective of whether maximal inhibition was produced before or after desensitization. A decrease in the fraction of CPT I that is inhibitable by malonyl-CoA during starvation has been sug-

gested to occur by other workers (Saggerson & Carpenter, 1981; Bremer, 1981), and may contribute towards the apparently lower inherent sensitivity of the enzyme at zero time in mitochondria from starved rats. The CPT I/CPT II (latent carnitine palmitoyltransferase) activity ratio (average 0.45, $n = 3$) was higher in mitochondria from starved rats (as judged by CPT activity in intact and sonicated mitochondria) than in mitochondria from fed animals (average 0.28, $n = 3$). This observation was in agreement with previous work (Saggerson & Carpenter, 1983; Stakkestad & Bremer, 1983).

Time courses for [^{14}C]malonyl-CoA binding to mitochondria

Under the conditions used, the [^3H]sucrose space of pelleted rat liver mitochondria was identical with the extramitochondrial space estimated by using excess non-labelled malonyl-CoA (see Table 1). The routine use of [^3H]sucrose enabled the effects of a greater number of conditions on binding to be studied on the same preparation of mitochondria within a reasonable time of their preparation. Time-dependence of [^{14}C]malonyl-CoA binding was studied at 0°C and at 37°C for two reasons: (i) to test our previous suggestion that loss of sensitivity of CPT I on incubation of rat liver mitochondria at 37°C could be accompanied by loss of malonyl-CoA-binding ability, whereas incubation at 0°C could result in a much slower loss (see Zammit, 1984); and (ii) to obtain information which would be required to devise future experimental protocols suitable for the study of the concentration-dependence of binding and any time-dependent changes thereof.

The time-courses illustrated in Figs. 2–4 were obtained by a combination of studies on instantaneous (5s) binding and that obtained after the

required period of incubation of the mitochondria with $3.5\ \mu\text{M}$ [^{14}C]malonyl-CoA. That binding obtained after 5s was likely to represent the instantaneous equilibration of malonyl-CoA binding at one or more classes of binding sites on the mitochondria was indicated by the observation that the degree of binding so obtained at zero time for mitochondria added directly to malonyl-CoA-containing medium was quantitatively similar whether determined at 0°C or at 37°C [starved 25.5 ± 2.3 ($n = 4$) at 37°C and 24.1 ± 2.6 ($n = 3$) pmol/mg of mitochondrial protein at 0°C; fed, 15.6 ± 2.0 ($n = 3$) at 37°C, and 14.5 ± 1.0 ($n = 3$) at 0°C; see also detailed time course of binding at the two temperatures, Figs. 2–4]. If equilibration between bound and free malonyl-CoA were not instantaneous, binding would be expected to be much higher at 37°C than at 0°C after only 5s. This conclusion was also supported by the observation that the concentration-dependence for instantaneous binding at 0°C and 37°C was virtually identical (Fig. 5).

(i) *Binding at 37°C.* The most striking feature of the time courses for [^{14}C]malonyl-CoA binding to mitochondria at 37°C (Figs. 3 and 4) was their general reciprocity to those obtained for the changes in inhibition of CPT I at the same concentration of malonyl-CoA (Fig. 1). The decreased sensitivity of CPT I during the first 2 min of incubation of mitochondria in the absence of malonyl-CoA was accompanied by a decreased ability to bind [^{14}C]malonyl-CoA. The subsequently induced increase in sensitivity on addition of malonyl-CoA was also mirrored by an increase in

Table 1. Representative comparison of measurement of non-specific malonyl-CoA association with rat liver mitochondria by using excess unlabelled malonyl-CoA (2mM) and [^3H]sucrose (0.45 $\mu\text{Ci/ml}$; 9.8 mCi/ μmol)

In the experiment illustrated, mitochondria were from a fed rat and binding was allowed to proceed for 1 min at 0°C before centrifugation. Routinely the sucrose space of the mitochondrial pellets was 6–7 μl /mg of mitochondrial protein.

$10^{-3} \times ^{14}\text{C}$ in supernatant (d.p.m./ml)	Non-specific binding of [^{14}C]malonyl-CoA (d.p.m.)	
	^{14}C in pellet	Calculated ^{14}C in sucrose space
38.2	390	373
120.0	1347	1373
247.3	2375	2388
632.5	6161	6097
1289.2	13679	13741

Fig. 2. Detailed time course of binding of [^{14}C]malonyl-CoA ($3.5\ \mu\text{M}$) to mitochondria from liver of a fed rat. Mitochondria were added to the malonyl-CoA-containing medium at 0°C (○) or 37°C (●) and mixed. After the indicated times, the tubes were centrifuged and the amount of specific [^{14}C]malonyl-CoA binding was measured as described in the Materials and methods section.

Fig. 3. Time course of binding of [^{14}C]malonyl-CoA ($3.5\mu\text{M}$) to mitochondria from livers of fed rats

Experiments were performed at 0°C (open symbols) or 37°C (closed symbols). Mitochondria were either incubated in the absence of malonyl-CoA ($\bullet$, $\circ$) followed after 2 or 8 min by addition of malonyl-CoA ($\blacktriangle$, $\blacklozenge$ and $\triangle$ respectively) or incubated directly in malonyl-CoA-containing medium ($\blacksquare$, $\square$). Separation of the mitochondria from the medium was achieved by centrifugation at $12000g$ for 90 s in Eppendorf 5412 centrifuges, the rotors of which were kept ice-cold before use. The supernatant was immediately removed and retained. The mitochondrial pellet and the sides of the tube were freed as much as possible from adhering medium. The pellets were resuspended in 0.3 ml of ice-cold 6% HClO_4 , followed by centrifugation. Samples of the original supernatant and of that of the acidified pellets were taken and measured for their contents of ^{14}C and ^3H radioactivity in order to obtain a measure of specific binding of malonyl-CoA to the rat liver mitochondria. The experiment here is typical of results obtained with mitochondrial preparations from three different animals. Averages of values for the amount of malonyl-CoA bound per mg of mitochondrial protein at zero time are given in the text.

the degree of binding, with a similar time-dependence. It is noteworthy that the final extent of binding attained after several minutes in the presence of malonyl-CoA was identical irrespective of whether the mitochondria had been incubated with malonyl-CoA from the start or only after 2 or 8 min incubation in the absence of malonyl-CoA.

The exception to these generally parallel (but reciprocal) changes in sensitivity of the enzyme and [^{14}C]malonyl-CoA binding to mitochondria was the observation that increased inhibition of CPT I on incubation of mitochondria with malonyl-CoA at zero time (Fig. 1) was not accompanied by any apparent increase in the

Fig. 4. Time course of binding of [^{14}C]malonyl-CoA ($3.5\mu\text{M}$) to mitochondria from livers of 48 h-starved rats

The notation used is explained in the legend to Fig. 3. The experiment here is typical of results obtained with mitochondrial preparations from three different animals. Averages of values for the amount of malonyl-CoA bound per mg of mitochondrial protein at zero time are given in the text.

Fig. 5. Scatchard plots for instantaneous (5 s) binding of [^{14}C]malonyl-CoA to rat liver mitochondria

Mitochondria were added to medium either at 0°C ($\circ$) or at 37°C ($\bullet$) containing various concentrations of malonyl-CoA. The medium was mixed for 5 s and centrifuged. The two plots shown were obtained for the same preparation of mitochondria; very similar plots were obtained with several other preparations.

degree of binding of [^{14}C]malonyl-CoA to mitochondria (Fig. 3). The reason for this apparent discrepancy is not known. However, as shown in Fig. 5, the concentration-dependence of [^{14}C]-

malonyl-CoA binding to rat liver mitochondria suggests that binding is probably due to two or more components with different affinities. It is possible that, although the observed degree of binding at $3.5\mu\text{M}$ -malonyl-CoA was apparently little changed with time at 37°C , the relative contributions of these components (with affinities of $<1\mu\text{M}$ and $>10\mu\text{M}$ respectively) may have varied reciprocally with time. The possibility that increased binding was not observed owing to rapid loss of malonyl-CoA from the medium cannot be entirely discounted. However, if this were the sole cause, it would have been expected to prevent also the increased binding which was observed after 2 and 8 min of incubation of the mitochondria in the absence of malonyl-CoA (Figs. 3 and 4). Moreover, in experiments performed at $7\mu\text{M}$ -malonyl-CoA, there was also no increase in [^{14}C]malonyl-CoA binding with time at 37°C (results not shown), whereas the loss of malonyl-CoA from the medium at this concentration is only 10–15% even after 6 min of incubation (see also Zammit, 1984).

(ii) *Binding at 0°C .* The time courses for binding of [^{14}C]malonyl-CoA to rat liver mitochondria at 0°C were very different from those obtained at 37°C . Thus at 0°C there was little decrease in the amount of instantaneous binding by mitochondria incubated in the absence of malonyl-CoA for up to 8 min, whereas there was a marked increase in binding over a period of several minutes in the presence of malonyl-CoA (Figs. 3 and 4). Therefore at 0°C the time courses of binding were exactly the opposite to those observed at 37°C (see above). As a result the maximal degree of binding observed was about 2-fold higher at 0°C than at 37°C . {This observation was in contrast with those of McGarry *et al.* (1983), who studied [^{14}C]malonyl-CoA binding to rat heart mitochondria.} It is suggested that the different time courses obtained at the two temperatures may have been due to differences in time-dependent changes (at 0°C and 37°C) in the relative contributions of the two or more binding components with different binding affinity, the presence of which was indicated by the curvilinear Scatchard plots for instantaneous binding at the two temperatures (Fig. 5). If this suggestion is valid, it could provide a possible mechanism whereby desensitization of CPT I occurs readily at 37°C , but not at 0°C , in isolated mitochondria (see also Zammit, 1984).

Although the general pattern of time-dependent changes in [^{14}C]malonyl-CoA binding by mitochondria from fed or starved animals was similar, the degree of binding was approx. 1.5-fold higher (expressed per mg of mitochondrial protein) for mitochondria from starved animals (see above). It is noteworthy that this corresponds to a similar order of difference between the control activities of

CPT I in the two types of mitochondria (see legend to Fig. 1, and Zammit, 1984). This similarity between the two parameters suggests that malonyl-CoA binding to CPT I may account for a substantial part of the binding of malonyl-CoA to rat liver mitochondria. However, there were no obvious correlations between the differences in the sensitivity of CPT I to malonyl-CoA inhibition in the two types of mitochondria and their malonyl-CoA-binding behaviour, although loss of malonyl-CoA-binding ability occurred faster in mitochondria from starved rats than in those from fed animals (cf. Figs. 3 and 4).

Conclusions

We have verified that desensitization of CPT I to inhibition by malonyl-CoA occurs *in vitro* and that it is fully reversible by subsequent incubation with a physiological concentration of malonyl-CoA. We have also obtained evidence in support of the suggestion (Zammit, 1984) that changes in the binding of malonyl-CoA to mitochondria may occur at 37°C that do not occur at 0°C . A detailed analysis of the correspondence between changes in [^{14}C]malonyl-CoA binding to mitochondria and changes in sensitivity of CPT I will require the parallel determination of $I_{0.5}$ values for malonyl-CoA inhibition of CPT I and of the affinity constants for malonyl-CoA binding at different stages during the corresponding time courses. The multi-component Scatchard plots shown in Fig. 5 suggest that different malonyl-CoA-binding sites with very different affinities (from $<1\mu\text{M}$ to $>10\mu\text{M}$) for malonyl-CoA exist in rat liver mitochondria. If CPT I accounted for a major part of this binding (see above), this would be expected, in view of the similarly large difference in $I_{0.5}$ values for malonyl-CoA observed for inhibition of CPT I activity before and after malonyl-CoA-induced sensitization (Zammit, 1984).

The physiological implications of time- and malonyl-CoA-dependent changes in affinity of CPT I for malonyl-CoA have been discussed in detail elsewhere (Zammit, 1984) and are reinforced by the present direct demonstration of the occurrence of time-dependent desensitization and its reversal *in vitro*. As mentioned above, studies in which CPT I activity and [^{14}C]malonyl-CoA binding to rat liver mitochondria are studied at a range of malonyl-CoA concentrations will be required to confirm the conclusions derived from the present study. The practical experimental implications of the present observations are self-evident: caution will need to be exercised in experiments in which malonyl-CoA binding to rat liver mitochondria is studied. Large differences in binding can be obtained, depending on the order of addition of malonyl-CoA and mitochondria to the

incubation medium as well as on the temperature at which binding studies are performed.

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