

Binding of [¹⁴C]malonyl-CoA to rat liver mitochondria after blocking of the active site of carnitine palmitoyltransferase I

Displacement of low-affinity binding by palmitoyl-CoA

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1. The active site of the overt activity of carnitine palmitoyltransferase (CPT I) in rat liver mitochondria was blocked by the self-catalysed formation of the *S*-carboxypalmitoyl-CoA ester of (–)-carnitine, followed by washing of the mitochondria. CPT I activity in treated mitochondria was inhibited by 90–95%. 2. Binding of [¹⁴C]malonyl-CoA to these mitochondria was not inhibited as compared with that of control mitochondria. 3. When CPT I activity was inhibited, palmitoyl-CoA could markedly displace [¹⁴C]malonyl-CoA binding from the low-affinity site for the inhibitor [Zammit, Corstorphine & Gray (1984) *Biochem. J.* **222**, 335–342], but not from the high-affinity site for malonyl-CoA binding. The saturation characteristics of the malonyl-CoA-binding component lost in the presence of palmitoyl-CoA were sigmoidal, and thus suggestive of co-operative binding at this site. 4. It is suggested that the site hitherto considered to be a low-affinity malonyl-CoA-binding site may be effectively a second, allosteric, acyl-CoA-binding site on CPT I under conditions that prevail *in vivo*, whereas the high-affinity site for malonyl-CoA may be exclusive to the inhibitor. 5. The possibility that the competitive-type interactions of malonyl-CoA and acyl-CoA on CPT I activity could arise from the effects of separate malonyl-CoA and acyl-CoA allosteric sites is considered. The possible significance of the large difference in the capacity of the two sites and their different saturation kinetics is also discussed.

INTRODUCTION

The initial categorization of inhibitory effect of malonyl-CoA on overt activity of carnitine palmitoyltransferase (CPT I) in mitochondria as competitive with respect to acyl-CoA substrate was made on the basis of the antagonization of inhibition by increasing concentrations of long-chain acyl-CoA. It has become evident, however, that the interaction of malonyl-CoA with CPT I must be more complex than could be accounted for solely by binding of the inhibitor at the active site of the enzyme. The observations that, when solubilized from the mitochondrial inner membrane, CPT I displays no inhibition by malonyl-CoA suggest that either malonyl-CoA never interacts with the active site of the enzyme (even when CPT I resides in the membrane) and/or that an allosteric-type effect of the inhibitor that permits such interaction is lost upon solubilization (Fiol & Bieber, 1984). From studies on the pH-dependence of the affinity of CPT I for palmitoyl-CoA as a substrate and of the ability of palmitoyl-CoA to displace [¹⁴C]malonyl-CoA bound to skeletal-muscle mitochondria, Mills *et al.* (1984) have hypothesized that binding of palmitoyl-CoA at the active site is distinct from that which occurs at a second, malonyl-CoA-binding, site. In these mitochondria only one, high-affinity, class of binding sites for malonyl-CoA was observed (Mills *et al.*, 1983). In rat liver mitochondria there are at least two malonyl-CoA-binding sites, which have a greater than 10-fold difference in avidity for malonyl-CoA (Zammit *et al.*, 1984; Bird & Saggerson, 1984).

In spite of these indications of separate regulatory and

catalytic sites on CPT I, there is no direct evidence for such an arrangement. No studies have been performed to ascertain the physical identity or otherwise of either, or both, of the malonyl-CoA-binding sites with the active site of CPT I in rat liver mitochondria (or mitochondria from other tissues). We were therefore interested in establishing directly whether blocking of the active site of CPT I affected the binding of [¹⁴C]malonyl-CoA to rat liver mitochondria and whether any residual malonyl-CoA binding could be displaced by palmitoyl-CoA, which, owing to the inaccessibility of the active site, would have to interact directly with the malonyl-CoA-binding site(s). The results suggest that malonyl-CoA binding to rat liver mitochondria is not decreased after blocking the active site by the self-catalysed formation of the *S*-carboxypalmitoyl-CoA ester of carnitine. In addition it is shown that palmitoyl-CoA only effectively displaces malonyl-CoA bound to the low-affinity site and not that bound to the high-affinity site on rat liver mitochondria. It is suggested that the regulatory site with low affinity for malonyl-CoA can be considered to be primarily a long-chain-acyl-CoA-binding site *in vivo*.

MATERIALS AND METHODS

The sources of animals and chemicals were as described previously (Zammit, 1980, 1984). In addition, 2-bromopalmitate was obtained from Aldrich Chemical Co. (Milwaukee, WI, U.S.A.), and CoA and ATP were from Sigma (Poole, Dorset, U.K.).

Mitochondria were isolated by differential centrifugation in a medium containing 300 mM-sucrose, 5 mM-

Tris/HCl and 1 mM-EGTA (pH 7.4 at 0 °C) as described previously (Zammit, 1980). They were suspended in 7.5 ml of medium containing 150 mM-KCl, 5 mM-Tris/HCl and 1 mM-EGTA (pH 7.4 at 0 °C). Samples (3.5 ml) of this suspension were added to two tubes containing 22 ml of KCl medium (pH 7.4 at 25 °C), which contained combinations of 2-bromopalmitate (20 μ M), ATPMg (1 mM) and CoA (100 μ M) as indicated in the text and legends to Figures. The mitochondria were incubated at 25 °C for 5 min, and for a further 5 min after the addition of L-carnitine (0.25 ml; final concn. 2 mM) or KCl medium (controls). The mitochondria were subsequently sedimented by centrifugation at 2000 *g* for 10 min. They were resuspended in 25 ml of KCl medium (at 0 °C) and washed once before final sedimentation and resuspension in 1.5–2.0 ml of KCl medium. They were kept on ice until used (within 30 min). The temperature used for these incubations (25 °C) was the highest at which subsequent binding of [¹⁴C]malonyl-CoA to the mitochondria was unaffected and which gave self-catalysed inhibition of CPT I within a reasonably short time.

Specific binding of [¹⁴C]malonyl-CoA to mitochondria was measured as described previously (Zammit *et al.*, 1984). Mitochondria (1–1.5 mg of protein) were incubated at 0 °C in a medium containing 150 mM-KCl, 5 mM-Tris/HCl, 1 mM-EGTA, 1 mM-dithiothreitol, 10 mg of albumin/ml, 1 μ g of antimycin A/ml and 1 μ g of rotenone/ml. The final pH was 7.4. The medium contained the appropriate amount of [¹⁴C]malonyl-CoA (5.8 μ Ci/ μ mol) and 50 μ M-[³H]sucrose (9.8 mCi/ μ mol). After sedimentation of the mitochondria by centrifugation at 12000 *g* for 90 s in an Eppendorf 4312 centrifuge, ¹⁴C and ³H radioactivities were measured in the pellet and supernatant (Rial & Nicholls, 1983). As described previously, initial rapid binding was followed by a slower increase in binding over the next 2–3 min (Zammit *et al.*, 1984). Thereafter there was no further increase in binding. Therefore, routinely, binding was performed for 5 min. CPT I activity was measured as described previously (Zammit *et al.*, 1984). Measurement of the rate of O₂ uptake by mitochondria was performed polarographically also as described previously (Zammit, 1980). Protein concentrations were measured as described by Lowry *et al.* (1951).

RESULTS AND DISCUSSION

Effects of blocking of CPT I active site on [¹⁴C]malonyl-CoA binding to rat liver mitochondria

In the present study CPT I activity was inhibited through the self-catalysed formation of the *S*-carboxypalmitoyl-CoA ester of carnitine, thus blocking the active site of the enzyme virtually irreversibly over the time scale of the experiments (Chase & Tubbs, 1969, 1972). This was achieved by incubating the mitochondria with 2-bromopalmitate, CoA, ATPMg and carnitine at 25 °C. Under these conditions the acyl-CoA synthetase activity of the outer membrane of the mitochondria catalyses the formation of 2-bromopalmitoyl-CoA, which is a substrate for CPT I. On subsequent addition of carnitine, CPT I becomes inhibited through the formation of the *S*-carboxypalmitoyl-CoA ester of (–)-carnitine at the active site (Chase & Tubbs, 1972). The enzyme has affinity for both the carnitine and the acyl-CoA moieties of this compound at the active site. This double affinity results

in very tight binding of the compound; reversibility of the effect is extremely slow (Chase & Tubbs, 1969, 1972). Consequently the method provides a mechanism whereby an active-site-directed inhibitor can be used effectively to block further binding of acyl-CoA substrate or of any putative competitive ligand at the active site.

Inhibition of CPT I activity resulting from this treatment of the mitochondria was routinely over 90% [controls, 8.10 ± 1.26 , treated, 0.80 ± 0.29 ($n = 6$) nmol/min per mg of protein at 37 °C]. The effect of 2-bromopalmitate had an absolute requirement for CoA and L-carnitine. In the presence of CoA, but in the absence of added carnitine, some inhibition (about 20%) occurred. This is thought to have resulted from the efflux of carnitine from the mitochondrial matrix during incubation of the mitochondria at 25 °C, owing to the uni-directional leak of carnitine mediated by the carnitine–acylcarnitine translocase of rat liver mitochondria (Pande & Parvin, 1980). Therefore, routinely control mitochondria were treated with bromopalmitate and ATP only, whereas ‘treated’ mitochondria were incubated, in addition, with CoA and carnitine (see the Materials and methods section). Experiments in which bromopalmitate was replaced by an equivalent concentration of palmitate (in the presence of CoA, ATP and carnitine) resulted in no inhibition of CPT I activity, thus establishing the requirement for bromopalmitate in order to observe inhibition of CPT I. This method minimized the amount of acyl-CoA to which the mitochondria were exposed during the preincubation period at 25 °C and ensured that the mitochondria could subsequently be washed free of the low concentration (20 μ M) of bromopalmitate. Because bromopalmitoyl-CoA may itself be an inhibitor of acyl-CoA synthetase (Mahadevan & Sauer, 1971), only minimal amounts of bromopalmitoyl-CoA are likely to be synthesized by the mitochondria, but these are sufficient to induce inhibition of CPT I on further addition of carnitine (Chase & Tubbs, 1972). That the mitochondria were functionally intact after this treatment was confirmed by measurement of their rate of O₂ consumption under different conditions. Treated mitochondria showed normal, high, respiratory control ratios (7–10) with glutamate and malate as substrates. Oxidation of palmitoyl-CoA in the presence of carnitine was almost totally abolished (> 95%; cf. CPT I activity above), whereas oxidation of palmitoyl-L-carnitine was unaffected. The State-3 rate of respiration with glutamate and malate as substrates was also unaffected (results not shown).

Inhibition of CPT I did not result in inhibition of the binding of [¹⁴C]malonyl-CoA to rat liver mitochondria (Fig. 1). The complex bimodal concentration-dependence of malonyl-CoA binding (cf. Zammit *et al.*, 1984; Bird & Saggerson, 1984) was also unaffected by CPT I inhibition (see also Fig. 3). These experiments therefore established that blocking of the active site of CPT I did not prevent malonyl-CoA binding and, consequently, that malonyl-CoA does not compete with palmitoyl-CoA for binding at the active site. It is important to stress that these experiments were performed on intact mitochondria and that lack of binding of malonyl-CoA at the active site is thus established for the enzyme under conditions in which malonyl-CoA is capable of exerting its inhibitory effect on CPT I activity.

These experiments also suggested that, if palmitoyl-CoA displaces malonyl-CoA from its binding site(s)

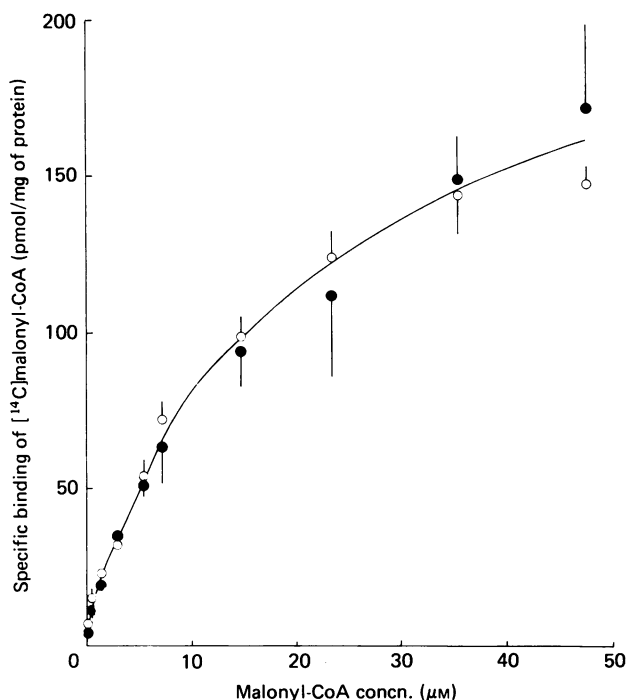


Fig. 1. Concentration-dependence of [2-¹⁴C]malonyl-CoA binding to rat liver mitochondria in which CPT I was active (○) or inhibited (●)

Control mitochondria in which CPT I was active were preincubated with 2-bromopalmitate and ATPMg only. To inhibit CPT I, mitochondria were incubated, in addition, with CoA and carnitine (see the Materials and methods section). Values are means (\pm S.E.M.) for three sets of paired observations on separate mitochondrial preparations.

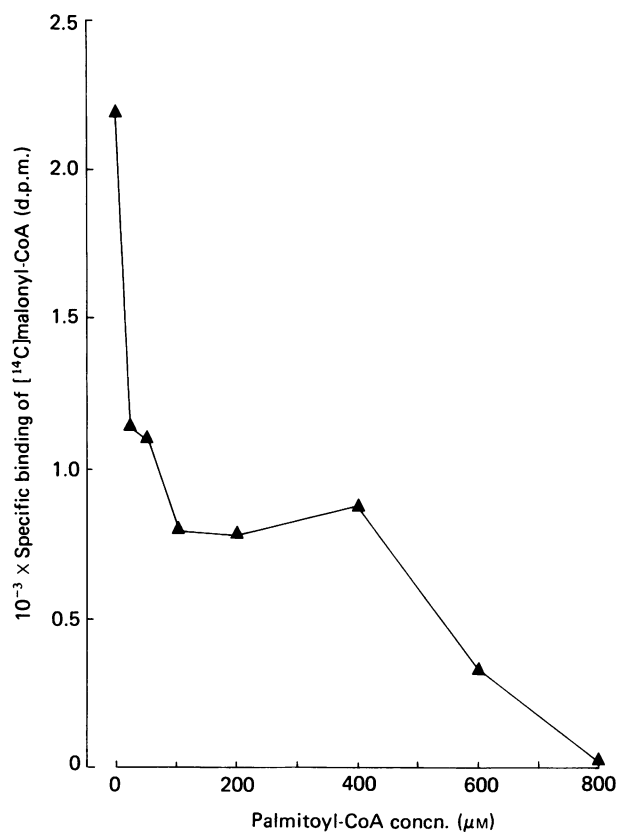


Fig. 2. Effect of palmitoyl-CoA concentration (in the presence of 10 mg of albumin/ml) on the binding of [2-¹⁴C]malonyl-CoA (45 μ M) to rat liver mitochondria

The results shown are representative of those obtained in two similar experiments.

on mitochondria (Mills *et al.*, 1984; Bird & Saggerson, 1984), it should be able to do so through direct competition with this allosteric site(s), since it became obvious that occupation of the active site was not by itself sufficient to affect displacement. In order to test this inference directly, we performed experiments in which binding of [¹⁴C]malonyl-CoA to mitochondria that had been treated with bromopalmitate (plus CoA, ATPMg and carnitine) was measured in the presence and absence of palmitoyl-CoA. Preliminary experiments had established that 100 μ M-palmitoyl-CoA gave near-maximal displacement (Fig. 2). Much higher concentrations (> 400 μ M) of palmitoyl-CoA resulted in total loss of specific association of malonyl-CoA with the mitochondria; this was probably due to detergent effects, since it also resulted in a large increase in the activity of CPT that could be measured (results not shown). The results showed that palmitoyl-CoA was only partially able to displace malonyl-CoA (Fig. 2). It displaced the inhibitor from its low-affinity site, but was largely, if not totally, ineffective in displacing malonyl-CoA from its high-affinity site (Fig. 3). Consequently the bimodal, two-site, binding pattern obtained in the absence of palmitoyl-CoA (see above) was transformed by palmitoyl-CoA into a unimodal pattern characteristic of high-affinity binding which approached saturation hyperbolically (Fig. 3a). This differential effect of palmitoyl-CoA on the two components is more strikingly obvious from Scatchard-type plots of the data

(Fig. 3b; but see below). Only low-affinity binding was lost in the presence of palmitoyl-CoA, as indicated from the Scatchard plot for the degree of binding lost at different malonyl-CoA concentrations (Fig. 3b).

When the characteristics of the [¹⁴C]malonyl-CoA-binding capacity lost in the presence of palmitoyl-CoA were examined quantitatively by subtraction of the respective mean values abstracted from the two curves in Fig. 3(a), evidence emerged that the low-affinity, high-capacity, component of [¹⁴C]malonyl-CoA binding approached saturation in a sigmoidal manner. This was also evident from the Scatchard plot for this component (Fig. 3b). Therefore the two components of malonyl-CoA binding to rat liver mitochondria (Zammit *et al.*, 1984; Bird & Saggerson, 1984) appear to differ not only in relative affinity and capacity but also in their saturation kinetics. It is possible that the sigmoidal nature of the low-affinity component could result from genuine allosteric-type co-operative interaction between the protomers of CPT I, which is known to be a tetrameric protein (Miyazawa *et al.*, 1983). We have obtained further evidence for these characteristics of the two components, using a different experimental approach (Zammit & Corstorphine, 1985b).

If the suggestion that one of the two components of malonyl-CoA binding to rat liver mitochondria is saturated in a sigmoidal manner is valid, then the use of Scatchard plots for the quantitative evaluation of the

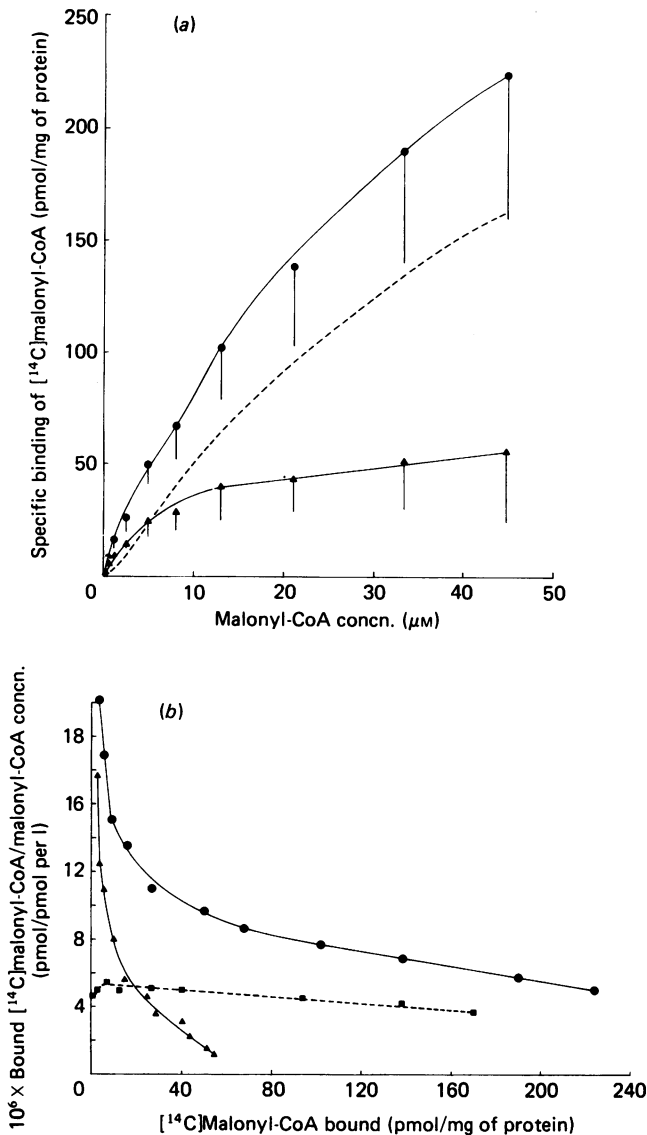


Fig. 3. Effect of palmitoyl-CoA on the concentration-dependence of [2-¹⁴C]malonyl-CoA binding to rat liver mitochondria

(a) Mitochondria in which CPT I was inhibited (see legend to Fig. 1) were incubated with the indicated concentrations of malonyl-CoA in the absence (●) or presence (▲) of palmitoyl-CoA (100 μM, plus 10 mg of albumin/ml). Values are means (± S.E.M.) for three paired determinations performed on separate mitochondrial preparations. The broken line represents the difference obtained between the two experimental curves, i.e. the concentration-dependence of the malonyl-CoA binding lost in the presence of palmitoyl-CoA. (b) Scatchard plots of the data in (a). The same notation is used.

respective kinetic parameters would present problems. This is because implicit in such a mathematical treatment is the assumption that saturation kinetics of the multiple binding components all obey Michaelis-Menten-type equations. Consequently we would like to restrict the use of Scatchard plots to illustrative purposes (Fig. 3b) rather than use them for quantitative derivation of binding parameters. In this respect, the present observation of sigmoidal saturation kinetics for the low-affinity compo-

nent may partly explain the paradoxical apparent 5-fold increase in the number of high-affinity binding sites in the presence of palmitoyl-CoA (Bird & Saggerson, 1984). If palmitoyl-CoA primarily displaces [¹⁴C]malonyl-CoA from a low-affinity sigmoidally saturating site, a two-hyperbola model of binding would interpret the effect as an increase in the amount of binding by the high-affinity component, owing to a smaller than expected effect of palmitoyl-CoA on total [¹⁴C]malonyl-CoA binding at low concentrations of malonyl-CoA. These characteristics of the high- and low-affinity components may also explain why the degree of inhibition of CPT I by different malonyl-CoA concentrations and the site-occupancy of the high-affinity site calculated from the two-hyperbola model are only modestly correlated for rat liver mitochondria (Bird & Saggerson, 1984).

Physiological considerations

The present findings establish that malonyl-CoA does not bind to the active site of CPT I. Moreover they raise the possibility that there are two regulatory sites associated with the enzyme: one that binds malonyl-CoA with high affinity and appears not to be susceptible to appreciable competition by acyl-CoA, and a second site that must be considered as essentially an acyl-CoA-binding site under conditions *in vivo*. Thus the half-maximally effective concentration of malonyl-CoA in saturating this low-affinity site is apparently > 25 μM (cf. Zammit *et al.*, 1984; Bird & Saggerson, 1984), i.e. well above the physiological range of malonyl-CoA concentrations (see, e.g., Zammit, 1981). Conversely, palmitoyl-CoA displaces malonyl-CoA from this site very effectively even at low concentrations (Fig. 2). The concentrations of palmitoyl-CoA required to achieve virtually total displacement are likely to occur *in vivo*, as indicated by the fact that 100 μM-palmitoyl-CoA (in the presence of 10 mg of albumin/ml) gives about 70% of maximal CPT I activity *in vitro* (results not shown), whereas it is known that, under conditions characterized by increased delivery of fatty acids to the liver *in vivo*, the CPT I reaction appears to attain saturation with acyl-CoA substrate (see Zammit, 1984, for review).

It is noteworthy that half-maximal residual binding of malonyl-CoA in the presence of 100 μM-palmitoyl-CoA occurred at 2–4 μM-malonyl-CoA (Fig. 3a). This observation is significant in terms both of the similarity of this value to the corresponding concentration required to achieve half-maximal inhibition of CPT I *in vitro* under similar conditions (Zammit & Corstorphine, 1985a) and of the fact that it is within the range of malonyl-CoA concentrations that occur in the liver *in vivo* (Zammit, 1981). The question arises as to whether the allosteric binding sites for malonyl-CoA and acyl-CoA occur on the same protein species ('allosteric' is used to denote non-active-site binding). It has been hypothesized that malonyl-CoA may exert its effects on CPT I activity by binding to a separate regulatory protein associated with CPT I in the mitochondrial inner membrane (Bieber & Farrell, 1983; Foster, 1984). Several aspects of the present findings can be interpreted as providing support for such a hypothesis. Thus, whereas the concentration-dependence of the malonyl-CoA binding to the high-affinity site was hyperbolic, there was evidence that the site with low affinity for malonyl-CoA approached saturation sigmoidally (Fig. 3a). It is possible therefore that the sites reside on two different proteins, only one

of which is capable of exhibiting co-operative-type interactions between its component promoters. In this respect it is noteworthy that CPT activity exhibits co-operative-type kinetics with respect of acyl-CoA substrate both when the enzyme resides in the mitochondrial inner membrane (CPT I; Saggerson & Carpenter, 1981; Cook, 1984) and after purification (at least for the bovine heart enzyme; Fiol & Bieber, 1984). Therefore the sigmoidal characteristics of acyl-CoA saturation at the active site and of the low-affinity malonyl-CoA (high-affinity acyl-CoA) binding to rat liver mitochondria may derive from the fact that they both reside on the same oligomeric protein (CPT has been shown to be a tetramer; Miyazawa *et al.*, 1983). However, although the data presented in this paper lend support to the concept of a separate monomeric malonyl-CoA-binding protein that interacts with an oligomeric CPT I within the mitochondria inner-membrane environment, it must be emphasized that such evidence is entirely circumstantial. Nevertheless, the demonstration that blocking the active site of CPT I does not interfere with malonyl-CoA (and acyl-CoA) binding at allosteric sites is physiologically significant. It provides the basis for a mechanism whereby changes in both malonyl-CoA and palmitoyl-CoA concentrations *in vivo* can exert their respective effects on the catalytic activity of CPT I without the requirement for direct competition between the two ligands for the active site.

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