Re-evaluation of the interaction of malonyl-CoA with the rat liver mitochondrial carnitine palmitoyltransferase system by using purified outer membranes

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1. The interaction of malonyl-CoA with the outer carnitine palmitoyltransferase (CPT) system of rat liver mitochondria was re-evaluated by using preparations of highly purified outer membranes, in the light of observations that other subcellular structures that normally contaminate crude mitochondrial preparations also contain malonyl-CoA-sensitive CPT activity. 2. In outer-membrane preparations, which were purified about 200-fold with respect to the inner-membrane-matrix fraction, malonyl-CoA binding was largely accounted for by a single high-affinity component ($K_D = 0.03 \mu\text{M}$), in contrast with the dual site (low- and high-affinity) previously found with intact mitochondria. 3. There was no evidence that the decreased sensitivity of CPT to malonyl-CoA inhibition observed in outer membranes obtained from 48 h-starved rats (compared with those from fed animals) was due to a decreased ratio of malonyl-CoA binding to CPT catalytic moieties. Thus CPT specific activity and maximal high-affinity [^{14}C]malonyl-CoA binding (expressed per mg of protein) were increased 2.2- and 2.0-fold respectively in outer membranes from 48 h-starved rats. 4. Palmitoyl-CoA at a concentration that was saturating for CPT activity ($5 \mu\text{M}$) decreased the affinity of malonyl-CoA binding by an order of magnitude, but did not alter the maximal binding of [^{14}C]malonyl-CoA. 5. Preincubation of membranes with either tetradecylglycidyl-CoA or 2-bromopalmitoyl-CoA plus carnitine resulted in marked ($> 80\%$) inhibition of high-affinity binding, concurrently with $> 95\%$ inhibition of CPT activity. These treatments also unmasked an effect of subsequent treatment with palmitoyl-CoA to increase low-affinity [^{14}C]malonyl-CoA binding. 6. These data are discussed in relation to the possible mechanism of interaction between the malonyl-CoA-binding site and the active site of the enzyme.

INTRODUCTION

Studies on the overt carnitine palmitoyltransferase system (CPT I) of mitochondria from mammalian tissues have been largely indirect because the enzyme is difficult to solubilize in its active state (Murthy & Pande, 1987a). This complicates any quantitative analysis of the regulatory properties of the protein and, in particular, of its inhibition by malonyl-CoA. Consequently, studies on the mechanism of this inhibition, as well as quantitative measurement of radiolabelled malonyl-CoA binding and its displacement by competing ligands, have had to be performed on relatively crude preparations, such as mitochondrial fractions prepared by differential centrifugation. In view of relatively recent observations which suggest that CPT activity exists in other subcellular structures that may contaminate mitochondria (Ramsay *et al.*, 1987; Lilly *et al.*, 1989), it is desirable that, in the absence of the purified system, some of the parameters described for crude whole mitochondrial fractions should be re-evaluated in much purer preparations. The observation that CPT I resides in the mitochondrial outer membrane (Murthy & Pande, 1987b) has provided the opportunity for this to be achieved, as it is now possible to study CPT I catalytic activity and malonyl-CoA-binding characteristics with very pure outer-membrane preparations that are virtually free of CPT II (latent mitochondrial enzyme) and CPT of peroxisomal and microsomal origin (Zammit *et al.*, 1989). Since the enzyme from both these latter sources too is inhibited by malonyl-CoA (Lilly *et al.*, 1989; Derrick & Ramsay, 1989), purification of outer membranes presumably would also minimize interference from these sources with the measurement of malonyl-CoA binding.

In the present paper we have set out to re-evaluate data that have proven to be contentious in previous studies, by using purified outer membranes from rat liver mitochondria. Several

previous observations have been either disputed or deemed to be of little physiological relevance, or are inexplicable on the basis of current ideas about the CPT I system. In view of the difficulties that arise from the use of relatively crude whole mitochondrial preparations, it is likely that most of these problems and uncertainties can be resolved with the use of purified outer-membrane preparations. We have identified the following aspects as needing more definitive studies using outer membranes. (i) The relationship between changes in sensitivity of CPT I to malonyl-CoA in different physiological conditions (e.g. starvation) and the affinity and maximal extent of malonyl-CoA binding (see Bird & Saggerson, 1984). This is important, as it has a bearing on the mechanism of malonyl-CoA inhibition of CPT I, i.e. whether by direct interaction or through binding to a regulatory subunit. (ii) The significance of the presence of more than one class of malonyl-CoA binding site in rat liver mitochondria (see Zammit *et al.*, 1984; Bird & Saggerson, 1984). This dual binding is not a universal property of mitochondria from other tissues (see Mills *et al.*, 1983). (iii) The apparent increase in high-affinity malonyl-CoA binding by palmitoyl-CoA (Bird & Saggerson, 1984). (iv) The relationship between the active site of the enzyme and the malonyl-CoA-binding site as determined by displacement of malonyl-CoA by active-site-directed inhibitors (Grantham & Zammit, 1986a; Declercq *et al.*, 1987).

The present findings indicate that aspects of the mechanism of interaction of malonyl-CoA with CPT I may need to be re-appraised, as well as the modulation of these parameters by starvation.

MATERIALS AND METHODS

Animals

These were male Wistar rats (300–400 g) that had been

maintained on standard laboratory chow diet (Biosure, Poole, Dorset, U.K.). Starved animals had their food withdrawn for 48 h before use. They had continuous access to water.

Preparation of mitochondrial outer membranes

Six to eight rats were used for each preparation of outer membranes. Mitochondria were prepared by differential centrifugation in a medium containing 300 mM-sucrose, 10 mM-Tris and 1 mM-EGTA (pH 7.4 at 0 °C) and further purified by Percoll-density-gradient centrifugation after resuspension in 31 % (w/v) Percoll (Zammit *et al.*, 1989). Outer membranes were prepared from mitochondria by a modification of standard methods as described elsewhere (Zammit *et al.*, 1989) to ensure minimal contamination (< 0.5%) with mitochondrial inner membrane, microsomes and peroxisomes. Membranes were stored at 8–10 mg of protein/ml in batches frozen at –80 °C until used. When required, samples were thawed by placing them on ice for 10–20 min, and the membranes were recovered by centrifugation at 30 lb/in² (207 kPa) in a Beckman Airfuge for 15 min, and subsequently resuspended in medium containing 150 mM-KCl, 1 mM-dithiothreitol and 1 mM-EGTA (pH 7.4 at 0 °C). In our experience it was much more difficult to prepare pure outer-membrane preparations from livers of fed rats. The yield of outer-membrane protein was 10–20 mg per 70 g of liver starting from starved rats, but only 2–4 mg for fed-rat liver.

Assay of CPT activity and [¹⁴C]malonyl-CoA binding

When malonyl-CoA sensitivity was studied, CPT activity was measured at 37 °C under the high-albumin-concentration conditions described by Grantham & Zammit (1986b, 1988). These conditions were used in order to replicate those used for studies on intact mitochondria in our laboratory, thus enabling a direct comparison of the results. For the studies with other inhibitors, the low-albumin (0.13%) conditions described by Saggerson & Carpenter (1981) were used and CPT was assayed at 100 μ M-palmitoyl-CoA to yield maximal activity. All assay media contained 150 mM-KCl, 1 mM-dithiothreitol and 1 mM-EGTA (pH 7.4 at 37 °C). Outer membranes (20–40 μ g of protein) were added to the medium for 2 min before addition of [³H]carnitine (0.5 mM, 0.36 Ci/mol) to start the reaction, which was terminated after 2 min by addition of HCl. Acylcarnitine formation was quantified as described previously (Robinson & Zammit, 1982).

Malonyl-CoA binding was quantified by a filtration assay (Zammit *et al.*, 1989). The binding mixture containing outer membrane (2–4 mg of protein) was incubated at 0 °C for 20 min. It was then rapidly filtered (0.2 μ m-diameter pore size; Millipore) and the filters were washed with 2 ml of ice-cold KCl medium. Non-specific association of malonyl-CoA with the filters was assessed by the inclusion of [³H]inulin and by routine checking that excess unlabelled malonyl-CoA (100 μ M) totally displaced specific [¹⁴C]malonyl-CoA binding (see Zammit *et al.*, 1989).

The effects of 2-bromopalmitoyl-CoA with or without carnitine and of tetradecylglycidyl-CoA (TDG-CoA) were studied by incubating outer membranes with the inhibitors for 10 min at 20 °C, followed by separation of the membranes by centrifugation at 180000 g for 15 min (Beckman Airfuge, 30 lb/in²). The membranes were then washed in KCl medium (see above) supplemented with 1 % fatty-acid-poor albumin, re-sedimented, and then used for assay of CPT activity and [¹⁴C]malonyl-CoA binding.

Other methods

Marker-enzyme activities for peroxisomes, microsomes and outer and inner mitochondrial membrane were assayed as described elsewhere (Zammit *et al.*, 1989). Protein was measured

by the method of Lowry *et al.* (1951) with bovine serum albumin as standard.

Materials

[2-¹⁴C]Malonyl-CoA, [³H]carnitine and [³H]inulin were obtained from Amersham International (Aylesbury, Bucks., U.K.). 2-Bromopalmitoyl-CoA was synthesized as described by Chase & Tubbs (1972). Tetradecylglycidic acid was a gift from McNeil Corp. (Milwaukee, WI, U.S.A.) and the CoA ester was synthesized as described by Kiorpes *et al.* (1984).

Statistical analysis

The malonyl-CoA-binding data (Scatchard plots) were analysed statistically by using maximum-likelihood estimation (see Munson & Rodbard, 1980). Models implying (i) one binding site, (ii) one site with non-specific binding and (iii) two independent sites were fitted to the data. The choice of the appropriate model was made on the basis of goodness-of-fit tests, residuals and standard errors of parameter estimates. In all cases model (ii) was judged to be the most appropriate.

RESULTS AND DISCUSSION

Characteristics of CPT activity and malonyl-CoA binding in outer membranes

Since the observation that CPT 1 resides in the mitochondrial outer membrane (Murthy & Pande, 1987b) reports have appeared that the difference in sensitivity of the enzyme to malonyl-CoA observed with intact mitochondria from fed, starved or diabetic rats was also observed with outer membrane preparations (Murthy & Pande, 1988; Cook *et al.*, 1988; Kashfi *et al.*, 1988). However, these reports are either in abstract form or relate to data not shown. Consequently, to our knowledge, the data in Fig. 1 are the first detailed results on this phenomenon to be

Fig. 1. Difference in sensitivity of CPT activity to malonyl-CoA inhibition in mitochondrial-outer-membrane preparations obtained from fed or 48 h-starved rats

Outer membranes were prepared from livers of fed ($\bullet$, $n = 6$) or 48 h-starved ($\circ$, $n = 7$) rats, as described in the Materials and methods section. The values shown are means ($\pm$ S.E.M.) of CPT activities expressed as a percentage of those obtained in the absence of malonyl-CoA (control). The absolute values of control CPT activities for all the preparations studied were fed 84.5 ± 17.1 ($n = 11$) and starved 184.7 ± 8.4 ($n = 13$) nmol/min per mg of protein at 37 °C.

Fig. 2. Scatchard plots for the specific binding of [^{14}C]malonyl-CoA to mitochondrial outer membranes from 48 h-starved rats

Malonyl-CoA binding to membranes was measured in control preparations either in the absence of palmitoyl-CoA (●) or in the presence of $5\ \mu\text{M}$ -palmitoyl-CoA (○). Binding of malonyl-CoA by membranes that had been incubated with TDG-CoA ($3\ \mu\text{M}$) and subsequently washed with 1% albumin is also shown (■). The values shown are representative of three experiments using separate membrane preparations.

reported for outer-membrane preparations. The difference in sensitivity of the enzyme activity as well as the concentrations of malonyl-CoA required to inhibit the enzyme by 50% were almost identical with those reported previously by Grantham & Zammit (1986b, 1988) for CPT I activity, assayed under the same conditions, in whole mitochondria. The specific activity of CPT (per mg of membrane protein) in the absence of malonyl-CoA was also higher for outer-membrane preparations from starved rats (see the legend to Fig. 1).

Contrary to those of CPT activity, the characteristics of [^{14}C]malonyl-CoA binding in outer-membrane preparations were quantitatively substantially different from those found with intact mitochondria (Zammit *et al.*, 1984; Bird & Saggerson, 1984). Thus the number of high-affinity binding sites relative to low affinity ones was much enhanced in outer membranes (Fig. 2, and Zammit *et al.*, 1989) compared with intact mitochondria (Zammit *et al.*, 1984; Bird & Saggerson, 1984). This observation suggests that most of the low-affinity binding observed with intact rat liver mitochondria may be totally unrelated to the CPT I system, and may be associated with other proteins present in the mitochondrial inter-membrane space or inner membrane, as well as other structures that contaminate crude mitochondrial preparations. When binding data were analysed for goodness of fit, a model assuming one class of (high-affinity) binding sites and steadily increasing non-specific binding invariably fitted the data better than if a model with two independent classes of binding sites with different affinities were assumed (see Zammit *et al.*, 1984; Bird & Saggerson, 1984). Consequently the data generated binding parameters for only one type of binding site (high-affinity). In this respect, therefore, the liver system would now appear more similar to that of skeletal muscle, in which no interference by a low-affinity component is observed even in intact mitochondrial preparations (Mills *et al.*, 1983). The effects of palmitoyl-CoA on the degree of low-affinity binding (see below) may provide clues as to its origin. The K_D value for high-affinity binding to outer membrane (Table 1) was very similar to that computed by Bird & Saggerson (1984) for intact mitochondria.

Effects of palmitoyl-CoA

A second aspect in which the present data differ from those obtained with [^{14}C]malonyl-CoA binding to intact mitochondria was in the effects of palmitoyl-CoA. In a previous study using whole mitochondria, we had observed that, although palmitoyl-CoA abolished the low-affinity binding, maximal high-affinity binding appeared to be unaffected (Grantham & Zammit, 1986a). On the other hand, Bird & Saggerson (1984), who analysed their data mathematically, using a two-site model, found that, paradoxically, palmitoyl-CoA increased the number of high-affinity malonyl-CoA binding sites by about 5-fold concomitantly with increasing the K_D value by 30-fold. Mills *et al.* (1983) had reported that palmitoyl-CoA decreased malonyl-CoA binding to skeletal-muscle mitochondria by a relatively small extent (about 40%). However, from those latter studies it was not possible to

Table 1. Characteristics of high-affinity binding of malonyl-CoA to mitochondrial outer membranes from fed or 48 h-starved rats, and the effects of palmitoyl-CoA, carnitine and preincubation with TDG-CoA and bromopalmitoyl-CoA plus carnitine

The values shown are means $\pm$ S.E.M. for the numbers of separate membrane preparations shown in parentheses. Data for experiments in which additions or preincubations were involved are shown paired to the relevant control values obtained on the same batches of membranes. Values that are statistically significantly different (paired *t* test) from the relevant control values are indicated by (*).

Animals	Addition or preincubation	Maximal [^{14}C]malonyl-CoA binding (pmol/mg of protein)	K_p (μM)
Fed (6)	—	41.0 ± 4.1	0.03 ± 0.01
48 h-starved (16)	—	$81.2 \pm 8.0^*$	0.03 ± 0.01
48 h-starved (3)	—	91.3 ± 14.8	0.03 ± 0.01
	Palmitoyl-CoA ($5\ \mu\text{M}$)	108.3 ± 26.6	$0.30 \pm 0.06^*$
48 h-starved (3)	—	65.1 ± 5.9	0.03 ± 0.01
	TDG-CoA ($3\ \mu\text{M}$)	$8.7 \pm 0.9^*$	$0.24 \pm 0.01^*$
	Bromopalmitoyl-CoA ($5\ \mu\text{M}$) + carnitine (4 mM)	$10.4 \pm 3.3^*$	$0.28 \pm 0.18^*$

ascertain whether the effect was on maximal binding or on the value of K_D . In the present experiments, we used a palmitoyl-CoA concentration ($5 \mu\text{M}$, in the absence of albumin) which under the same conditions gave maximal CPT activity and was, consequently, not inhibitable by excess malonyl-CoA (results not shown). The data in Fig. 2 and Table 1 show that palmitoyl-CoA had no effect on the maximal extent of high-affinity [^{14}C]malonyl-CoA binding, but increased the K_D 10-fold, from $0.03 \pm 0.01 \mu\text{M}$ to $0.30 \pm 0.16 \mu\text{M}$. These data suggest that palmitoyl-CoA has no effect on the maximal binding capacity at the high-affinity malonyl-CoA site of rat liver mitochondria. This is in agreement with our previous conclusion (Grantham & Zammit, 1986a), but differs from the large increase in binding reported by Bird & Saggerson (1984) in the presence of palmitoyl-CoA.

The restriction of the palmitoyl-CoA effect entirely to that observed on K_D also raises an important question as to the mechanism of the antagonism between malonyl-CoA and palmitoyl-CoA. If the two molecules bind at the same site on CPT I, it would be expected that, at a concentration of palmitoyl-CoA that totally negates malonyl-CoA inhibition, the [^{14}C]malonyl-CoA binding would have been totally abolished (i.e. palmitoyl-CoA would increase the K_D for malonyl-CoA indefinitely). Consequently, the data indicate that palmitoyl-CoA does not compete sterically for binding, but rather changes the conformation of CPT I in such a way as to make malonyl-CoA binding less favourable. The fact that at sufficiently high malonyl-CoA concentrations the full binding capacity is expressed in the absence of any inhibition of enzyme activity (see above) also indicates that the effect of palmitoyl-CoA is not limited to decreasing the affinity of malonyl-CoA binding, but also totally negates the transmission of the malonyl-CoA effect to the active site. These phenomena could occur whether the malonyl-CoA site is juxtaposed to the active site (as suggested by Declercq *et al.*, 1987) or exists either on a transmembrane domain of the CPT I molecule (see, e.g., Murthy & Pande, 1987a) or on a totally separate regulatory subunit (see Bieber & Farrell, 1983; Bird & Saggerson, 1984).

On a more practical level, the above observations indicate that previous inconsistencies in the effects of palmitoyl-CoA on malonyl-CoA binding were probably due to the interference from the large amount of low-affinity binding present in intact mitochondria. Indeed in the present experiments palmitoyl-CoA was found to increase the extent of non-specific binding (see below). In this respect, it is also noteworthy that we were unable to confirm the observation by Bird & Saggerson (1985) that L-carnitine decreases the K_D of the high-affinity malonyl-CoA site in rat liver mitochondria (Fig. 3).

Effects of starvation

The first evidence for the existence of a separate malonyl-CoA-binding regulatory subunit was the observation by Bird & Saggerson (1984) that liver mitochondria isolated from rats in different physiological conditions had the same number of high-affinity binding sites (per mg of mitochondrial protein). This was in spite of the 7-fold difference in CPT I activity that was found for the same mitochondria. We have re-investigated this phenomenon, using fed and starved male rats as sources for outer-membrane preparations. The data obtained suggested that, as described by Bird & Saggerson (1984), starvation had no statistically significant effect on K_D of malonyl-CoA binding (Table 1). However, the use of outer membranes enabled us to discern a significant 2-fold increase in the number of high-affinity binding sites in preparations from starved rats. Moreover, this increase was very similar to that observed for specific CPT activity in outer membranes. Consequently, these data do not provide evidence that diminished sensitivity of CPT I to malonyl-

Fig. 3. Scatchard plots for the specific binding of [^{14}C]malonyl-CoA to mitochondrial outer membranes from 48 h-starved rats, illustrating the lack of effect of L-carnitine

Binding was measured as described in the Materials and methods section, in either the absence (●) or the presence (Δ) of 4 mM-L-carnitine. The experiment was repeated twice with very similar results.

CoA in starved rats is due to either (i) diminished affinity of the malonyl-CoA-binding species for the ligand or (ii) a lower ratio of malonyl-CoA binding subunits to CPT I catalytic subunits. The diminished sensitivity of CPT I to malonyl-CoA under conditions such as starvation must, consequently, be ascribed to changes in the transmission of the conformational changes induced by malonyl-CoA to the active site (see also Zammit, 1987).

Effects of carnitine S-carboxypalmitoyl-CoA and of TDG-CoA

In a previous study (Grantham & Zammit, 1986a) we were unable to observe any effect of the formation of the S-carboxypalmitoyl-CoA ester of carnitine at the CPT I active site on [^{14}C]malonyl-CoA binding to intact rat liver mitochondria. This was interpreted as indicating that the malonyl-CoA-binding site is distinct from the active site of CPT I. Subsequently, Declercq *et al.* (1987) published data which showed that incubation of rat liver mitochondria with 2-bromopalmitoyl-CoA and carnitine did depress malonyl-CoA binding in rat liver mitochondria. By analogy with data they obtained on skeletal-muscle mitochondria, in which only high-affinity sites are present (see above), they concluded that the active-site-directed suicide inhibitor physically displaces malonyl-CoA binding from a site very close to the sites of carnitine and palmitoyl-CoA binding (Declercq *et al.*, 1987). Since TDG-CoA had the same effect, those authors concluded that all these ligands (including malonyl-CoA) bind at or very close to the active site of CPT I.

It was therefore desirable to ascertain whether the discrepancies between our data and those of Declercq *et al.* (1987) were due to artefacts arising out of high levels of low-affinity binding in our intact mitochondrial preparations. The effects of TDG-CoA and 2-bromopalmitoyl-CoA plus carnitine were investigated with increasing concentrations of the inhibitors. Maximal inhibitory effects on both CPT activity and [^{14}C]malonyl-CoA binding were

obtained at $5\text{ }\mu\text{M}$ for 2-bromopalmitoyl-CoA and $2\text{ }\mu\text{M}$ -TDG-CoA under the conditions used. Whereas the maximal degree of CPT inhibition was always reproducibly above 90% (see also Grantham & Zammit, 1986a), the effects of the two inhibitors on malonyl-CoA binding (at a single concentration, $4\text{ }\mu\text{M}$) were more variable, but never exceeded 70% inhibition. Analysis of the residual malonyl-CoA binding revealed that this was almost entirely due to non-specific binding (Fig. 2 and Table 1). Any residual 'high-affinity' sites (about 15% of original) now displayed the same order of affinity for malonyl-CoA as in the presence of palmitoyl-CoA (see above). These observations therefore suggest that in our earlier findings these effects were obscured by the presence of high levels of low-affinity binding present in intact mitochondrial fractions, and that the decrease in total binding observed by Declercq *et al.* (1987) was indeed due to inhibition of high-affinity binding of malonyl-CoA to an outer-membrane protein.

The effect of palmitoyl-CoA on [^{14}C]malonyl-CoA binding after inhibition by either TDG-CoA or 2-bromopalmitoyl-CoA plus carnitine (Fig. 4) is noteworthy. In these experiments membranes were incubated with the inhibitors and washed; malonyl-CoA binding to the resuspended membranes was then measured in either the absence or the presence of $5\text{ }\mu\text{M}$ -palmitoyl-CoA. Inclusion of palmitoyl-CoA markedly increased binding, even though the effects of the two inhibitors were retained during the experimental procedure (Table 1). However, the K_D of this renewed binding was high ($> 0.3\text{ }\mu\text{M}$). The effect is therefore of doubtful physiological significance, but it suggests a possible reason for the spuriously high binding values obtained in the presence of palmitoyl-CoA (Bird & Saggerson, 1984) observed with intact mitochondrial preparations, especially as the degree of low-affinity binding was initially already very high and the data were analysed by a model that assumed two saturable sites.

Fig. 4. Malonyl-CoA binding to rat liver outer membranes at increasing concentrations of malonyl-CoA

Membranes from 48 h-starved rats were incubated either in the absence (●, ◆) or in the presence (▲, ■) of $5\text{ }\mu\text{M}$ -TDG-CoA. They were then sedimented, and washed by resuspension in KCl medium containing 1% BSA. They were re-sedimented, resuspended and used for measurement of binding in the absence (●, ▲) or presence (◆, ■) of palmitoyl-CoA (PCoA). This experiment is representative of six experiments in which either TDG-CoA (3) or bromopalmitoyl-CoA (BrPCoA) plus carnitine (3) were used and which gave very similar results.

It may be relevant that the magnitude of this palmitoyl-CoA effect was the same irrespective of whether the membranes had been preincubated with inhibitors (Fig. 4). It is possible that palmitoyl-CoA partitions in the membrane and disrupts the lipid environment of the membrane sufficiently to give an increase in low-affinity binding. Whether this is related to the CPT system is difficult to ascertain.

General conclusions

The use of highly pure preparations of rat liver mitochondrial outer membranes have enabled the clarification of several ambiguities in the data obtained previously on the relationship between CPT I activity and malonyl-CoA binding (Zammit *et al.*, 1984; Bird & Saggerson, 1984; Declercq *et al.*, 1987). First, we have shown that the apparently high capacity for low-affinity malonyl-CoA binding present in liver mitochondrial preparations is much diminished in outer-membrane preparations, thus raising the possibility that most, if not all of it, may be unrelated to the CPT I system. Second, our experiments revealed that in at least one physiological transition (fed to starved) there is a parallel increase in CPT I activity and malonyl-CoA capacity for high-affinity binding. This observation suggests that either the two parameters reside in the same protein, or that, if two subunits are involved (catalytic and regulatory), they are co-ordinately induced. Third, palmitoyl-CoA only affects the affinity of binding of malonyl-CoA and not the maximal capacity. This has implications for both the modelling of the relationship between the active and regulatory sites (see above) and the re-assessment of previous data indicating a palmitoyl-CoA-induced increase in high-affinity binding capacity in rat liver mitochondria (Bird & Saggerson, 1984). Fourth, 2-bromopalmitoyl-CoA plus carnitine and TDG-CoA were shown to inhibit high-affinity binding virtually completely, contrary to our previous observations on whole mitochondria (Grantham & Zammit, 1986a) but in confirmation of the inference of Declercq *et al.* (1987).

The effects of TDG-CoA and 2-bromopalmitoyl-CoA plus carnitine are totally different from those of palmitoyl-CoA. This strengthens the concept put forward by Declercq *et al.* (1987) that TDG-CoA and the *S*-carboxypalmitoyl-CoA ester of carnitine interfere with malonyl-CoA binding because of the covalent and bidentate nature of their binding respectively. However, whether this binding interferes directly (sterically) with the malonyl-CoA binding site, or whether it changes the enzyme conformation to a state in which it cannot bind malonyl-CoA at an allosteric site (either on the same protein or on a regulatory subunit), is still to be determined. Any model of the action of these ligands would have to accommodate the observations by Murthy & Pande (1987a,b) that (i) the active site of CPT I and the malonyl-CoA-binding site appear to reside on the opposite sides of the mitochondrial outer membrane, and that (ii) treatment of mitochondria with proteases differentially affects the action of malonyl-CoA and TDG-CoA on CPT I when either is present during the assay of the activity of the enzyme.

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