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Interaction of malonyl-CoA and related compounds with mitochondria from different rat tissues

Relationship between ligand binding and inhibition of carnitine palmitoyltransferase I

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The sensitivity of carnitine palmitoyltransferase I (CPT I; EC 2.3.1.21) to inhibition by malonyl-CoA and related compounds was examined in isolated mitochondria from liver, heart and skeletal muscle of the rat. In all three tissues the same order of inhibitory potency emerged: malonyl-CoA ≫ succinyl-CoA > methylmalonyl-CoA ≫ propionyl-CoA > acetyl-CoA. For any given agent, suppression of CPT I activity was much greater in skeletal muscle than in liver, with the heart enzyme having intermediate sensitivity. With skeletal-muscle mitochondria a high-affinity binding site for [^{14}C]malonyl-CoA was readily demonstrable (K_d approx. 25 nM). The ability of other CoA esters to compete with [^{14}C]malonyl-CoA for binding to the membrane paralleled their capacity to inhibit CPT I. Palmitoyl-CoA also competitively inhibited [^{14}C]malonyl-CoA binding, in keeping with its known ability to overcome malonlyl-CoA suppression of CPT I. For reasons not yet clear, free CoA displayed anomalous behaviour in that its competition for [^{14}C]malonyl-CoA binding was disproportionately greater than its inhibition of CPT I. Three major conclusions are drawn. First, malonyl-CoA is not the only physiological compound capable of suppressing CPT I, since chemically related compounds, known to exist in cells, also share this property, particularly in tissues where the enzyme shows the greatest sensitivity to malonyl-CoA. Second, malonyl-CoA and its analogues appear to interact with the same site on the mitochondrial membrane, as may palmitoyl-CoA. Third, the degree of site occupancy by inhibitors governs the activity of CPT I.

CPT I catalyses the first step specific to mitochondrial long-chain fatty acid oxidation and, in liver at least, is believed to play a key regulatory role in this process by virtue of its sensitivity to inhibition by malonyl-CoA (McGarry & Foster, 1980). Although widely distributed in the body, CPT I displays strikingly different characteristics, depending on the tissue examined. For example, in mitochondria from rat heart and skeletal muscle the K_m of CPT I for carnitine is some 5- and 20-fold greater, respectively, than that of the liver enzyme (Long *et al.*, 1982; McGarry *et al.*, 1983). Sensitivity to malonyl-CoA spans an even wider range and appears to be greatest in those tissues exhibiting the highest K_m for carnitine. Thus the inhibitory potency of malonyl-CoA towards CPT I of rat heart and skeletal muscle is respectively about 40 and 200 times that with rat liver mitochondria (Saggerson & Carpenter, 1981*a*; McGarry *et al.*, 1983).

The mechanism by which malonyl-CoA interacts with the mitochondrial inner membrane to suppress CPT I activity is not known. To begin to explore this question, we considered the possibility that compounds structurally related to malonyl-CoA, although having little effect on the liver enzyme, might exert significant inhibition on CPT I in tissues where the sensitivity to malonyl-CoA is much greater. The studies outlined below establish that this is the case. Furthermore, using mitochondria from rat skeletal muscle we also demonstrate the presence of a high-affinity binding site for malonyl-CoA. The inhibitory potency of other CoA esters towards CPT I is shown to be directly related to their ability to compete with malonyl-CoA for binding to this site.

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

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Experimental

Animals

Male Sprague–Dawley rats (body wts. 130–160g) were maintained on a high-sucrose low-fat diet as previously described (McGarry *et al.*, 1978*b*) and were used for experiments in the fed state.

Preparation of mitochondria

Mitochondria were isolated from liver, heart and skeletal muscle by the procedures described by McGarry *et al.* (1983) (methods A and C were used with similar results), and were finally suspended in 0.15M-KCl. Protein was measured by the method of Lowry *et al.* (1951).

Assay of carnitine palmitoyltransferase

CPT I was measured in intact mitochondria in the direction palmitoyl-CoA + [^{14}C]carnitine → palmitoyl[^{14}C]carnitine + CoA, as described by McGarry *et al.* (1983). Final concentrations of palmitoyl-CoA, L-carnitine and fatty acid-free bovine serum albumin were 50μM, 200μM and 1%, respectively. With 0.2–0.4mg of mitochondrial protein/ml of assay mixture at 30°C, reaction rates were linear with time over the time periods studied (generally 5–8min). The concentrations of inhibitors are given in the legends to Figures and Tables. Some mitochondrial preparations, particularly those from heart and skeletal muscle, displayed small and variable quantities of CPT activity that was insensitive to malonyl-CoA. As discussed by McGarry *et al.* (1983), this probably stemmed from exposure of some CPT II as a result of damage to the mitochondria during their preparation. The extent of this contamination was calculated from a plot of (percentage CPT suppression by malonyl-CoA)$^{-1}$ versus [malonyl-CoA]$^{-1}$ and was used to obtain a more accurate estimate of the malonyl-CoA-sensitivity of CPT I (McGarry *et al.*, 1983). Uncorrected data are shown in the Figures, and Table 1 gives both uncorrected and corrected values.

Malonyl-CoA binding to skeletal-muscle mitochondria

Assays were performed in glass centrifuge tubes (10mm × 75mm) in a final volume of 1ml containing: Tris/HCl (pH7.4), 90mM; KCl, 60mM; reduced glutathione, 0.25mM; $MgCl_2$, 4mM; fatty acid-free albumin, 1%, w/v; 0.01–0.4μM-[1,3-^{14}C]-malonyl-CoA (50μCi/μmol) in the absence or presence of 100μM unlabelled malonyl-CoA (to obtain non-specific binding or trapping of ^{14}C); and about 3mg of mitochondrial protein. Incubations were started by addition of the mitochondria and were usually continued for 20min at 0°C with mixing at 3–4min intervals. The tubes were then centrifuged at 15000*g* for 10min at 4°C, the supernatants were aspirated and 0.25ml of 1M-KOH was immediately added to the pellets. Solubilization of the mitochondria was facilitated by heating at 50°C for 30min, after which time the contents of the tube, together with a 0.8ml water wash, were transferred to counting vials and assayed for radioactivity after addition of 10ml of Aquasol II (New England Nuclear). Non-specific binding seldom exceeded 20% of the total. The difference between total and non-specific binding was used to calculate the specific-binding data. Competition studies were performed in a similar manner, except that the concentration of labelled malonyl-CoA was fixed at 0.25μM and unlabelled ligands were present from zero time at the indicated concentrations.

Materials

CoA and all CoA esters were from P-L Biochemicals (Milwaukee, WI, U.S.A.). Highly purified malonyl-CoA decarboxylase was kindly provided by Dr. P. E. Kolattukudy (Washington State University, Seattle, WA, U.S.A.). The sources of other materials have been given previously (McGarry *et al.*, 1983).

Results

Effects of malonyl-CoA and related compounds on mitochondrial CPT I activity

Fig. 1 depicts the sensitivity of CPT I to inhibition by CoA and various CoA esters in mitochondria from rat liver, heart and skeletal muscle. In keeping with previous findings (McGarry *et al.*, 1983), sensitivity to malonyl-CoA was markedly different in the three tissues, and increased in the order liver < heart < skeletal muscle. The same pattern was seen with the other CoA esters tested, and in all three systems the same order of inhibitory potency emerged, i.e. malonyl-CoA ≫ succinyl-CoA > methylmalonyl-CoA ≫ propionyl-CoA > acetyl-CoA. In other words, the greater the sensitivity to malonyl-CoA, the greater was the sensitivity to the other agents, and, of these, succinyl-CoA and methylmalonyl-CoA were the most inhibitory. Table 1 shows the I_{50} values (concentration required for 50% inhibition of CPT I) for malonyl-CoA, succinyl-CoA and methylmalonyl-CoA under our standard assay conditions. Although the superiority of malonyl-CoA as an inhibitor of CPT I is clearly evident in all tissues, the potency of succinyl-CoA and methylmalonyl-CoA also becomes impressive in the non-hepatic tissues, particularly skeletal muscle. To rule out the possibility that these effects were due to slight contamination of succinyl-CoA and methylmalonyl-CoA with malonyl-CoA, all three compounds were tested on skeletal-muscle mitochondria before and after brief exposure to highly purified malonyl-CoA decarboxylase. As shown in Fig. 2, such treatment had no impact on the response of

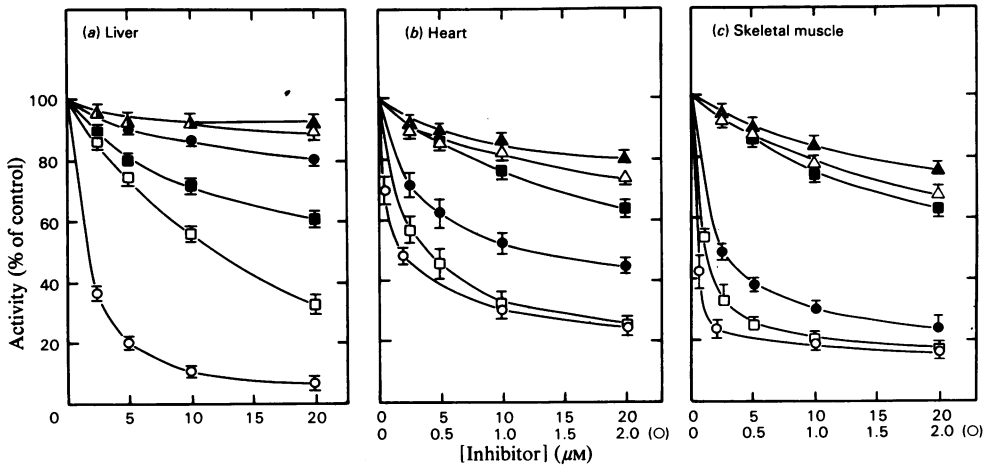

Fig. 1. *Effect of malonyl-CoA and related compounds on CPT I from different tissues*
Enzyme activity was measured in mitochondria from liver, heart and skeletal muscle as described in the Experimental section. Sufficient mitochondrial protein was used to give uninhibited rates of 0.3–0.5 nmol of palmitoylcarnitine formed/min. The inhibitors tested were: ○, malonyl-CoA; □, succinyl-CoA; ●, methylmalonyl-CoA; △, propionyl-CoA; ▲, acetyl-CoA; ■, CoA. Note the different scales for malonyl-CoA concentration. Values are means ± S.E.M. for three experiments.

Table 1. *Sensitivity of CPT I in mitochondria from rat liver, heart and skeletal muscle to various CoA esters*
The term I_{50} refers to the concentration of inhibitor required for 50% suppression of enzyme activity. Uncorrected values (a) are taken directly from Fig. 1. Corrected values (b), given as means ± S.E.M., are adjusted for CPT activity that was non-suppressible by malonyl-CoA (see the Experimental section).

	I_{50} (μM)					
	Liver		Heart		Skeletal muscle	
CoA ester	(a)	(b)	(a)	(b)	(a)	(b)
Malonyl-CoA	1.7	1.7 ± 0.12	0.19	0.08 ± 0.04	0.04	0.023 ± 0.007
Succinyl-CoA	11.6	11.6 ± 0.18	4.4	2.8 ± 1.1	1.24	0.85 ± 0.18
Methylmalonyl-CoA	≫50	≫50	13.1	5.9 ± 1.8	2.5	1.7 ± 0.02

CPT I to succinyl-CoA and methylmalonyl-CoA, whereas it almost completely abolished the suppressive effect of malonyl-CoA. We conclude that the findings with succinyl-CoA and methylmalonyl-CoA represent genuine properties of these compounds.

Methylmalonyl-CoA is unique among the CoA esters tested in that it exists in two stereoisomeric forms (*R* and *S*). To determine whether inhibition of CPT I is specific for one or other of the isomers, the *R,S* mixture was incubated with malonyl-CoA decarboxylase for 26h as described by Kim & Kolattukudy (1980). After this time it could be shown that 50% of the starting material had been converted into propionyl-CoA [the latter was detected by reduction of 5,5′-dithiobis-(2-nitrobenzoic acid) in the presence of carnitine and carnitine acetyltransferase]. When tested against CPT I of skeletal-muscle mitochondria, the resulting solution (containing only the *R* isomer of methylmalonyl-CoA) exhibited 50% of the inhibitory potency of the original *R,S* mixture (results not shown). It thus seems likely that both isomers of methylmalonyl-CoA are equally effective inhibitors of CPT I.

Fig. 1 shows that, in contrast with the CoA esters examined, free CoA inhibited the CPT I reaction to a similar degree in all three tissues. This point is elaborated upon below.

Binding of malonyl-CoA to skeletal-muscle mitochondria

It seemed reasonable to assume that malonyl-CoA must first bind to some site on the outer aspect of the mitochondrial inner membrane (the presumed

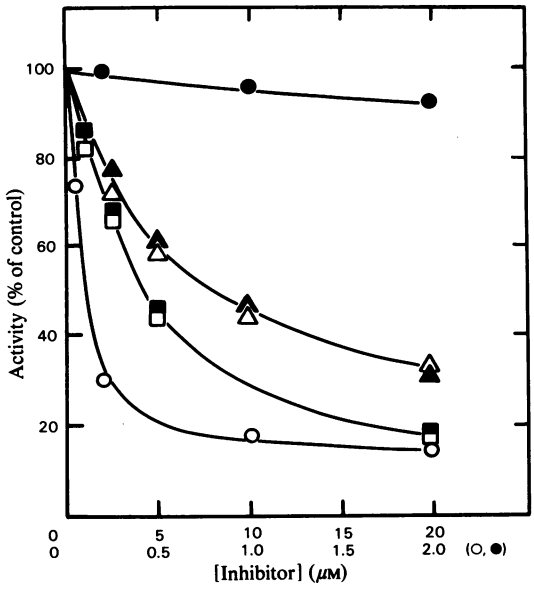

Fig. 2. *Response of skeletal-muscle CPT I to CoA esters in the absence or presence of malonyl-CoA decarboxylase*

Assays were performed as described for Fig. 1 in the absence (open symbols) or presence (closed symbols) of malonyl-CoA decarboxylase (8 μg), which was added to the incubation mixture 20 min before addition of the mitochondria. The inhibitors tested were: O, ●, malonyl-CoA; □, ■, succinyl-CoA; and △, ▲, methylmalonyl-CoA. The results of one experiment are shown. Note different scale for malonyl-CoA concentration.

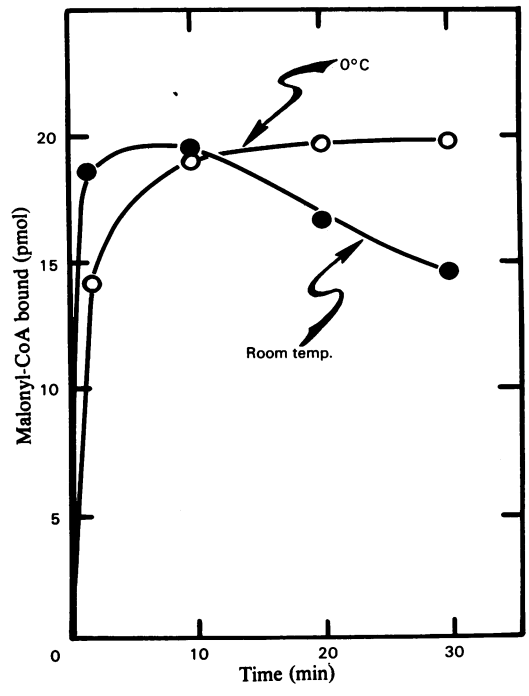

Fig. 3. *Time course of [^{14}C]malonyl-CoA binding to skeletal-muscle mitochondria*

Mitochondria (approx. 3 mg of protein) were incubated at 0°C or room temperature with 0.25 μM-[1,3-^{14}C]malonyl-CoA in the presence or absence of 100 μM unlabelled malonyl-CoA as described in the Experimental section. Specific binding is shown for a representative example of several closely agreeing experiments.

location of CPT I) to effect inhibition of the enzyme. It also seemed likely that, because of their extreme sensitivity to the inhibitor, mitochondria from skeletal muscle would prove useful for the direct demonstration of such binding. This turned out to be the case. As shown in Fig. 3, in an incubation mixture similar to that employed for studies of CPT I activity, mitochondria from skeletal muscle were found to bind malonyl-CoA in a time-dependent manner. At room temperature binding reached a maximum between 2 and 10 min, but thereafter declined. Separate experiments suggested that this loss of bound radioactivity might have been due to small quantities of malonyl-CoA decarboxylase, since pelleted mitochondria bearing [1,3-^{14}C]-malonyl-CoA gradually lost radioactivity when left at room temperature before addition of the KOH. At 0°C, however, binding of malonyl-CoA was maximal at 10 min and remained constant for at least 30 min. Subsequent experiments were therefore performed at 0°C for 20 min.

The kinetics of malonyl-CoA binding to skeletal-muscle mitochondria are shown in Fig. 4. The system was saturable at a malonyl-CoA concentration of 0.2 μM (Fig. 4*a*). Linearity of the Scatchard plot (Fig. 4*b*) indicates that only one class of binding site was present and that the affinity of malonyl-CoA for this site was not affected by the extent of ligand binding. Under the conditions of assay the K_d for malonyl-CoA was approx. 25 nM. A similar value was obtained when binding was measured over a period of 5 min at 30°C.

The ability of compounds other than malonyl-CoA to inhibit CPT I, particularly in mitochondria from skeletal muscle (Fig. 1), suggested that they too must be capable of binding to the mitochondrial inner membrane. To address this question, the various agents used in the experiments of Fig. 1 were tested for their ability to compete with [^{14}C]-malonyl-CoA for binding to skeletal-muscle mitochondria. The results are shown in Fig. 5. The profile of labelled-ligand displacement was qualitatively

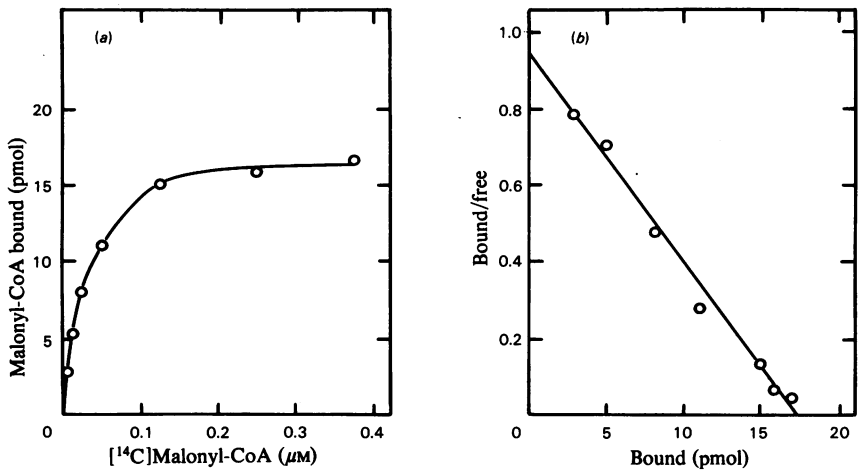

Fig. 4. *Kinetics of [14C]malonyl-CoA binding to skeletal-muscle mitochondria*
Mitochondria (approx. 3mg of protein) were incubated at 0°C for 20min with the indicated concentration of [1,3-14C]malonyl-CoA in the presence or absence of 100μM-unlabelled malonyl-CoA. Total binding ranged from 700 to 3800c.p.m.; equivalent values for non-specific binding were 100–1000c.p.m. Panel (*a*) shows the quantity of labelled ligand specifically bound. Panel (*b*) represents a Scatchard analysis of the data from panel (*a*): a typical result is shown. From five similar experiments a K_d value of 0.026 ± 0.002 μm (mean ± S.E.M.) was calculated.

similar to that for CPT I inhibition (Fig. 1*c*), i.e. the order of effectiveness was malonyl-CoA ≫ succinyl-CoA > methylmalonyl-CoA ≫ propionyl-CoA = acetyl-CoA. This relationship is illustrated in numerical terms in Table 2, which shows that for the CoA esters studied a direct relationship emerged between binding affinity and inhibition of CPT I activity. Free CoA, however, again displayed anomalous behaviour, in that its ability to compete for malonyl-CoA binding was disproportionately greater than its inhibition of CPT I (compare the effects of CoA and methylmalonyl-CoA in Fig. 1*c*, Fig. 5 and Table 2).

Because palmitoyl-CoA and malonyl-CoA interact in a competitive-like manner in the CPT I reaction (McGarry *et al.*, 1978*a*, 1983; Saggerson & Carpenter, 1981*b*), it was decided to test the effects of fatty acyl-CoA species on malonyl-CoA binding to mitochondria. As shown in Fig. 6, palmitoyl-CoA and oleoyl-CoA were moderately effective competitors for malonyl-CoA binding; octanoyl-CoA was more potent. With all three fatty acyl-CoA species, however, the concentrations needed for significant displacement of malonyl-CoA were much higher than those found with succinyl-CoA and methylmalonyl-CoA (Fig. 5). Palmitoylcarnitine was ineffective and, if anything, seemed to enhance the binding of malonyl-CoA slightly.

From the data of Figs. 4–6 and additional experiments with glutaryl-CoA and 3-hydroxy-3-methylglutaryl-CoA (results not shown), K_d values for the interaction of the various CoA esters with the malonyl-CoA-binding site could be calculated by using the equation derived by Cheng & Prusoff (1973):

$$K_d \text{ (competing ligand)} = \frac{I_{50} \text{ (binding)}}{1 + \dfrac{[[^{14}\text{C}]\text{malonyl-CoA}]}{K_d \text{ (malonyl-CoA)}}}$$

where I_{50} (binding) represents the concentration of competing ligand needed to decrease the binding of 0.25μM-[14C]malonyl-CoA by 50%. The K_d for malonyl-CoA (0.025μM) was obtained directly from Fig. 4. The results, shown in Table 3, establish three points. First, the calculated K_d for unlabelled malonyl-CoA as the competing ligand agrees with the measured K_d for [14C]malonyl-CoA binding (Fig. 4*b*). Second, the K_d values for malonyl-CoA, succinyl-CoA and methylmalonyl-CoA derived from binding studies (Table 3) are similar to the I_{50} values measured in the CPT I reaction (Table 1). Third, the calculated K_d for palmitoyl-CoA binding (approx. 12μM) is not greatly different from the apparent K_m for this substrate in the CPT I reaction (approx. 35μM) observed under similar experimental conditions (McGarry *et al.*, 1983).

The general shapes of the curves in Figs. 5 and 6 suggested that those agents capable of decreasing [14C]malonyl-CoA binding to mitochondria did so by interacting in competitive fashion with the

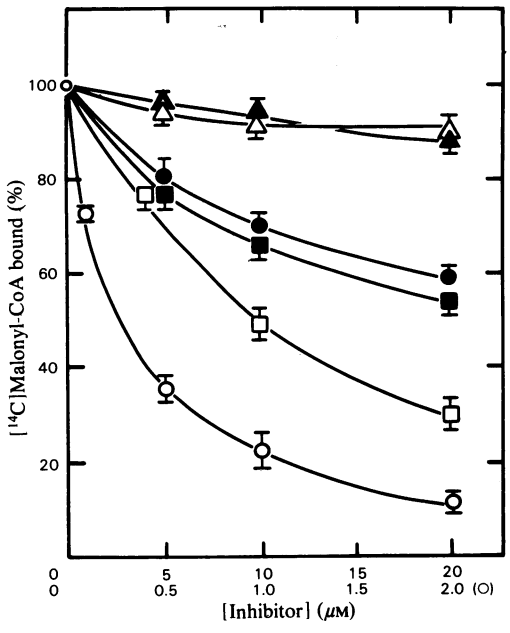

Fig. 5. *Effects of CoA and CoA esters on [^{14}C]malonyl-CoA binding to skeletal-muscle mitochondria*

Mitochondria (approx. 3 mg of protein) were incubated at 0°C for 20 min with 0.25 μM-[1,3-^{14}C]-malonyl-CoA in the presence or absence of the following unlabelled competing agents: ○, malonyl-CoA; □, succinyl-CoA; ●, methylmalonyl-CoA; △, propionyl-CoA; ▲, acetyl-CoA; ■, CoA (note different scale for malonyl-CoA). The 100% value represents the quantity of labelled ligand specifically bound (generally 15–20 pmol) as determined from incubations in the presence and absence of 100 μM unlabelled malonyl-CoA. Values are means ± S.E.M. for four experiments.

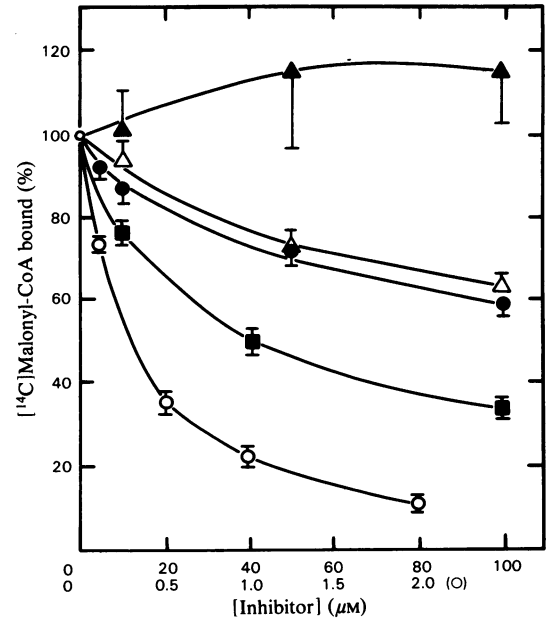

Fig. 6. *Effects of acyl-CoA species and acylcarnitine on [^{14}C]malonyl-CoA binding to skeletal-muscle mitochondria*

Experiments were performed as described for Fig. 5, with the following competing agents: ○, malonyl-CoA; ■, octanoyl-CoA; ●, palmitoyl-CoA; △, oleoyl-CoA; ▲, palmitoylcarnitine. Note different scale for malonyl-CoA concentration. Values are means ± S.E.M. for three experiments.

Table 2. *Relationship between malonyl-CoA displacement from skeletal muscle mitochondria and inhibition of CPT I by various compounds*

For malonyl-CoA and succinyl-CoA the data were derived from the experiments of Figs. 1(*c*) and 5. For methylmalonyl-CoA and CoA additional experiments, performed over a broader concentration range (results not shown), were used for calculation purposes. Values represent the relative concentration of each compound needed for 50% inhibition of CPT I or 50% displacement of [^{14}C]malonyl-CoA binding. The potency of malonyl-CoA has been set at 100%.

	Potency (%)	
Compound	CPT I inhibition	[^{14}C]Malonyl-CoA displacement
Malonyl-CoA	100	100
Succinyl-CoA	2.8	2.5
Methylmalonyl-CoA	1.25	0.9
CoA	0.12	1.2

Table 3. *Binding constants for free CoA and CoA esters towards skeletal-muscle mitochondria*

Concentrations of competing agents giving 50% displacement of [^{14}C]malonyl-CoA [I_{50} (binding)] were obtained from curves such as those shown in Figs. 5 and 6. Additional experiments using higher concentrations of competing ligands were performed with CoA, methylmalonyl-CoA and palmitoyl-CoA (results not shown). K_d values were calculated as described in the text.

Compound	I_{50} (binding) (μM)	K_d (μM)
Malonyl-CoA	0.25	0.023
Glutaryl-CoA	2	0.18
Succinyl-CoA	10	0.91
CoA	20	1.8
Methylmalonyl-CoA	30	2.7
3-Hydroxy-3-methyl-glutaryl-CoA	40	3.6
Octanoyl-CoA	50	4.5
Palmitoyl-CoA	130	11.8

malonyl-CoA-binding site. Supportive evidence for this thesis is provided by the data shown in Fig. 7. In these experiments binding of ^{14}C was measured over

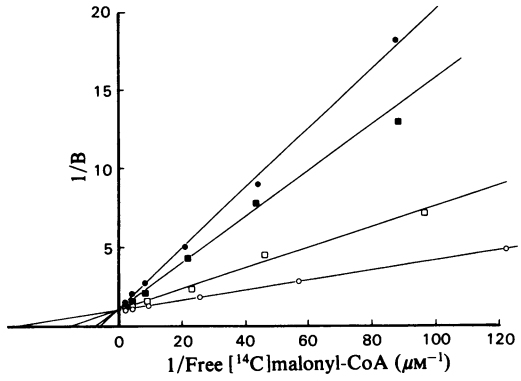

Fig. 7. *Kinetics of the effects of competing agents for [^{14}C]malonyl-CoA binding to skeletal-muscle mitochondria*

Mitochondria (approx. 3 mg of protein) were incubated at 0°C for 20 min with [1,3-^{14}C]malonyl-CoA over the concentration range shown in Fig. 4(*a*). Other additions were: ○, none; □, 5 μM-succinyl-CoA; ■, 10 μM-CoA; ●, 100 μM-palmitoyl-CoA. Specific binding (B) of the labelled ligand was determined as described in the Experimental section. Because the data were derived from more than one experiment, they have been normalized such that maximal binding is set at unity. The reciprocal of B, expressed relative to 1, is plotted against the reciprocal of the concentration of unbound [^{14}C]-malonyl-CoA.

a range of [^{14}C]malonyl-CoA concentrations in the absence or presence of a fixed concentration of palmitoyl-CoA, succinyl-CoA or free CoA. All three compounds increased the apparent K_m for malonyl-CoA binding, but had no effect on the maximal binding capacity of the mitochondria for this ligand.

Anomalous behaviour of free CoA

As noted above, the relationship between malonyl-CoA displacement from mitochondria (Fig. 5) and inhibition of CPT I activity (Fig. 1) seen with free CoA did not fit the pattern observed with the other compounds examined. The fact that CoA, unlike the CoA esters, is a product of the CPT I reaction, coupled with the observation that its effect on enzyme activity was approximately the same in liver, heart and skeletal muscle (Fig. 1), raised the possibility that the nucleotide was working through mass action and not via a malonyl-CoA-type of mechanism. The equilibrium constant of the CPT reaction is close to unity (Norum, 1964). From additional experiments (results not shown), the following observations, consistent with the above interpretation, were made. First, increasing the carnitine concentration in the assay by 10-fold (from 0.2 to 2 mM) had no effect on the malonyl-CoA-sensitivity of CPT I in skeletal-muscle mitochondria, whereas it decreased the inhibitory potency of CoA by 50%. Second, after detergent solubilization and subsequent dialysis, the enzyme from liver and skeletal muscle lost all sensitivity to malonyl-CoA, in keeping with previous reports (McGarry *et al.*, 1978*a*; Bremer, 1981), but suppression by CoA was still evident. That other factors were at work, however, is suggested from the results of numerous experiments in which CPT I activity of skeletal-muscle mitochondria was measured over a range of malonyl-CoA concentrations in the absence or presence of free CoA. The concentrations of CoA used were such that they should have displaced a large fraction of the malonyl-CoA from the mitochondrial membrane without causing major inhibition of CPT I. Accordingly, we expected the free nucleotide to protect the enzyme from malonyl-CoA inhibition, particularly at the lowest concentrations of the CoA ester tested. No such protection was ever seen.

Discussion

A new development in the area of mammalian fatty acid metabolism and its regulation has been the observation that the sensitivity of mitochondrial CPT I to inhibition by malonyl-CoA is far more pronounced in non-hepatic tissues than in liver (Saggerson & Carpenter, 1981*a*; McGarry *et al.*, 1983). This prompted us to select for further study three rat tissues (liver, heart and skeletal muscle) in which mitochondrial CPT I exhibits low, intermediate and high sensitivity, respectively, to inhibition by malonyl-CoA. Specifically, we sought answers to the following questions. (1) Might compounds structurally related to malonyl-CoA also inhibit CPT I, particularly in mitochondria showing the greatest sensitivity to the malonyl ester? (2) If so, will the variation between tissues noted for malonyl-CoA-sensitivity of CPT I also hold for malonyl-CoA analogues? (3) Can a malonyl-CoA-binding site be demonstrated in a super-sensitive tissue such as skeletal muscle? (4) Do malonyl-CoA analogues interact with the malonyl-CoA-binding site in a pattern commensurate with their ability to suppress CPT I activity? The answer to all of these questions appears to be affirmative, although, as discussed below, certain peculiarities of the system are clearly evident, and a number of caveats must be expressed in interpreting some of the findings.

As expected on the basis of earlier studies (McGarry *et al.*, 1977), of all the CoA esters examined malonyl-CoA proved to be the most effective inhibitor of CPT I. In all three tissues the

same order of potency emerged, namely malonyl-CoA ≫ succinyl-CoA > methylmalonyl-CoA ≫ propionyl-CoA ≥ acetyl-CoA. Moreover, as the sensitivity to malonyl-CoA increased in the order liver < heart < skeletal muscle, so too did the sensitivity to the other agents. For example, the respective I_{50} values (μM) for malonyl-CoA, succinyl-CoA and methylmalonyl-CoA shifted from about 2, 12 and ≫50 for liver to 0.02, 1 and 2 for skeletal muscle, with heart mitochondria displaying intermediate values. Thus, in an absolute sense malonyl-CoA can no longer be considered the only physiological compound capable of inhibiting CPT I, since structurally related CoA esters, known to be present in mammalian cells, also share this property. However, the potency of CPT I inhibition by the other CoA esters is impressive only in those tissues where the enzyme shows extreme sensitivity to malonyl-CoA.

With skeletal-muscle mitochondria, which responded to nanomolar concentrations of malonyl-CoA in the CPT I assay, a high-affinity (K_d approx. 25 nM) saturable binding site for [^{14}C]malonyl-CoA could be readily demonstrated. In addition, [^{14}C]-malonyl-CoA was displaced from this site in competitive fashion by unlabelled malonyl-CoA, succinyl-CoA, methylmalonyl-CoA, propionyl-CoA and acetyl-CoA over concentration ranges entirely consistent with their respective abilities to inhibit CPT I. In other words, for each compound the calculated K_d from binding studies approximated to the observed I_{50} value for CPT I inhibition (absolute equivalence would not be expected, since I_{50} values will be influenced by the concentration of palmitoyl-CoA used in the CPT I assay). We must emphasize that these relationships have been established only with mitochondria from skeletal muscle, although it seems reasonable to suppose that they will also apply to other tissues.

Taken together, these observations strongly suggest that malonyl-CoA and related compounds bind to a common site on the mitochondrial inner membrane and that for any given agent the efficiency of binding to this site determines its potency as an inhibitor of CPT I. Examination of structure–function relationships between the CoA esters provides insight into the molecular requirements for interaction with the membrane and inhibition of CPT I. Thus the CoA moiety appears to be essential, since neither free malonic acid nor succinic acid affects malonyl-CoA binding or CPT I activity. The most potent compounds (malonyl-CoA, glutaryl-CoA and succinyl-CoA) are straight-chain dicarboxylic acid derivatives. Substitution within these chains (methylmalonyl-CoA, 3-hydroxy-3-methylglutaryl-CoA) markedly diminishes binding affinity and inhibitory potency towards CPT I. Removal of the free carboxy group [propionyl-CoA, acetyl-CoA, isovaleryl-CoA, tiglyl-CoA (2-methylbut-2-enoyl-CoA] further decreases effectiveness. Presumably palmitoyl-CoA represents a special case, since, unlike the other CoA esters studied, it is a substrate for CPT I. In the malonyl-CoA-binding assay it acted in competitive fashion, with an apparent K_d value similar to its apparent K_m value in the CPT I reaction. It is thus tempting to speculate that at least one of the sites on the mitochondrial membrane to which palmitoyl-CoA, malonyl-CoA and malonyl-CoA analogues bind is in fact the active site of CPT I itself. That the situation must be more complicated, however, is suggested by the fact that the presence of malonyl-CoA introduces sigmoidicity into the relationship between [palmitoyl-CoA] and CPT I activity in intact mitochondria or mitochondrial membranes such that the substrate–inhibitor interaction does not conform to classical competitive kinetics (McGarry *et al.*, 1978*a*, 1983; Saggerson & Carpenter, 1981*b*; Saggerson, 1982). Moreover, when released from its membrane environment CPT I loses all sensitivity to malonyl-CoA and related compounds (McGarry *et al.*, 1978*a*), suggesting that a regulatory subunit on the membrane might also be operative. In this regard, although we have interpreted our competitive binding data as being indicative of a single binding site for malonyl-CoA and other CoA esters, the presence of two or more adjacent sites than can influence each other's binding characteristics cannot be rigorously excluded.

A particularly puzzling feature of the present studies is the anomalous behaviour of free CoA. Although it was able to displace labelled malonyl-CoA from the mitochondrial membrane and to suppress CPT I activity, the two events were not related in the manner seen with malonyl-CoA and its analogues. As noted in the Results section, there is reason to believe that free CoA might have inhibited CPT I through a mass-action effect (since it is a substrate in the reverse reaction) and not via a malonyl-CoA-type of mechanism. But if this were the sole explanation, why did CoA fail to protect the enzyme from malonyl-CoA inhibition when used in concentrations expected to displace a major fraction of the malonyl-CoA from the mitochondrial membrane? At the present time we have no explanation for this paradox.

Concerning the properties and potential regulatory roles of mitochondrial CPT, many other questions, not addressed here, remain to be answered. For example, are CPT I and CPT II of a given tissue different proteins, or are they identical enzymes with functionally different properties imposed by their geometrical distribution on the mitochondrial inner membrane? Is CPT I in different tissues of the same species a distinct isoenzyme, or is it the same protein whose kinetic charac-

teristics are grossly altered by variations in membrane environment? Extension of the studies described here to mitochondrial preparations from which CPT I has been selectively removed, coupled with reconstitution experiments involving mitochondrial membranes and solubilized enzyme, could provide useful insight into these questions. From a physiological standpoint, the notion that malonyl-CoA or related intermediates might under certain circumstances exert control over CPT I in non-hepatic tissues (McGarry *et al.*, 1983) remains an intriguing prospect. However, progress on this front must await the technology needed for accurate determination of the intracellular location and concentrations of such compounds.

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