Binding of malonyl-CoA to isolated mitochondria

Evidence for high- and low-affinity sites in liver and heart and relationship to inhibition of carnitine palmitoyltransferase activity

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1. [^{14}C]Malonyl-CoA bound to intact mitochondria isolated from rat liver and heart in a manner consistent with the presence of two independent classes of binding sites in each tissue. 2. The binding characteristics for mitochondria obtained from fed male rats were: for heart, $K_{D(1)} = 11\text{--}18\text{ nM}$, $K_{D(2)} = 30\text{ }\mu\text{M}$, $N_1 = 7\text{ pmol/mg}$ of protein, $N_2 = \text{approx. } 660\text{ pmol/mg}$ of protein; for liver, $K_{D(1)} = 0.1\text{ }\mu\text{M}$, $K_{D(2)} = 5.6\text{ }\mu\text{M}$, $N_1 = 11\text{ pmol/mg}$ of protein, $N_2 = 165\text{ pmol/mg}$ of protein. In the presence of $40\text{ }\mu\text{M}$ -palmitoyl-CoA the characteristics of binding at the high-affinity sites were changed, so that for heart $K_{D(1)} = 0.26\text{ }\mu\text{M}$, with no change in N_1 and for liver $K_{D(1)} = \text{approx. } 2\text{ }\mu\text{M}$, with N_1 increased to approx. 40 pmol/mg of protein. Differences between the two tissues in tightness of malonyl-CoA binding at the high-affinity sites explains the considerably greater sensitivity of heart CPT₁ (overt form of carnitine palmitoyltransferase) to inhibition by malonyl-CoA [Saggerson & Carpenter, (1981) *FEBS Lett.* **129**, 229–232; McGarry, Mills, Long & Foster (1983) *Biochem. J.* **214**, 21–28]. 3. Starvation (24 h) did not change the characteristics of [^{14}C]malonyl-CoA binding to liver mitochondria and did not alter the I_{50} (concentration giving 50% inhibition) for displacement of [^{14}C]malonyl-CoA by palmitoyl-CoA. Therefore the decreased sensitivity of liver CPT₁ to inhibition by malonyl-CoA in starvation [Saggerson & Carpenter (1981) *FEBS Lett.* **129**, 225–228; Bremer (1981) *Biochim. Biophys. Acta* **665**, 628–631] is not explained by differences in malonyl-CoA binding. 4. Percentage occupancy of the high-affinity sites in heart mitochondria by malonyl-CoA correlated closely with percentage inhibition of CPT₁ measured under similar conditions. This finding supports the proposal that the high-affinity binding sites are the functional sites mediating inhibition of CPT₁ by malonyl-CoA. Similar experiments with liver mitochondria also suggested that the occupancy of high-affinity sites by malonyl-CoA regulates CPT₁ activity. 5. 5,5'-Dithiobis(2-nitrobenzoic acid), which decreased the sensitivity of heart or liver CPT₁ to inhibition by malonyl-CoA [Saggerson & Carpenter (1982) *FEBS Lett.* **137**, 124–128], also decreased [^{14}C]malonyl-CoA binding to the high-affinity sites of heart mitochondria. 6. N_1 values for [^{14}C]malonyl-CoA binding to high-affinity sites in liver mitochondria were determined in various physiological states which encompassed a 7-fold range of CPT₁ maximal activity (fed, starved, pregnant, hypothyroid, foetal). The N_1 value did not change in these states. This finding supports the proposal that the high-affinity site is unlikely to be the catalytic unit of CPT₁. 7. The physiological importance of the low-affinity binding sites for malonyl-CoA is unknown.

Abbreviations used: CPT₁ and CPT₂, the overt and latent forms respectively of carnitine palmitoyltransferase (EC 2.3.1.21); Nbs₂, 5,5'-dithiobis(2-nitrobenzoic acid); I_{50} , the concentration of malonyl-CoA

required to inhibit CPT₁ activity by 50%; $K_{D(1)}$, $K_{D(2)}$, N_1 and N_2 , the dissociation constants and maximal binding capacities of the high-affinity (1) and low-affinity (2) malonyl-CoA sites.

Malonyl-CoA is a potent inhibitor of CPT₁ activity, an enzyme located on the outer aspect of the mitochondrial inner membrane (Hoppel & Tomec, 1972). As a consequence of this inhibition, malonyl-CoA is thought to play an important role in regulating the partitioning of hepatic fatty acid metabolism between oxidation and esterification (McGarry & Foster, 1980).

Subsequent evidence has revealed unexpectedly that the inhibitory potency of malonyl-CoA is 20–100-fold greater in heart and skeletal muscle, where I_{50} values are of the order 0.1–0.2 μM , than in tissues such as liver and kidney cortex, where I_{50} values are in the micromolar range (Saggerson & Carpenter, 1981b; McGarry *et al.*, 1983; Mills *et al.*, 1983). Although the subcellular distribution of malonyl-CoA is unknown, total tissue contents of this metabolite for liver, heart and skeletal muscle in fed rats are in the range 2–8 nmol/g wet wt. (McGarry *et al.*, 1983), implying a possible regulatory role for malonyl-CoA in the muscle tissues as well as in liver.

In starvation and some other physiological states the sensitivity of hepatic CPT₁ to inhibition by malonyl-CoA is considerably altered (Ontko & Johns, 1980; Cook *et al.*, 1980; Saggerson & Carpenter, 1981a,c; Robinson & Zammit, 1982). This change in sensitivity is not, however, seen in other tissues (Veerkamp & Van Moerkerk, 1982; Saggerson & Carpenter, 1983).

There is considerable uncertainty regarding the mechanism by which malonyl-CoA exerts its inhibitory effect on CPT₁ activity. Skeletal-muscle mitochondria have been shown to contain saturable high-affinity binding sites for malonyl-CoA (Mills *et al.*, 1983). However, the nature of the binding site remains unclear. McGarry *et al.* (1978) and Mills *et al.* (1983) have suggested that malonyl-CoA could interact in a competitive-like fashion with the active site of CPT₁ itself, although these authors also acknowledge that this mechanism is unlikely to be consistent with malonyl-CoA increasing the sigmoidicity of CPT₁ kinetics with respect to palmitoyl-CoA concentration (McGarry *et al.*, 1978; Saggerson & Carpenter, 1981c; Saggerson, 1982). Alternatively, malonyl-CoA could bind to a separate regulatory component or to an allosteric site on CPT₁, a hypothesis that is more attractive in several ways, since solubilization of the membrane-bound enzyme with detergent renders it insensitive to malonyl-CoA (McGarry *et al.*, 1978; Bremer, 1981; Saggerson, 1982). Presumably the detergent either could cause a conformational change in the enzyme structure or could result in the dissociation of a regulatory component from the catalytic unit of the enzyme.

In this study we have determined the characteristics of specific binding of [¹⁴C]malonyl-CoA to

rat liver and heart mitochondria. We have attempted to relate the malonyl-CoA-binding characteristics to the inhibitory effect of this metabolite against CPT₁. Comparisons have been made between liver and heart on the one hand, and between liver mitochondria in the fed and starved states on the other, in an attempt to relate differences in sensitivity of the enzyme to changes in the malonyl-CoA-binding characteristics. We have also related the number of hepatic mitochondrial binding sites to CPT₁ maximal activity in various physiological states in which CPT₁ activity changes. This was done to gain insight into the possible nature of the malonyl-CoA-binding sites.

Materials and methods

Animals

Sprague-Dawley rats were bred in the animal colony at University College, London. Normal fed males (160–180 g) and pregnant females received GR3-EK cube diet (E. Dixon and Sons, Ware, Herts., U.K.) *ad libitum*. Hypothyroidism was produced by maintaining rats for 4 weeks on an iodine-deficient diet (Special Diet Services Ltd., Witham, Essex, U.K.) with drinking water containing 6-n-propyl-2-thiouracil (0.01% w/v) and ethanol (0.25% v/v). Euthyroid controls were fed on a comparable diet containing normal iodine obtained from the same suppliers and also had 0.25% ethanol in their drinking water. Average rates of growth over the 4-week period were euthyroid rats, from 76 to 225 g; hypothyroid rats, from 112 to 175 g. Tri-iodothyronine (T_3) and thyroxine (T_4) concentrations in plasma at the time of death were determined at University College Hospital by radioimmunoassay and were found to be (means $\pm$ S.E.M. for the numbers of animals shown): euthyroid animals (21), $T_4 = 32.6 \pm 2.4$ nm, $T_3 = 1.06 \pm 0.04$ nm; hypothyroid animals (18), $T_4 \leq 10$ nm (below the limits of the assay), $T_3 = 0.17 \pm 0.01$ nm. Pregnant rats were judged to be within 48 h of term, and foetal liver was obtained from rats at the same stage of pregnancy. Starved animals received only water for 24 h before the experiments, and all animals were killed between 09:30 and 11:00 h.

Chemicals

[2-¹⁴C]Malonyl-CoA and DL-[Me-³H]carnitine hydrochloride were obtained from Amersham International (Amersham, Bucks., U.K.). Sources of other chemicals have been described previously (Saggerson *et al.*, 1982); solvents were of analytical-reagent grade.

Experimental methods

Liver and heart mitochondria were obtained as described by Saggerson (1982), and were resus-

pended in 0.3 M-sucrose medium containing 10 mM-Tris/HCl buffer (pH 7.4), 1 mM-EGTA and 1 mM-dithiothreitol (except in experiments with Nbs₂) to give a protein concentration of approx. 6 mg/ml (liver) or 3 mg/ml (heart).

In malonyl-CoA-binding experiments, incubations were performed in 1.5 ml plastic stoppered centrifuge tubes. The incubation medium contained, in a final volume of 1.05 ml, 185 μ mol of sucrose, 25 μ mol of Tris/HCl (pH 7.4), 40 μ mol of KCl, 1 μ mol of EGTA, 1 μ mol of dithiothreitol, 1.3 mg of defatted bovine serum albumin, 5 pmol-30 nmol of [¹⁴C]malonyl-CoA (sp. radioactivity was initially approx. 13 Ci/mol, but was varied in later experiments) plus any further additions indicated in the legends to the Figures and Tables. The medium was equilibrated to 0–4°C for 1 h, and the experiments were started by the addition of approx. 3 mg (liver) or 1.5 mg (heart) of mitochondrial protein (at 0–4°C), followed by mixing. After the incubation period (usually 20 min) on ice, the tubes were rapidly centrifuged for 2 min at 6500 *g*_{av.} in an Eppendorf 5412 centrifuge (at 5°C), and the supernatant was discarded. Any remaining supernatant fluid was removed by drying the inside walls of the tubes with tissue paper held in forceps. The mitochondrial pellets were solubilized in 0.5 ml of 10% (v/v) Triton X-100 at 30°C and were then quantitatively transferred to vials containing 13 ml of scintillation fluid [80 g of naphthalene plus 4 g of 2,5-bis-(5-*t*-butylbenzoxazol-2-yl)thiophen in 600 ml of toluene plus 400 ml of 2-methoxyethanol] for scintillation counting of radioactivity. Non-specific entrapment of [¹⁴C]malonyl-CoA was calculated by including 500 nmol of unlabelled malonyl-CoA in five or six incubations covering the range of test malonyl-CoA concentrations used. The amount of specifically bound malonyl-CoA was calculated by subtracting the non-specific radioactivity (d.p.m.) associated with the pellet from the total in the pellet. This procedure was more reliable than using [³H]sucrose to calculate the entrapment of aqueous phase in the pellet. The centrifugation procedure was shown to sediment over 95% of the glutamate dehydrogenase activity in the mitochondrial fraction. The experimental design was similar in many aspects to that described by Mills *et al.* (1983). In competition experiments, the [¹⁴C]malonyl-CoA concentration was fixed, and the concentration of the competing agent was varied, as indicated in the legends to the Figures and Tables.

CPT₁ assays were performed at 25°C in the incubation medium described above, except that malonyl-CoA concentrations were varied over the range 0–50 μ M and protein concentration was approx. 0.2 mg/ml. After a 4 min preincubation period in the presence of palmitoyl-CoA (40 or

100 μ M) and, where appropriate, malonyl-CoA, at 25°C, reactions were started by the addition of 0.4 μ mol of L-carnitine plus 1 μ Ci of DL-[Me-³H]carnitine and were continued for 4 min. Palmitoyl[³H]carnitine was extracted into butanol-1-ol and determined by scintillation counting as described by Saggerson *et al.* (1982). Under these conditions, assays were linear with time and protein concentration. Protein concentration was determined by the method of Lowry *et al.* (1951) with bovine serum albumin as a standard.

Analysis of malonyl-CoA-binding data

The binding data were analysed according to a mathematical model describing two independent classes of binding sites and represented by the equation.

$$\frac{B}{L} = \frac{L(N_1 + N_2) + N_1 K_{D(2)} + N_2 K_{D(1)}}{K_{D(1)} K_{D(2)} + L(K_{D(1)} + K_{D(2)}) + L^2}$$

where $K_{D(1)}$ and $K_{D(2)}$ are dissociation constants and N_1 and N_2 are maximal binding capacities for each site, L = free ligand concentration and B = bound ligand concentration. Values for $K_{D(1)}$, N_1 , $K_{D(2)}$ and N_2 were estimated by using a non-linear least-squares fitting program FACSIMILE (Chance *et al.*, 1977), available on the University College London computer. Data were input in the form of free ligand concentration and bound/free ligand ratio. There was a close correlation between experimental values and theoretical values for the model, and the residual sum of squares was generally within the range expected for a 1% experimental error. The analysis was sensitive to the accuracy of experimental determination of the initial points (i.e. at low free ligand concentrations) and, for consistency, analysis of heart data was commenced with the 5 nM point, and that of liver data with the 50 nM point. However, in some later experiments, it was observed that lower $K_{D(1)}$ values could be obtained if ligand binding to liver mitochondria was determined at concentrations below 50 nM. Simple graphical Scatchard analysis of the heart data gave similar $K_{D(1)}$ and N_1 values to those determined by computer analysis (see the Results and discussion section).

Results and discussion

Binding of [¹⁴C]malonyl-CoA to liver and heart mitochondria

Time courses for binding at low (0.05 μ M and 0.25 μ M for heart and liver respectively) and high (20 μ M) concentrations of the ligand were measured in preliminary experiments. These were chosen as representative of binding with the high-affinity site alone or with the high-affinity + low-affinity

sites, respectively (see below). At 0–5°C binding was constant between 10 and 20 min, and there were no apparent differences in the rates of equilibration with either the low or the high concentrations of ligand (results not shown). Non-specific binding (not shown) was essentially constant with 2 min, and the non-specifically bound radioactivity generally represented 20–40% of the total, depending on the ligand concentration. All subsequent binding studies therefore involved incubations for 20 min at 0–5°C. This procedure permitted large numbers of samples to be processed at any one time with little timing error. The

time courses were similar to those obtained by Mills *et al.* (1983) in similar incubations with rat skeletal-muscle mitochondria.

The concentration-dependence of specific binding of [14 C]malonyl-CoA to liver and heart mitochondria is shown in Figs. 1(a) and 1(c). Total saturation of binding sites was not apparent, even at a malonyl-CoA concentration of 30 μ M. Scatchard plots of [14 C]malonyl-CoA binding to liver and heart mitochondria were non-linear (Figs. 1b and 1d). These and other data were analysed on the basis of a non-interacting two-site model of binding (see the Materials and methods section).

Fig. 1. Concentration-dependence of [14 C]malonyl-CoA binding to liver and heart mitochondria. Binding was measured after a 20 min incubation. The values are means of several separate experiments as indicated. The bars represent s.e.m.; where not shown these lie within the symbol. (a) $\circ$, Liver, fed (6); $\bullet$, liver, 24 h-starved (5) (b) Scatchard analysis of data from (a). (c) Heart, fed (3). (d) Scatchard analysis of data from (c).

For heart mitochondria the Scatchard plots were readily resolved into two approximately linear components (Fig. 1d). The binding data for liver mitochondria were also satisfactorily fitted to the same two-site model, although it is appreciated that other, more complex, models may be consistent with the data. Estimated values for K_D and N are shown in Table 1. For fed male rats there is a 10-fold difference between the $K_{D(1)}$ values for malonyl-CoA binding in heart and liver mitochondria. Differences in binding characteristics at these high-affinity sites could therefore contribute appreciably to the greater sensitivity of heart CPT₁ to inhibition by malonyl-CoA compared with the liver enzyme. The possible role of these high-affinity sites is considered further below. Table 1 also shows that there is no appreciable difference in the number of high-affinity binding sites between liver and heart mitochondria, although the heart appeared to contain a larger number of poorly resolved low-affinity sites. The complexity of Scatchard plots for binding to liver and heart mitochondria (Figs. 1b and 1d) may be contrasted with the findings of Mills *et al.* (1983), who have concluded that malonyl-CoA binds to a single class of sites on skeletal-muscle mitochondria. The high-affinity binding to heart mitochondria observed here ($K_D = 11 \pm 2$ nM) is appreciably tighter than that demonstrated with skeletal-muscle mitochondria ($K_D = 26 \pm 2$ nM; Mills *et al.*, 1983). With heart mitochondria a simple graphical determination of $K_{D(1)}$ and N_1 could be performed by linear regression using all data points up to $0.1 \mu\text{M}$ malonyl-CoA. These values were not significantly different from those obtained by the computer analysis of all the data (comparisons were made in four separate experiments). The simple graphical approach was not applicable with the liver mitochondria.

Figs. 1(a) and 1(b) and Table 1 show that starvation resulted in no significant differences in the characteristics of malonyl-CoA binding to liver mitochondria. Control CPT₁ assays were performed in parallel with these binding studies to check that the expected loss of sensitivity of the enzyme to inhibition by malonyl-CoA was observed in these preparations. Starvation caused the expected loss of sensitivity (results not shown), confirming previous observations (Saggerson & Carpenter, 1981a, 1982a,c; Saggerson *et al.*, 1982). It is concluded that the diminished sensitivity of hepatic CPT₁ to malonyl-CoA in starvation cannot readily be attributed to differences in the binding of the inhibitor to CPT₁ or to an associated regulatory component.

In some instances, longer-term changes did result in some alteration in the tightness of malonyl-CoA binding for the high-affinity sites in liver mitochondria (Table 1). This may be seen when the fed pregnant animal is compared with the fed male, and when euthyroid males are compared with normal fed males. The latter change could be attributable to differences in dietary regimen [the euthyroid rats were fed on a different standard cube diet and received 0.25% (v/v) ethanol in drinking water]. Also $K_{D(1)}$ was lower in foetal rat liver than in the adult male, although the foetal value was not different from $K_{D(1)}$ in the fed pregnant mothers of these animals (Table 1). CPT₁ in liver mitochondria is considerably more sensitive to inhibition by malonyl-CoA in foetal animals compared with their mothers (Saggerson & Carpenter, 1982c). Again it seems that changes in sensitivity of the enzyme do not correlate with changes in binding characteristics.

The conditions shown in Table 1 encompass a several-fold range of hepatic CPT₁ maximal activities, as shown previously (Saggerson &

Table 1. Dissociation constants and maximal binding capacities for [¹⁴C]malonyl-CoA binding to liver and heart mitochondria. Binding was measured after a 20 min incubation. The values are means $\pm$ S.E.M. for the indicated numbers of separate experiments. $K_{D(1)}$, $K_{D(2)}$ and the maximum numbers of high- and low-affinity sites (N_1 and N_2 respectively) were computed from Scatchard analysis of the data by using a two-site model for binding.

	$K_{D(1)}$ (μM)	N_1 (pmol/mg of protein)	$K_{D(2)}$ (μM)	N_2 (pmol/mg of protein)
Liver				
Fed (8)	0.096 ± 0.017	10.9 ± 1.8	5.6 ± 0.4	165 ± 15
24h-Starved (6)	0.103 ± 0.033	10.4 ± 2.1	5.1 ± 0.7	155 ± 14
Pregnant, fed (3)	0.049 ± 0.011	8.0 ± 1.6	11.6 ± 5.0	123 ± 25
Pregnant, 24h-starved (3)	0.044 ± 0.004	8.1 ± 1.2	3.8 ± 0.1	140 ± 13
Euthyroid, fed (4)	0.058 ± 0.016	8.3 ± 2.0	11.2 ± 3.8	134 ± 16
Hypothyroid, fed (4)	0.049 ± 0.010	7.4 ± 2.0	9.7 ± 2.4	133 ± 7
Hypothyroid, 24h-starved (5)	0.153 ± 0.051	9.2 ± 1.6	5.7 ± 1.9	120 ± 16
Foetal, fed (4)	0.048 ± 0.010	8.1 ± 1.5	6.7 ± 1.5	76 ± 12
Heart				
Fed (4)	0.011 ± 0.002	7.2 ± 0.7	29.9 ± 11.3	665 ± 202

Carpenter, 1981a, 1982a,c; Saggerson *et al.*, 1982). Fig. 2 demonstrates clearly that the computed N_1 values are essentially invariant in different physiological states, whereas the $V_{\max}$ for CPT₁ changes over a 7-fold range. The same lack of correlation is seen when N_2 values are correlated with $V_{\max}$ of CPT₁ (plot not shown). Variations in $V_{\max}$ of CPT₁ could be attributable either to alterations in the amount of enzyme protein in different states or to modification of the activity of an invariant amount of enzyme protein. If the several-fold activity changes are due to alteration in amount of enzyme (this is not resolved), this then occurs without any change in the concentration of malonyl-CoA-binding sites and could be taken as strong

Fig. 2. Relationship between CPT₁ maximum activity and concentration of high-affinity malonyl-CoA-binding sites in liver mitochondria obtained from rats in various physiological states

$V_{\max}$ of CPT₁ was estimated in the presence of 100 μM -palmitoyl-CoA and 400 μM -L-carnitine. Malonyl-CoA binding was measured after a 20 min incubation with a range of concentrations of 0.05–30 μM , and the number of high-affinity sites was computed by using a two-site model for binding. The values are means of several separate experiments as indicated. The bars represent S.E.M. Only results where binding and enzyme assays are performed in the same mitochondrial preparations are included. $\circ$, 24 h-starved (4); $\square$, pregnant, 24 h-starved (3); $\bullet$, fed (6); $\triangle$, euthyroid, fed (4); $\blacksquare$, pregnant, fed (3); ∇ , hypothyroid, 24 h-starved (5); $\blacktriangle$, foetal, fed (4); $\blacktriangledown$, hypothyroid, fed (4). Further details concerning the treatment of animals are given under Materials and methods.

evidence that malonyl-CoA does not bind to CPT₁ itself.

Effect of starvation on the displacement of [¹⁴C]-malonyl-CoA by palmitoyl-CoA

Palmitoyl-CoA can displace malonyl-CoA from high-affinity binding sites on skeletal-muscle mitochondria (Mills *et al.*, 1983), and these two agents have competitive-like actions with regard to CPT₁ activity (McGarry *et al.*, 1978; Saggerson & Carpenter, 1981c). Since, in the absence of palmitoyl-CoA, the binding characteristics for [¹⁴C]malonyl-CoA were similar in liver mitochondria from both fed and starved rats (Figs. 1a and 1b, Table 1), it was considered that the altered sensitivity of CPT₁ activity to malonyl-CoA in these two states might be caused by palmitoyl-CoA being more effective at displacing [¹⁴C]malonyl-CoA from its binding sites in liver mitochondria from starved rats. Therefore the concentration of palmitoyl-CoA was varied over the range 0–100 μM , and [¹⁴C]malonyl-CoA concentration was fixed at 0.1, 1 or 10 μM . There was no apparent difference in the competitive-like effects of palmitoyl-CoA on binding in mitochondria from fed and starved rats, and the concentrations of palmitoyl-CoA giving 50% displacement of [¹⁴C]malonyl-CoA binding were not significantly affected by starvation (Table 2).

Effect of Nbs₂ on malonyl-CoA binding to heart mitochondria

The thiol-group reagent Nbs₂ considerably decreases the sensitivity of liver or heart CPT₁ to inhibition by malonyl-CoA (Saggerson & Carpenter, 1982b). The experiment shown in Fig. 3 suggests that this effect may, at least in part, be attributable to Nbs₂ decreasing malonyl-CoA binding. However, the effect appeared to be com-

Table 2. Effect of palmitoyl-CoA on [¹⁴C]malonyl-CoA binding to liver mitochondria from fed and starved rats

Binding was measured after a 20 min incubation with the indicated concentrations of [¹⁴C]malonyl-CoA and 0, 5 μM , 10 μM , 20 μM , 30 μM , 40 μM , 60 μM , 80 μM , or 100 μM -palmitoyl-CoA. The concentration of palmitoyl-CoA giving 50% displacement of [¹⁴C]malonyl-CoA (I_{50}) was determined graphically. The values are means $\pm$ S.E.M. for four separate experiments in each case.

[¹⁴ C]Malonyl-CoA concn. (μM)	I_{50} for palmitoyl-CoA (μM)	
	Fed	Starved (24 h)
0.1	22 $\pm$ 3	24 $\pm$ 2
1	48 $\pm$ 8	43 $\pm$ 2
10	47 $\pm$ 6	47 $\pm$ 2

Fig. 3. Effect of Nbs_2 on $[^{14}C]$ malonyl-CoA binding to high-affinity sites in heart mitochondria

Binding was measured after a 20 min incubation in the presence (●) or absence (○) of 0.25 mM- Nbs_2 . The values are means $\pm$ S.E.M. for four separate experiments. The $K_{D(1)}$ value in the absence of Nbs_2 was 16.0 nM.

plex, causing the Scatchard plot to become convex curvilinear (results not shown), and was not analysed further.

Direct correlation of occupancy of malonyl-CoA-binding sites with inhibition of CPT₁ activity

We have attempted, over a range of malonyl-CoA concentrations, to correlate binding at low- and high-affinity sites with inhibition of CPT₁ activity. Since, of necessity, the measurement of activity required the presence of the substrate palmitoyl-CoA (40 μ M), we therefore firstly investigated the effect of 40 μ M-palmitoyl-CoA on malonyl-CoA binding. Palmitoyl-CoA had a competitive-like interaction at the high affinity malonyl-CoA-binding sites in heart mitochondria, increasing the apparent value of $K_{D(1)}$ without changing the value of N_1 (Fig. 4). Palmitoyl-CoA (40 μ M) also increased the K_D values by approx. 20-fold at both the high- and the low-affinity sites in the liver (Fig. 5). Unexpectedly, the apparent concentration of the higher-affinity binding sites was also increased by approx. 5-fold. Fig. 6 shows that there was an excellent correlation between malonyl-CoA binding at the high-affinity sites in heart mitochondria and inhibition of the enzyme. At-

Fig. 4. Effect of palmitoyl-CoA on $[^{14}C]$ malonyl-CoA binding to high-affinity sites in heart mitochondria

Binding was measured after a 20 min incubation in the absence (○) or presence of (●) 20 μ M or (□) 40 μ M-palmitoyl-CoA. The values are means of four separate experiments and are shown in the form of a Scatchard analysis of the data. $K_{D(1)}$ values were: zero palmitoyl-CoA, 18 nM; 20 μ M-palmitoyl-CoA, 63 nM; 40 μ M-palmitoyl-CoA, 262 nM.

Fig. 5. Effect of palmitoyl-CoA on $[^{14}C]$ malonyl-CoA binding to liver mitochondria

Binding was measured after a 20 min incubation in the absence (○) or presence (●) of 40 μ M-palmitoyl-CoA. The values represent means of three separate experiments and are shown in the form of a Scatchard analysis of the data. Computer analysis of individual Scatchard plots, using a two-site model for binding, revealed the following apparent effects of palmitoyl-CoA: (i) $K_{D(1)}$ increased from $0.072 \pm 0.015 \mu$ M to $2.02 \pm 0.26 \mu$ M; (ii) number of high-affinity sites (N_1) increased from 7.0 ± 0.3 to 38.7 ± 3.9 pmol · mg of protein⁻¹; (iii) $K_{D(2)}$ increased from $5.2 \pm 0.4 \mu$ M to 44 μ M; (iv) number of low-affinity sites (N_2) non-significantly changed from 159 ± 23 to 190 pmol · mg of protein⁻¹. (Only two determinations of N_2 and $K_{D(2)}$ were made in the presence of palmitoyl-CoA.)

Fig. 6. Relationship between occupancy of high-affinity [^{14}C]malonyl-CoA-binding sites on heart mitochondria and inhibition of CPT_1 by malonyl-CoA

Binding was measured after a 20 min incubation in the presence of $40\text{ }\mu\text{M}$ -palmitoyl-CoA: 100% occupancy of the sites was determined by Scatchard analysis of the binding data. CPT_1 activity was measured with $40\text{ }\mu\text{M}$ -palmitoyl-CoA and $400\text{ }\mu\text{M}$ -L-carnitine. The values are means of two separate but similar experiments. ●, Percentage inhibition of CPT_1 ; ○, percentage occupancy of high-affinity malonyl-CoA sites ($100/N_1 \times \text{amount bound}$). The two sets of values are linearly correlated (slope = 1.01, $r = 0.99$).

tempts at similar correlations with liver mitochondria were less satisfactory (Fig. 7). Under the experimental conditions, percentage occupancy of the low-affinity sites alone was insufficient to explain the percentage inhibition of CPT_1 activity. On the other hand, percentage occupancy of the higher-affinity sites exceeded the resulting percentage inhibition of CPT_1 activity. If one considers it most likely that the higher- rather than the lower-affinity sites are effective in achieving inhibition of CPT_1 by malonyl-CoA, then the association between ligand binding and effect is very close in the heart, but less so in the liver. This looser association between binding and effect in the liver may be the basis for the change in sensitivity of the enzyme to malonyl-CoA that is seen in starvation and other physiological states. Obviously, in the starved state, since there is no change in malonyl-CoA binding (Table 2 and Fig. 1a), correlation between binding and effect would be expected to be even looser than in the fed state. A loose association between binding and effect is consistent with the model in which it is envisaged that malonyl-CoA does not bind directly to the catalytic

Fig. 7. Relationship between occupancy of [^{14}C]malonyl-CoA-binding sites on liver mitochondria and inhibition of CPT_1 by malonyl-CoA

Binding was measured after a 20 min incubation in the presence of $40\text{ }\mu\text{M}$ -palmitoyl-CoA with 16 different concentrations of [^{14}C]malonyl-CoA ($0.05\text{--}30\text{ }\mu\text{M}$). To simplify the Figure, not all of these values are shown. Scatchard analysis of the binding data using a two-site model showed: $K_{D(1)} = 2.5\text{ }\mu\text{M}$; $K_{D(2)} = 41\text{ }\mu\text{M}$; number of high-affinity sites (N_1) = $44\text{ pmol}\cdot\text{mg of protein}^{-1}$; number of low-affinity sites (N_2) = $126\text{ pmol}\cdot\text{mg of protein}^{-1}$. These values were used to calculate the expected percentage occupancy of sites: ●, percentage occupancy of high-affinity sites; ○, percentage occupancy of low-affinity sites; □, percentage occupancy of all sites ($100 \times \text{measured } [^{14}\text{C}]\text{malonyl-CoA binding}/(N_1 + N_2)$). ▲, Percentage inhibition of CPT_1 .

unit of CPT_1 itself. This loose association could allow the amount of CPT_1 protein to be altered in different physiological states without altering the number of malonyl-CoA-binding sites (Fig. 2).

General discussion

This study establishes that malonyl-CoA binds to heart or liver mitochondria in a manner consistent with the presence of two classes of binding sites. However, in both the presence and the absence of palmitoyl-CoA, the binding of malonyl-CoA at the high-affinity sites is an order of magnitude tighter in mitochondria from heart compared with liver, in accord with the known differential tissue sensitivity for inhibition of CPT_1 activity by malonyl-CoA (Saggerson & Carpenter, 1981b; Mills *et al.*, 1983). This observation raises the question therefore of whether the protein(s) to which malonyl-CoA binds are different in the two

tissues, or whether there exists in the liver a mechanism to attenuate the inhibitory potency of malonyl-CoA.

It seems probable that the high- rather than the low-affinity sites are functional in inhibiting CPT₁ activity, since $K_{D(1)}$ values for binding in the presence of 40 μ M-palmitoyl-CoA (Figs. 4 and 5) are similar to I_{50} values for inhibition of liver and heart CPT₁ activity under comparable conditions (Saggerson & Carpenter, 1981b). Indeed, in heart mitochondria, there is a direct correlation between occupancy of the high-affinity sites and inhibition of enzyme activity (Fig. 6). Further support for this argument can be derived from the data of Clarke & Bieber (1981), who purified bovine heart total CPT (CPT₁ + CPT₂) to homogeneity, thereby increasing the specific activity relative to total mitochondrial protein by 1600-fold and resolving the activity as a M_r -67000 monomer. From these findings it can be calculated that the approximate concentration of total CPT catalytic units in bovine heart mitochondria should be $10^9/(1600 \times 67000) = 9.3$ pmol/mg of mitochondrial protein, or approx. 6 pmol/mg if one assumes that in these heart mitochondria 60% of the activity represents the overt form (CPT₁) (Saggerson & Carpenter, 1981b). This value is remarkably similar to the concentration of heart high-affinity binding sites for malonyl-CoA (7.2 pmol/mg of protein) that we have measured here (Table 1).

The physiological role of the large number of low-affinity binding sites in liver and heart mitochondria is unclear, and the contrast with skeletal muscle, where Mills *et al.* (1983) have reported the presence of only a single class of high-affinity sites, is noteworthy.

As discussed above, a two-site model may not be adequate to describe malonyl-CoA binding to liver mitochondria. One must consider the possibilities of there being multiple classes of binding sites or of co-operativity between sites. We cannot distinguish between these possibilities at present. The change in the characteristics of malonyl-CoA binding when palmitoyl-CoA is added to liver mitochondria (Fig. 5) suggests a considerable degree of interconversion between binding sites of different affinities. In this context it is noteworthy that Zammit and co-workers (Robinson & Zammit, 1982; Zammit, 1983a,b) have proposed that the malonyl-CoA sensitivity of hepatic CPT₁ is positively correlated with the ambient malonyl-CoA concentration. It will be of interest to

establish whether this phenomenon might be related to interconversion of malonyl-CoA-binding sites with different affinities.

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