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Carnitine palmitoyltransferase (CPT_2) from liver mitochondrial inner membrane becomes inhibitable by malonyl-CoA if reconstituted with outer membrane malonyl-CoA binding protein

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A soluble extract was obtained on treatment of rat liver mitochondrial outer membranes with cholate which bound [^{14}C]malonyl-CoA but was essentially free of carnitine palmitoyltransferase (CPT) activity. Extraction of mitochondrial inner membranes with cholate readily solubilized a CPT activity which was insensitive to malonyl-CoA. Combination of these two extracts caused the CPT derived from inner membranes to become inhibitable by malonyl-CoA.

Carnitine palmitoyltransferase; Malonyl-CoA; Liver; Mitochondria

1. INTRODUCTION

Mitochondrial utilization of long chain fatty acids requires the participation of carnitine palmitoyltransferases (CPT). Two forms, CPT_1 and CPT_2 have been localized to mitochondrial outer and inner membranes respectively [1,2]. CPT_1 is inhibited by malonyl-CoA in many mammalian tissues [3,4] whereas CPT_2 is not [5]. A high affinity binding site for malonyl-CoA has been recognised in mitochondria [6] and mitochondrial outer membranes [7,8]. This binding site can be discriminated kinetically [9,10] and topographically [1] from the catalytic site of CPT_1. Radiation inactivation analysis [7] or antibodies [2] have suggested that these regulatory and catalytic sites reside on separate polypeptides; although this conclusion is not universally accepted [11]. At present there is no concensus as to similarity or dissimilarity of CPT_1 and CPT_2 (see [2] for discussion of this controversy). This study shows that a malonyl-CoA binding site may be extracted from liver mitochondrial outer membranes and then reconstituted with CPT_2 from inner membranes to confer sensitivity to malonyl-CoA.

2. EXPERIMENTAL

2.1. *Membrane preparations*

Total membranes were obtained from sonicated rat liver mitochondria as described in [2]. Purified outer membrane fractions were obtained essentially as described in [12] with minor modifications [2]. 100 μM Malonyl-CoA inhibited CPT_1 in these preparations by 98 ± 1% in a radiochemical assay [13] and by 75 ± 2% in a 4,4′-dithiopyridine-linked spectrophotometric assay [2] (means ± SE, n = 4 in each case). Based on succinate dehydrogenase measurements [14] these membranes had less than 2% contamination by mitochondrial inner membranes. Rat liver mitochondrial inner membrane fractions were obtained from sonicated mitoplasts as described in [2]. Measurements of rotenone-insensitive NADH cytochrome c reductase indicated a 12% contamination of these preparations with outer membranes. CPT activity in these inner membrane preparations was completely insensitive to malonyl-CoA in either the radiochemical or the spectrophotometric assay.

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2.2. *Detergent treatment of membranes*

Total mitochondrial membranes, inner membranes or outer membranes were suspended (approx. 4 mg of protein/ml) in 20 mM potassium phosphate buffer (pH 7.2) containing phenylmethylsulphonyl fluoride (0.1 mg/ml). 15 mM sodium cholate solution was added to each preparation to give a final ratio of 10:1 in terms of μl of detergent solution per mg of protein. These mixtures were kept on ice for 1 h with frequent mixing. In some instances samples were then taken for analysis. The remainder of the detergent-treated preparations were then centrifuged at 115 000 × g_{av} for 1 h and the supernatants collected and taken directly for analysis or for treatment with polyethylene glycol (see below).

2.3. *Analytical methods*

CPT was assayed at 25°C and pH 6.8 by a 4,4′-dithiopyridine-linked spectrophotometric assay [2] in the presence and absence of 100 μM malonyl-CoA. Binding of malonyl-CoA was measured using 200 nM [^{14}C]malonyl-CoA with correction for non-specific binding [2,6]. Protein was measured [15] using bovine albumin as a standard.

3. RESULTS AND DISCUSSION

Several studies have shown disruption of the CPT_1 system by detergents. Often detergent treatment of mitochondria or mitochondrial membranes results in loss of sensitivity to malonyl-CoA [16,17] and in extreme instances CPT_1 activity is drastically inhibited

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[18]. In the course of attempts to solubilize and purify a malonyl-CoA responsive CPT we noted a phenomenon which is summarized in Table I. Total mitochondrial membranes were obtained from sonicated rat liver mitochondria [2]. This preparation contains both CPT_1 and CPT_2 and therefore, typically, approx. 40% of the total CPT activity was inhibitable by 100 μM malonyl-CoA under our assay conditions. Treatment of these membranes with sodium cholate did not result in any appreciable gain or loss of CPT activity (results not shown), but it is evident that sensitivity of the CPT_1 component to malonyl-CoA was totally abolished (Table I). After high speed centrifugation almost 40% of the available CPT activity was found to have been solubilized by these mild detergent conditions. The CPT in this soluble fraction also exhibited no sensitivity to malonyl-CoA (Table I) although the supernatants did contain material that bound [^{14}C]malonyl-CoA (approx. 30 pmol/mg of protein at 200 nM malonyl-CoA). Surprisingly, addition of polyethyleneglycol (PEG) 6000 to these supernatants conferred malonyl-CoA sensitivity on the solubilized CPT, to a level at least comparable with that found in untreated membranes. A qualitatively similar phenomenon was observed if CPT was solubilized using the non-ionic detergent Tween 20 except that the reestablishment of malonyl-CoA sensitivity was less (only 23 ± 1% inhibition was observed in 3 experiments). The phenomenon was optimal using PEG 6000 over the range of 80–110 mg/ml. Other grades of PEG (1500, 4000 or 8000) were far less effective. We have no rational explanation for this convenient effect of PEG 6000 but, possibly by decreasing hydration in the local environment or by removing detergent, it appears to cause reorientation of CPT catalytic and regulatory components in micelles so that a kinetically productive interaction is achieved. It might casually be concluded from this experiment that we had solubilized and then reconstituted the catalytic entity of CPT_1 with the malonyl-CoA binding entity. However, outer membrane CPT_1 is not readily solubilized by a variety of anionic, cationic or non-ionic detergents [17]. By contrast, inner membrane CPT_2 is readily soluble [18]. We therefore performed the experiment shown in Fig. 1. Using purified outer membranes we again found that mild treatment with cholate did not cause appreciable change in either the CPT activity or the binding of [^{14}C]malonyl-CoA. Also in agreement with previous work (see above) cholate only solubilized 3.0 ± 0.1% of the outer membrane CPT_1, leaving 95 ± 8% of the activity in the 115 000 × *g* pellet. By contrast, cholate extracted 36 ± 7% of the [^{14}C]malonyl-CoA binding at an average specific activity of 56 pmol/mg of protein. We used 200 nM malonyl-CoA because, with purified outer membranes, essentially all binding at this concentration of ligand is at high affinity sites [7,8]. From Fig. 1 it was concluded that the experiment in Table I was not a reconferment of malonyl-CoA sensitivity on CPT_1 since virtually none was solubilized (Fig. 1). This led to the novel idea that a malonyl-CoA binding site extracted from outer membranes must be able to confer malonyl-CoA sensitivity on solubilized CPT_2 derived from the inner membrane. Fig. 2 substantiates this conjecture. Malonyl-CoA insensitive CPT_2 was extracted from inner membrane preparations using cholate. Addition of PEG 6000 alone to these extracts conferred no sensitivity to malonyl-CoA. However, if concentrated solubilized outer membrane protein was also added to the reconstitution mixture, sensitivity to malonyl-CoA

Table I

Apparent disengagement and reconstitution of malonyl-CoA sensitivity of CPT in liver mitochondrial total membranes

Preparation	% Inhibition of CPT activity by 100 μM malonyl-CoA
Membranes	36 ± 1
Membranes + cholate	0
115 000 × *g* supernatant	0
115 000 × *g* supernatant + PEG 6000	53 ± 13

Liver total mitochondrial membranes were treated with sodium cholate as described under Experimental and then centrifuged at 115 000 × g_{av} for 1 h. This treatment solubilized 38 ± 7% of the available CPT activity. Polyethyleneglycol (PEG) 6000 was added to the resulting supernatants to give a final concentration of 80 mg/ml. After standing on ice for approx. 1 h, aliquots were taken for assay of CPT in the presence and absence of 100 μM malonyl-CoA. The values are means ± SE for 3 separate experiments.

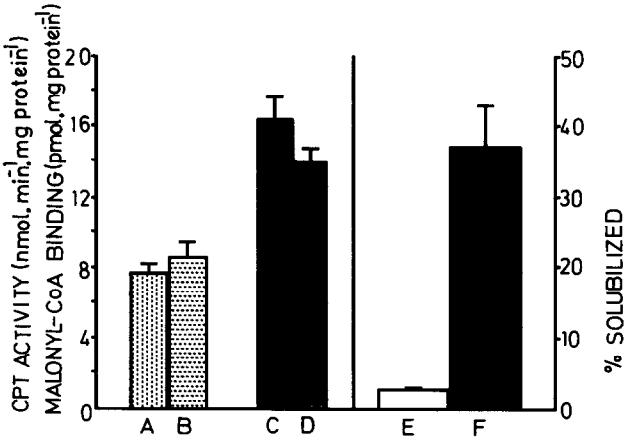

Fig. 1. Effect of treating liver mitochondrial outer membranes with cholate. Outer membrane fractions were obtained from rat liver mitochondria as described under Experimental. A, C: CPT activity and [^{14}C]malonyl-CoA binding respectively with untreated outer membranes. B, D: CPT activity and [^{14}C]malonyl-CoA binding respectively with outer membranes after treatment with cholate for 1 h. E, F: Aliquots of membranes treated with cholate (see B and D) were centrifuged at 115 000 × g_{av} for 1 h. The resulting supernatants and resuspended pellets were then assayed for CPT activity (E) and [^{14}C]malonyl-CoA binding (F) followed by calculation of the percentage of each that was solubilized.

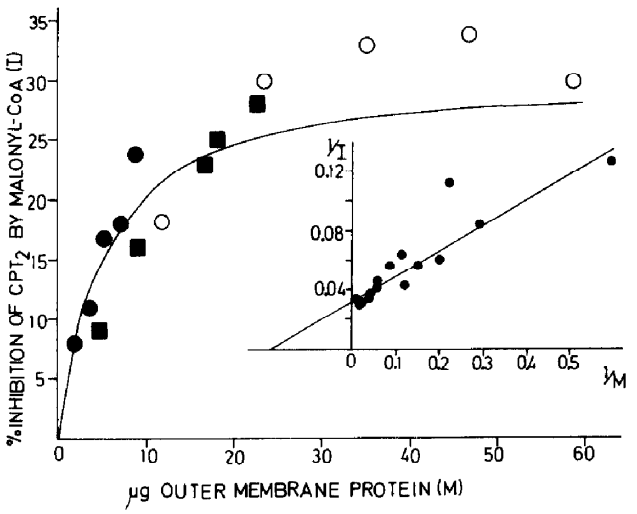

Fig. 2. Conferment of malonyl-CoA sensitivity on CPT from mitochondrial inner membranes. Three separate preparations of rat liver mitochondrial inner and outer membranes were obtained, treated with cholate, centrifuged at 115 000 × g_{av} for 1 h and the supernatants were collected as described under Experimental. Supernatants from outer membranes were concentrated approx. 30-fold by ultrafiltration over 15 min. Incubation mixtures were set up containing in a final volume of 0.5 ml: approx. 0.3 mg of solubilized inner membrane protein (2.5–4.5 nmol/min of CPT_2 activity), the indicated amounts of concentrated solubilized outer membrane protein and PEG 6000 (final concentration = 107 mg/ml). These mixtures were stood on ice for ca 1 h before 20–40 μl aliquots were taken for assay of CPT (± 100 μM malonyl-CoA) in a final volume of 1.0 ml. The symbols ○,•, ■ indicate data from 3 separate experiments. The inset graph is a double reciprocal plot of all the data (r = 0.90).

was observed. This was a saturable phenomenon needing 5.5 μg of outer membrane protein for half of the maximal effect (31% inhibition by 100 μM malonyl-CoA). We believe that 100% inhibition of the CPT_2 is not achieved in this experiment for two reasons. First, it is unlikely that all available CPT catalytic units are correctly oriented with a malonyl-CoA binding entity in the cholate micelles. Second, for convenience in monitoring this experiment we used a spectrophotometric assay that always underestimates the malonyl-CoA sensitivity of CPT compared with the less versatile radiochemical assay (see Experimental). We considered the possibility that this experiment simply represented reconferment of malonyl-CoA sensitivity on the small amount of solubilized CPT_1 that might contaminate the reconstitution mixture. This might come from two sources, but both can be discounted as insignificant. First, marker enzyme distributions indicated that the inner membrane preparations were 12% contaminated by outer membranes. It was therefore calculated that solubilized CPT_1 obtained from this source could contribute 0.3% of the CPT activity in the reconstitution mixture. Second, each 10 μg of concentrated solubilized outer membrane had 0.045 nmol/min of CPT activity which would represent 1.5% of the total CPT activity in the reconstitution mixture.

There are three conclusions from this study. The first is to strengthen proposals that CPT_1 catalytic and malonyl-CoA binding entities are discrete [2,7]. This is based on the selective extraction of the latter into cholate as shown in Fig. 1. The second conclusion is that cholate permits the extraction of the malonyl-CoA binding entity in a functional form since it can be used to confer malonyl-CoA sensitivity. The third is the most important and novel conclusion; namely that CPT_2 is potentially sensitive to malonyl-CoA. Normally the segregation of CPT_2 to the inner membrane should ensure avoidance of interaction with the malonyl-CoA binding entity from the outer membrane. It is to be expected that CPT_1 and CPT_2 should have a common domain that can make a kinetically productive interaction with the malonyl-CoA binding protein. In support of this it has been implied that the two forms of mitochondrial CPT have some immunological epitopes in common [2]. At present it is not known which component of the inner membrane will normally interact with CPT_2 at the domain that can be occupied by the malonyl-CoA binding protein in these reconstitution experiments.

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