Roles of the N- and C-terminal domains of carnitine palmitoyltransferase I isoforms in malonyl-CoA sensitivity of the enzymes: insights from expression of chimaeric proteins and mutation of conserved histidine residues

S. Todd SWANSON*, Daniel W. FOSTER*, J. Denis MCGARRY*† and Nicholas F. BROWN*¹

*Department of Internal Medicine, University of Texas Southwestern Medical Center, 5323 Harry Hines Boulevard, Dallas, TX 75235-9135, U.S.A., and †Department of Biochemistry, University of Texas Southwestern Medical Center, Dallas, TX 75235-9135, U.S.A.

The mitochondrial outer membrane enzyme carnitine palmitoyltransferase I (CPT I) plays a major role in the regulation of fatty acid entry into the mitochondrial matrix for β -oxidation by virtue of its inhibition by malonyl-CoA. Two isoforms of CPT I, the liver type (L) and muscle type (M), have been identified, the latter being 100 times more sensitive to malonyl-CoA and having a much higher K_m for the substrate carnitine. Here we have examined the roles of different regions of the CPT I molecules in their response to malonyl-CoA, etomoxir (an irreversible inhibitor) and carnitine. To this end, we analysed the properties of engineered rat CPT I constructs in which (a) the N-terminal domain of L-CPT I was deleted, (b) the N-terminal domains of L- and M-CPT I were switched, or (c) each of three conserved histidine residues located towards the N-terminus in L-CPT I was mutated. Several novel points emerged: (1) whereas the N-terminal domain is critical for a normal malonyl-CoA response,

it does not itself account for the widely disparate sensitivities of the liver and muscle enzymes to the inhibitor; (2) His-5 and/or His-140 probably play a direct role in the malonyl-CoA response, but His-133 does not; (3) the truncated, chimaeric and point-mutant variants of the enzyme all bound the covalent, active-site-directed ligand, etomoxir; and (4) only the most radical alteration of L-CPT I, i.e. deletion of the N-terminal 82 residues, affected the response to carnitine. We conclude that the N-terminal domain of CPT I plays an essential, but permissive, role in the inhibition of the enzyme by malonyl-CoA. By contrast, the larger C-terminal region dictates the degree of sensitivity to malonyl-CoA, as well as the response to carnitine; it is also sufficient for etomoxir binding. Additionally, further weight is added to the notion that one or more histidine residues may be involved in the CPT I–malonyl-CoA interaction.

INTRODUCTION

Carnitine palmitoyltransferase I (CPT I) is believed to play a primary role in the regulation of mitochondrial β -oxidation of fatty acids in all tissues of the body (reviewed in [1]). It represents the first element of a multi-component system that effects the net transport of long-chain acyl groups from the cytosol into the mitochondrial matrix where they can be oxidized as a direct source of cellular energy or, in the liver, used for the synthesis of ketone bodies. CPT I is an integral outer membrane protein that performs the *trans*-esterification of a long-chain acyl group from CoA to carnitine. The acylcarnitine formed can then cross the mitochondrial inner membrane via a specific transporter. A distinct gene product, CPT II, located on the inner face of the inner membrane, then reverses the *trans*-esterification reaction to regenerate acyl-CoA [1].

In addition to their separated physical locations, CPT I and II differ in other critical respects. First, CPT I is potently inhibited by malonyl-CoA, the product of the acetyl-CoA carboxylase (ACC) reaction and an intermediate in the pathway of *de novo* fatty acid synthesis. In lipogenic tissues this provides a simple mechanism to prevent oxidation of newly generated fatty acids [2,3]. However, ACC activity and malonyl-CoA are also present in a variety of non-lipogenic tissues, where they are presumed to play a purely regulatory role with regard to CPT I [4]. In sharp

contrast, CPT II is insensitive to malonyl-CoA. Secondly, whereas CPT II is expressed as the same gene product body wide, two isoforms of CPT I have been identified; they are denoted L-CPT I (liver-type) and M-CPT I (muscle-type) to reflect the sites in which they were originally characterized, although it has since been determined that each is expressed in a variety of tissues [5–9]. The two CPT I variants differ profoundly in certain kinetic properties, notably in their K_m for the substrate carnitine ($\approx 500 \mu\text{M}$ for M-CPT I and $\approx 30 \mu\text{M}$ for L-CPT I in the rat) and in their ability to be inhibited by malonyl-CoA (M-CPT I is 100 times more sensitive [1]).

The molecular basis for the kinetic differences between the CPT I isoforms remains completely unknown. For these reasons, and because CPT I continues to attract attention as a potential target in the pharmacological management of diabetes [10,11], understanding of how the enzyme interacts with its substrates and inhibitors (both physiological and artificial) takes on considerable significance. This in turn requires detailed knowledge of structure/function relationships surrounding the various CPT proteins. Helpful in this regard has been the cloning of cDNAs for each of the CPT isoforms [12–14], and subsequent expression/mutagenesis studies that have begun to delineate certain critical domains and residues within the enzyme molecules [15–19]. In addition, a plausible topology of the CPT I peptide backbone in relation to the mitochondrial outer membrane has been proposed

Abbreviations used: CPT I and CPT II, mitochondrial outer and inner membrane carnitine palmitoyltransferase enzymes respectively; L-CPT I and M-CPT I, liver and muscle type CPT I; LM, ML, chimaeric forms of CPT I; L- Δ (1–82), truncated variant of L-CPT I; ACC, acetyl-CoA carboxylase; DMEM, Dulbecco's Modified Eagle's Medium; FCS, fetal-calf serum; TBS, Tris-buffered saline; IC₅₀, concentration causing 50% inhibition.

¹ To whom correspondence should be addressed (e-mail nbrown@mednet.swmed.edu).

Figure 1 Schematic diagram of the CPT I molecule

The proposed membrane topology of CPT I is based largely on the work of Zammit and others [1,20–22]. Abbreviations: OMM, mitochondrial outer membrane; N (striped region) and C (clear region), N-domain and C-domain, respectively; shaded regions M1 and M2, proposed membrane-spanning domains (we have previously referred to these hydrophobic stretches as H1 and H2 [1,17]; however, in view of their recent designation as membrane-spanning domains [1,20–22], we here refer to them as M1 and M2); arrow, N-terminus of truncated enzyme L-Δ(1–82); H5, H133 and H140, mutated histidine residues. The expanded region indicates amino acid (beginning with residue 93 in each case) and corresponding cDNA sequences around the splice site used in the manufacture of chimaeric cDNAs. *Italicized* bases above the L (L-CPT I) and M (M-CPT I) sequences indicate positions mutated to introduce the *NotI* restriction sites (underlined). LM and ML show the sequences of the chimaeric molecules. Serine residues present in neither of the parent sequences are shown in **bold** face.

[1,20–22]. In this model, the protein contains a cytosolic N-terminal region (≈ 50 residues), two membrane spans connected by a loop of ≈ 30 amino acids in the intermembrane space, and a larger (≈ 650 residues), also extramitochondrial, C-terminal domain (Figure 1). Thus it appears that most of the protein resides on the external face of the mitochondrial outer membrane. Since the precise functions of each of these regions of the molecule are as yet not defined, we will refer to the first external domain and membrane span as the ‘N-domain’ and the second membrane span and the remaining portion of the molecule as the ‘C-domain’ (Figure 1).

In the present studies we address the question of which regions of the L- and M-CPT I molecules are primarily responsible for their very different responses to malonyl-CoA and carnitine. We were particularly interested in the role of the N-terminal domain, which we had previously implicated in the interaction of L-CPT I with malonyl-CoA [17]. To this end, we have generated chimaeric molecules in which the N-domains of the rat L and M isoforms have been exchanged. Also, because it has long been suspected that one or more histidine residues may be intimately involved in the interaction of CPT I with malonyl-CoA [23,24], we have also targeted three conserved histidines in the N-terminal region of the protein for mutagenesis (one, His-5, located in the N-domain; two, His-133 and His-140, towards the

amino end of the C-domain). The altered proteins were then expressed in COS cells and their properties compared. The results outlined below reveal that malonyl-CoA inhibition of CPT I hinges on a complex, and somewhat unexpected, involvement of both the N- and C-domains of the protein, and permit several important refinements to the current model of CPT I-inhibitor–substrate interactions.

MATERIALS AND METHODS

Manufacture of expression plasmids

Construction of plasmids pCMV6-rL-CPT I and pCMV6-rM-CPT I for the expression of rat L- and M-CPT I in COS cells has been described [8]. Deletion of the first 82 codons of the rat L-CPT I cDNA to allow initiation of translation at Met-83 has also been detailed previously [17]. The product was introduced into pCMV6 for expression of the truncated protein, L-Δ(1–82).

In the design of chimaeric molecules, the selection of a splice site was based on the proposed membrane topology of CPT I. We considered it highly probable that the exact sequence of the short stretch of amino acids between the two hydrophobic domains (M1 and M2 in Figure 1), which is believed to lie in the inter-membrane space and is poorly conserved between the two isoforms, is unlikely to be critical for enzyme function. Accordingly, a unique restriction site was introduced into the rat L- and M-CPT I cDNAs in this region. This was accomplished with minimal alteration in the amino acid sequences (Figure 1) and, as shown below, the chimaeric molecules generated retained catalytic activity and malonyl-CoA sensitivity. To facilitate mutagenesis, portions of pCMV6-rL-CPT I and pCMV6-rM-CPT I (an *EcoRI* fragment including the entire coding region of L-CPT I and a *KpnI* fragment encoding 900 bp at the 5′ end of the M-CPT I cDNA) were subcloned into M13. Oligonucleotide-directed mutagenesis was performed using the Muta-gene *in vitro* mutagenesis kit (Bio-Rad, Richmond, VA, U.S.A.) to introduce a *NotI* restriction site at corresponding positions in each cDNA. The mutant DNA was then returned to the original pCMV6 vectors to regenerate full-length expression constructs. These were cut with *NotI*, which also cuts the vector 3′ of the site of cDNA insertion, to release fragments containing the cDNAs 3′ of the artificial *NotI* sites. The purified fragments and plasmid backbones (still containing the 5′ portion of each cDNA) were then switched and ligated to generate the chimaeric molecules. These have been denoted as LM (L-CPT I N-domain, M-CPT I C-domain) and ML (M-CPT I N-domain, L-CPT I C-domain). For the purposes of the present paper, the terms ‘N-domain’ and ‘C-domain’ are used to denote those regions of the CPT I molecule located either side of this splice site.

Mutagenesis of histidine codons 5, 133 and 140 of L-CPT I to alanine (GCC) was also accomplished using the L-CPT I cDNA in M13 and the mutant sequences recloned back into pCMV6. To generate the H5A/H140A double mutant (His-5 → Ala/His-140 → Ala), a second round of mutagenesis was performed with the H140A construct, adding the H5A mutation.

Culture and transfection of mammalian COS cells

Simian COS-M6 cells were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM) containing 5% (v/v) fetal-calf serum (FCS). This concentration of serum maintains endogenous CPT I activity at a low level. Cells were plated at a density of 1.8×10^6 cells/15 cm dish. After 24 h, transfection with each expression plasmid was accomplished by the DEAE-dextran method [12] and the cells were then cultured for 3 days before harvest. Control cells were transfected with a plasmid containing rat L-

CPT I cDNA in the reverse orientation. Large-scale preparation of plasmids for transfection was accomplished with the Qiagen Maxi Kit (Qiagen, Chatsworth, CA, U.S.A.).

Cell harvest and CPT assay

Tissue-culture plates (15 cm) were washed with 10 ml of ice-cold Tris-buffered saline (TBS) and then scraped with a 'rubber policeman' in 5 ml of the same solution. Normally, three to six plates had been transfected with each construct and the cells were pooled at this point and sedimented by centrifugation at 1200 *g* for 5 min at 4 °C. After resuspension into 1 ml of 150 mM KCl/5 mM Tris/HCl, pH 7.2 (buffer A), they were broken with 10 cycles of a glass homogenizer fitted with a tight pestle. Further centrifugation was performed in a microfuge for 5 min at 4 °C at 16000 *g* and the final pellet was resuspended in the desired volume of buffer A.

CPT I was assayed in these preparations where the mitochondria remain largely intact. Activity was measured in the forward direction in the presence of 50 μ M palmitoyl-CoA, 1% (w/v) essentially fatty-acid-free BSA and 10–1000 μ M of L-[¹⁴C]carnitine [8]. To obtain malonyl-CoA response curves, the inhibitor was included as indicated. For assay of CPT II, a portion of the cell homogenate was made 1% (w/v) with respect to the detergent octyl glucoside and kept on ice for 30 min before assay, with frequent mixing. This procedure largely inactivates CPT I, but releases CPT II from the mitochondrial matrix in active form [25]. Protein was measured by the method of Lowry et al. [26].

[³H]Etomoxir labelling of CPT I in COS cells

At 3 days after cell transfection on 10 cm plates, the medium was aspirated and replaced with 4 ml of DMEM/5% (v/v) FCS. [³H]Etomoxir was added to a concentration of 3 μ M and the cells were returned to the incubator for a further 6 h. At this time the cells were harvested essentially as described above, except that the first resuspension and homogenization were done in 5 mM potassium phosphate buffer, pH 7.2, and the final resuspension was in 200 μ l of 150 mM NaCl/5 mM Tris/HCl, pH 7.2 (buffer B). Membrane suspensions were frozen in liquid nitrogen and stored at –70 °C. Thawed samples were made 1% (v/v) with respect to Tween-20 and kept on ice for 30 min with frequent mixing, then centrifuged for 2 min at 16000 *g* at 4 °C in a microfuge. The pellet was resuspended in 50 μ l of buffer B and made 1% (v/v) with Triton X-100. This was similarly kept on ice and then centrifuged. Portions of the supernatant were then taken for SDS/PAGE. After staining with Coomassie Blue and destaining, gels were impregnated with En³Hance (DuPont NEN, Boston, MA, U.S.A.), dried and exposed to X-ray film.

Generation of a polyclonal antibody to rat L-CPT I and immunoblotting

A portion of the rat L-CPT I cDNA encompassing amino acid residues 122–773 (i.e. the entire coding sequence downstream of the membrane-spanning domains) was amplified by PCR. The 5' oligonucleotide included an *Eco*RI restriction site and the 3' oligonucleotide contained bases encoding six tandem histidine residues, a stop codon and an *Eco*RI site. The product was ligated into pGEX-3X (Pharmacia, Piscataway, NJ, U.S.A.). The resulting construct encoded a glutathione *S*-transferase/partial rat L-CPT I fusion protein with an additional six histidine residues at the C-terminus. Transformation of this plasmid into *Escherichia coli* and induction of expression under recommended conditions yielded large amounts of the fusion protein in the

form of inclusion bodies. This material was solubilized in 6 M guanidinium chloride and purified over a Ni²⁺-nitrilotriacetate-agarose column according to the manufacturer's instructions (Qiagen). The product was used to inoculate a New Zealand White rabbit using TiterMax adjuvant (Vaxcel Inc., Norcross, GA, U.S.A.), resulting in antibody generation.

For immunoblot analysis, transfected cells were harvested and subjected to detergent extraction as described above for [³H]-etomoxir labelling experiments. After SDS/PAGE and electrotransfer to Immobilon PVDF membranes (Millipore, Bedford, MA, U.S.A.), incubation with the primary and secondary (goat anti-rabbit IgG; Bio-Rad, Richmond, VA, U.S.A.) antibodies was conducted according to conventional procedures. Detection was carried out with the Renaissance Western Blot Chemiluminescence Reagent (DuPont NEN).

Northern-blot analysis of untransfected COS cells

Two 15 cm plates of COS cells were seeded at the same density and grown under conditions similar to those used in the transfection experiments. Cell harvest was as for the CPT assay, but after the first centrifugation the pellet was resuspended in 1 ml of Trizol reagent (Gibco BRL, Grand Island, NY, U.S.A.) and homogenized for 30 s using a Polytron tissue disrupter at half power. Total RNA was prepared as recommended in the instructions provided with the Trizol reagent. Portions of RNA (20 μ g) were electrophoresed on 1% agarose gels and transferred to nylon membranes, which were then analysed with single-stranded ³²P-labelled DNA probes for human L-CPT I and M-CPT I [9], encompassing bases 464–1058 (L-CPT I) and 55–950 (M-CPT I) of the published sequences [27,28]. Human cDNAs were used to maximize the probability of cross-reaction, since COS cells are of monkey origin. RNA from human heart, a tissue known to express both CPT I isoforms [9], was used as a positive control. A 28 S ribosomal RNA probe served as a loading control [9].

RESULTS

Expression and analysis of chimaeric CPT I enzymes

Table 1 summarizes the CPT activities obtained from COS cells transfected with various expression plasmids. Control cells

Table 1 CPT activity in COS cells transfected with CPT I variant expression plasmids

Assays were performed in the presence of 500 μ M carnitine. See the Materials and methods section for details. Values shown are means $\pm$ S.E.M. for the number of independent experiments indicated (*n*). Statistical significance: **P* < 0.0005, ***P* < 0.01, ****P* < 0.05 versus control (paired samples).

Plasmid	Activity (nmol · min ^{–1} · mg of protein ^{–1})		<i>n</i>
	CPT I	CPT II	
Control	0.34 $\pm$ 0.02	3.77 $\pm$ 0.22	13
L-CPT I	2.97 $\pm$ 0.21	5.66 $\pm$ 0.30*	13
M-CPT I	1.63 $\pm$ 0.13	3.49 $\pm$ 0.26	4
LM	1.24 $\pm$ 0.04	3.41 $\pm$ 0.21	4
ML	2.83 $\pm$ 0.26	4.49 $\pm$ 0.47**	4
L-Δ(1–82)	0.66 $\pm$ 0.07	3.58 $\pm$ 0.33	8
L-H5A	2.54 $\pm$ 0.21	5.90 $\pm$ 0.36*	9
L-H133A	1.25 $\pm$ 0.11	3.70 $\pm$ 0.24	9
L-H140A	2.29 $\pm$ 0.24	4.89 $\pm$ 0.38***	9
L-H5A/H140A	1.95 $\pm$ 0.38	4.46 $\pm$ 0.5***	4

Figure 2 Effect of malonyl-CoA on activity of chimaeric and truncated rat CPT I variants

COS cells were transfected with a null construct (CONT) or cDNAs for rat L-CPT I (L), M-CPT I (M), chimaeras (LM, ML) or the truncated form of L-CPT I [L-Δ(1-82)]. In (B), activity attributable to the endogenous activity has been subtracted. Data are expressed relative to activity in the absence of malonyl-CoA (100%) and are the mean $\pm$ range for two independent measurements (errors shown in A only).

carrying a null construct in which the L-CPT I cDNA had been inserted in the reverse orientation displayed a low, but measurable, CPT I activity. Under the assay conditions used here, the endogenous level of CPT II was ≈ 10 times that of CPT I. Transfection with the liver or muscle forms of rat CPT I resulted in a several-fold increase in CPT I activity. A significant increase in apparent CPT II activity as measured was observed in transfections with L-CPT I and certain point mutant variants, a phenomenon that we have observed previously [8]. However, we suspect that this is due to incomplete inactivation of the overexpressed recombinant CPT I by octyl glucoside [8]. Transfections with the two chimaeric forms of CPT I, LM and ML, yielded activities similar to those obtained with the parental constructs, M and L respectively. In contrast, the L-Δ(1-82) protein was expressed poorly, giving an overall activity only about twice that of control cells. It should be noted that the data in Table 1 were obtained from assays containing 500 μ M carnitine, more than 10 times the K_m of rat L-CPT I, but only

Figure 3 Response to carnitine of chimaeric and truncated CPT I variants

See the legend to Figure 2 for explanation of labels. To allow direct comparison between enzyme variants, data are expressed relative to activity in the presence of 1 mM carnitine (100%). Data are the mean $\pm$ range for two independent experiments.

equalling that of the muscle enzyme. Also, the relative catalytic efficiencies of the two isoforms are unknown, and therefore the activities shown cannot be assumed to reflect actual expression levels of the various proteins.

To assess the malonyl-CoA sensitivity of the expressed CPT I variants, assays were performed in the presence of up to 200 μ M of the inhibitor (Figure 2). In this analysis, the L and M enzymes clearly displayed highly divergent sensitivities to malonyl-CoA, as we have shown previously [8]. The IC_{50} (concentration causing 50% inhibition) was approx. 7 and 0.07 μ M for the L- and M-isoforms, respectively (assuming that the non-suppressible activity in Figure 2 represented latent CPT II). The LM protein, consisting of the majority of the muscle isoform with a substituted liver-type N-domain, showed a sensitivity essentially identical with that of the M-form. Conversely, ML, composed primarily of L-CPT I, but with the muscle-type N-domain, generated a curve that was superimposable on that of L-CPT I. The L-Δ(1-82) construct appeared to show a reduced sensitivity relative to the full-length form of the enzyme. For comparison, in Figure 2(B), the endogenous CPT I activity of the control cells has been subtracted. The curves for L, M, LM and ML are essentially unchanged by this manoeuvre because of the relatively small contribution of endogenous enzyme to total CPT I activity (Table 1). However, in the case of the less well expressed L-Δ(1-82), its greatly reduced sensitivity compared with the parent enzyme becomes much more obvious, there being no inhibition at a malonyl-CoA concentration of up to 10 μ M. The endogenous activity measured in control cells displayed slightly greater sensitivity to malonyl-CoA than the recombinant L-CPT I or ML-CPT I (Figure 2A). This was surprising given the fact that the COS cells derive from monkey kidney and, in the rat and mouse at least, kidney is known to express the L-form of CPT I [9].

In additional studies with the chimaeric CPT I proteins, we measured activities in the presence of a wide range of concentrations of the substrate, carnitine (Figure 3). Again, as noted previously with this expression system [8], the radically different

Figure 4 [^3H]Etomoxir labelling of chimaeric and truncated CPT I variants expressed in COS cells

COS cells transfected with L-CPT I, M-CPT I, the chimaeric forms (LM, ML), the truncated construct [L- $\Delta(1-82)$] or the null construct (control) were processed as described in the Materials and methods section. The relative amount of protein loaded in each lane is indicated. L and M (at right) indicate the migration positions of L- and M-CPT I.

response of the two naturally occurring isoforms was clearly apparent, L-CPT I displaying much more rapidly saturating kinetics, i.e. a lower K_m . As was the case for malonyl-CoA sensitivity, the LM and ML chimaeras showed carnitine responses identical with those of the M and L isoforms, respectively. Once again, the endogenous COS cell activity exhibited kinetics intermediate between those of the rat L- and M-CPT I enzymes. L- $\Delta(1-82)$ behaved similarly to the COS cell enzyme. Due to the presence of endogenous COS cell CPT I activity, as well as the inevitable release of a small amount of CPT II during preparation of the cell homogenates and the presumed presence of some extramitochondrial acyltransferase activity, it is not meaningful to try to derive a true K_m from these data. However, when the carnitine curves are normalized in the manner used in Figure 3, the differing responses of the CPT I variants are immediately apparent.

Figure 4 shows the result of an experiment in which [^3H]etomoxir, a covalent ligand and inhibitor of CPT I, has been used to label the expressed enzymes. This type of analysis is facilitated by the differential migration of the L- and M-forms of CPT I on SDS/PAGE, despite their almost identical molecular masses [8]. Immediately apparent is that the larger, C-domain of each protein dictates its rate of migration on SDS/PAGE. Although predicted to differ by < 100 in relative molecular mass, the L and ML products ran more slowly than the M and LM species. Also, it should be noted that the gel in Figure 4 was loaded to equalize the labelled band intensities for the five exogenous proteins under study which required more of the M and LM products. The L- $\Delta(1-82)$ product ran further than L-CPT I itself, but still not as far as the M-isoform. COS cells transfected with the null construct showed only faint labelling in the position of rat L-CPT I. The anomalous relative migration of the L- and M-CPT I molecules has been noted in humans [1] as well as rodents [8] and the band observed in the control cells probably represents L-CPT I. In addition, in Northern-blot analysis of COS cell RNA using probes encoding human L- and M-CPT I only, the mRNA for the L-isoform could be detected (results not shown). Taken together, the Northern-blot and [^3H]etomoxir-labelling experiment (Figure 4) strongly indicate that endogenous COS cell CPT I is primarily of the L-type, although the malonyl-CoA inhibition data (Figure 2) may point to the presence of a small amount of the M-type enzyme. Indeed, in view of the poorer labelling of M-CPT I with etomoxir, and the possibility that the human cDNA probe for M-CPT I might have failed to cross-react well with the COS cell M-CPT I mRNA, we cannot totally discount the presence of a level of that isoform too low to be detected by these methods. Alternatively,

Figure 5 Effect of malonyl-CoA on activity of point mutants of rat L-CPT I

COS cells were transfected with a null construct (CONT) or cDNAs for wild-type rat L-CPT I, L- $\Delta(1-82)$ or point mutants H5A, H133A, H140A or H5A/H140A (designated H5,140A in the Figure). In (B), activity attributable to the endogenous activity has been subtracted. Data are expressed relative to control values in the absence of malonyl-CoA (100%) as the mean $\pm$ S.E.M. for three to six independent measurements (errors shown in A only).

monkey L-CPT I could simply be slightly more sensitive than the rat enzyme.

Expression and analysis of N-terminal histidine residue mutants of L-CPT I

Figure 5(A) shows a further series of malonyl-CoA curves performed with control COS cells and cells expressing rat L-CPT I, L- $\Delta(1-82)$ or one of three point mutant variants, H5A, H133A and H140A. Again, the endogenous activity displayed a sensitivity slightly greater than that of rat L-CPT I. Whereas H133A behaved no differently from the parent enzyme, both the H5A and H140A variants yielded inhibition curves that were shifted substantially to the right. Once again, to accentuate the properties of L- $\Delta(1-82)$, curves from which the endogenous activity has been subtracted are shown in Figure 5(B). In this representation,

it is clear that in this series of experiments mutants H5A and H140A caused a desensitization of a similar magnitude to the $\Delta(1-82)$ truncation.

In view of the partial malonyl-CoA-desensitizing effects of mutations H5A and H140A, we prepared an additional plasmid encoding the double mutant H5A/H140A. The double mutant was found to display a lower sensitivity to malonyl-CoA than either of the single mutants H5A or H140A, or the truncated form of the enzyme (Figure 5).

None of the single point mutations or the double mutant caused a significant change in the pattern of response to increasing concentrations of carnitine (results not shown).

[^3H]Etomoxir labelling experiments with COS cells expressing the mutants H5A, H133A and H140A in each case yielded a radioactive band the same size as that obtained with the wild-type L-CPT I enzyme (results not shown). However, mutant H133A yielded a much less strongly labelled band than did the other mutants. To exclude the possibility that the poor labelling of H133A was a direct effect of the mutation on etomoxir binding, we performed an immunoblot with extracts of COS cells expressing the wild-type or mutant L-CPT I. The polyclonal antibody raised against a portion of rat L-CPT I recognized a protein of the expected size in each case, but failed to detect any protein band in untransfected cells (results not shown). However, the signal with H133A was much weaker than that with the other recombinant proteins. We infer that the H133A mutant was not expressed well by the COS cells. This is consistent with the observation that, although the H133A mutant did not appear to have altered kinetics, it was invariably less active in the COS cells than the other point mutants or the parent L-CPT I construct (Table 1).

DISCUSSION

Our primary goal was to gain insight into which regions of the L- and M-CPT I molecules are responsible for inhibition of catalytic activity by malonyl-CoA and why sensitivity to the CoA-ester is so different between the two isoforms. The results were informative and somewhat surprising.

The strategy employed was to generate L- and M-CPT I cDNAs that had been structurally altered and to examine the effects of those changes on the properties of the expressed proteins. As an expression vehicle we employed COS cells in tissue culture rather than *E. coli* or yeast which we have used in previous CPT expression studies [16,17,29]. This had several advantages. First, it provides a mammalian mitochondrial membrane environment for housing of the recombinant CPT I proteins. It is unknown how dependent certain properties of the CPT I molecule may be on the composition of the membrane itself or on interaction with other membrane proteins, but alterations in membrane fluidity *in vitro* are known to affect the behaviour of L-CPT I in rat liver mitochondria [30]. Secondly, in our previous work, rat L-CPT I was expressed only at very low levels by *Saccharomyces cerevisiae* [17], in contrast with COS cells [8,13]. Thirdly, we have been unsuccessful in the use of [^3H]etomoxir as a labelling agent for CPT I expressed in yeast cells (N. F. Brown and J. D. McGarry, unpublished work), again unlike the situation with COS cells [8]. We recognize the one obvious disadvantage of the COS cell, namely the presence of a low level of endogenous CPT activity. However, under the culture conditions we have employed, this is minimized and does not affect analysis of the recombinant enzymes.

Before the present study was performed, various lines of evidence suggested that the N-terminal portion of CPT I was intimately involved in malonyl-CoA sensitivity. In particular,

studies examining the effects of partial proteolysis on rat CPT I as it exists in the mitochondrial membrane *in situ* had indicated that domains responsible for catalytic activity and malonyl-CoA sensitivity were spatially distinct [21,22,31–33]. Specifically, various proteases, notably aminopeptidase [32], were shown to diminish the potency of malonyl-CoA as an inhibitor, whereas catalytic activity was relatively unaffected. Furthermore, expression in *S. cerevisiae* of a truncated rat L-CPT I consisting of only the second membrane span and the C-terminus [construct L- $\Delta(1-82)$] generated an active enzyme, but one in which the IC_{50} for malonyl-CoA was increased 15-fold relative to the wild-type protein [17]. Consistent with these properties of the truncated enzyme is that CPT II, a smaller protein than CPT I and insensitive to malonyl-CoA, is similar to the C-terminal domain of CPT I (residues 170 onwards, i.e. it lacks the N-terminal region and the proposed membrane spans of CPT I). From these findings, it might have been expected that both the site and affinity of the malonyl-CoA interaction would be governed principally by the structure of the N-terminus of the CPT I protein. This is not true, however, as shown by the properties of L- and M-CPT I chimeras in which the two N-domains were swapped, i.e. constructs LM, containing the N-domain of L-CPT I and the C-domain of M-CPT I, and ML, having a muscle N-domain spliced to the C-domain of the liver enzyme. The finding that the malonyl-CoA response profiles of LM and ML were indistinguishable from those of the unaltered M-CPT I and L-CPT I proteins, respectively, establishes unambiguously that the differential sensitivity of the two CPT I variants to the CoA ester is determined primarily by the structure of the C-domain. However, from the behaviour of L- $\Delta(1-82)$ noted above, it is equally clear that the N-domain plays a critically important, albeit permissive, role in terms of malonyl-CoA inhibition of enzyme activity. In this regard, we had long suspected a histidine residue to be involved in malonyl-CoA binding to the enzyme, possibly forming an ionic interaction with the carboxy group of the inhibitor [23,24]. Studies with malonyl-CoA analogues have also emphasized the importance of the carboxy function in the binding reaction [34]. For these reasons, we performed site-directed mutagenesis to individually change three conserved histidine residues located towards the N-terminus of L-CPT I into an alanine. The first, His-5, is part of a stretch of 18 residues that is perfectly conserved at the extreme N-terminus of rat and human L- and M-CPT I [1], therefore falling within the N-domain. His-133 and His-140, while towards the N-terminus of the protein as a whole, lie immediately distal to the second proposed membrane span, just within the C-domain, and are therefore both still present at the proximal end of L- $\Delta(1-82)$. Although poorly expressed in COS cells (for reasons unknown), mutant H133A showed wild-type sensitivity to malonyl-CoA. By contrast, mutations H5A and H140A both caused a marked rightward shift in the inhibition curve and the effect was enhanced when both mutations were present (H5A/H140A). This adds weight to the notion that one or more histidine residue(s) may be involved in malonyl-CoA inhibition of CPT I and provides two candidates for further study. Perhaps not surprisingly, removal of these histidine residues did not entirely abolish the malonyl-CoA effect. One would predict that although an interaction between a histidine residue and the carboxy moiety of malonyl-CoA may be crucial to specific, high-affinity binding, the complete inhibitor binding site will involve a number of interactions with several amino acid residues and would likely retain some affinity for the inhibitor even if one component of that site is removed. Alternatively, if deletion of the entire N-terminal domain or removal of His-5 and His-140 resulted in loss of the allosteric site, the significant inhibition of these variants at very high

concentrations of malonyl-CoA (100–200 μ M) that we also observed in the yeast system with L- Δ (1–82) [17] might be due in part to competition with palmitoyl-CoA at the active site.

It is likely, however, that the major deletion has other effects on enzyme structure. This is evidenced by the fact that the L- Δ (1–82) construct also displayed an altered response to carnitine, showing a higher K_m than did the wild-type enzyme. This was not seen with any of the point mutants or the LM and ML chimaeras.

For the reasons detailed above, the focus of the present study was directed towards the N-terminal region unique to the CPT I molecules (specifically, the N-domain and histidine residues situated within or close to it in the primary sequence). It should be noted, however, that histidines located in other parts of the CPT I molecule (i.e. within the region of sequence similarity to CPT II) may nonetheless, by virtue of their molecular environment, play a role in conferring the property of malonyl-CoA inhibition uniquely to CPT I.

With a view towards the pharmacological use of CPT I inhibitors, it is essential to determine the nature of their interactions with the enzyme molecule. Information regarding the nature of inhibitor-binding sites came from the [3 H]etomoxir labelling experiments. Of particular note was the finding that manoeuvres which had such profound effects on the malonyl-CoA response, i.e. mutation of the conserved histidines or deletion of the entire N-terminal domain, did not prevent binding of the covalent inhibitor, which is believed to occur at the active site [1]. This is a further strong indication that the binding sites for these two inhibitors are distinct. Also, as well as harbouring the etomoxir-binding region, it is evident that the larger C-domains of the L- and M-CPT I isoforms are responsible for the anomalous differential migration of the two proteins on SDS/PAGE, since this still persisted with the LM and ML chimaeras. That both M-CPT I and LM labelled less well than L-CPT I and ML is consistent with previous observations that the muscle isoform is less susceptible to inhibitors of this class than is the liver enzyme [6].

In summary, the data strongly implicate the N-terminal domain of CPT I as having a crucial role in inhibition of the enzyme by malonyl-CoA. However, the widely disparate sensitivities of the liver and muscle forms of the protein stem primarily from differences in the two C-termini. It is likely that overall ability to inhibit is determined by the nature of the interaction between the N- and C-terminal regions and how profoundly the catalytic site of each isoform is altered by that interaction. Delineation of the precise function of the N-domain and its relationship with the catalytic C-domain should help explain not only the mechanism through which malonyl-CoA exerts its suppressive effect, but also the changes in inhibitor sensitivity that L-CPT I undergoes in certain physiological and pathophysiological states [35]. It will also aid in the rational design of pharmacological inhibitors should such agents be contemplated for clinical use.

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REFERENCES

- McGarry, J. D. and Brown, N. F. (1997) *Eur. J. Biochem.* **244**, 1–14
- McGarry, J. D. and Foster, D. W. (1980) *Annu. Rev. Biochem.* **49**, 395–420
- McGarry, J. D., Woeltje, K. F., Kuwajima, M. and Foster, D. W. (1989) *Diabetes Metab. Rev.* **5**, 271–284
- McGarry, J. D., Mills, S. E., Long, C. S. and Foster, D. W. (1983) *Biochem. J.* **214**, 21–28
- Weis, B. C., Esser, V., Foster, D. W. and McGarry, J. D. (1994) *J. Biol. Chem.* **269**, 18712–18715
- Weis, B. C., Cowan, A. T., Brown, N., Foster, D. W. and McGarry, J. D. (1994) *J. Biol. Chem.* **269**, 26443–26448
- Brown, N. F., Weis, B. C., Husti, J. E., Foster, D. W. and McGarry, J. D. (1995) *J. Biol. Chem.* **270**, 8952–8957
- Esser, V., Brown, N. F., Cowan, A. T., Foster, D. W. and McGarry, J. D. (1996) *J. Biol. Chem.* **271**, 6972–6977
- Brown, N. F., Hill, J. K., Esser, V., Kirkland, J. L., Corkey, B. E., Foster, D. W. and McGarry, J. D. (1997) *Biochem. J.* **327**, 225–231
- Foley, J. E. (1992) *Diabetes Care* **15**, 773–784
- Deems, R. O., Anderson, R. C. and Foley, J. E. (1998) *Am. J. Physiol.* **274**, R524–R528
- Woeltje, K. F., Esser, V., Weis, B. C., Sen, A., Cox, W. F., McPhaul, M. J., Slaughter, C. A., Foster, D. W. and McGarry, J. D. (1990) *J. Biol. Chem.* **265**, 10720–10725
- Esser, V., Britton, C. H., Weis, B. C., Foster, D. W. and McGarry, J. D. (1993) *J. Biol. Chem.* **268**, 5817–5822
- Yamazaki, N., Shinohara, Y., Shima, A. and Terada, H. (1995) *FEBS Lett.* **363**, 41–45
- Brown, N. F., Esser, V., Gonzalez, A. D., Evans, C. T., Slaughter, C. A., Foster, D. W. and McGarry, J. D. (1991) *J. Biol. Chem.* **266**, 15446–15449
- Brown, N. F., Anderson, R. C., Caplan, S. L., Foster, D. W. and McGarry, J. D. (1994) *J. Biol. Chem.* **269**, 19157–19162
- Brown, N. F., Esser, V., Foster, D. W. and McGarry, J. D. (1994) *J. Biol. Chem.* **269**, 26438–26442
- de Vries, Y., Arvidson, D. N., Waterham, H. R., Cregg, J. M. and Woldegiorgis, G. (1997) *Biochemistry* **36**, 5285–5292
- Zhu, H., Shi, J., Cregg, J. M. and Woldegiorgis, G. (1997) *Biochem. Biophys. Res. Commun.* **239**, 498–502
- Kolodziej, M. P. and Zammit, V. A. (1993) *FEBS Lett.* **327**, 294–296
- Fraser, F., Corstorphine, C. G. and Zammit, V. A. (1996) *Biochem. Soc. Trans.* **24**, 184S
- Fraser, F., Corstorphine, C. G. and Zammit, V. A. (1997) *Biochem. J.* **323**, 711–718
- Mills, S. E., Foster, D. W. and McGarry, J. D. (1984) *Biochem. J.* **219**, 601–608
- Stephens, T. W., Cook, G. A. and Harris, R. A. (1983) *Biochem. J.* **122**, 521–524
- Woeltje, K. F., Kuwajima, M., Foster, D. W. and McGarry, J. D. (1987) *J. Biol. Chem.* **262**, 9822–9827
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- Britton, C. H., Schultz, R. A., Zhang, B., Esser, V., Foster, D. W. and McGarry, J. D. (1995) *Proc. Natl. Acad. Sci. U.S.A.* **92**, 1984–1988
- Yamazaki, N., Shinohara, Y., Shima, A., Yamanaka, Y. and Terada, H. (1996) *Biochim. Biophys. Acta* **1307**, 157–161
- Brown, N. F., Sen, A., Soltis, D. A., Jones, B., Foster, D. W. and McGarry, J. D. (1993) *Biochem. J.* **294**, 79–86
- Kolodziej, M. P. and Zammit, V. A. (1990) *Biochem. J.* **272**, 421–425
- Murthy, M. S. R. and Pande, S. V. (1987) *Proc. Natl. Acad. Sci. U.S.A.* **84**, 378–382
- McGarry, J. D., Brown, N. F., Inthanousay, P. P., Park, D. I., Cook, B. A. and Foster, D. W. (1992) in *New Developments in Fatty Acid Oxidation* (Coates, P. M. and Tanaka, K., eds.), pp. 47–61, Wiley-Liss Inc., New York
- Kashfi, K. and Cook, G. A. (1992) *Biochem. J.* **282**, 909–914
- Kashfi, K., Mynatt, R. L. and Cook, G. A. (1994) *Biochim. Biophys. Acta* **1212**, 245–252
- Zammit, V. A. (1996) *Biochem. J.* **314**, 1–14