Identification of Positive and Negative Determinants of Malonyl-CoA Sensitivity and Carnitine Affinity within the Amino Termini of Rat Liver- and Muscle-type Carnitine Palmitoyltransferase I*

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The extreme amino terminus and, in particular, residue Glu-3 in rat liver (L) carnitine palmitoyltransferase I (CPT I) have previously been shown to be essential for the sensitivity of the enzyme to inhibition by malonyl-CoA. Using the Pichia pastoris expression system, we now observe that, although mutants E3A (Glu-3 → Ala) or Δ(3–18) of L-CPT I have markedly lowered sensitivity to malonyl-CoA compared with the wild-type protein, the mutant Δ(1–82) generated an enzyme that had regained much of the sensitivity of wild-type CPT I. This suggests that a region antagonistic to malonyl-CoA sensitivity is present within residues 19–82 of the enzyme. This was confirmed in the construct Δ(19–30), which was found to be 50-fold more sensitive than wild-type L-CPT I. Indeed, this mutant was >4-fold more sensitive than even the native muscle (M)-CPT I isoform expressed and assayed under identical conditions. This behavior was dependent on the presence of Glu-3, with the mutant E3AΔ(19–30) having kinetic characteristics similar to those of the E3A mutant. The increase in the sensitivity of the L-CPT I Δ(19–30) mutant was not due to a change in the mechanism of inhibition with respect to palmitoyl-CoA, nor to any marked change of the K_{I50} for this substrate. Conversely, for M-CPT I, a decrease in malonyl-CoA sensitivity was invariably observed with increasing deletions from Δ(3–18) to Δ(1–80). However, deletion of residues 9–18 from M-CPT I affected the K_{m} for carnitine of this isoform, but not of L-CPT I. These observations (i) provide the first evidence for negative determinants of malonyl-CoA sensitivity within the amino-terminal segment of L-CPT I and (ii) suggest a mechanism for the inverse relationship between affinity for malonyl-CoA and for carnitine of the two isoforms of the enzyme.

Carnitine palmitoyltransferase I (CPT I,1 malonyl-CoA-sensitive) is an integral membrane protein first identified in the outer membrane and contact sites of mitochondria (1, 2). The enzyme catalyzes the formation of acylcarnitines from long-chain acyl-CoA esters, thus enabling the movement of acyl moieties across intracellular membranes. CPT I exists in two isoforms (Liver and Muscle), which have considerable sequence similarity but differ greatly, and inversely, in their malonyl-CoA sensitivity and K_{m} for carnitine (3, 4). It is a polytopic protein, with two transmembrane (TM) segments and amino and carboxyl segments (approximately 46 and 652 residues, respectively) that are both exposed on the cytosolic aspect of the membrane (5). It has been shown that the extreme amino terminus of the nascent L-CPT I is retained in the mature protein (6) and, moreover, that it is essential for the expression of malonyl-CoA sensitivity (5, 7). Subsequent work with expressed CPT I constructs has confirmed these conclusions by showing that deletion of the amino-terminal highly conserved 6 amino acid residues of the L-isoform results in the loss of high affinity malonyl-CoA sensitivity (8). Glu-3, and to a much lesser extent His-5, have been identified as residues within the extreme amino terminus that enable L-CPT I to bind malonyl-CoA with high affinity (9, 10). Thus, the E3A (Glu-3 → Ala) mutant loses malonyl-CoA sensitivity, although it has not been ascertained whether this residue contributes directly to a malonyl-CoA binding site or is required to enable the amino-terminal segment to interact effectively with the much larger carboxyl-terminal segment and maintain it in a conformation that binds malonyl-CoA optimally. That interaction between the amino- and carboxyl-terminal segments is important for the expression of malonyl-CoA sensitivity in the L-isoform was demonstrated by work in which chimeras were constructed using combinations of three segments (amino-terminal plus TM1, loop plus TM2, and carboxyl-segment) from each of L- and M-CPT I (11). The precise amino-to-carboxyl pairings affected the sensitivity to malonyl-CoA and the K_{m} for palmitoyl-CoA of the chimeric CPTs, whereas TM1-TM2 pairings affected the affinity for carnitine (11). These studies highlighted our long-standing observations on the importance of the interaction of CPT I with the membrane, of which it is an integral protein, for the expression of its kinetic characteristics (12, 13) and which more recently have been confirmed by work on the reconstitution of the solubilized or purified recombinant protein in vesicles of different lipid molecular order (14, 15).

Larger truncations extending into the protein sequence from the amino terminus of L-CPT I have, in general, shown that there is very little additional loss of either malonyl-CoA sensitivity or maximal activity compared with those already achieved in the Δ(2–6) or E3A mutants (9). However, the observations made by two groups independently on the Δ(1–82) mutant of rat L-CPT I (which is active and can be considered to be the catalytic core) are at variance with each other. Thus, whereas, in work by Esser et al. (16) it was shown that this construct has considerable residual malonyl-CoA sensitivity, Shi et al. (8) could not detect any malonyl-CoA binding to a

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1 The abbreviations used are: L-CPT I, liver isoform of carnitine palmitoyltransferase; M-CPT I, muscle isoform; TM, transmembrane segment; IC_{50}, concentration of malonyl-CoA that gives 50% inhibition of CPT activity; K_{I50}, concentration of palmitoyl-CoA that gives half the maximal activity of CPT observed at the carnitine concentration used in the assay; PCR, polymerase chain reaction.
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mitochondrial fraction of yeast in which the mutant was expressed, and the IC$_{50}$ for malonyl-CoA was much higher than that for the parental L-CPT I. Unfortunately, these two sets of studies were performed using different yeast expression systems (Saccharomyces cerevisiae in Esser et al. (16) and Pichia pastoris in Shi et al. (8)) such that a strict comparison of the two sets of data is not possible. However, we made preliminary observations on the Δ(1–82) mutant expressed in P. pastoris, which suggested that, not only is the protein expressed by this construct sensitive to malonyl-CoA but it is highly significantly more so than the E3A mutant expressed in the same yeast expression system. This observation raised the prospect that the amino-terminal segment of L-CPT I may contain two types of elements: those that are positive for malonyl-CoA sensitivity (i.e. the extreme amino terminus) and those that are negative (i.e. sequences between the highly conserved amino terminus and residue 82). Therefore, we have generated internal deletions, within the 46-residue cytosolic amino-terminal segment, but with the retention of the fully conserved residues 1–18. We find that these internal deletions result in a 50-fold increase in the malonyl-CoA sensitivity of the relevant L-CPT I mutants, suggesting that, in addition to containing regions that are essential for malonyl-CoA sensitivity, the amino-terminal segment also contains other regions that strongly affect this parameter negatively. Of the many mutants of CPT I studied by various workers, these are the first to express a much more malonyl-CoA-sensitive enzyme.

EXPERIMENTAL PROCEDURES

All materials were obtained as described previously (11, 17) except where detailed. L-Δ(1–82) was generated by digesting L-CPT I in pGAPZ with Csp45I and SpH I. Following removal of the smaller restriction fragment, T4 DNA polymerase was used to convert the overhangs to blunt ends, and the plasmid was recircularized. The L-Δ(19–30) deletion mutant was generated by using two overlapping PCRs as described previously (11). Oligonucleotide pairs A and B, and C and D (see Table I) were used for the two half reactions, followed by A and D for the final PCR. The L-Δ(19–46) construct was generated in the same way using primer pairs A and E, and F and D, followed by A and D. In both cases, the PCR products were cloned with Csp45I and ApaI and used to replace the corresponding fragment of wild-type L-CPT I with an introduced silent ApaI site (11) cloned into the vector pGAPZ A. The E3A variants of full-length L-CPT I and the two deletion mutants were generated by PCR using primers G and H, with the appropriate clone as template in each case. Again the PCR products were inserted into CPT I as Csp45I-AflII fragments. L-Δ(3–82) was generated using self-annealing overlapping oligonucleotides I and J. The oligonucleotides were annealed and extended using T4 DNA polymerase. The product was cleaved with NotI and BglII and cloned into CPT I in pGAPZ.

The M-Δ(1–80) mutant was constructed by using the existing codon for methionine at position 81 in rat M-CPT I as the start codon. For deletion of the first 18 amino acids, codons for amino acids one and two were retained to maintain identical initiation codon context to the wild-type construct. M-Δ(1–80) was generated by replacing the Csp45I-HindIII fragment of M-CPT I in pGAPZ (11) with a cleaved PCR product generated with primers K and L. M-Δ(3–18) was generated by replacing the SpeI-AflII fragment of wild-type M-CPT I with a cleaved PCR product generated with primers M and N. M-Δ(19–30) was generated by the same means as its liver counterpart using primer pairs O and P, and Q and N. The final PCR product (primers O and N) was cloned in as a SpeI-AflII fragment as for M-Δ(3–18).

Transformation of P. pastoris strain X-33, CPT I expression, and preparation of cell-free extracts was as described previously (11, 17). L-CPT I and M-CPT I-derived mutants were selected with 0.1 and 0.5 mg of zeocin/ml, respectively, except for L-CPT I-Δ(1–82) for which use of 0.5 mg of zeocin/ml was required to obtain sufficient levels of CPT activity sufficient for assay. The lower expression levels of this mutant may be due to the fact that, although the other L-CPT I constructs had the same initiation codon context, with alanine as the second amino acid (ATGGA), in Δ(1–82) the presence of isoleucine as residue two defines the less optimal start codon context ATGGA (18).

Secondary structure predictions for the CPT I amino-terminal regions were performed using various methods, including a consensus prediction method on the Network Protein Sequence Analysis server on the Web. These methods predict that the amino-terminal 46 amino acids of L- and M-CPT I adopt similar structures.

CPT I activity was measured using cell-free yeast extracts as described previously (11, 17). Briefly, for determination of the $K_m$ for carnitine, palmitoyl-CoA concentration was fixed at 135 μM. For determination of $K_m$ for palmitoyl-CoA, carnitine concentration was fixed at 500 μM. For practical reasons this was the highest concentration of carnitine of sufficiently high specific activity that was feasible, but it was not always a saturating concentration. Therefore, the apparent $K_m$ for palmitoyl-CoA, or the concentration of substrate that gives half the maximum activity obtained under the assay conditions, is given. The IC$_{50}$ malonyl-CoA (i.e. the concentration of inhibitor that inhibits the CPT activity by 50%) was determined using 35 μM palmitoyl-CoA and 500 μM carnitine. Data were fitted to the Michaelis-Menten equation for palmitoyl-CoA or carnitine as variable substrates and to an equation for simple competitive inhibition with respect to palmitoyl-CoA to obtain IC$_{50}$ values for malonyl-CoA. The mechanism of inhibition by malonyl-CoA was studied by performing assays at different palmitoyl-CoA concentrations in the absence and presence of two different malonyl-CoA concentrations. Malonyl-CoA concentration dependence of activity at three different concentrations of palmitoyl-CoA (20, 35, and 70 μM) was used to construct Dixon plots and to obtain K values.

Curve fitting was carried out using Sigma-Plot software with nonlinear regression analysis and Excel software using linear regression analysis. Statistical analysis was calculated using two-tailed Student’s t tests.

RESULTS

Comparison of the Properties of Native L-CPT I with Those of Δ(1–82), E3A, and Δ(3–18) Mutants

Malonyl-CoA Sensitivity—The most marked kinetic difference between the Δ(1–82) protein and the E3A and Δ(3–18) mutants was in the IC$_{50}$ values for malonyl-CoA inhibition (Table II). That of the Δ(1–82) mutant (68 ± 1 μM) was much more similar to that of the parental protein (only 1.8-fold higher) than were those of the other mutants (5-fold higher than for native L-CPT I). This was in disagreement with previous observations of other authors (8, 9) who have reported that the IC$_{50}$ was raised to the same levels by both Δ(1–82) and E3A mutations.

Affinities for Carnitine and Palmitoyl-CoA—Construct Δ(1–82) also had distinctive characteristics with respect to the affinities for the reaction substrates. The $K_m$ for carnitine was 300% higher, at 484 ± 39 μM, than that of the wild-type protein (p = 0.001), whereas its $K_m$ for palmitoyl-CoA was 25% lower (Table II). By contrast, the kinetic characteristics of the Δ(3–18) mutant were similar to those of E3A for all kinetic parameters, including >1.5-fold higher values (relative to the wild-type) for palmitoyl-CoA and carnitine.

Mechanism of Malonyl-CoA Inhibition—The kinetic basis for the large loss in malonyl-CoA sensitivity of the E3A mutant was studied. Calculation of the K values for malonyl-CoA for the three constructs (native, E3A, and Δ(1–82); see Fig. 1 and Table II) showed that malonyl-CoA increased the K values for palmitoyl-CoA in all three mutants, with very similar K values despite the 5-fold range in IC$_{50}$ values (Table II). This was in contrast to the parallel increase in K and IC$_{50}$ reported for mutants E3A and Δ(1–82) previously (9). For all three constructs, the pattern of inhibition was competitive (Fig. 2). However, although for native L-CPT I the effect of malonyl-CoA was limited to raising the K values for palmitoyl-CoA (Fig. 2, a and b) for mutants E3A and Δ(1–82), there was, in addition, a reproducible increase in the V$_{max}$ (Fig. 2, c and d). This type of behavior (hyperbolic inhibition) is rare for native enzymes but has been discussed in detail previously (19).
Properties of L-CPT I Mutants Bearing Internal Deletions within the Cytosolic Amino-segment: Δ(19–30) and Δ(19–46)

The above data indicated that, apart from the extreme amino terminus, elements within the cytosolic amino-segment may influence malonyl-CoA sensitivity such that their deletion, as in Δ(1–82), has a rescuing effect on this parameter. The existence of such a negative element in L- but not M-CPT I could potentially provide the basis for the 50- to 100-fold difference in the IC50 for malonyl-CoA displayed by the two isoforms. Therefore, we wanted to determine which features of the cytosolic amino-terminal segment could be responsible. Algorithms for prediction of transmembrane regions, suggest that residue 46 of L-CPT I defines the limit of the extra-membranous cytosolic amino-segment before the start of the hydrophobic TM1 segment. Because residues 1–18 are fully conserved between L- and M-CPT I, we concentrated on residues 19–46. All secondary structure prediction methods based on single or multiply aligned sequences agree that residues 15–19 (TPDG) constitute a random coil or β-turn. The region between this proposed turn and the membrane-embedded sequence is predicted to form an α-helix, broken by a random coil for residues 35–38, based on the consensus of nine different prediction methods. Therefore, two mutants of L-CPT I were constructed in which residues up to, and including, the turn region (amino acids 1–18) were retained. Deletions were made between residues (19–30 or 19–46) such that in these mutants the extreme amino terminus, including Glu-3, is predicted to be physically closer to the aqueous-phase/membrane interface.

Malonyl-CoA Sensitivity—The most striking property of the L-CPT I-Δ(19–30) and Δ(19–46) mutants was that they had IC50 values for malonyl-CoA inhibition that were manyfold lower than that for the parental protein (Table II). This was especially evident for the Δ(19–30) protein, which had an IC50 value that was almost 50-fold lower than that of L-CPT I (0.8 μM versus 38 μM malonyl-CoA; see Fig. 3).

Affinities for Carnitine and Palmitoyl-CoA—There were modest, but statistically significant, increases in the K0.5 for palmitoyl-CoA and K for carnitine with respect to the wildtype protein (up to 2-fold; see Table II). Therefore, mutations Δ(19–30) and Δ(19–46) had effects similar to E3A and Δ(3–18) on the increases in the values for both the reaction substrates,
but markedly opposite effects on the $K_i$ for malonyl-CoA (above).

Mechanism of Malonyl-CoA Inhibition—To ascertain that the observed 50-fold decrease in $I_{C50}$ for malonyl-CoA for the Δ(19–30) mutant did not result from the change in the $K_m$ for palmitoyl-CoA, we performed kinetic analyses of the dependence of the activities of the mutants and of the parental protein, with respect to palmitoyl-CoA and malonyl-CoA concentrations. The $K_i$ value for the Δ(19–30) mutant was almost two orders of magnitude lower than that for native L-CPT I (Table II). The data in Fig. 2 (a and b) show that this decrease in $K_i$ was not accompanied by any change in the kinetic mechanism of malonyl-CoA inhibition of the enzyme activity of Δ(19–30) compared with the parental protein (both competitive). A similar result was obtained for the Δ(1–46) mutant (not shown). Thus, the decrease in $K_i$ for malonyl-CoA could account entirely for the decreased $I_{C50}$ of these internal deletion mutants (Table II).

Interaction between the Effects of the E3A Mutation and Deletions Internal to the Cytosolic Amino-segment of L-CPT I

In view of the opposing effects on malonyl-CoA sensitivity of the E3A and of the Δ(19–30) and, to a lesser extent, the Δ(19–46) mutations, we investigated whether the enhanced malonyl-CoA sensitivity demonstrated by the deletion mutants was conditional on the presence of Glu-3. Therefore, two mutants, E3A-Δ(19–30) and E3A-Δ(1–46) were constructed. The data in Table II show that the E3A-Δ(19–30) mutant was two orders of magnitude less sensitive to malonyl-CoA than the Δ(19–30) mutant, indicating that the lowered $I_{C50}$ induced by the internal deletion does not obviate the requirement for the presence of Glu-3. However, despite its increase, the $I_{C50}$ value was significantly lower (by 25%) than that of the E3A mutant ($p = 0.008$). By contrast, the effect of deletion of residues 19–46 on malonyl-CoA sensitivity was fully reversed by simultaneous mutation of Glu-3. Both the E3A derivatives of the internal deletion mutants showed very similar kinetic parameters for palmitoyl-CoA and carnitine to those displayed by the proteins expressed from mutants with the parental deletions and the

E3A mutant itself (Table II) indicating that the effects of the combined mutations were not additive.

Comparison of the Properties of Native M-CPT I to Those of the Δ(1–80) and Δ(3–18) Mutants Derived from It

Previous work (20) has shown that deletion of the amino-terminal first 18 residues of human M-CPT I causes only a relatively minor increase in $I_{C50}$ for malonyl-CoA, whereas further amino-terminal truncation of the protein by 28, 39, 51, or 72 residues gives a 10-fold greater loss in sensitivity. (It is to be noted, however, that the $I_{C50}$ is still lower than that for full-length L-CPT I.) Therefore, in human M-CPT I any effect of the sequence between residues 19–30 on malonyl-CoA sensitivity would be expected to be positive, rather than negative. Therefore, we decided to test this possibility to ascertain whether the 19–30 region of the amino terminus of M-CPT I also acts as an internal determinant of malonyl-CoA sensitivity. As an initial step, we made the rat M-CPT I-Δ(1–80) construct. We found that, contrary to the equivalent human M-CPT I mutant, which was reported to be inactive (20), the rat M-CPT I-Δ(1–80) mutant resulted in an enzyme that had about the same activity as the full-length protein (Table II). This enabled us to obtain detailed kinetic data and to compare them with those of the analogous liver-type truncation mutant (above). The kinetic parameters for the M-CPT I-Δ(1–80) and -Δ(3–18) mutants expressed in P. pastoris are reported in Table II, along with data for the wild-type enzyme.

$I_{C50}$ for Malonyl-CoA—The protein resulting from the expression of M-CPT I-Δ(1–80) showed a larger increase (3.2-fold) in $I_{C50}$ for malonyl-CoA compared with native M-CPT I and to that of the deletion mutant M-CPT I-Δ(3–18), which showed minimal change in $I_{C50}$ for malonyl-CoA compared with M-CPT I (cf. equivalent mutation in human M-CPT I (20)). These observations were in sharp contrast to L-CPT I-Δ(3–18) for which the $I_{C50}$ value was increased 5-fold compared with its parental protein and to L-CPT I-Δ(1–82), which had an $I_{C50}$ intermediate between that of the wild-type and of L-CPT I-Δ(3–18). Although the mutant M-CPT I-Δ(19–30) expressed very low CPT activity, even after selection for a high copy number of genomic integration with high zeocin concentration (1 mg/ml), we were able to demonstrate that deletion of residues 19–30 did not result in any marked increase in malonyl-CoA sensitivity (not shown). Thus, it appears that, contrary to those of L-CPT I, the two amino-terminal sequences 1–18 and 19–30 of M-CPT I do not interact either with each other or with the rest of the M-CPT I molecule to provide positive or negative determinants of sensitivity to inhibition by malonyl-CoA. However, evidently when they are both simultaneously truncated, malonyl-CoA sensitivity is markedly decreased (20). Due to the low
CPT activity of this construct, we were unable to perform a detailed kinetic analysis.

Affinities for Palmitoyl-CoA and Carnitine—The effects of the Δ(1–80) truncation of M-CPT I to lower the $K_m$ for palmitoyl-CoA were similar to those observed for the equivalent truncation in L-CPT I (Table II). However, the effect on the $K_m$ for carnitine was very different for the two isoforms, in that the value of this parameter was unaffacted relative to that of the native M-CPT I protein (641 ± 52 and 779 ± 80 μM, respectively), whereas the equivalent truncation of L-CPT I resulted in an increase of the $K_m$ for carnitine by 3-fold (see above). As a result, it emerged that the $K_m$ for carnitine of the catalytic cores (TM2 plus carboxyl-segment) of the L- and M-isoforms were very similar to each other (Table II). Moreover, there was a marked increase in the $K_m$ for carnitine after deletion of residues 3–18 in the M-isoform, whereas the equivalent mutation in L-CPT I did not alter the $K_m$ for carnitine significantly (Table II). These data indicate that it is not the catalytic domains per se but their interaction with the respective amino-terminal regions (cytosolic segment plus TM1) that impart to the parental proteins the very different $K_m$ values of native L- and M-CPT I.

**DISCUSSION**

The present observations on the mutants L-CPT I-Δ(1–82) and M-CPT I-Δ(1–80) give rise to the concept of a core within CPT I (comprising TM2 and the carboxyl-segment) that is catalytically active and sensitive to inhibition by concentrations of malonyl-CoA of the same order of magnitude as those that inhibit the full-length parental proteins. Although the original concept (5), that the cytosolic amino-terminal segment is essential for the expression of malonyl-CoA sensitivity, evidently applies to the native L-CPT I (as confirmed by truncation mutants; this study and Ref. 8), it is obvious from the present data that this overall effect results from a far more complex interaction than has hitherto been envisaged, between positive and negative determinants of malonyl-CoA sensitivity within the amino-terminal segment of the protein. Moreover, the data reveal the dichotomy of the effects of the amino-terminal segment on malonyl-CoA sensitivity in the L-isoform on the one hand, and on the affinity for carnitine in the M-isoform, on the other. This may be the cause of the inverse relationship between the IC$_{50}$ for malonyl-CoA and the $K_m$ for carnitine of the two isoforms.

**Negative Determinants of Malonyl-CoA Sensitivity within the Cytosolic Amino-Terminal Region of L-CPT I**—This is the first report that a short sequence (residues 19–30) within the cytosolic amino-terminal segment of L-CPT I has a negative effect on the malonyl-CoA sensitivity of L-CPT I. The effect is very marked: deletion of this region increases malonyl-CoA sensitivity by 50-fold, in effect lowering the IC$_{50}$ and $K_i$ of L-CPT I for malonyl-CoA inhibition to the same order of magnitude as that of the native M-isoform. These data, in conjunction with the observations that the extreme amino terminus is essential for malonyl-CoA sensitivity of any construct of L-CPT I that contains TM1 and the amino-segment, show that this cytosolic amino-terminal 46-residue sequence contains both highly positive and highly negative determinants of the malonyl-CoA sensitivity of L-CPT I. The fact that the catalytic core, Δ(1–82), has an inherent malonyl-CoA sensitivity that is intermediate between that of the E3A and Δ(3–18) mutants on the one hand, and of the native L-CPT I and the Δ(19–30) mutant on the other, emphasizes the potential for the generation of a range of malonyl-CoA sensitivities in the L-isoform (see Fig. 4) but not the M-isoform. It is suggested that in the intact protein in vivo such a balance between the positive and negative effects of the two sequences (residues 1–18, and residues 19–30, respectively) would arise from conformational changes that alter their mutual interaction and/or interaction with the rest of the protein. Such conformational changes have previously been suggested to occur in response to changes in the lipid molecular order of the membrane in which the TM segments reside (12, 13) and to account for the changes in the IC$_{50}$ for malonyl-CoA observed for L-CPT I in mitochondria isolated from rats in different physiological states (see Ref. 21 for review).

The relatively high sensitivity of the catalytic cores of both L- and M-CPT I indicate that the extreme amino terminus, and Glu-3 in particular, does not participate directly in malonyl-CoA binding but, rather, that it interacts with the carboxyl-segment to optimize its malonyl-CoA sensitivity, as originally suggested (5, 7, 22). In the case of L-CPT I this interaction alone (i.e. in the absence of the negative effect of residues 19–30) enables the protein to adopt a configuration that is as sensitive to malonyl-CoA as that of native M-CPT I. It is the presence of residues 19–30 within intact CPT I that tempers this effect, so as to result in the well-documented 50- to 100-fold lower malonyl-CoA sensitivity displayed by native L-CPT I compared with M-CPT I (3, 4). The fact that the negative effect of residues 19–30 is only evident in the context of the pro-
The inhibitory action of Glu-3 suggests that (i) residues 19–30 are likely to act by interfering with the ability of residues 3–18, and Glu-3 in particular, to interact with the rest of the molecule, and/or (ii) that Glu-3 determines not only the point contact(s) in which it itself is involved, but also additional interactions in which other regions of the amino-terminal segment normally participate in the wild-type protein.

Nothing is known from previous studies about the molecular interactions in which Glu-3 is involved. It is likely that this residue interacts with the carboxyl-segment of CPT I, although in the absence of experimental evidence, involvement of other regions of CPT I, or components other than those within CPT I itself (e.g. membrane phospholipid head groups) cannot be ruled out. Deletion of residues 19–30 or 19–46 from L-CPT I both lead to the same phenomenon of enhanced malonyl-CoA sensitivity, while still retaining dependence on the presence of Glu-3. If Glu-3 interacts with the same components within the carboxyl-segments of the two mutants as those in the wild-type protein, then the fact that a more extensive deletion Δ19–46 reduces the distance between the extreme amino terminus and the membrane by an additional 16 residues only changes the effect on malonyl-CoA sensitivity marginally suggests that interaction between the amino terminus and the carboxyl-segment is highly flexible. Candidates for the origin of such flexibility are the mutual and/or membrane interactions of the two TM segments through which the amino- and carboxyl-segments of the molecule are connected.

The fact that the antagonism between the extreme amino terminus and the 19–30 sequence occurs solely for L-CPT I, and not for the M-isoform, is in agreement with many previous observations establishing that (i) changes in malonyl-CoA sensitivity occur under different physiological states in vitro only for L-CPT I and not for M-CPT I (20); (ii) these changes are reflected in the unique hysteresis properties of the isoform in vitro (24); and (iii) they can be mimicked by altering the lipid molecular order of mitochondrial outer membranes in vitro (13, 25) or after reconstitution of the solubilized or purified protein into liposomes (14, 15, 26). Therefore, it is suggested that the interplay between the effects of the structural motifs on either side of the turn predicted for residues 15–19 (TDPG, see above) enables L-CPT I to respond to changes in the fluidity of the membrane in which TMAs 1 and 2 are embedded, and with/within which they are presumed to interact (7, 11, 22, 27).

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**Different Roles of the Amino-Terminal Segment in Determining the Kinetics of M- and L-isoforms—**Our data show that the amino-terminal 3–18 amino acid residues influence the kinetic characteristics of the M- and L-isoforms reciprocally with respect to carnitine and malonyl-CoA. When the amino terminus and TM1 were removed from L- and M-CPT I (i.e. in constructs L-CPT I-Δ1–82 and M-CPT I-Δ1–80), respectively) the difference in the $K_m$ for carnitine was greatly narrowed, such that the values for the catalytic cores of the two isoforms were not statistically significantly different (Table II). This suggests that the large difference between native L- and M-CPT I in their $K_m$ for carnitine (3) results not from the intrinsic properties of the carboxyl-segments themselves, but from the influence of their respective cytosolic amino-terminal segment and/or TM1. This agrees with our previous conclusions, deduced from studies with L/M chimeras, which revealed that TM1-TM2 interactions are major determinants of the $K_m$ for carnitine in both isoforms (11). In the case of L-CPT I, the identity of the amino terminus plus TM1 and TM2 was unimportant so long as native TM pairings were retained (11). In the same study it was shown that, for M-CPT I, the $K_m$ for carnitine was influenced not only by inter-TM interactions but also by the identity of the amino-segment itself (11). The present data show that residues 1–18 of L-CPT I are important for this effect to occur. Because this linear sequence is identical between L- and M-isoforms, it is suggested that it is its interaction with the rest of the cytosolic amino-terminal segment that differs between L- and M-CPT I. In this respect, it is noteworthy that the $K_m$ for carnitine of the M-Δ1–80 mutant is the same as that of the full-length M-CPT I (Table II), suggesting that a sequence within residues 19–80 cancels out the effect of residues 1–18 on this parameter. It was previously reported (20) that the $K_m$ for carnitine is the same for wild-type human M-CPT I and the Δ(2–28) truncation mutant. Therefore, this
provides circumstantial evidence for the possibility that residues 19–28 within this region are responsible for countering the effects of residues 1–18 on the $K_m$ for carnitine of the muscle isoform. Thus, just as residues in the regions 1–18 and 19–30 have opposite influence on malonyl-CoA sensitivity in L-CPT I, the same two regions in M-CPT I appear to have opposing effects on the $K_m$ for carnitine.

**Reciprocal relationship between $K_m$ for carnitine and IC$_{50}$ for Malonyl-CoA in L- and M-CPT I**—A hitherto unexplained feature of L- and M-CPT I isoforms is the inverse relationship between their respective values for the IC$_{50}$ for malonyl-CoA inhibition and $K_m$ for carnitine, when measured for the native enzymes in isolated rat liver and muscle mitochondria (3). This inverse relationship is retained in the recombinant proteins expressed in yeast (11, 28, 29) or mammalian cells (10). The present data suggest that it arises from the opposite effects of the amino-terminal segment, and the extreme amino terminus in particular, on these parameters in the two isoforms. Thus, as shown in Fig. 4, the removal of residues 3–18 from L-CPT I raises its malonyl-CoA IC$_{50}$ significantly (cf. Ref. 8), but the equivalent mutation has no effect on malonyl-CoA sensitivity of M-CPT I (Table II). By contrast, the presence of the cytosolic amino-terminal segment has the effect of lowering the $K_m$ for carnitine for L-CPT I (compare the wild-type with $\Delta$(1–82) in Fig. 5) but has no effect on the $K_m$ for carnitine of M-CPT I unless residues 3–18 are deleted (compare M-CPT I-$\Delta$(3–18) with wild-type M-CPT I in Fig. 5) when the $K_m$ for carnitine is increased severalfold. Therefore, the 3–18 sequence, although entirely conserved between the two isoforms, has reciprocal effects on affinities for carnitine and for malonyl-CoA: it controls the IC$_{50}$ for malonyl-CoA in L-CPT I (but not M-CPT I), whereas it controls the $K_m$ for carnitine in M-CPT I but not in L-CPT I. This correlates with the inverse relationship between the IC$_{50}$ for malonyl-CoA and $K_m$ for carnitine for the two isoforms. Thus, the major kinetic differences between L- and M-CPT I are not primarily due to a large difference in the catalytic properties of the carboxyl-segment plus TM2, but to the way in which these catalytic cores are influenced by the respective amino termini. Presumably, subtle structural differences between the two isoforms, which are highly related in sequence, result in interaction of the amino terminus with different regions of the apposed substrate binding regions.

**Mechanism of Malonyl-CoA Inhibition**—The relatively high malonyl-CoA sensitivity of the catalytic cores (TM2 plus carboxyl-segments) of L- and M-CPT I indicates that malonyl-CoA binds to the same region of the carboxyl-segment both in these mutant proteins and in intact, full-length CPT I. Previous studies have indicated that the malonyl-CoA and palmitoyl-CoA sites overlap; blocking the active site with a bi-dentate ligand species, formed by incubating mitochondria in the presence of Br-palmitoyl-CoA and carnitine, abolishes high affinity malonyl-CoA binding (30, 31). In the present study we observed that L-CPT I-$\Delta$(3–18) and L-CPT I-$\Delta$(1–82) both display hyperbolic inhibition (19); i.e. the inhibitor not only increased the $K_m$ for palmitoyl-CoA but also increased $V_{max}$. This supports the suggestion that palmitoyl-CoA and malonyl-CoA bind to the same or overlapping sites, because hyperbolic inhibition is thought to result from the ability of a small inhibitor molecule, binding at

the active site, to induce a conformation more favorable for the much larger substrate molecule to bind (19).

In conclusion, the present studies have revealed that the interaction between the amino- and carboxyl-segments of L-CPT I is more complex than has hitherto been envisaged and that a short sequence (residues 19–30) within the amino terminus of L-CPT I acts negatively to prevent this isofrom from attaining the high malonyl-CoA sensitivity shown by M-CPT I. The results add to our knowledge of the structural basis for the differences between the kinetic characteristics of native L- and M-CPT I isoforms, especially with respect to their reciprocal sensitivities and affinities for malonyl-CoA and carnitine, respectively. Elucidation of the molecular mechanisms underlying these differences may facilitate the design of specific inhibitors of the respective isoforms of the enzyme, thus enabling tissue selectivity of action in vivo.

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Identification of Positive and Negative Determinants of Malonyl-CoA Sensitivity and Carnitine Affinity within the Amino Termini of Rat Liver- and Muscle-type Carnitine Palmitoyltransferase I
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