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Structure-Function Studies with the Mitochondrial Carnitine Palmitoyltransferases I and II

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Summary. Mitochondrial carnitine palmitoyltransferases I and II (CPTI and CPTII), together with the carnitine carrier, transport long-chain fatty acyl-CoAs from the cytosol to the mitochondrial matrix for β-oxidation. As an enzyme that catalyzes the rate-limiting step in fatty acid oxidation, CPTI is inhibited by malonyl-CoA, the first intermediate in fatty acid synthesis. Our development of a high level of expression for CPTI and CPTII in *P. pastoris*, a yeast with no endogenous CPT activity has enabled us to map the malonyl-CoA and substrate binding sites by mutational analysis. Using deletion and substitution mutants of L-CPTI expressed in *P. pastoris*, we have shown that Glu3 and His5 are necessary for malonyl-CoA inhibition and high-affinity binding of L-CPTI but not for catalysis. Similar studies of M-CPTI clearly establish that the N-terminal residues Glu3, Val19, Leu23, and Ser24 in M-CPTI are important for malonyl-CoA inhibition and binding, but not for catalysis. Furthermore, using chimeras between rat and pig L-CPTI, and deletion mutation analysis, we demonstrated that the differences in malonyl-CoA sensitivity observed between the pig and rat L-CPTI were due to differences in the interaction of the first 18 N-terminal amino acid residues with the C-terminal region of the respective enzymes. Consistent with this, the conserved C-terminal residues R601, E603, R606, and K560 were found to be important for L-CPTI activity, malonyl-CoA inhibition and binding, because mutation of these residues decreased malonyl-CoA sensitivity and enzyme activity. We also identified two conserved C-terminal residues in L-CPTI, D567, and E590, that when mutated to alanine cause a substantial increase in malonyl-CoA sensitivity, suggesting a structural basis for the differences in malonyl-CoA sensitivity between L-CPTI and M-CPTI. Our cysteine-scanning mutagenesis of M-CPTI revealed that a single Cys residue, Cys305, was essential for catalysis. In addition, deletion and substitution analysis of the extreme C-terminal region of M-CPTI, suggest that L764 may be important for proper folding and optimal activity. In summary, our structure-function studies with the mitochondrial carnitine palmitoyltransferases I and II have identified critical residues for inhibitor and substrate binding and catalysis.

Keywords. Structure-activity relationships; Enzymes; Fatty acids; Oxidations; Carnitine.

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Introduction

Transport of long-chain fatty acids from the cytosol to the mitochondrial matrix for β-oxidation involves the conversion of long-chain fatty acyl-CoA to acylcarnitines by carnitine palmitoyltransferase I (CPTI), translocation across the inner mitochondrial membrane by the carnitine carrier, and reconversion to long-chain fatty acyl-CoA by carnitine palmitoyltransferase II (CPTII) [1, 2]. Mammalian mitochondrial membranes have two active but distinct carnitine palmitoyltransferases (CPT): a malonyl-CoA-sensitive, detergent-labile, integral membrane protein, CPTI, located on the outer mitochondrial membrane, and a malonyl-CoA-insensitive, detergent-stable CPTII, loosely associated on the matrix side of the inner mitochondrial membrane (Fig. 1). A current model for the membrane topology of CPTI predicts exposure of the N- and C-terminal domains crucial for activity and malonyl-CoA sensitivity on the cytosolic side of the outer mitochondrial membrane (Fig. 2) [3].

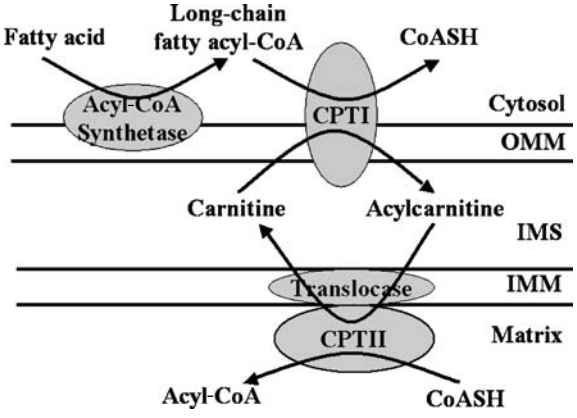

Fig. 1. Reactions catalyzed by mitochondrial carnitine palmitoyltransferase (CPT) I and II; abbreviations: OMM, outer mitochondrial membrane; IMS, intermembrane space; IMM, inner mitochondrial membrane

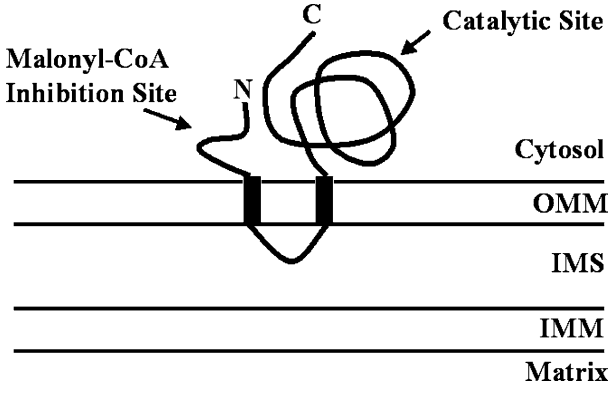

Fig. 2. Model for carnitine palmitoyltransferase I (CPTI) membrane topology (see Fig. 1 for abbreviations)

As an enzyme that catalyzes the rate-limiting step in fatty acid oxidation, CPTI is tightly regulated by its physiologic inhibitor, malonyl-CoA, the first intermediate in fatty acid synthesis. This is an important regulatory mechanism in fatty acid oxidation and suggests coordinated control of fatty acid oxidation and synthesis. In heart, more recent studies suggest that high levels of long-chain fatty acids activate muscle CPTI expression *via* the peroxisome proliferator activated receptor α (PPARα), and its coactivator, PGC-1α (PPAR Gamma Coactivator-1α), resulting in increased fatty acid oxidation [4–8]. Long-chain fatty acids are thus regulators of their own metabolism. Regulation of CPTI by malonyl-CoA and long-chain fatty acids is a major property of CPTI and confers to the enzyme the ability to signal to the cell the relative availability of carbohydrates and lipid fuels. Understanding the molecular mechanism of the regulation of the CPT system by malonyl-CoA and long-chain fatty acids is crucial in the design of drugs for control of excessive fatty acid oxidation in diabetes mellitus, in myocardial ischemia in which accumulation of acylcarnitines has been associated with arrhythmias, in obesity, and in human inherited CPT deficiency diseases [9–12].

The Role of the CPT System in Cellular Fatty Acid Metabolism

Liver Mitochondrial L-CPTI

Mammalian tissues express three isoforms of CPTI, liver (L-CPTI) and heart/muscle (M-CPTI) that are 62% identical in amino acid sequence, and a brain isoform, CPTIC, that is 54% identical to L- and M-CPTI [13–15]. L-CPTI is the predominant isoform expressed in a wide variety of tissues including the pancreas, kidney, lung, spleen, intestine, ovary, and human fibroblasts [2, 13]. The human L-CPTI gene is composed of 18 exons and 17 introns spanning 60 kb of DNA with two untranslated exons upstream of the initiator codon [16]. Compared to M-CPTI, L-CPTI has a much higher affinity for carnitine but is less sensitive to malonyl-CoA inhibition. All of the CPTI isoforms are expressed in the brain with the expression of L-CPTI being greater than that of M-CPTI and CPTIC in all regions of the brain [17]. CPTIC is only expressed in the brain and testis, and M-CPTI is very highly expressed in the cerebellum. The role of CPTIC in transport of long-chain fatty acids is unclear, because CPTIC has not been demonstrated to have catalytic activity, although it was reported to display high-affinity malonyl-CoA binding [15]. The human CPTIC is located on chromosome 19q, but the organization of the gene structure is not known.

Heart Mitochondrial M-CPTI

The cDNAs and the genes for human, rat, and mouse M-CPTI have been independently cloned and sequenced by several laboratories [13, 18–23]. In human, rat, or mouse tissues, M-CPTI is only expressed in heart, skeletal muscle, brown and white adipose tissue, and testis [2, 13, 24]. The genomic DNA for human, rat, and mouse M-CPTI has also been isolated and characterized [18–20, 24]. The human, rat, and mouse M-CPTI genes are approximately 10 kb in length. The marked difference in size between the human M-CPTI (10 kb) and L-CPTI (60 kb) genes is

due to variations in some intron sizes. The structural gene for human M-CPTI is composed of 18 exons interrupted by 17 introns spanning more than 8576 bp with an additional 1.0 kb of 5′ untranslated sequences [19, 20]. The human M-CPTI gene 5′-flanking DNA contains two untranslated exons (1A and 1B) that extend from −746 to −633 and −523 to −470 bp upstream of the initiator codon, respectively [13, 19, 20]. The 5′ ends of exon 1A and 1B function as independent transcription start sites [24, 25]. A putative gene encoding a "choline kinase homologue" is located only 300 bp upstream from exon A of the human M-CPTI gene [19, 20].

The genes for human heart M-CPTI and L-CPTI are located on chromosomes 22q and 11q, respectively [19, 21, 24]. Thus, human liver and skeletal muscle CPTI are different proteins encoded by separate genes. In addition, three novel M-CPTI isoforms generated by alternative splicing of the M-CPTI transcript which are expressed at very low levels in most tissues compared to M-CPTI have been reported [25]. However, when expressed in the yeast *P. pastoris*, none of the splice variants had CPTI activity indicating that they do not play a role in the modulation of malonyl-CoA inhibition of fatty acid oxidation [24]. Adult rat heart expresses both L-CPTI and M-CPTI, but the predominant isoform is M-CPTI [14, 26]. In heart, isoform switching occurs during growth [27]. CPTI thus belongs to a growing list of cardiac proteins that are developmentally regulated. M-CPTI is expressed throughout perinatal development, making it a primary target for metabolic modulation of myocardial fatty acid oxidation [28]. In the neonatal heart, L-CPTI contributes approximately 25% of total CPT activity, and this value falls during growth to its adult level of 2–3% [27, 28]. Electrical stimulation of neonatal cardiac myocytes also induces isoform switching by turning on expression of M-CPTI mRNA [29, 30].

The K_m for carnitine for M-CPTI is ∼20-fold higher than that of L-CPTI, which is ascribed to the higher levels of carnitine in heart compared to liver [27]. M-CPTI is also much more sensitive to malonyl-CoA inhibition than L-CPTI, although there is no significant difference in the malonyl-CoA concentration between the two tissues. Since the IC_{50} for malonyl-CoA inhibition of human or rat M-CPTI is at least 30-fold lower than that of L-CPTI, the level of malonyl-CoA reported in heart is adequate to significantly inhibit fatty acid oxidation [2]. It is estimated that about 60–80% of the energy requirement of the heart is derived from fatty acid oxidation [99]. The important question of how fatty acid oxidation can proceed in heart in the presence of high tissue levels of malonyl-CoA appears to be resolved, in part, by the more recent reports of the transcriptional regulation of the M-CPTI gene expression by long-chain fatty acids *via* the PPARα [4–6].

Cloning and Expression of the cDNAs for the CPT System

CPTII

Both the human and the rat L-CPTII cDNAs have been cloned and sequenced [31, 32]. The cDNA sequences predicted proteins of 658 amino acid residues (71 kDa). CPTII is a catalytically active, malonyl-CoA-insensitive, distinct enzyme, because a rat liver cDNA encoding CPTII synthesizes an active protein when expressed in *E. coli* [33, 34], in the yeasts *Saccharomyces cerevisiae* [35]

and *P. pastoris* [36], in baculovirus [37], or in COS cells (Simian Virus 40 Transformed African Green Monkey Kidney Fibroblast Cells) [32].

L-CPTI

The cDNAs for rat and human L-CPTI have been cloned and sequenced [36, 38, 39]. The cDNA sequences predicted proteins of 773 amino acid residues (88 kDa) with an overall identity of about 30% to CPTII. A novel high level of expression of the rat L-CPTI cDNA in *P. pastoris*, a yeast with no endogenous CPT activity, established for the first time that L-CPTI is a catalytically active, malonyl-CoA-sensitive, distinct enzyme that is reversibly inactivated by detergents [36]. The kinetic characteristics of the yeast-expressed L-CPTI were similar to those of rat liver mitochondrial L-CPTI. An improved method for expression of L-CPTI cDNA in *S. cerevisiae* was later developed that confirmed the properties of the *P. pastoris* expressed L-CPTI [40].

Heart M-CPTI

The rat brown adipose tissue [22], and the human M-CPTI cDNAs have been cloned and sequenced [13, 19]. These cDNAs encode a protein of 772 amino acid residues (88 kDa) with 62% identity to rat L-CPTI at the amino acid level. The human heart M-CPTI cDNA was expressed in *P. pastoris*, and isolated mitochondria from the M-CPTI expression strain exhibited a malonyl-CoA-sensitive CPT activity that is reversibly inactivated by detergents [13, 41]. Both the rat L-CPTI and M-CPTI cDNAs were expressed in *P. pastoris* strain X-33 using the multicopy expression vector pGAPZ-B [42, 43], but the IC_{50} for malonyl-CoA inhibition of the expressed CPTIs was 10–50-fold higher than that obtained using the single copy vector pHW010 in the *P. pastoris* strain GS115 [13, 36], or *S. cerevisiae*-expressed CPTI [40], or the rat liver and heart mitochondrial CPTI [44]. M-CPTI was also expressed in COS cells, but, due to the high endogenous levels of L-CPTI present in COS cells, it was not possible to determine the IC_{50} for malonyl-CoA inhibition of M-CPTI or the K_m for the substrates [44].

Structure-Function Studies with the Yeast-Expressed CPT System

M-CPTI and L-CPTI are integral membrane proteins located in the outer mitochondrial membrane with two predicted membrane-spanning α-helices within the 125 N-terminal amino acid residues. A current model for the membrane topology of CPTI predicts exposure of the N- and C-terminal domains crucial for activity and malonyl-CoA sensitivity on the cytosolic side of the outer mitochondrial membrane (see Fig. 2) [3].

CPTII

With the yeast-expressed CPTII, mutations of conserved residues H372, D376, and D464 to alanine resulted in complete loss of CPTII activity, suggesting that these residues may be essential for catalysis [35]. When the N-terminal domain of L-CPTI (residues 1–150), which contains the two transmembrane domains and the mitochondrial targeting sequence, was fused to the N-terminus of the

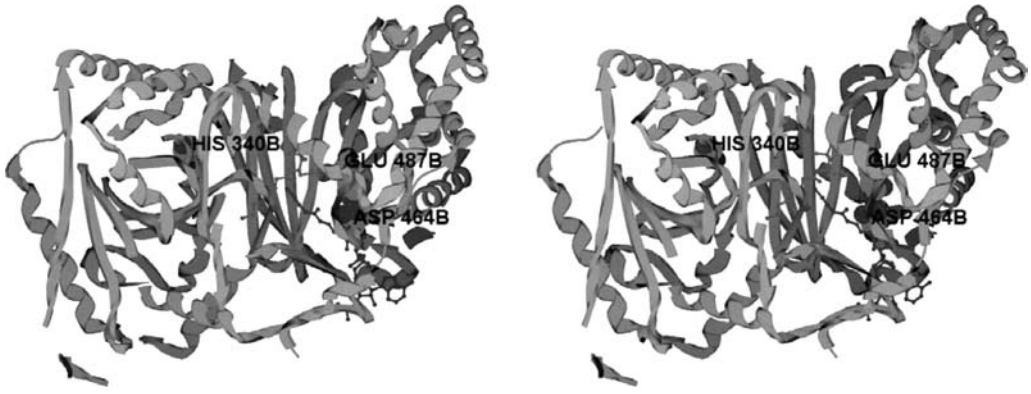

Fig. 3. RL-CPTII homology model; a homology model for RL-CPTII was constructed using a threading server (proteins.msu.edu) with the coordinates for carnitine octanoyltransferase as template (COT, 1×l7.pdb); no attempt was made to add missing features to the model; the backbone is shown as a yellow cartoon; several amino acid residues discussed in the text are labeled with their residue number and are shown with their atoms depicted as solid spheres; octanolycarnitine is shown as a green ball-and-stick figure and is positioned based on a COT structure (1×l8.pdb); CoA is shown as a magenta ball-and-stick figure and is positioned based on a carnitine acyltransferase structure (CAT, 1ndi.pdb)

malonyl-CoA-insensitive CPTII, the active chimeric CPTII was anchored at the outer mitochondrial membrane [45]. However, the chimeric CPTII was insensitive to malonyl-CoA inhibition, indicating that the N-terminal domain of L-CPTI cannot confer malonyl-CoA sensitivity to CPTII [45]. More recent site-directed mutagenesis studies with the *E. coli* expressed CPTII from our lab demonstrate that Glu487 in CPTII is essential for catalysis because substitution of Glu487 with alanine, aspartate, or lysine resulted in almost complete loss in CPTII activity [34]. Because a conservative substitution mutation of this residue, E487D, resulted in almost complete loss in activity, we predicted that Glu487 is at the active-site pocket of CPTII. The substantial loss in CPTII activity observed with the E487K mutant, along with the previously reported loss in activity observed in a child with a CPTII deficiency disease [46] establishes that Glu487 is crucial for maintaining the configuration of the liver CPTII active site (Fig. 3). Substitution of the conserved Glu500 in CPTII with alanine or aspartate reduced the *V*max for both substrates, suggesting that Glu500 may be important in stabilization of the enzyme-substrate complex [34].

Mapping of N-terminal Residues in CPTI Important for Malonyl-CoA Inhibition and Binding

Our development of a high level of expression for CPTI in *P. pastoris* has enabled us to map the malonyl-CoA and substrate binding sites by mutational analysis [13, 36, 41, 47–49]. Amino acid sequence alignment of both human and rat M-CPTIs, L-CPTIs, and CPTII reveals the presence of a conserved N-terminal sequence of 124 amino acid residues with two putative transmembrane domains in all CPTIs, which are absent from CPTII, the malonyl-CoA-insensitive enzyme (Fig. 4A). It has been hypothesized that some of the residues important for malonyl-CoA sensitivity of CPTI may reside within these 124 N-terminal amino acid residues. Using deletion

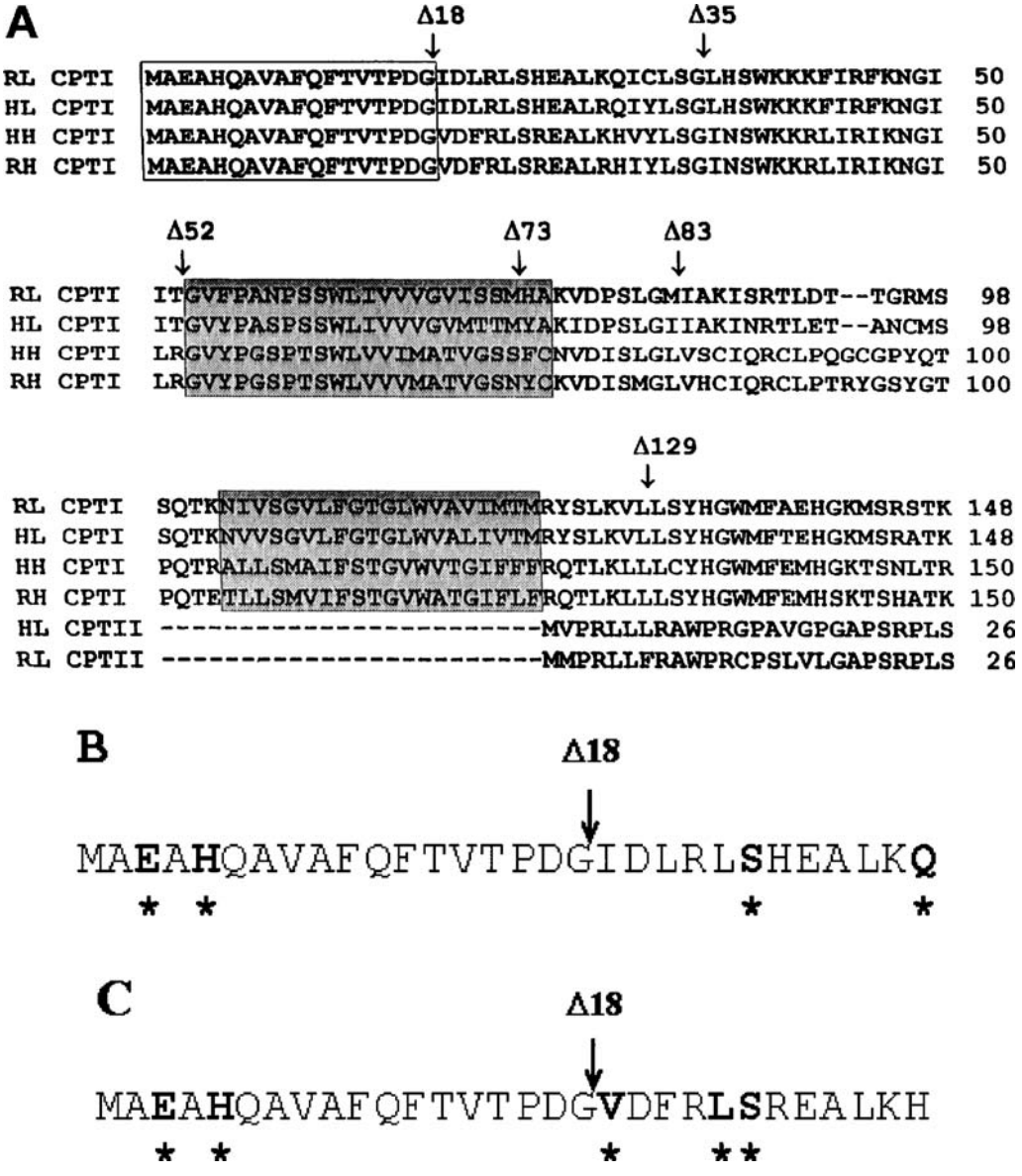

Fig. 4. A: Amino acid sequence of the first 150 N-terminal residues of human and rat liver CPTs; the shaded areas represent the positions of the two predicted membrane-spanning domains of all known CPTIs; the position of each of the deletion mutants is shown by an arrow; amino acid sequence of the first 30 N-terminal residues of (B) rat L-CPTI and (C) human heart M-CPTI; the position of each of the mutations to alanine is shown by a star; HH, RH = human, rat heart; HL, RL = human, rat liver

and substitution mutants of L-CPTI expressed in *P. pastoris*, we have demonstrated that Glu3 and His5 are necessary for malonyl-CoA inhibition and high-affinity binding of L-CPTI but not for catalysis [47, 48]. Since Asp can only partially substitute for Glu3, whereas the Glu3Gln mutation has the same effect as the Glu3Ala mutation, these studies suggest that the negative charge and the longer side chain of glutamate is essential for optimal malonyl-CoA sensitivity [50]. We hypothesize that

the Glu3 to Ala substitution may disrupt hydrogen bonding network or a salt bridge, perhaps to a residue near the active site of L-CPTI. Within the N-terminal region of L-CPTI, in addition to Glu3, the positive determinant of the malonyl-CoA sensitivity of the enzyme, two N-terminal residues, Ser24 and Gln30 were reported to be negative determinants of the malonyl-CoA sensitivity of L-CPTI, because separate or combined mutation of these residues to Ala increased the malonyl-CoA sensitivity of the enzyme, but their effect was entirely dependent on the presence of Glu3, because mutation of Glu3 to Ala overrides their effect [48, 50, 51].

Thus, a short sequence (residues 19–30) within the N-terminus of L-CPTI acts negatively to prevent this isoform from attaining the highest malonyl-CoA sensitivity shown by M-CPTI [42], and the same short sequence within the N-terminus of M-CPTI acts positively to confer the highest malonyl-CoA sensitivity shown by M-CPTI, because deletion of this peptide sequence abolished malonyl-CoA sensitivity (see Fig. 4B, C) [49, 51]. Furthermore, our data clearly establish that residues essential for malonyl-CoA inhibition and binding in M-CPTI are located within the first 28 N-terminal amino acids [49]. Deletion of the first 18 N-terminal residues combined with substitution mutations V19A, L23A, and S24A resulted in a mutant M-CPTI with an over 140-fold decrease in malonyl CoA sensitivity and a significant loss in both high- and low-affinity malonyl CoA binding [51]. A change of Glu3 to Ala resulted in a 60-fold decrease in malonyl CoA sensitivity and loss of high-affinity malonyl-CoA binding. Specifically, the N-terminal residues Glu3, Val19, Leu23, and Ser24 in M-CPTI were found to be important for malonyl-CoA inhibition and binding, but not for catalysis (Fig. 4) [51]. As with L-CPTI, in M-CPTI the effect of Val19, Leu23, and Ser24 on malonyl-CoA sensitivity was entirely dependent on Glu3. These findings were independently confirmed by others [42]. In summary, mutagenesis studies of the N-terminal region of L-CPTI and M-CPTI show that the conserved first 18 N-terminal residues in M-CPTI and L-CPTI have reciprocal effects on the affinity for carnitine and malonyl-CoA sensitivity [42, 47, 48]. As first reported by us, this region controls the IC_{50} for malonyl-CoA in L-CPTI but not in M-CPTI [47, 48], whereas it controls the K_m for carnitine in M-CPTI but not in L-CPTI [42], thus establishing the inverse relationship between the IC_{50} for malonyl-CoA inhibition and the K_m for carnitine for the two isoforms (see Fig. 4A–C).

It has been hypothesized that the major kinetic differences observed between M-CPTI and L-CPTI may be due to interactions between the C- and N-terminal regions that determine the malonyl-CoA sensitivity of L-CPTI as shown by the chimera studies [43, 49, 52]. Replacement of the different segments of the N-terminal domain of L-CPTI with the corresponding N-terminal domain of M-CPTI lowered the affinity for the substrates and the malonyl-CoA sensitivity in the chimeric L-CPTI [43, 49, 52]. In addition, replacement of the N-terminal domain of L-CPTI by a specific outer mitochondrial membrane signal anchor sequence [45], or removal of the two transmembrane domains and the linker region [53] resulted in a partially active protein that is less sensitive to malonyl-CoA inhibition. Thus, the N-terminal domain of L-CPTI is essential for maintaining optimal conformation necessary for both catalysis and malonyl-CoA sensitivity. In the newborn pig liver, perinatal changes in lipid metabolism differ markedly from those described in other mammals, which may be due to very low rates of β-oxidation and ketogenesis

[54–57]. Earlier reports and our more recent studies with the yeast-expressed pig L-CPTI have demonstrated that unlike the rat liver L-CPTI, the pig liver L-CPTI, has a much higher sensitivity to malonyl-CoA inhibition that is characteristic of human or rat M-CPTI enzymes, although the K_m for the substrates are similar for both enzymes [58]. Using chimeras between rat and pig L-CPTI, and deletion mutation analysis, we showed that the difference in malonyl-CoA sensitivity observed between the pig and rat L-CPTI is due to the differences in interaction between the first 18 N-terminal amino acid residues with the C-terminal region of the respective enzymes [59]. The role of the N- and C-terminal interactions as determinant of malonyl-CoA sensitivity was recently confirmed by cross-linking studies using yeast-expressed and native rat liver mitochondrial L-CPTI based on the trypsin resistant folded state of L-CPTI [60]. Therefore, in terms of malonyl-CoA sensitivity, pig L-CPTI behaves like a natural chimera of the L- and M-CPTI isoforms. In contrast, our more recent studies with yeast-expressed pig M-CPTI demonstrate that unlike the corresponding human or rat enzyme, the pig M-CPTI has a high affinity for carnitine and low sensitivity to malonyl-CoA inhibition [61], characteristics similar to those of human or rat liver CPTI, confirming the unusual kinetic properties of the pig CPTI isoforms compared to other mammals that makes them useful models to study the structure-function relationship of the CPTI enzymes.

Identification of C-terminal Residues in CPTI Important for Activity and Malonyl-CoA Sensitivity

Within the C-terminal domain of L-CPTI, three conserved residues, Glu603, Arg601, and Arg606 were found to be essential for malonyl-CoA inhibition, because mutation of these residues significantly decreased malonyl-CoA sensitivity [62]. Since a conservative substitution of Glu603 to aspartate or glutamine resulted in partial loss of activity and malonyl-CoA sensitivity, our data suggest that the negative charge and the longer side chain of glutamate are essential for catalysis and malonyl-CoA sensitivity. We predict that this region of L-CPTI spanning the conserved C-terminal residues may be involved in binding the CoA moiety of palmitoyl-CoA and malonyl-CoA and/or may be the putative low affinity acyl-CoA/malonyl-CoA binding site [62]. This is the first report to demonstrate that the conserved C-terminal residues R601, E603, and R606 are important for L-CPTI activity, malonyl-CoA inhibition and binding (Fig. 5). Our mutagenesis studies further demonstrate that substitution of K560 with Ala resulted in a 13-fold decrease in malonyl-CoA sensitivity and 50% loss in L-CPTI activity (unpublished data). Mutation of the C-terminal Met593 to Ser, a conserved residue in the malonyl-CoA sensitive acyltransferases, CPTI and COT, was also reported to abolish malonyl-CoA sensitivity in L-CPTI [63].

Determination of the Structural Basis for the Differences in Malonyl-CoA Sensitivity Between Rat Liver L-CPTI and Human Heart M-CPTI

The highly conserved C-terminal acidic residues, Glu590 and Asp567 were found to be negative determinants of L-CPTI malonyl-CoA sensitivity, because mutation of these residues to Ala increased the malonyl-CoA sensitivity of the enzyme to that of the wild-type muscle, M-CPTI, suggesting a structural basis for the differences in

Fig. 5. RL-CPTI homology model; a homology model for RL-CPTI was constructed using a threading server (proteins.msu.edu) with the coordinates for carnitine octanoyltransferase as template (COT, 1×l7.pdb); no attempt was made to add missing features to the model; the backbone is shown as a yellow cartoon; several amino acid residues discussed in the text are labeled with their residue number and are shown with their atoms depicted as solid spheres; octanolycarnitine is shown as a green ball-and-stick figure and is positioned based on a COT structure (1×l8.pdb); CoA is shown as a magenta ball-and-stick figure and is positioned based on a carnitine acyltransferase structure (CAT, 1ndi.pdb)

malonyl-CoA sensitivity between the muscle, M-CPTI and liver, L-CPTI isoforms of the enzyme [64]. A change of Glu590 to alanine, glutamine, and lysine in L-CPTI resulted in only a partial decrease in catalytic activity, but caused a significant 9- to 16-fold increase in malonyl-CoA sensitivity close to the level observed with M-CPTI, suggesting the importance of neutral and/or positive charges in the switch of the kinetic properties of L-CPTI to that of M-CPTI (see Fig. 5).

To examine the role of the second highly conserved C-terminal acidic residue, Asp567, on catalysis and malonyl-CoA sensitivity, we separately changed the residue to alanine, histidine, and glutamate. Substitution of Asp567 with glutamate resulted in activity and malonyl-CoA sensitivity similar to the wild-type L-CPTI. A change of Asp567 to alanine or histidine caused a significant 14- to 25-fold increase in malonyl-CoA sensitivity, a 10-fold increase in the K_m for carnitine and a substantial loss in catalytic activity (Table 1, unpublished data). Substitution of Asp567 with the neutral uncharged residue (alanine) and/or a basic positively charged residue (histidine)

Table 1. Activity, malonyl-CoA sensitivity, and kinetic characteristics of wild type and mutant L-CPTI

Strain	Activity	IC_{50}	Carnitine		Palmitoyl-CoA	
	nmol/mg · min	μM	K_m	V_{max}	K_m	V_{max}
			μM	nmol/mg · min	μM	nmol/mg · min
WT	6.0 ± 0.5	2.1	43.3	11.6	95.5	45.1
D567E	6.0 ± 0.7	1.9	86.3	9.9	39.2	14.8
D567A	0.7 ± 0.1	0.08	465.8	6.4	5.0	1.9
D567H	0.6 ± 0.1	0.15	570.0	4.3	4.1	0.9

significantly increased L-CPTI malonyl-CoA sensitivity and decreased its affinity for carnitine to the level observed with the muscle isoform of the enzyme, suggesting the importance of neutral and/or positively charged residues in the switch of the kinetic properties of L-CPTI to that of M-CPTI. A change of the highly conserved Q571 to Ala also caused a 4-fold increase in malonyl-CoA sensitivity and a 50% decrease in L-CPTI activity. The double L-CPTI mutant, E3AE590A was inactive. Our studies identify specific conserved residues in L-CPTI (Q571, D567, and E590) that when mutated to alanine cause a substantial increase in malonyl-CoA sensitivity. This is the first demonstration whereby mutation of two highly conserved acidic residues in the C-terminal region of L-CPTI resulted in a switch of its kinetic properties close to that of the M-CPTI and provides the structural basis for the differences in malonyl-CoA sensitivity between L-CPTI and M-CPTI.

Mapping of C-terminal Residues Important for Substrate Binding and Catalysis

Mutagenesis of the highly conserved arginine and tryptophan residues in the C-terminal region of L-CPTI demonstrated that these conserved residues in L-CPTI are important for catalysis, because they stabilize the enzyme-substrate complex by charge neutralization and hydrophobic interactions [65]. The predicted secondary structure of the 100 amino acid residue region of L-CPTI, containing arginines 388 and 451 and tryptophans 391 and 452, consists of four α-helices similar to the known 3D-structure of the acyl-CoA binding protein [65]. We predict that this C-terminal region spanning the 100 amino acid residues constitutes the putative palmitoyl-CoA binding site in L-CPTI (Fig. 6). Our cysteine-scanning mutagenesis study with the human heart M-CPTI revealed for the first time that a single residue, Cys305 was essential for catalysis [66] (see Fig. 6). A complete loss in L-CPTI activity due to a change of Cys305 to Trp was recently reported in a patient with CPTI deficiency disease [67]. A single change of Cys305 to Ala also inactivated L-CPTI. For M-CPTI, separate substitution mutation of the conserved C-terminal

Fig. 6. Sequence alignment of portions of the C-terminal region of various acyltransferases; abbreviations: L-CPTI, carnitine palmitoyltransferase I liver isoform; M-CPTI, carnitine palmitoyltransferase I muscle isoform; CPTII, carnitine palmitoyltransferase II; OCTC, peroxisomal carnitine octanoyltransferase; CAT, carnitine acetyltransferase; CLAT, choline acetyltransferase

residues D454, H473, and E590 with alanine resulted in complete loss in activity suggesting that these residues may be important for substrate binding and catalysis [unpublished data]. Furthermore, our data demonstrate that deletion of the C-terminal residue L764 or substitution with arginine inactivated M-CPTI, suggesting that L764 may be important for proper folding and optimal activity of M-CPTI [68]. Substitution mutation of the highly conserved C-terminal His473 to Ala resulted in complete loss in catalytic activity in L-CPTI and CPTII suggesting that His473 may be at the catalytic site pocket of CPTI and CPTII [35, 69]. Mutation of the highly conserved Arg601, Arg606, and Arg655 to Ala resulted in a major loss in catalytic activity [65]. Thus, the presence of these conserved arginine residues is probably crucial for maintaining the configuration of the L-CPTI active site. Sequence alignment of the acyltranferase family of proteins shows the presence of a conserved signature motif between R601 and R606, (TET) for the CPTs and carnitine octanyltransferase (COT), (TDT) for the carnitine acetyltransferases (CAT), and (VDN) for the choline acetyltransferases (CLAT) (see Fig. 6). In addition, in the CLATs, R655 is replaced by asparagine. A change of the conserved signature motif VDN to TET together with mutation of N655R in the CLATs altered the substrate specificity of the CLATs from choline to carnitine, suggesting that the carnitine binding site resides between the conserved C-terminal residues R601 and R655 in the CPT and CAT family of carnitine acyltransferases [70]. Our studies show that the mutant L-CPTI with a change of TET to VDN, TET/R to VDN/N, and R655 to asparagine have no L-CPTI activity [unpublished data]. Mutation of the conserved Arg505 in bovine liver carnitine octanoyltransferase (COT), corresponding to Arg655 in CPTI, was found to increase the K_m for carnitine by several fold [71]. Based on the R505N mutation in COT and the recently published COT 3D-structure [72], it was suggested that this conserved arginine residue in carnitine acyltransferases contributes to substrate binding by forming a salt bridge with the carboxylate moiety of carnitine. The recently published 3D-structure of choline acetyltransferase which is similar to the CAT 3D-structure revealed that both lack of electrostatic interaction and steric hindrance to carnitine binding at the active site are important determinants for the choline over carnitine substrate specificity of the enzyme [73]. The L-CPTI mutant with a change of Arg655 to Ala had insufficient activity to allow measurement of the K_m value for carnitine.

A substantial decrease in L-CPTI activity was reported in a patient with CPTI deficiency disease homozygous for the missense mutation of the conserved C-terminal residue D454 to Gly [74]. The 3D-structure of CAT shows that the side chain of Met564 is located within the acyl binding pocket, suggesting that this residue may be an important determinant of substrate acyl chain length specificity because glycine replaces Met564 in the CPTs [75]. Recently, mutation of the CAT residue M564 to glycine was reported to change its substrate specificity from a short chain to that of a medium chain but not long-chain acyl-CoA, indicating the presence of other residues that play a critical role in substrate specificity [76, 77]. Additional C-terminal residue mutations that have been identified to cause a significant decrease in activity in L-CPTI deficient patients include, R357W, L484P, C304W, delR395, P479L, A275T, A414V, Y498C, G709E, and G710E [78, 79]. Based on a CPTI model derived from the CAT 3D-structure, some of the mutations were reported to affect the stability of the protein and/or the enzyme-substrate

complex (A275T, A414V, and Y498C), whereas others (G709E and G710E) were found to be important for catalysis and substrate chain length specificity [75, 79]. Since the primary structure of CAT does not contain the first N-terminal 160 amino acid residues present in L-CPTI which includes the two transmembrane domains, that are essential for activity and stability of the enzyme, conclusions drawn from a CPTI model based on the CAT structure should be viewed with caution, and will have to await confirmation by the actual 3D-structure of CPTI.

Human Inherited CPT Deficiency Diseases

Human genetic defects in fatty acid oxidation specifically ascribed to CPTI, CPTII, and carnitine translocase have been reported [12, 80]. CPTII deficiency, the most common inherited disorder of lipid metabolism affecting the skeletal muscle, is an autosomal recessive disorder with three distinct clinical phenotypes [81–84]. The classic adult muscular form, usually induced by prolonged exercise, exposure to cold, fasting, or infection, is characterized by recurrent episodes of muscular weakness, pain, rhabdomyolysis, and myoglobinuria [85]. A severe early-onset form of the disease, characterized by respiratory distress, hypoglycemia with seizures and hepatomegaly and cardiomegaly with cardiomyopathy, has also been reported in children and newborns [84]. A third infantile form, in some cases triggered by fasting or febrile illness which is characterized by recurrent attacks of acute liver failure with hypoketotic hypoglycemia, coma, seizures, hepatomegaly, and dilated hypertrophic cardiomyopathy and arrhythmias also exists [86, 87]. The human CPTII gene is 20 kb in length, contains five exons, and is located at chromosome Ip32 [88]. More than 25 different mutations and three polymorphisms have been identified in the CPTII gene [12, 89, 90]. In the less severe form of the disease, all mutations reported thus far are missense with a common Ser113Leu mutation, whereas in the infantile form, frameshift and missense mutations have been reported [82–85]. The missense mutations are found in exons 1, 3, 4, and 5 [91]. One missense mutation, S113L, accounts for $\sim$50% of the mutant alleles responsible for the adult myopathic form of the disease [92–94]. Recently, an E487K missense mutation in conjunction with S113L, characterized by recurrent episodes of myalgia and myoglobinuria triggered by fever was reported in a child with CPTII deficiency disease [46]. L-CPTI deficiency diseases have been reported in both fibroblasts and liver cells of infants and children and as maternal illness in pregnancy [95–97]. L-CPTI deficiency, a rare disorder of hepatic mitochondrial long-chain fatty acid transport into the mitochondria, which characteristically presents as recurrent attacks of fasting hypoketotic hypoglycemia has so far been ascribed to 21 homozygous and heterozygous missense and nonsense mutations, two deletions, and an insertion in the L-CPTI gene [98]. To date, there have been no reported cases of patients with M-CPTI deficiency.

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