STRUCTURE-FUNCTION STUDIES WITH MITOCHONDRIAL CARNITINE PALMITOYLTRANSFERASE I AND II

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ABSTRACT

Structure-Function Studies with Mitochondrial Carnitine Palmitoyltransferase I and II

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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 in the presence of L-carnitine 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). As an enzyme that catalyzes the first step in fatty acid oxidation, CPTI is regulated by its physiological inhibitor, malonyl-CoA, and by hormonal and dietary factors. In this study, we identified specific conserved amino acid residues important for activity and malonyl-CoA sensitivity in L-CPTI, M-CPTI and CPTII.

In L-CPTI, we separately conducted substitution mutation analysis on five conserved arginines (R388, R451, R601, R606 and R655), two tryptophans (W391 and W452) and two glutamates (E590 and E603). Our data suggested that conserved arginine and tryptophan residues in L-CPTI contribute to the stabilization of the enzyme-substrate complex by charge neutralization and hydrophobic interactions. A conservative substitution of E603 to aspartate or glutamine resulted in partial loss of activity and

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malonyl-CoA sensitivity. Replacement of the conserved R601 or R606 with alanine also showed over 40-fold decrease in malonyl-CoA sensitivity. We predict that this region of L-CPTI spanning these conserved C-terminal residues may be the region of the protein involved in binding the CoA moiety of palmitoyl-CoA and malonyl-CoA and/or the putative low affinity acyl-CoA/malonyl-CoA binding site. Substitution of E590 with alanine, glutamine and lysine significantly increased L-CPTI malonyl-CoA sensitivity to the level observed with the muscle isoform of the enzyme.

In M-CPTI, deletion and substitution mutation analysis of the extreme C-terminal revealed a conserved leucine residue (L764) is essential for catalysis but not for malonyl-CoA inhibition and binding. In the N-terminal region of M-CPTI, site-directed mutagenesis studies demonstrate that E3, V19, L23, and S24 in M-CPTI are important for malonyl-CoA inhibition and binding. Cysteine-scanning mutagenesis of M-CPTI identified that a single cysteine residue (C305) is important for catalysis. In the liver isoform of CPTII, E487 is essential for catalysis and E500 may play a role in substrate binding and catalysis.

CHAPTER 1 INTRODUCTION

1.1 FATTY ACID SYNTHESIS

Fatty acids are major energy-rich molecules and their oxidation provides a major energy source in most tissues. Fatty acids are also an integral part of cells as membrane components and influence membrane fluidity and receptor or channel function. Fatty acid synthase (FAS) knockout mice including most of the heterozygotes die in utero, suggesting that fatty acid synthesis is very important in embryonic development [56]. Not only are both fatty acids synthesis and oxidation tightly controlled through various pathways, fatty acids also act as signaling molecules regulating the expression of genes involved in fatty acids transport or metabolism in an adaptive manner. Fatty acids are primarily synthesized in the cytoplasm of tissues such as liver, adipose (fat), central nervous system and lactating mammary gland. The rate-limiting step of fatty acid synthesis is the formation of malonyl-CoA from acetyl-CoA by acetyl-CoA carboxylase (ACC) [1]

1.2 FATTY ACID OXIDATION

There are two major sources of fatty acids, the dietary lipid and fatty acids stored in adipocytes as triacylglycerol from *de novo* biosynthesis (Fig. 1.1) [77]. Fatty acids stored in adipocytes as triacylglycerol are mobilized in response to energy demands by enzymes like triacylglycerol lipase, diacyclglycerol lipase, and monoacylglycerol lipase under the control of hormones such as epinephrine, glucagon and insulin. Epinepherine and glucagon activate lipolysis while insulin inhibits fat breakdown. The free fatty acids then diffuse from adipose cells, are loosely bound to albumin in the blood, and are

1



Fig. 1.1 Fatty acid synthesis, transport, and metabolic pathways. FA: fatty acid, ALB: albumin, FAT: fatty acid transporter, FABP: fatty acids binding protein, PL: phospholipids, FA-CoA: fatty acyl-CoA. [Adapted from reference 77]

transported to other tissues for oxidation. These fatty acids either passively diffuse through the plasma membrane as a result of their hydrophobicity or enter the membrane with the aid of transporter proteins. There are at least six known different fatty acid transporter (FAT) proteins: translocase (FAT-CD36), the fatty acid transport protein (FATP), the mitochondrial aspartate aminotransferase, caveolin, the adipose differentiation-related protein and the fatty acid-binding protein (FABP) [77]. In the cell, free fatty acids can be signaling molecules that regulate the expression of specific genes. Once inside the cytoplasm, fatty acids are transported to specific organelles for different uses through the mediation of cytosolic fatty-acid binding proteins (FABP) (Fig. 1.1) or other proteins such as sterol carrier protein-2 (SCP-2) [175]

Fatty acid oxidation is the major energy producer for most tissues. Fatty acids are first activated into acyl-CoAs by the acyl-CoA synthetase in the outer mitochondrial membrane. There are five different acyl-CoA synthetase have been cloned, each producing a distinct enzyme [94, 95, 138, 203, 251].

The longer chain fatty acids cannot diffuse across the mitochondrial membrane so they are transported by the carnitine palmitoyltransferase (CPT) system [176]. Longchain fatty acids binding protein (FABP) promotes fatty acids oxidation by binding and delivering long-chain fatty acids directly to the mitochondrial membrane [268]. Longchain fatty acyl-CoA binding protein (ACBP) interacts with CPTI of the CPT system to facilitate the transfer of the acyl-CoA to CPTI [141]. ACBP also modulates the concentration of free acyl-CoA within the cell. Inside the mitochondria matrix, even number fatty acids are broken down to acetyl-CoA by β -oxidation.

Fatty acids are also oxidized in peroxisomes [152, 548]. This process is catalyzed by a different set of enzymes. It plays functionally complementary but very different roles from those of mitochondrial fatty acids oxidation [109, 110, 171, 228].

1.3 REGULATION OF FATTY ACID METABOLISM

Acetyl-CoA carboxylase (ACC) is the major site of regulation of fatty acid synthesis because it catalyzes the synthesis of malonyl-CoA, the rate-limited step of fatty

acid synthesis [1]. Malonyl-CoA is not only the precursor of fatty acid synthesis but also a critical regulator of mitochondrial fatty acid β -oxidation because it is an inhibitor of carnitine palmitoyltransferase-I (CPTI), which catalyzes the rate-limited step in fatty acid oxidation [112, 176]. Two ACC isoforms, ACC α and ACC β have been cloned and sequenced [8, 1, 2, 107, 166, 255]. Though they catalyze the same reaction, the two isoforms have different physiological roles. This difference is indicated by their tissue distributions and kinetic properties. ACC α is found mainly in lipogenic tissues such as white adipose tissue and lactating mammary gland, while ACCB is highly expressed in energy-producing tissues such as heart and skeletal muscle [20, 129, 231]. Both isoforms are found in liver, where both fatty acid synthesis and oxidation are important [2, 263, 290]. It is hypothesized that ACC α controls fatty acid synthesis and ACC β regulates fatty acids oxidation. Evidence supporting that ACC β controls fatty acids oxidation comes from ACCB knockout mice that exhibit an elevated rate of fatty acid oxidation and less fat accumulation with a normal life span [4]. The malonyl-CoA levels in heart and muscle of knockout mice are 10 and 30 fold lower respectively than those of wild-type mice [4]. It is interesting that the knockout mice have increased sensitivity to insulin as well [4]. When both are fed high-fat/high-carbohydrate diets, the knockout mice weigh less and maintain normal levels of insulin and glucose, while the wild type mice become diabetic with hyperglycemic and hyperinsulinemic status [5]. Not surprisingly, the expression levels of uncoupling proteins (UCPs) were remarkably higher in adipose, heart (UCP2), and muscle (UCP3) tissues of mutant mice than those of the WT mice [5]. Subcellular localization studies of acetyl-CoA carboxylase by immunofluorescence microscopic analysis using affinity-purified anti-ACC2-specific antibodies and transient expression of the green fluorescent protein fused to the C terminus of the N-terminal sequences of ACC α and ACC β show that ACC α is a cytosolic protein and that ACC β is associated with the mitochondria [3]. Immunocolocalization of ACC β with a known human mitochondria-specific protein and the integral mitochondrial membrane associated enzyme, carnitine palmitoyltransferase I further confirmed this conclusion [3]. All these findings support the hypothesis that ACCB regulates mitochondrial fatty acid oxidation

through inhibition of carnitine palmitoyltransferase I by malonyl-CoA and it is a potential drug target for therapy against obesity and related diseases.

1.3.1 Regulation of Gene Transcription by Fatty Acids

Fatty acids play another very important role in the cells as signal molecules regulating the expression of various genes, especially those that are involved in fatty acids transport and metabolism. Fatty acid induced gene up-regulation has been found in various tissues including liver, adipose tissue and small intestine and gene downregulation is seen mainly in the liver [78]. Their effects depend on their chain length and degree of saturation. Saturated and unsaturated long-chain fatty acids or their CoAderivatives are major inducers and polyunsaturated fatty acids are the major repressors of gene expression [78]. Intermediary products of fatty acids metabolism can also regulate gene expression [225]. As a result of the regulation, dietary fatty acids are related to the onset and progression of various diseases, such as cancer [51, 284], atherogenesis [55], hyperlipidaemia [106], insulin resistance [250] and hypertension [190], as well as blood coagulability and fibrinolytic defects [124].

FABP binds free LCFAs with high affinity in cytosol, playing an important role in the control of the cellular fatty acid (FA) flux and protection of cells against the adverse effects of free fatty acids (FFAs) [19, 75, 269]. FABP is abundant in both the liver and small intestine. Experiments conducted in the well-differentiated FAO rat hepatoma cell line showed LCFAs (palmitic acid, oleic acid, linoleic acid, linolenic acid, etc.) stimulated the expression of liver fatty acid-binding protein (L-FABP) in a timedependent and dose-dependent manner [181]. Preincubation of FAO cells with cycloheximide blocks this stimulation, indicating an indirect mechanism and that other protein synthesis is involved [78]. Transcription factors such as peroxisome-proliferatoractivated receptors (PPARs) are possible candidates [78]. Poirier *et al* found out that PPAR δ/α agonist GW2433 up-regulates the expression of L-FABP in the intestine of PPAR α -null mice [216]. In adipocyte, PPAR β/δ is responsible for FA-controlled adipogenesis and adipocyte lipid binding protein (ALBP) induction [105]. Lipogenic genes such as FAS and S14 have sterol regulatory element-binding proteins (SREBP) response elements (SRE) in their promoters [77]. Polyunsaturated fatty acid (PUFA) like linoleate (C18:2 N-6) can suppress transcription of FAS [25] through reducing the level of mature SREBP [294]. In hepatoma cells, unsaturated FAs competitively suppressed the activation of liver X receptor (LXR), an agonist of SREBP 1c gene, thereby down regulating some lipogenic genes [209]. Oleate (C18:1 N-9) also inhibits SREBP 1c expression, but it is unclear why it does not affect lipogenic gene expression [78]. Acyl-CoA oxidase (AOX) catalyzes the first reaction of peroxisomal fatty acid β -oxidation [67]. Its gene has a canonical peroxisome-proliferator-responsive element (PPRE) in the promoter region [78]. After activation by FAs, PPAR α binds to PPRE and enhances the expression of the AOX gene [149]. Rats fed with a high fat diet exhibit an increased AOX mRNA level. Nuclear run-on experiments also demonstrate that fenofibric acid and alpha-bromopalmitate increase AOX gene expression at the transcriptional level [78].

Dietary lipids like fibrates and LCFAs, saturated or unsaturated, induce the expression of L-CPTI [30, 31, 53]. But available evidence suggests their mechanisms are quite different. First, LCFAs stimulates L-CPTI but not CPTII gene expression, while clofibrate induces the expression of both genes [53]. Second, lipooxygenase inhibitors prevent clofibrate from inducing L-CPTI gene transcription, but they have no effect on the regulation of the L-CPTI gene by LCFAs [78]. Third, the effect of LCFA on L-CPTI is reduced by insulin in a dose-dependent manner, while the PPAR α -mediated clofibrate stimulation is insensitive to insulin [78]. Fourth, LCFAs induction of L-CPTI gene expression both in wild type and PPAR \square -null mice [168]. The effect of clofibrate on L-CPTI gene expression is known to be PPAR α -dependent. Further study revealed that the clofibrate-responsive element is a classical direct repeat 1 (DR1) motif in the promoter of the L-CPTI gene, while LCFAs act on elements in the first intron of the gene [168]. It is thus clear that regulation of gene expression by dietary lipids is very complex.

Besides PPARs, SREBP 1 and LXR discussed above, other transcription factors such as ChREBP, HNF4, thyroid hormone receptor (TR) have been identified in FAs-

mediated gene regulation (Fig. 1.2) [78]. Hepatocyte nuclear factor-4 (HNF-4) is an orphan transcription factor of the steroid hormone receptor superfamily with an unusual amino acid in the conserved "knuckle" of the first zinc finger (DGCKG) [246]. HNF-4 is found in kidney, intestine and liver. Acyl-CoA thioesters of long-chain fatty acids bind directly to the ligand-binding domain of HNF-4, which results in the change of oligomeric-dimeric equilibrium of HNF-4 or the affinity of HNF-4 for its cognate promoter element [78]. The change then activates or inhibits the targeted genes depending on the chain length and the degree of saturation of the fatty acyl-CoA ligands. For example, oleate and PUFAs prevent HNF4 from activating the human apolipoprotein CIII gene, while the saturated LCFA palmitate enhances HNF4's ability to promote gene expression [77]. PUFAs were also reported to repress the expression of glucose-6 phosphatase through HNF4, a similar mechanism reported in thyroid hormone receptor (TR)-mediated gene regulation [128, 226]. Free fatty acids (FFAs) and their CoA esters competitively bind TR against its natural ligand triiodothyronine (T3), and thus inhibit the expression of T3-dependent genes [128]. In vivo, the inhibition is more prominent in the heart than in the liver and kidney [300]. It is proposed that there might be a crosstalk between TR and PPARs in regulating peroxisome proliferator-responsive genes because of the competition between TR and PPAR for limiting amounts of the heterodimerization partner RXR and for binding to PPRE [125]. However, in vivo, TR appears to have positive effects with RXR and PPAR in promoting peroxisome proliferator-dependent trans-activation [78]. Docosahexaenoic acid (DHA), a long-chain PUFA abundant in the adult mammalian brain, is found to bind all three RXR isoforms and enhances the expression of RXR-responsive genes, suggesting that TR might play a role in PUFA regulation [71]. The above discussions clearly indicate that FA regulation of gene transcription is very complicated and various pathways act independently or collectively to provide macro- and micro-adaptation to the changing environment in different tissues. Among them, the most important one is the PPAR-mediated pathway.





1.3.2 Introduction to PPARs

Peroxisome proliferator-activated receptors (PPARs) are nuclear receptors present in several organs and cell types, which bind to specific DNA sequences and regulate gene expression when they are activated by physiological or pharmacological ligands [101]. Issemann & Green first reported PPAR in 1990 as a ligand-activated transcription factor, which specifically interacted with drugs that were known to cause peroxisomal proliferation in liver [101]. PPARs are involved in the differentiation of many cell types (hepatocyte, fibroblast, adipocyte, keratinocyte, myocyte, and monocyte/macrophage) and these nuclear receptors are now attracting the attention of many medical specialties and the pharmaceutical industry. There are four different PPAR isoforms (PPAR α , PPARy, PPAR δ and PPAR β) and each isoform is encoded by a different gene. These isoforms share a very close structural homology [264]. They have a common transcription function (termed AF-1) at the least conserved N-terminal region containing putative phosphorylation sites; a highly conserved DNA-binding domain in the Cterminal region consisting of two zinc fingers; and a dimerization domain and a hydrophilic pocket, which binds to the ligand (Fig. 1.3) [101]. In order to bind to DNA, PPARs first form dimers with RXR, another nuclear receptor that is itself activated by 9cis-retinoic acid [142]. PPAR isoforms have different tissue distributions. PPARa is relatively abundant in tissues with a high oxidative capacity, such as intestine, pancreas, liver, skeletal muscle, kidney, heart and adrenals [101]. PPARy is primarily expressed in adipose tissue, intestine, mammary gland, endothelium, smooth muscle cells and macrophage/foam cells [101]. The presence of PPARy in cardiac muscle cells is very low [9, 32, 143, 256]. PPARy mRNA in isolated cardiac myocytes can only be detected by RT-PCR [101]. PPAR8 has a widespread tissue distribution in embryonic kidney and lipid metabolizing tissues such as small intestine, heart, adipose tissue, skeletal muscle, and developing brain [101]. All three PPAR isoforms are found in skeletal and cardiac muscle cells, suggesting that each isoform could potentially play a role in the regulation of muscle metabolism. PPAR α and γ are the two main categories of these receptors, which are both characterized by their ability to influence lipid metabolism, glucose



Fig. 1.3 Domain structure of the nuclear hormone receptor. AF-1: ligand-independent transactivation function, DBD: DNA-binding domain. LBD: ligand-binding domain, AF-2: ligand-dependent transactivation function. [Adapted from reference 101]

homeostasis, cell proliferation, differentiation and apoptosis, as well as the inflammatory response, by transcriptional activation of target genes [101].

PPARs are activated by PPAR ligands. PPAR ligands exist naturally (such as medium and long chain fatty acids and eicosanoids) or can be synthesized (such as peroxisome proliferators, and hypolipidemic, anti-inflammatory and insulin-sensitizing drugs). PPAR α is activated by fatty acids, eicosanoids and fibrates, while PPAR γ activators include arachidonic acid metabolites, oxidized low-density lipoprotein and thiazolidinediones [101]. In vitro acyl-CoA esters inhibit PPAR α activity by inducing recruitment of the co-repressor protein NcoR [81]. Hence the fatty acid and fatty acyl-CoA ester ratio may function as agonists and antagonists and thus determine the transactivating activity of PPAR α within the cell [101].

Once activated, PPAR heterodimerizes with the retinoic-acid X receptor (RXR). The heterodimer subsequently interacts with a peroxisome proliferator response element (PPRE) or fatty acid response element (FARE) in the target genes (Fig. 1.4) [140]. Binding of liganded PPAR results in the recruitment of the basic transcriptional machinery and activation of transcription. The interaction of nuclear receptors with the RNA-synthesizing sequence requires co-activators that can specifically and differentially modulate the biological activity of PPAR. PPAR co-activators include the steroid receptor co-activator SRC-1, the PPAR binding protein (PBP), the PPARγ co-activators PGC-1 and PGC-2, and the integrator protein p300 [97, 222, 317, 318]. PGC-1 is one of the most important co-activators not only because it is relatively abundant in heart and slow-twitch skeletal muscle and but also because it interacts with several other nuclear hormone receptors, including the nuclear respiratory factors NRF-1 and NRF-2 that are important for mitochondrial biogenesis [159, 160]. In liver, PGC-1 expression is strongly stimulated by cAMP suggesting that this second messenger affects PPAR transcriptional activity via PGC-1 [308].

PPAR activity is tightly regulated by the availability of ligands, competition for the mutual dimerization partner RXR and for PPRE-sites, the recruitment of co-activators and repressors, and the degree of phosphorylation to adjust the level of lipid metabolism to variations in physiological and pathological conditions [12, 14, 49, 50, 137].



Fig. 1.4 Proposed mechanism for the PPAR-complex mediated transcriptional regulation. PPAR: peroxisome-proliferator-activated receptor, PPRE: peroxisome proliferator response element, RXR: retinoid X receptor. [Adapted from reference 140]

PPAR regulates genes encoding proteins directly involved in inter-organ lipid transport, lipid uptake or fatty acid metabolism such as carnitine palmitoyltransferase I (CPTI) [265, 297, 309]. Peroxisome proliferator-activated receptors regulate energy homeostasis and various metabolic processes by controlling the expression of these genes. They are regarded as appealing therapeutic targets for inflammation and atherosclerosis, obesity, insulin resistance, etc [215].

1.3.3 PPARa

The receptor PPARa is found in liver and heart. It promotes genes in liver for lipid metabolism such as acyl-CoA oxidase for β-oxidation of fatty acids and cytochrome P450 for omega oxidation. PPARa's ligands, apart from synthetic compounds of the fibrate class, include polyunsaturated fatty acids and eicosanoids, which are precursors of prostaglandins [101]. Many enzymes involved in fatty acid oxidation are reported to be regulated by PPARa (Fig. 1.5) [88]. In the starved state, PPARa's activation causes a shift from carbohydrate to fat oxidation [279]. When fed a high-fat diet, PPARa-null mice accumulate fat in their livers. The pivotal role of the PPARa isoform in cardiac fatty acid metabolism has been confirmed in PPARa-null mice [279]. mRNA levels of MCPT-I. long chain acyl-CoA dehydrogenase (LCAD), medium chain acyl-CoA dehydrogenase (MCAD) and acyl-CoA oxidase in the hearts of PPARa-null mice is lower than those in wild-type mice under baseline conditions [279]. Overall myocardial β -oxidation of the medium and long chain fatty acids is significantly reduced in the PPAR-null mice as compared with the wild-type mice, suggesting that mitochondrial fatty acid oxidation is impaired in the absence of PPARa [279]. However, overall myocardial β -oxidation of the very long chain fatty acid (lignoceric acid) remains virtually unchanged, indicating expression of enzymes involved in peroxisomal β -oxidation is independent of PPAR [279]. PPARa-null mice are found to have the same phenotype as that of humans with PPARa genetic defects, suggesting PPARa plays a pivotal role in the cellular metabolic response to fasting [156]. Enzymes normally induced during fasting in the heart and liver, such as medium-chain acyl-CoA dehydrogenase, CPT-I, acyl-CoA oxidase and cytochrome P450 4A3 are not induced in PPARa-null mice [156]. The results provide





compelling evidence for the regulatory role of PPAR α in the expression of genes encoding proteins involved in cardiac fatty acid metabolism.

Recent study on the role of PPARa in fuel metabolism in skeletal muscle was also conducted using PPARa-null mice [192]. Levels of glucose, lactate, ketones and nonesterified fatty acids were found to be lower in PPARa-null mice than those in wildtype mice after exhaustive exercise or starvation [192]. KO mice exhausted earlier than wild-type and exhibited greater rates of glycogen depletion in liver but not skeletal muscle during exercise [192]. There was no significant decrease in fatty acid oxidative capacity and in PDK4 and UCP3 expression between skeletal muscle of KO and wildtype mice under baseline conditions, though they are markedly different between genotypes in heart [192]. Real time quantitative PCR analyses showed that PPAR δ is much more abundantly present in skeletal muscle than either PPAR α or PPAR γ , which is very different from that in liver and heart [192]. PPARδ agonist GW742 increased fatty acid oxidation about 2-fold and induced expression of several lipid regulatory genes, including PDK4 and UCP3. The response is very similar to that induced by the PPAR\delta agonist GW647 [192]. These results suggest regulation by PPARa in skeletal muscle is possibly redundant and the lack of PPARa in knockout mice could be compensated by the abundance of PPAR δ [192].

1.3.4 PPARy

PPAR γ is the most extensively studied member of the PPAR family. It is expressed mainly in adipose tissue [101]. PPAR γ not only functions as a central transcriptional regulator of both adipogenic and lipogenic genes, but also regulates glucose homeostasis and insulin sensitivity [249]. It promotes adipocyte differentiation from fibroblasts and promotes lipid storage in mature adipocytes by up-regulating genes for fatty acid-binding protein and acyl CoA synthetase [140]. It is considered to play a pivotal role in syndrome X, a collective metabolic syndrome including obesity, insulin resistance (IR), hyperlipidemia, and hypertension [155]. Thiazolidinediones (TZDs), a class of antidiabetic agents used to improve insulin sensitivity in human patients suffering from type 2 diabetes, are high-affinity PPAR γ ligands, suggesting the importance of PPAR γ in syndrome X [155]. TZDs also enhance adipocyte differentiation [261, 204] and ameliorate insulin resistance [153, 199]. Another evidence is that point mutations in the ligand-binding domain of PPAR γ result in diabetes and hypertension in humans [16]. Adipose-specific PPAR γ -null mice exhibit markedly less adipocytes and hypertrophy, elevated levels of plasma free fatty acids and triglyceride, and decreased levels of plasma leptin and ACRP30 [116]. Though their blood glucose, glucose and insulin tolerance, and insulin-stimulated muscle glucose uptake remain unchanged, the PPAR γ -null mice are much more susceptible to high-fat diet-induced steatosis, hyperinsulinemia, and insulin resistance [116]. The cAMP-responsive transcription factor CREB also controls hepatic lipid metabolism through nuclear hormone receptor PPAR γ [118]. In response to pancreatic glucagon and adrenal cortisol CREB inhibits the expression of PPAR γ and induces PGC-1 (PPAR γ coactivator 1) during fasting to coordinate hepatic lipid and glucose metabolism [117, 224, 270, 308].

1.3.5 PPARδ

PPAR δ exists in many tissues, especially where fatty acids are a preferred energy source, such as muscle [101]. Most of PPAR δ -null mice do not survive due to placental defects and the few survivors show systemic disturbances in lipid metabolism [11]. PPAR δ transgenic mice selectively expressing a high level of PPAR δ in their adipocytes showed significant reduction in body weight compared to wild-type [278]. Some mice did not have any visible white fat at all, indicating higher-level fatty acid oxidation [278]. There was no reduction in the number of adipocytes; however, the size and triglyceride content of adipocytes were reduced [278]. Serum triglyceride levels were much reduced, although cholesterol levels remained unchanged [278]. Transgenic mice also gained less than half the body weight that wild-type mice fed with the same high fat diet gained [278]. At the same time, serum triglycerides and free fatty acids in the transgenic mice fed the high fat diet remained at a low level [278]. The experiments supported the conclusion that PPAR δ expression in adipose tissue increased fatty acids oxidation and prevented diet-induced obesity. A synthetic agonist named GW 501516 can activate the PPAR δ receptor of adipocytes preventing obese db/db mice from accumulation of fat in brown fat and liver, suggesting PPAR δ is an ideal target for anti-obesity drug screening [205]. Both in vivo and vitro studies revealed that PPAR δ not only up-regulates fatty acid oxidation directly, but also uncouples ATP formation in brown fat, leading to heat production at the expense of ATP [278]. In heart, cre-loxP-mediated cardiomyocyte-restricted deletion of PPAR δ was shown to downregulate constitutive expression of key fatty acid oxidation genes such as M-CPTI and decrease basal myocardial fatty acid oxidation [54]. Similar to PPAR α knockout mice, such a decrease in fatty acid oxidation gene transcription is reversibly restored by PPAR α -selective ligand but not PPAR δ -selective ligand [278].

1.3.6 Regulation of CPTI Expression by PPARs

PPARα and its coactivator, PGC-1α, regulate CPTI expression [33, 270, 154]. PGC-1a plays an important role in mitochondrial biogenesis and energy metabolism in brown adipose tissue, skeletal muscle, and heart through activation of specific transcription factors [224]. Both calcineurin and CaMK directly activate transcription of the M-CPTI gene, but only calcineurin activates the L-CPTI and CPTII genes [236]. Long-chain fatty acids are among the ligand activators of PPARa and the fatty acid response element (FARE-1) in the M-CPTI gene is located between -775 and -763 bp upstream of the initiator codon [33, 311]. FARE-1 interacts with the PPAR α and the retinoic acid receptor heterodimers to confer transcriptional activation of the M-CPTI gene. High levels of long-chain fatty acids thus stimulate their own metabolism by transcriptional activation of PPARa. PPARa- and PGC-1a-mediated control of cardiac metabolic gene expression is activated during postnatal development, short-term starvation, diabetes, and in response to exercise training [13, 89]. Administration of the M-CPTI inhibitor, etomoxir, to PPARa-null mice resulted in death of all male mice, compared to 25% of females, which were rescued by estrogen treatment [74]. PGC-1a and MEF2A synergistically activate M-CPTI promoter activity, which is repressed by overexpression of USF proteins [185]. PGC-1a regulates L-CPTI gene expression by cAMP in combination with HNF4a and CREB [167]. More recently, PGC-1a was reported to activate L-CPTI gene expression primarily through multiple sites in the first

intron [248]. In cardiomyocyte-restricted PPARδ knockout mice, both transcription of the MCPTI gene and the protein level were significantly decreased [54].

1.4 THE ROLE OF THE CPT SYSTEM IN CELL METABOLISM

Mitochondrial β -oxidation of long-chain fatty acids (LCFA) is the major source of energy production in animals. The primary sources of fatty acids for oxidation are dietary and mobilization from cellular stores. Fatty acids from the diet are delivered from the gut to cells via transport in the blood [77]. Fatty acids are stored in the form of triacylglycerols primarily within adipocytes of adipose tissue. In response to energy demands, the fatty acids stored in the form of triacylglycerols can be mobilized for use by peripheral tissues. The release of metabolic energy, in the form of fatty acids, is controlled by a complex series of interrelated cascades that result in the activation of hormone-sensitive lipase [77]. Fatty acids must be activated in the cytoplasm before being oxidized in the mitochondria. Activation is catalyzed by fatty acyl-CoA synthase (also called acyl-CoA ligase or thiokinase). The net result of this activation process is the consumption of 2 molar equivalents of ATP.

Oxidation of fatty acids occurs in the mitochondria. How the activated fatty acids are transported from cytoplasm to the mitochondria was a mystery before Fritz revealed the essential role of carnitine in the oxidation of long-chain fatty acids in the mid-1950s [92, 93, 34]. It turned out that the transport of fatty acyl-CoA into the mitochondria is accomplished via an acyl-carnitine intermediate, which itself is generated and reconverted by the action of the CPT system [176].

The CPT system is composed of three major proteins: carnitine palmitoyltransferase I (CPTI), carnitine/acyl-carnitine translocase and carnitine palmitoyltransferase II (CPTII). CPTI is an integral membrane protein located on the outer mitochondria membrane, while CPTII is an enzyme loosely associated on the matrix side of the inner mitochondria membrane [21, 98, 108, 120, 179, 193, 194]. Carnitine-acyl-carnitine translocase is an integral inner mitochondrial transport protein. CPTI converts long-chain fatty acyl-CoAs to acyl-carnitine in the presence of Lcarnitine:

palmitoyl-CoA + L-carnitine $\leq >$ CoA + L-palmitoylcarnitine The acyl-carnitine is then transported across the inner mitochondria membrane by the translocase to the site of CPTII. CPTII catalyzes the regeneration of the fatty acyl-CoA molecule [176]. The overall function of the CPT system is to transport activated LCFA from the cytosol to the mitochondrial matrix where β -oxidation takes place. Fritz and Yue first hypothesized the presence of this carnitine-dependent multi-step process for the transport of activated fatty acids to mitochondria. (Fig. 1.6) [176].

Two major developments in the 1970s highlighted the biomedical relevance of the CPT system. First, it was recognized that the genetic defects related to the mitochondria CPT system cause some serious human deficiency diseases (HDD) [73]. Second, It was revealed that CPTI plays a critical role in the regulation of fatty acid oxidation at least in liver [22, 180]. These important observations stimulated intense interest in the structure/function studies of the CPT system. The cloning, expression and mutagenesis studies of the CPT genes in recent years have provided much deeper understanding of the structure/function relationship, regulation and physiological role of the CPT system.

As the rate-limiting enzyme regulating fatty acid oxidation in mitochondria, CPTI is inhibited by malonyl-CoA, the first intermediate in fatty acid synthesis, suggesting coordinated control of fatty acid oxidation and synthesis [177]. This inhibition can be overcome by high levels of long chain fatty acyl-CoA, suggesting competitive displacement of malonyl-CoA by long chain fatty acyl-CoA from the M-CPTI binding site [35, 178]. The malonyl-CoA/CPTI interaction plays an important role in metabolic signaling in mamalian tissues such as the liver, heart, skeletal muscle and pancreatic β -cells [10, 164, 165, 179, 235].

Malonyl-CoA inhibition of CPTI plays a critical regulatory role during exercise. Pre-exercise carbohydrate ingestion increases the availability and utilization of carbohydrate during exercise, thus suppressing lipolysis at the point that limits the uptake and oxidation of FFA by muscle mitochondria [61, 121, 243, 244,285]. This effect is



Fig. 1.6 Reactions catalyzed by mitochondrial carnitine palmitoyltransferase (CPT) I and II. OMM: outer mitochondrial membrane, IMS: intermembrane space, IMM: inner mitochondrial membrane. [Adapted from reference 176]

possibly mediated by an increase in muscle malonyl-CoA concentration due to the higher rate of glycolysis [80, 234, 287, 288, 289].

1.5 THE PRIMARY STRUCTURE OF CPT

CPTI and CPTII are catalytically distinct enzymes encoded by different genes. There is only a single form of CPTII expressed in all oxidative tissues. The cDNAs of human and rat liver CPTII have been cloned and sequenced [90, 292]. CPTII from human and rat have 87% identity at the cDNA and 82% identity at the amino acid level. The human liver CPTII mRNA is approximately 0.5 kb larger than rat liver CPTII mRNA. The open reading frame (ORF) of the CPTII cDNA is 1974 bp encoding 658 amino acids. The 25 amino acid N-terminal leader sequence of CPTII is cleaved upon mitochondrial import and the mature CPTII is approximately 71 kDa [44, 292].

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 [219, 282, 314]. 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 [219]. L-CPTI and M-CPTI are encoded by different genes localized on chromosome 11q13.1-13.5 and 22q13.31-13.32, respectively [6, 36, 37, 267]. The human CPTIC is located on chromosome 19q, but the organization of the gene structure is not known. 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 [150]. CPTIC is only expressed in the brain and testis, and M-CPTI is very highly expressed in the cerebellum. L-CPTI is expressed in the liver, brain, pancreatic beta cells, kidney, fibroblast, heart and non-adipocytes of brown adipose tissue [36, 83, 84, 211, 282, 304, 314]. M-CPTI is found in skeletal muscle, heart, testis, and white and brown adipocytes [83, 304, 305, 315]. The liver isoform of CPTI makes up only 2-3% of the total CPTI in
the adult heart and approximately 25% in the newborn [293, 299]. The CPTI cDNA from rat and human liver, heart, skeletal muscle and brown adipose tissue have been cloned and sequenced [83, 84, 282, 304, 306, 314, 315]. The open reading frame of rat and human M-CPTI is 2316 bp encoding a protein of 772 amino acids and the open reading frame of rat and human L-CPTI is 2319 bp encoding a protein of 773 amino acids. L-CPTI mRNA is 4.7 kb with a long 2 kb 3'-untranslated region and M-CPTI mRNA is 3 kb. The human and rat L-CPTI or M-CPTI have 86% identity at the amino acid level. However, M-CPTI and L-CPTI of the same species have only 63% identity [72, 314]. Interestingly, all isoforms of CPTI from different species have the same first 18 Nterminal amino acids.

Based on limited proteolysis studies, Fraser has proposed a model for the membrane topology of CPTI predicting 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. 1.7) [91]. In this model, most of the protein (90%) is located in the cytosol.

The L-CPTI gene is composed of 20 exons, spanning 60 kb of DNA. Within the 5' upstream region of the gene, two alternate promoters and numerous transcription factorbinding sites were identified. In the 3' untranslated region, the major polyA signal was suggested to lie about 2 kb downstream of the stop codon. Six new mutations in four liver CPTI-deficient patients, namely Q100X (exon 4), A414V (exon 11), Y498C (exon 13), 1876-1G>A (intron 15), a 113-bp intronic insertion in the mature CPT1A mRNA (exon 13-14 junction), and a large 8-kb deletion encompassing intron 14 to exon 17 were characterized based on the genomic information [104]. For M-CPTI, the mouse and rat genes both contain 19 exons and 18 introns. The introns of these compact genes are 80% (mouse versus rat) and 60% (mouse versus human) identical. For some species (sheep and goat), the promoter sequences contain a short interspersed repeated sequence (SINE) upstream of highly conserved regulatory elements. These conserved elements constitute two promoters in humans, sheep, mice, and rats. Except for some differences in splicing, the transcriptional organization of these genes is very uniform. In humans extensive splicing and splice variation is found in the 5'- and 3'-untranslated regions [266]. This



Fig. 1.7 Model for carnitine palmitoyltransferase (CPT) I membrane topology. OMM: outer mitochondrial membrane, IMS: intermembrane space, IMM: inner mitochondrial membrane. [Adapted from reference 293]

valuable information will help us understand how CPT activities are regulated at the transcription level.

The genomic DNA encoding mouse M-CPTI has been isolated and its transcription initiation site is compared with that of its counterparts in human and rats [302]. The mouse M-CPTI gene appears to have multiple initiation sites similar to the rat and human genes. The upstream fatty acid response element for peroxisome proliferators is also found in the mouse M-CPTI gene.

1.6 THE ROLE OF THE CPT SYSTEM IN HUMAN HEALTH AND DISEASES

1.6.1 Ishemic, Ischemic-Reperfused Heart

Plasma long-chain fatty acids are the primary source for energy production in the heart. Fatty acid oxidation normally provides 60-70% energy requirements of the heart [23, 52, 148, 196, 232]. Utilization of fatty acids is reduced in the ischemic, and ischemic-reperfused myocardium because of the limited supply of O₂ for oxidation and impaired ATP production. Furthermore, perfusion with fatty acids results in high rates of fatty acid oxidation, and accumulation of harmful metabolic intermediates that cause in situ membrane damage and cardiac dysfunction. It is very common that levels of fatty acids become higher following a myocardial infarction [191, 206, 208] or during and following cardiac surgery [165, 252]. These high levels of fatty acid oxidation could result from decrease in malonyl-CoA levels and increase in M-CPTI activity. High rates of fatty acid oxidation stimulate the 5'-AMP-activated protein kinase, which in turn inhibits acetyl-CoA carboxylase resulting in decreased malonyl-CoA levels, uncontrolled M-CPTI activity and fatty acid oxidation. Low ACC activity could be reversed in vitro by addition of 10 mM citrate, suggesting that the phosphorylated enzyme was in the inhibited state during reperfusion. Activation of AMPK during ischemia and reperfusion may result in the phosphorylation and inhibition of ACC during reperfusion [148]. The decrease in the sensitivity of CPTI to malonyl-CoA inhibition may also contribute to the high level of fatty acid oxidation. Heart contains an active malonyl-CoA decarboxylase,

which could potentially decrease malonyl-CoA levels and increase fatty acid oxidation [309]. Whereas decreased synthesis and decarboxylation may play a role in lowering malonyl-CoA levels and increasing fatty acid oxidation in the ischemic-reperfused heart, recent reports indicate reperfusion with fatty acids could also induce PPAR α , a transcriptional activator of CPTI gene resulting in increased fatty acid oxidation [163]. High rates of fatty acid oxidation in the ischemic-reperfused heart would result in accumulation of acylcarnitines, which have been associated with cardiac arrhythmias [60]. Inhibition of CPTI prevents increase in long-chain acylcarnitines and reduces the incidence of malignant cardiac arrhythmias, thus confirming the central role CPTI plays in lipid metabolism, making it a possible target for pharmacological intervention in the prevention of sudden cardiac death in patients with ischemic heart disease [99].

1.6.2 The Role of the CPT System in NIDDM and Obesity

Pancreatic islets express the liver isoform of CPTI (L-CPTI) [10]. In the pancreatic β -cells, glucose is a potent stimulator of insulin secretion. Glucose stimulates insulin secretion in part by increasing cellular ATP resulting in closure of the β -cell plasma membrane K_{ATP} channel [218]. Glucose may also enhance insulin secretion by regulating lipid metabolism since glucose causes an increase in malonyl-CoA levels in β cells followed by a rise in insulin secretion [218]. Inhibition of CPTI, a key enzyme in fatty acid oxidation, by high levels of malonyl-CoA results in a rise in long-chain fatty acyl-CoAs. Higher levels of cytosolic long-chain acyl-CoAs in turn stimulate insulin secretion possibly through interaction with the K_{ATP} channel. This concept links fuel metabolism to insulin secretion in β -cell signal transduction [218]. It suggests that acetyl-CoA carboxylase and CPTI act as fuel sensors in the β -cell, monitoring the concentration of all circulating fuel levels in the β -cell, liver, muscle and adipose tissue. This hypothesis suggests that changes in malonyl-CoA and long-chain acyl-CoA metabolism contribute to altered insulin release and sensitivity [218].

1.6.3 CPT and Human Deficiency Diseases (HDD)

CPTII deficiency is the most common disorder in CPT related human deficiency diseases, resulting in the inability of muscle cells to metabolize long chain fatty acids. The most common disorder of CPTII deficiency is the 'adult onset' form, but there are also two rare infantile forms of CPTII deficiency [27, 69, 259, 274]. Symptoms of CPTII deficiency consist of attacks of myalgia, cramps, muscle stiffness, painful muscles or muscle weakness [271, 260]. In the case of a severe attack, these symptoms progress to myoglobinuria, caused by rhabdomyolisis [245].

CPT deficiency was first reported in the 1970's [73, 82]. Amino acid substitution or missense mutations cause most of the CPTII deficiency. One missense mutation, S113L, accounts for ~50% of the mutant alleles responsible for the adult myopathic form of the disease [26, 258, 276]. Some other CPTII mutations also have been identified, including: R631C [258, 259], P50H [271], D553N [271], Y628S [27], E174K [280, 301], F383Y [280, 301], R124Stop [306, 307], E489K [45], Y120C, I502T and a 36-38 insGC mutation that results in early termination of translation [172]. These mutations result in partial or total loss of CPT activity. Recently, several new forms of CPT deficiency were found in patients with P50H, S113L, V3681, M647V and two truncation mutations [275]. A novel splice site mutation (a new g->a splice-site mutation in the splice-acceptor site of intron 2 was also identified [247].

CPTI deficiency is one of several life-threatening disorders of long-chain fatty acid oxidation and energy metabolism. Most of the case reports of CPTI deficiency are of the L-CPTI since M-CPTI may be too critical for life because the importance of the enzyme for normal heart function. CPTI deficiency can be treated with medium-chain triglycerides, which bypass the CPT-mediated, carnitine-dependent mitochondrial transport system [85, 86, 114, 115].

CPTI deficiency is more rare or possibly under-diagnosed than CPTII deficiency. Dozens of cases of CPTI deficiency have been described since the first report of CPTI deficiency in 1981 [18, 38, 73, 29]. It was reported that a CPTI deficiency disease due to a D454G substitution mutation resulted in 98% loss of the CPT activity [126]. Recently, CPTI mutations G710E (2129G \rightarrow A) [217, 220], R357W, L484P, R123C, P479L, C304W, A275T and deletion of R395 [39] were found in patients. Among these mutations, some decreased CPTI activity (R357W, L484P, R123C, P479L), and others caused total loss of activity (G710E, C304W and deletion of R395). However, the mutant A275T had no effect on activity. The mutant P497L also decreased malonyl-CoA sensitivity, which is similar to the effect observed with mutants R601A, R655A, E603D that were constructed in our lab [262].

Recently, five missense mutations, namely A275T, A414V, Y498C, G709E, and G710E were mapped to a 3-D structure model of the human L-CPT1 based on the crystal structure of homologous enzyme mouse carnitine acetyltransferase (CAT) [103]. Some amino acids were close to the active site (G709E and G710E), forming the catalytic core of the enzyme. Some were not in the vicinity of the active site (A275T, A414V and Y498C), but they are important in maintaining the natural structure of the protein and the stability of enzyme-substrate complex. Mutations in functional determinants result in dramatic loss of activity or inactive enzyme, while mutations in structural determinants mostly decrease enzyme efficiency [103].

1.7 FUNCTIONAL EXPRESSION AND PURIFICATION OF CPTs

1.7.1 Expression and Purification of CPTII

CPTII is loosely associated on the matrix side of the inner mitochondrial membrane, which makes it much easier to express and purify than CPTI. cDNAs corresponding to the precursor and mature forms of rat CPTII have been expressed in *E. coli* [43]. Rat CPTII expressed in *E. coli* is active and has characteristics similar to those of the enzyme in mitochondria. The human CPTII cDNA contain an artifactual termination signal for T3 RNA polymerase that could be bypassed by the T7 polymerase. The cDNA subcloned into the expression vector, pCMV4 was transfected into COS cells, resulting in a 6-fold induction of mitochondrial CPTII catalytic activity [292]. The cDNA encoding rat liver CPTII was heterologously expressed in a recombinant baculovirus/insect cell system [136]. The recombinant CPTII (rCPTII) expressed in baculovirus-infected insect cells is mostly soluble, which is different from the expression in *E. coli*. Milligram quantities (up to 1.8 mg/l of culture) of active rCPTII were chromatographically purified from large-scale cultures of insect cells infected with the recombinant baculovirus [136]. In *S. cerevisiae*, the expression of CPTII cDNA yielded high CPT activity [41]. Yeast *P. pastoris*, a system devoid of endogenous CPT activity was also used to express CPTII [72]. The kinetic studies of the yeast expressed CPTII showed that the properties of the expressed CPTII are similar to those of the intact inner mitochondrial protein.

1.7.2 Expression and Purification of Liver and Muscle CPTI

The cDNA encoding rat liver CPTI subcloned into the expression vector, pCMV6, was transfected into COS cell, resulting in a selective and 10-20-fold induction of a malonyl-CoA- and etomoxir-CoA-sensitive CPTI activity [84]. Rat liver CPTI cDNA also was expressed in S. cerevisiae and in the yeast P. pastoris, producing an expected size, membrane-bound, malonyl-CoA sensitive and detergent-labile protein [42, 72]. The M-CPTI cDNA isolated from rat brown adipose tissue and heart has been expressed in COS cell [83]. The human heart/skeletal muscle isoform of CPTI has only been expressed in P. pastoris [314, 315], a yeast with no endogenous CPT activity, producing an 80-kDa protein that was located in the mitochondria. Isolated mitochondria from the M-CPTI expression strain exhibited a malonyl-CoA-sensitive CPTI activity that was detergent labile. This is the first report of the expression of a heart CPTI in a system devoid of endogenous CPT activity and the functional characterization of a human heart M-CPTI in the absence of the liver isoform and CPTII. Later on, the human heart M-CPTI gene was expressed at high levels from a strain of the P. pastoris containing approximately 24 copies of the expression plasmid [315]. Levels of M-CPTI were high, more than ten-fold higher than those with a single-copy strain and were sufficient to perform reconstitution studies on the membrane protein, a key step in purification and structural analysis of the enzyme [315].

The successful expression of CPTII and CPTI isoforms made the structure/function study of these enzymes much more convenient. Before the expression of those enzymes, the relationship between CPTI and CPTII and the characteristic differences between liver CPTI and heart/skeletal muscle CPTI have long been a controversy. These studies established unequivocally that CPTII and CPTI are distinct proteins.

1.8 STRUCTURE AND FUNCTION STUDIES OF THE CPT SYSTEM

The structure-function relationship and regulation of CPTI have generated much interest, but has been limited due to the lack of the 3-D structure of the CPTs and other acytransferases. The cloning and sequencing of cDNAs encoding CPTI, the elucidation of the membrane topology, combined with site-directed mutagenesis and the recently developed bioinformatic techniques are giving us an opportunity to unveil the secrets of CPTI. Several groups are concurrently conducting mutagenesis studies to map the malonyl-CoA binding and the catalytic sites.

1.8.1 Malonyl-CoA (Inhibitor) Binding Site

All known CPTI sequences from different species contain a conserved N-terminal sequence of 124 residues with two putative transmembrane domains, which is absent from CPTII. It is reasonable to hypothesize that some of the residues important for malonyl-CoA binding may reside in this region since CPTI is malonyl-CoA sensitive and CPTII is not.

Amino acid residues E3 and H5 were found to be critical amino acid residues for malonyl-CoA binding of L-CPTI [241, 242]. Residues H5 and H140 were also reported to be involved in malonyl-CoA inhibition of L-CPTI [253]. M-CPTI is much more sensitive to malonyl-CoA inhibition than L-CPTI. By deletion mutation analysis of human M-CPTI, our lab suggested that some of the malonyl-CoA binding resides are located between amino acid residues 18 and 28 in human M-CPTI which is different from that of L-CPTI [253]. However, chimera studies suggested that the N-terminal regions of L-CPTI and M-CPTI are not the only regions responsible for malonyl-CoA inhibition and it is possible that the N-terminal residues which are important for malonyl-CoA binding and inhibition interact with the C-terminal residues to bind malonyl-CoA cooperatively

[197, 253]. A systematic study on six chimeric proteins constructed from combinations of three linear segments of rat L- and M-CPTI revealed that alterations in the combinations of the N-terminal plus TM1 (transmembrane segment) and C-terminal domains as well as in the N terminus plus TM1/TM2 pairings resulted in changes in the K_m values for carnitine and palmitoyl-CoA [133]. The sensitivity to malonyl-CoA of the L-type catalytic domain and the changes in affinity for malonyl-CoA and palmitoyl-CoA occurred independently of changes in the affinity for carnitine [133, 240]. For M-CPTI, a decrease in malonyl-CoA sensitivity was invariably observed with increasing deletions from $\Delta(3-18)$ to $\Delta(1-80)$ [133]. However, deletion of residues 3-18 from M-CPTI affected the K_m for carnitine of this isoform, but not of L-CPTI [133, 240].

With 62% identity at the cDNA level and the similar overall structure (membrane topology), liver and muscle CPTI isoforms have different kinetic characteristics, particularly, with respect to their affinity for one of the substrates (L-carnitine) and the inhibition by malonyl-CoA. This provides the opportunity to elucidate the roles of different regions of the protein by constructing chimeric enzymes. Our lab was the first to show that replacement of the N-terminal domain of L-CPTI with the N-terminal domain of M-CPTI does not change the malonyl-CoA sensitivity of the chimeric L-CPTI, suggesting that the N-terminal and C-terminal interaction is responsible for the differences in sensitivity to malonyl-CoA [240]. A natural chimera of L- and M-CPTI with low K_m for carnitine and high sensitivity to malonyl-CoA inhibition, pig liver CPTI, was recently identified [197, 198]. Chimeras were constructed between different regions of the pig and rat L-CPTI to investigate the role of the C-terminal domain in malonyl-CoA sensitivity, since pig and rat L-CPTI differ only in this property [197, 198]. This study indicated that the C-terminal domain controls the malonyl-CoA sensitivity of the overall protein through the interaction with the first 18 N-terminal residues, which is in agreement with our previous chimera study [197]. This was confirmed by similar studies from other labs [132]. All these chimera studies suggested that the amino acid residues essential for the differences in malonyl-CoA sensitivity between M- and L- CPTI are distributed throughout the proteins.

1.8.2 Substrate Binding Sites

Amino acid residues H372 as well as D376 and D464 were demonstrated to be essential for CPTII catalytic activity by a combination of chemical modification of *S. cerevisiae* expressed CPTII with diethyl pyrocarbonate (DEPC) and substitution mutations of conserved residues [42].

The first 3D structure of the carnitine acyltransferases family was reported by the recent crystallization of Carnitine Acetyltransferase (CAT) alone and in complex with the substrate carnitine or CoA at 1.8 Å resolution. [135, 295] The structure of CAT contains two domains consisting of 16 β strands (β 1- β 16) and 20 α helices (α 1- α 20) [135]. The C domain contains a six-stranded mixed β sheet, together with eleven α helices [135]. The N domain contains an eight-stranded mixed β sheet, which is covered on both sides by eight α helices [135]. α Helix 13, residues 386–487, forms the long connection between the N and the C domains [135]. The active site, H343, is in the junction between β 8 and α 12 in the N domain [135]. The carnitine binding site is formed by the β sheet (strands $\beta 11 - \beta 14$) in the C domain and residues in $\alpha 5 - \beta 1$ and $\beta 8 - \alpha 12$ in the N domain [135]. The CoA binding site is on the opposite side of the H343 side chain from the carnitine binding site [135]. In the active site, the thiol group forms a hydrogen bond with the side chain N ϵ 2 atom of the catalytic H343 residue [135]. The acetyl group of acetylcarnitine, modeled based on the structure of the carnitine complex, points toward a hydrophobic pocket that is enclosed by the intersection of the two β sheets in the enzyme (strands β 1 and β 8 in the N domain, strands β 13 and β 14 in the C domain) and helix α 12 [135]. Many important residues are identified such as R518, Y452, S454, T465, W102, Y107, E347, F566, V569, S552 [135]. Most of these structures and residues can be mapped correspondingly onto CPTI and CPTII. Computer-generated rat liver CPTI and mouse CPTII 3-D model structures using SwissModel show significant similarity to the CAT crystal structure.

Based on the crystal structure of the homologous enzyme, mouse carnitine acetyltransferase (CAT), a 3-D structural model for the liver L-CPTI was proposed [186]. This model lacks the first N-terminal 166 amino acids, including the crucial transmembrane domains of CPTI. The N-terminal is essential for both activity and malonyl-

CoA sensitivity [240-242]. A mutagenesis study based on the model revealed that the conserved STS (685-687) motif is indispensable for enzyme activity [186]. Replacement of T686 and S687 with alanine abolished the enzyme activity and a change of S685 with alanine slightly increased K_m for canitine and decreased the catalytic efficiency for both substrates [186]. To identify amino acids that participate in malonyl-CoA inhibition in the C-terminal of all carnitine acyltransferases, five conserved amino acids (T314, N464, A478, M593, and C608 in rat L-CPTI) common to malonyl-CoA senstive acyltransferases (carnitine octanoyltransferase and CPTI) were mutated to their counterparts in malonyl-CoA insensitive acyltransferases (CPTII, carnitine acetyltransferase (CAT) and choline acetyltransferase (ChAT)) [187]. All mutations had a minimal effect on L-CPTI activity [187]. However, the majority of them have a significant impact on the IC₅₀ for malonyl-CoA inhibition of CPTI [187]. Substitution of M593 to Serine almost abolished malonyl-CoA sensitivity and a mutation of A478 to Glycine increases IC₅₀ for malonyl-CoA by more than 3-fold [187]. M593 is very near the tripeptide TET602-604, which has been reported to play an important role in the binding of carnitine in catalysis [62].

Sequence alignment of the acyltranferase family of proteins shows the presence of a conserved signature motif, (--RTETXR--) for the CPTs and carnitine octanyltransferase, (--RTDTXR--) for the carntine acetyltransferases, and (--RVDNXR--) for the choline acetyltransferases (X is V or I). Replacement of VDN in the signature motif in choline acetyltransferase (ChAT) to TET together with a change of N655 to arginine conferred upon ChAT the ability to accept carnitine rather than choline as a substrate [62]. Choline acyltransferases have an Asn residue at this location while carnitine acyltransferases has an Arg residue because the choline substrate lacks a carboxylate group compared to the carnitine substrate. In the 3D structure of carnitine acetyltransferase (CAT), there is an electrostatic interaction between the Arg residue and the carboxylate group of carnitine [135].

1.9 CPT REGULATION

1.9.1 Metabolic Regulation of the CPTI Gene in Different Tissues

In human skeletal muscle, the mRNA level of CPTI did not change significantly with either a high-fat diet or an isoenergetic high-carbohydrate diet [47]. This increased CPTI activity is probably achieved by changing the concentration of its physiological inhibitor, malonyl-CoA [47]. The same mechanism is primarily responsible for the control of CPTI in the newborn heart where the increase in fatty acid oxidation has been observed [207]. Malonyl CoA levels dramatically decreased, due in part to an increase in malonyl CoA decarboxylase activity in these cases [47, 207]. On the contrary, hyperglycemia with hyperinsulinemia increases malonyl-CoA concentration, inhibits functional CPTI activity, and shunts LCFA away from oxidation toward storage in human muscle [227]. Short-term fasting/refeeding in humans affects the transcription of M-CPTI [214]. During short-term fasting, substrate utilization in skeletal muscle shifts from predominantly carbohydrate to fat as a means of conserving glucose [214]. M-CPTI remained elevated after either a high-carbohydrate or a high fat meal following the fasting [214]. The expression of M-CPTI is increased in type I (soleus) but not type II (extensor digitorum longus) skeletal muscle [174].

1.9.2 Regulation of the CPTI Gene by cAMP

The cAMP response unit of the L-CPTI gene is composed of a cAMP-response element motif and a DR1 sequence located 3 kb upstream of the transcription start site [167]. The coactivator PGC-1 is involved in the regulation of L-CPTI gene expression by cAMP in combination with HNF4 alpha and cAMP-response element-binding protein (CREB) [117, 118, 167]. In rat, high fat diet increases the expression of L-CPTI gene [230]. Nitric oxide/cyclic GMP signaling pathway is found to regulate fatty acid metabolism in rate hepatocytes [96]. 8-Br-cGMP inhibits ACC and fatty acid synthesis, thus decreasing malonyl-CoA concentration. It also activates CPT-I by malonyl-CoAindependent mechanism [96].

1.9.3 Hormonal Regulation of the CPTI Gene

17β-estradiol (E2) was reported to increase the transcription of the CPTI genes [48]. Also L-CPTI is expressed at a higher level in the liver of hyperthyroid animals. Thyroid hormone (T3) is more efficient in stimulating L-CPTI transcription in the liver than in non-hepatic tissues [128]. A thyroid hormone response element (TRE), which binds thyroid hormone receptor (TR), is located in the L-CPTI promoter [130]. The first intron of the L-CPT-I gene appears to be necessary for full thyroid hormone (T3) induction [130]. Chromatin immunoprecipitation assay show this region binds to proteins involved in thyroid hormone (T3) induction, such as CCAAT enhancer-binding proteins (C/EBP) and upstream stimulatory factor (USF-1 and USF-2) that physically interact with TR [130]. In vivo study using transgenic mice carrying different length of first intron fragments also demonstrates the importance of the first intron in response to thyroid hormone (T3) [130].

Neonatal rat cardiomyocytes displayed a higher fatty acid oxidation rate and transcription from the metabolic genes with exposure to PPAR α and PPAR β/δ ligands [101, 102]. M-CPTI promoter is induced by PPARα and PPARβ/δ ligands in a dosedependent manner [101, 102]. PPARy ligands do not have a similar effect as PPARa and PPAR β/δ ligands. This study suggests that PPAR α and PPAR β/δ , but not PPAR γ , are involved in the regulation of cardiac M-CPTI [101, 102]. An in vivo study of human skeletal muscle shows that the level of M-CPTI mRNA positively correlates with that of PPARα [312]. Peroxisomal proliferator-activated receptor gamma coactivator (PGC-1), a coactivator for many factors in the nuclear hormone receptor family including PPARa, the glucocorticoid receptor, the thyroid hormone receptor, and several orphan receptors, up-regulates the expression of the M-CPTI gene [185]. Moore et al. reported that PGC-1 stimulates M-CPTI expression through interactions with myocyte enhancer factor 2A (MEF2A) [185]. The induction can be inhibited by upstream stimulatory factor (USF), a member of the basic helix-loop-helix leucine zipper family that preferentially binds to the E-box consensus CANNTG with CG as interior nucleotides [185]. PCG-1 and USF proteins are found to physically interact with each other and the interaction reduces MEF2A and PGC-1 synergy, which results in lower efficiency in stimulating M-CPTI

gene expression [185]. This study suggests that PCG-1 is an important stimulator of CPTI in the heart and USF represses the expression of CPTI through modulating PGC-1 action on the M-CPTI gene [185].

Chromatin immunoprecipitation assays suggested that the 5'-flanking region of the CPTII gene binds PPAR α in vivo [15]. A novel peroxisome proliferator-responsive element (PPRE) was located in the proximal promoter of the CPTII gene [15]. This PPRE is different from others in containing only one half-site which is a perfect consensus sequence (TGACCT) but no clearly recognizable second half-site (CAGCAC) [15]. CPTII gene is also negatively regulated by other members of the nuclear receptor superfamily, which also bind to this element and repress the activation mediated by PPAR α [15]. This indicates that the entry of fatty acids into the mitochondria, a critical step in their oxidation is highly regulated through the interplay between several nuclear receptors [15].

CPTs are potential targets for the development of therapeutic agents against diseases like obesity and diabetes. Reversible CPTI inhibitors such as long-chain carbamoyl aminocarnitine derivatives are being screened as potential antiketotic and antidiabetic drugs [99]. DRF 2655, a PPARα and PPARγ agonist, is being screened as a body-weight lowering, hypolipidemic and euglycemic agent. Db/db mice treated with DRF 2655 showed 651% increase in CPT activity [273]. Selective decrease of lipid oxidation in the hypothalamus through genetic or biochemical inhibition of hypothalamic CPT1 activity resulted in substantially low food intake and endogenous glucose production [200]. The rate of lipid oxidation in selective hypothalamic neurons could be a sensor of nutrient availability to the hypothalamus, based on the regulation of exogenous and endogenous inputs of nutrients into the circulation [200].

1.10 HIGH-LEVEL EXPRESSION SYSTEM FOR THE CPTS IN YEAST *P. PASTORIS*

The expression vector pHWO10 was used in our lab to express both the CPTI and CPTII enzymes [72, 314, 315]. This vector has the *P. pastoris* glyceraldehyde-3-

phosphate dehydrogenase (pGAP) gene promoter, ampicillin resistance gene and HIS4 gene [281]. An *EcoR*I site was introduced by PCR immediately 5' of the ATG start codons of the CPTI and CPTII cDNAs to enable cloning into the unique EcoRI sites located just 3' of the glyceraldehyde-3-phosphate dehydrogenase gene promoter (pGAP) in plasmid pHWO10. Bacterial colonies bearing the expression vector were screened for their resistance to ampicillin. The HIS4 gene was used to introduce the expression vector to yeast *P. pastoris* GS115 through recombination with its his4 locus. The expression plasmid was linearized by the restriction enzyme *BspE*I within the HIS4 gene before electroporation. A crossover between the his4 locus in the chromosome and the HIS4 gene on the pGAP vector inserted the vector at the his4 locus (Fig. 1.8). The yeast strain GS115 has a mutation in the histidinol dehydrogenase gene (his4), which prevents it from synthesizing histidine. Positive transformants had duplicate copies of the HIS4/his4 genes and were selected for their ability to grow on histidine-deficient medium because pGAP vector complements his4 in the host.

1.11 CPT ASSAY

CPT activity was assayed by the forward exchange method using L-[³H]carnitine [35, 72]. The standard enzyme assay mixture has a total volume of 0.5 mL containing 0.2 mM L-[³H]carnitine (~10,000 dpm/nmol), 50 μ M palmitoyl-CoA, 20 mM HEPES (pH 7.0), 1 or 2% fatty acid-free albumin, and 40-75 mM KCl, with or without 10-100 μ M malonyl-CoA. The mixture was pre-incubated for 2-3 minutes at 30°C on a water bath in a glass tube. Reaction was initiated by the addition of mitochondria followed by 3 min incubations at 30°C and stopped by addition of 4 ml 6% perchloric acid. The glass tube was centrifuged at 2000 rpm for 7 minutes and the resulting pellet was resuspended in 1.6 ml water and the product, [³H]palmitoylcarnitine was vortexed again. Finally, the reaction mixture was centrifuged for 2 minutes at 2000 rpm and 0.5 ml of the upper phase (butanol phase) was transferred to a vial for radioactive counting.



Fig. 1.8 Integration of pHWO10 expression vector at his4 locus. [Based on *Pichia* expression kit manual]

Malonyl-CoA was included in the reaction mixture at the desired concentration to measure malonyl-CoA sensitivity. The K_m value for palmitoyl-CoA was determined by varying the palmitoyl-CoA concentration at a fixed molar ratio (6.1:1) of palmitoyl-CoA to albumin and a fixed carnitine concentration [241]. Similarly, The K_m for carnitine was determined by varying the carnitine concentration at a fixed palmitoyl-CoA concentration.

CHAPTER 2

IDENTIFICATION BY MUTAGENESIS OF CONSERVED ARGININE AND TRYPTOPHAN RESIDUES IN RAT LIVER CARNITINE PALMITOYLTRANSFERASE I IMPORTANT FOR CATALYTIC ACTIVITY*

2.1 INTRODUCTION

Transport of long-chain fatty acids from the cytoplasm to the mitochondrial matrix involves the conversion of their acyl-CoA derivatives to acylcarnitines, translocation across the inner mitochondrial membrane, and reconversion to acyl-CoA [21,180]. Carnitine palmitoyltransferase I (CPTI) catalyzes the conversion of long-chain fatty acyl-CoAs to acylcarnitines in the presence of carnitine. Mammalian tissues express two isoforms of CPTI, a liver isoform (L-CPTI) and a heart/skeletal muscle isoform (M-CPTI), that are 62% identical in amino acid sequence [40, 282, 283, 304, 305, 314] (GenBankTM accession number U62317). As an enzyme that catalyzes the first rate-limiting step in β -oxidation, CPTI is tightly regulated by its physiological inhibitor, malonyl-CoA, the first intermediate in fatty acid synthesis, suggesting coordinated control of fatty acid oxidation and synthesis [21, 108]. Because of its central role in fatty acid metabolism, a good understanding of the molecular mechanism of the regulation of the CPT system is an important first step in the development of treatments for diseases, such as myocardial ischemia and diabetes, and in human inherited CPT deficiency diseases [9, 27, 60].

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Dai, J., Zhu, H., Shi, J., and Woldegiorgis, G. (2000) Identification by mutagenesis of conserved arginine and tryptophan residues in rat liver carnitine palmitoyltransferase I important for catalytic activity. J. Biol. Chem. 275, 22020-22024.

Contribution: I designed and conducted all the experiments. I collected and analyzed all the data. I prepared all figures for publication.

We developed a novel high level expression system for human heart M-CPTI, rat L-CPTI, and CPTII in the yeast *Pichia pastoris*, an organism devoid of endogenous CPT activity [72, 314, 315, 293]. Furthermore, by using this system, we have shown that CPTI and CPTII are active distinct enzymes and that L-CPTI and M-CPTI are distinct malonyl-CoA-sensitive CPTIs that are reversibly inactivated by detergents. More recent sitedirected mutagenesis studies from our laboratory have demonstrated that glutamic acid 3 and histidine 5 in L-CPTI are necessary for malonyl-CoA inhibition and high affinity binding but not for catalysis [241, 242]. For M-CPTI, our deletion and substitution mutation analyses to date indicate that, in addition to E3 and H5, other specific residues within the 19-28 N-terminal amino acids are necessary for malonyl-CoA inhibition and high affinity binding, in agreement with the differences in malonyl-CoA sensitivity observed between M-CPTI and L-CPTI [240]. In this work, site-directed mutagenesis studies of conserved residues in the predicted C-terminal catalytic domain of L-CPTI demonstrate for the first time that conserved arginine and tryptophan residues are important for catalysis.

2.2 EXPERIMENTAL PROCEDURES

2.2.1 Construction of Rat Liver CPTI Mutants

Mutants of L-CPTI were constructed using the Quick Change mutagenesis procedure (Stratagene) with the pYGW11 (pGAP-L-CPTI) plasmid DNA as template. For example, to construct R388A, the forward primer R388AF, 5'-CCCTCACTGCTGCAGACGCGGTGCCCTGGGGCAA-3', and the reverse primer R388AR, 5'-TTGCCCAGGGCACCGCGTCTGCAGCAGTGAGGGG-3', were used for mutagenesis. Mutants R451A, R601A, R606A, R655A, W391A, and W452A were constructed as above but with the following pairs of primers: R451AF, 5'-GGAAGATGCTTTGACGCGTGGTTTGACAAGTCC-3'; R451AR, 5'-GGACTTGTCAAACCACGCGTCAAAGCATCTTCC-3'; R601AF, 5'-GGTCTTCCGAGAAGGGGCGACAGAGACTGTACG-3'; R601AF, 5'-GGTCTTCCGAGAAGGGGCCCCTTCTCGGAAGAGCC-3'; R606AF, 5'-GGACAGAGACTGTAGCGTCCTGCACTATGGAGTCC-3'; R606AR, 5'-GGACTCCATAGTGCAGGACGCTACAGTCTCTGTCC-3'; R655AF, 5'-CGCCGGCATCGACGCCCATCTCTTCTGCC-3'; R655AR, 5'-GGCAGAAGAGAGAGGGCGTCGATGCCGGCG-3'; W391AF, 5'-GCAGACAGAGTGCCCGCGGGCAAAGTGTCG-3'; W391AR, 5'-CGACACTTTGCCGCGGGCACTCTGTCTGC-3'; W452AF, 5'-GGAAGATGCTTTGACAAGGCCTTTGACAAGTCC-3'; and W452AR, 5'-GGACTTGTCAAACGCCCTGTCAAAGCATCTTCC-3'.

Bacterial colonies obtained upon transformation of the mutagenesis reactions were screened for the ability to productively serve as templates for polymerase chain reaction using forward primers with 3' ends specific to each of the above mutations. For example, the R388A mutant was screened for with the R388ACKR: 5'-TTGCCCAGGGCACCGC-3' primer. The mutation-specific 3' bases are indicated in bold. The DNA sequences of positive colonies were then confirmed by DNA sequencing.

The expression plasmids were linearized and integrated into the HIS4 locus of *P. pastoris* GS115 by electroporation [241] Histidine prototrophic transformants were selected on YND plates and grown on YND medium. Mitochondria were isolated by disrupting the yeast cells with glass beads as described previously [72]

2.2.2 CPT Assay

CPT activity was assayed by the forward exchange method using L-[³H]carnitine [35, 72]. The K_m value for palmitoyl-CoA was determined by varying the palmitoyl-CoA concentration at a fixed molar ratio (6.1:1) of palmitoyl-CoA to albumin as described previously [241]

2.2.3 Chemical Modification of Yeast-expressed L-CPTI

Mitochondria (200 μ g) from the yeast strains expressing L-CPTI, CPTII, and M-CPTI were incubated with 10 mM phenylglyoxal in disruption buffer (pH 6.0) at 25 °C for 30 min as described by Shanmugasundaran et al. [238, 239]. For the NBS treatment, 200 μ g of mitochondria were incubated with 0.4 mM NBS on ice for 30 min.

2.2.4 Western Blot

Proteins were separated by SDS-polyacrylamide gel electrophoresis in a 7.5% gel and transferred onto nitrocellulose membranes. Immunoblots were developed by incubation with the L-CPTI-specific antibodies as described previously [241]. Sources of other materials and procedures were as described in our previous publication [241].

2.3 RESULTS

2.3.1 Effect of Phenylglyoxal and N-Bromosuccinimide on CPT Activity

Preincubation of isolated mitochondria from the yeast strains expressing the CPTs at room temperature with 10 mM phenylglyoxal, an arginine-specific modifying reagent, resulted in an irreversible loss of 70% of both CPTI and CPTII activity. The inactivation was concentration- and time-dependent. These chemical modification studies with phenylglyoxal provided evidence that conserved arginine residue(s) is/are important for maximal CPT activity. Similarly, treatment of isolated mitochondria from the yeast strains expressing L-CPTI and CPTII with N-bromosuccinimide, a tryptophan-specific reagent, resulted in loss of 50 and 59% of L-CPTI and CPTII activity, respectively, indicating that conserved tryptophan residue(s) may be very important for L-CPTI activity.

Alignment of the sequences of all carnitine and choline transferases from different species showed the presence of five conserved arginine and three conserved tryptophan residues (Fig. 2.1). For L-CPTI, these are arginine residues 388, 451, 601, 606, and 655 and tryptophan residues 236, 391, and 452. To determine the role of these conserved arginine and tryptophan residues in L-CPTI on catalytic activity, they were each separately mutated to alanine (R388A, R451A, R601A, R606A, R655A, W391A, and W452A).

2.3.2 Generation of Mutations and Expression in *P. pastoris*

Construction of plasmids carrying substitution mutations R388A, R451A, R601A, R606, R655A, W391A, and W452A was performed as described under "Experimental

		388		451		601	606	655	
RLCPTI	L]	AADRV	PWA	FDRWFDK	FR	EGRTE'	TVRSCT	GIDRHLH	CL
HHCPTI	L1	AGGRV	EWA	YNRWFDK	FR	EGRTEI	TVRSCT	GIDRHLE	CL
DMELCPTI	LI	AWNRS	KWA	YNRWFDK	FR	EGRTEI	EVRPCT	GIDRHLF	CL
RLCPTII	L1	SENRD	VWA	TNRWFDK	FK	HGRTET	CIRPAS-	GFDRHLY	(AL
HCPTII	L'I	SENRD	IWA	TNRWFDK	FK	HGRTET	CIRPAS-	GFDRHLE	AL
CELEGANS	LI	HDTRD	SWH	TNRWVDK	FA	NSRTE1	LRTVN	GVDRHLE	VL
BLCOT	L1	TEERTI	RWA	RWGDK	FY.	HGRTET	VRPCT	GFDRHLI	GL
RCOT	LI	SEERTI	RWA	RWGDK	FY	HGRTET	VRSCT	GFDRHLI	GL
YCAT	F1	TESRR	/WIS	LNRWYDK	FQ	NGRTE?	AIRSVT	GQDRHLY	'AL
MCAT	LI	SNHRN	rwa	GNRWFDK	FH	LGRTDI	TIRSAS	AFDRHLL	.GL
HCAT	LI	SNHRNS	SWA	GNRWFDK	FH	LGRTDI	TIRSAS-	AFGRHLI	GL
PCHAT	LT	SDGRSI	AW3	ANRWYDK	FH	BGRVDN	IIRSAT-	AIDNHLL	.GL
DCHAT	LI	AEPRS	[WA	GNRWFDK	FL	HGRVDO	IRAAS-	GIDIPLL	GL
	.*	*	*.	** **	*	**	.*	*	.*

RLCPTI: rat liver carnitine palmitoyltransferase I (P32198)* HHCPTI: human heart carnitine palmitoyltransferase I (Q92523) DMELCPTI: D.melanogaster carnitine palmitoyltransferase I (AJ010150.1) RLCPTII: rat liver carnitine palmitoyltransferase II (P18886) HCPTII: human carnitine palmitoyltransferase II (P23786) CELEGANS: C.elegans carnitine palmitoyltransferase I-like protein (U64852.1) BLCOT: bovine liver carnitine octanoyltransferase (O19094) RCOT: rat carnitine octanoyltransferase (P1466) YCAT: yeast carnitine acetylcoatransferase (P80235) MCAT: murine house mouse carnitine acetylcoatransferase (P47934) HCAT: human carnitne acetyltransferase (P13222) DCHAT: pig choline acetyltransferase (P07668)

*GenBank Accession Numbers

Fig. 2.1 Sequence alignment of portions of the C-terminal region of various acyltransferases.

Procedures." Mutations were confirmed by DNA sequencing. *P. pastoris* was chosen as an expression system for L-CPTI and the mutants, because it does not have endogenous CPT activity [72, 242, 241, 293, 314, 315]. The *P. pastoris* expression plasmids expressed L-CPTI under control of the *P. pastoris* glyceraldehyde-3-phosphate dehydrogenase gene promoter [72, 281]. Yeast transformants with the wild type L-CPTI gene and the mutants were grown in liquid medium supplemented with glucose. As previously reported, no CPT activity was found in the control yeast strain with the vector but without the CPTI cDNA insert [72].

Western blot analysis of wild type L-CPTI (88 kDa) and the mutants using a polyclonal antibody directed against a maltose-binding protein-L-CPTI fusion protein [72] is shown in Fig. 2.2, A and B. For the wild type and all the conserved arginine substitution mutations R388A, R451A, R601A, R606A, and R655A (Fig. 2.2A) and for the wild type and the tryptophan mutants W391A and W452A (Fig. 2.2B), proteins of predicted sizes were synthesized with similar steady-state levels of expression.

2.3.3 Effect of Mutations on L-CPTI Activity and Malonyl-CoA Sensitivity

Substitution mutants R388A, R451A, and R606A had activity 7.0-18.0% of the wild type L-CPTI activity that was malonyl-CoA-sensitive; the R606A mutant exhibited the lowest activity (Table 2.1). Mutants R601A and R655A had less than 2% of the wild type L-CPTI activity and were less sensitive to malonyl-CoA inhibition than the wild type. Replacement of W391 and W452 with alanine resulted in 50 and 93% loss of L-CPTI activity, respectively (Table 2.1), but the residual activity was sensitive to malonyl-CoA inhibition.

2.3.4 Kinetic Characteristics of Mutant L-CPTIs

Mutants R388A, R451A, and R606A exhibited normal saturation kinetics when the carnitine concentration was varied relative to a constant second substrate, palmitoyl-CoA (Fig. 2.3A), a property identical to that of the wild type L-CPTI. For the R388A, R451A, and R606A mutants, the calculated K_m values for carnitine were similar to the







Fig. 2.3(A, B) Kinetic analysis of wild type and mutant L-CPTI activities. Isolated mitochondria (150 μ g of protein) from the yeast strains expressing the wild type (circle) and R388A (triangle), R451A (square), and R606A (asterisk) mutants were assayed for CPT activity in the presence of increasing concentrations of carnitine. Inset, expanded dose-response curve for the arginine substitution mutants. B, same as A, except CPT activity was measured in the presence of increasing concentrations of palmitoyl-CoA.



Fig. 2.4(A, B) Kinetic analysis of wild type and mutant L-CPTI activities. Isolated mitochondria (150 μ g of protein) from the yeast strains expressing the wild type (circle) and W391A (square) and W452A (triangle) mutants were assayed for CPT activity in the presence of increasing concentrations of carnitine. B, same as in A, except CPT activity was measured in the presence of increasing concentrations of palmitoyl-CoA.

wild type value as shown in Table 2.2, and the V_{max} values were only 6-13% of the wild type value (Table 2.2), indicating a major effect of the mutations on catalytic activity.

The catalytic efficiency as estimated by V_{max}/K_m for R388A, R451A, and R606A was decreased by 91, 89, and 95%, respectively. Due to the extremely low residual activity in mutants R601A and R655A, it was not possible to perform saturation kinetics and determine the K_m or the V_{max} values for carnitine or palmitoyl-CoA. With respect to the second substrate, palmitoyl-CoA, mutants R388A, R451A, and R606A exhibited normal saturation kinetics similar to the wild type (Fig. 2.3B) when the molar ratio of palmitoyl-CoA: albumin was fixed at 6.1:1. The calculated K_m value for palmitoyl-CoA for mutants R388A and R451A was about 50% lower than the wild type, whereas for mutants R388A, R451A, and R606A were 2-8% of the wild type values (Table 2.2), and the catalytic efficiency was decreased by 90, 83, and 74%, respectively. Thus, substitution of the conserved arginine residues 388, 451, and 606 with alanine caused a substantial loss in catalytic activity but not in malonyl-CoA sensitivity. Substitution of arginines 601 and 655 with alanine resulted in nearly complete loss in CPTI activity, which was accompanied by loss in malonyl-CoA sensitivity.

With respect to carnitine and palmitoyl-CoA, substitution mutants W391A and W452A exhibited normal saturation kinetics similar to the wild type (Fig. 2.4, A and B). Mutants W391A and W452A caused a 2-4-fold increase in the K_m for carnitine, respectively, but decreased the K_m value for palmitoyl-CoA (Table 2.2). However, both mutations resulted in significant loss in the V_{max} for carnitine and palmitoyl-CoA (Table 2.2). For mutants W391A and W452A, the catalytic efficiency decreased by 80 and 98%, respectively, when the carnitine concentration was varied, and 55 and 90%, respectively, when the palmitoyl-CoA concentrations were varied relative to a second substrate.

2.4 DISCUSSION

The site-directed mutagenesis study described here is aimed at elucidating the function of several strictly conserved basic and aromatic amino acid residues found at the

Table 2.1

CPT activity and malonyl-CoA sensitivity in yeast strains expressing wild-type and mutant L-CPTI.

Strain	Activity nmol/mg · min (%)	Malonyl-CoA Inhibition		
Strain		2 μΜ	100 µM	
Wild-type L-CPTI	12.32 ± 0.361 (100)	8.31	1.44	
R388A	1.46 ± 0.030 (11.8)	0.36	0	
R451A	2.16 ± 0.100 (17.5)	1.04	0	
R601A	0.23 ± 0.030 (1.9)	0.25	0.10	
R606A	0.85 ± 0.070 (6.9)	0.79	0.21	
R655A	0.18 ± 0.070 (1.5)	0.10	0.08	
W391A	6.040 (49)	1.53	0.21	
W452A	0.850 (6.9)	0.17	0	

Mitochondria (150 μ g of protein) from the yeast strains expressing wild-type L-CPTI, arginine, and tryptophan substitution mutations were assayed for CPT activity and malonyl-CoA sensitivity as described under "Experimental Procedures." The results are the means \pm S.D. of at least three independent experiments with different mitochondrial preparations. Numbers in parentheses represent percent of CPT activity in the mutants compared to the wild type (100%).

Table 2.2

	C	arnitine	Palmitoyl-CoA		
Strain	K _m	V _{max}	K _m	V _{max}	
	μМ	nmol/mg · min	μМ	nmol/mg · min	
Wild-type	100.3	20.00	43.05	49.23	
R388A	79.5	1.43	22.90	2.52	
R451A	121.3	2.64	20.70	3.99	
R606A	114.1	1.20	3.74	1.13	
W391A	235.8	10.38	29.53	14.99	
W452A	419.4	1.80	11.58	1.23	

Kinetic characteristics of yeast-expressed wild-type and mutant L-CPTIs

Isolated mitochondria (150 μ g of protein) from the yeast strains expressing the wild type, arginine, and tryptophan substitution mutations were assayed for CPT activity in the presence of increasing concentrations of carnitine or palmitoyl-CoA. Values are averages of two independent experiments with different mitochondrial preparations.

proximity of the active site of L-CPTI. Earlier chemical modification studies with CoAmetabolizing enzymes suggested that adjacent arginine and tryptophan residues located at the active site might be involved in CoA binding [238, 239]. Studies with other enzyme systems using the arginine-specific reagent, phenylglyoxal, and the tryptophan-specific reagent, NBS, have shown that the negatively charged pyrophosphate group and the adenine moiety of CoA bind to adjacent positively charged arginine and hydrophobic (aromatic) tryptophan residues of the enzymes, respectively [68, 238, 239, 296]. We found that chemical modification of isolated mitochondria from the yeast strain expressing L-CPTI by phenylglyoxal and NBS resulted in loss of catalytic activity. Five arginine and three tryptophan residues are fully conserved throughout the family of acyltransferases with known primary sequences. In this study, we separately changed each of the five conserved arginine and two of the conserved tryptophan residues to alanine and determined the CPTI activity of the mutant proteins. Arginine residues 388, 451, and 606, and tryptophan residues 391 and 452, when changed to alanine, resulted in mutant proteins that had considerably reduced L-CPTI activity that was malonyl-CoAsensitive. Substitution mutation of arginine residues 601 and 655 with alanine resulted in mutant proteins that had little or no detectable L-CPTI activity. Despite the differences in enzyme activity observed between the mutants and the wild type, the immunoblots with L-CPTI-specific antibodies revealed that all the mutants were expressed at similar steadystate levels as the wild type.

The reaction catalyzed by L-CPTI at the catalytic pocket, conversion of palmitoyl-CoA to palmitoylcarnitine in the presence of L-carnitine, has been hypothesized to involve deprotonation of the hydroxyl group of carnitine by a catalytic base (-proton abstraction by His, Glu, or Gln) and attack by the resultant oxyanion at the carbonyl of the thioester of palmitoyl-CoA to generate palmitoylcarnitine and free CoA [66]. Since the CPT system has two substrates (palmitoyl-CoA and carnitine) with different physical properties, the active site pocket of the enzymes is predicted to contain separate or only partially overlapping binding pockets. Thus, a mutation that affects only the K_m for one of the substrates might be predicted. No such mutation was found in this

study. In fact, no change in K_m of more than ~10-fold was observed for any of the mutant L-CPTIs in this study.

Mutants R388A, R451A, R606A, W391A, and W452A had little effect on the K_m values for carnitine or palmitoyl-CoA but caused a considerable decrease in the V_{max} for both substrates, suggesting that the main effect of the mutations was to decrease the stability of the enzyme-substrate complex. However, it is also possible that the mutations could lead to misfolding in 90% of the molecules, producing a lower *kcat* and unchanged K_m values. Since the mutations had minimal effect on the K_m , such a lack of K_m alteration would suggest that separate substitution of the arginine and tryptophan residues is not sufficient to alter carnitine or palmitoyl-CoA binding. However, since these mutations decreased the V_{max} by 10-40-fold, the substantial decrease in V_{max} could be related to the alteration of intrinsic L-CPTI stability. In CPTII, a single nucleotide missense mutation of the conserved R503 to cysteine, which corresponds to the conserved R606 in CPTI, is the cause for CPTII deficiency disease in humans [254].

On the other hand, the R601A and R655A mutants were devoid of detectable activity. Thus, the presence of these conserved arginine residues is probably crucial for maintaining the configuration of the L-CPTI active site. Substitution mutation of the highly conserved R505 to asparagine in bovine liver carnitine octanoyltransferase, corresponding to the conserved Arg-655 in CPTI, was found to increase the K_m for carnitine by more than 1650-fold [63]. Based on the R505N mutation in carnitine octanoyltransferase, it was suggested that this conserved arginine residue in carnitine octanoyltransferase and other acytransferases contributes to substrate binding by forming a salt bridge with the carboxylate moiety of carnitine [63]. Mutant L-CPTI with a change of R655 to Ala had insufficient activity to allow measurement of its K_m value for carnitine. We suggest that conserved arginine and tryptophan residues in L-CPTI contribute to the stabilization of the transition state by charge neutralization and hydrophobic interactions, respectively.

Alignment of residues 381-481 of L-CPTI that contain R388, R451, W391, and W452 with a protein of known three-dimensional structure in the GenBankTM by the Swiss model software resulted in a known secondary structure of the acyl-CoA-binding

protein (ACBP), a protein with 86 amino acid residues, being the best fit for the predicted secondary structure of the 100 C-terminal amino acids that constitute the putative palmitoyl-CoA binding region of L-CPTI [145, 146]. ACBP and the 100-amino acid fragment of L-CPTI showed 32% similarity. The three-dimensional structure of the acyl-CoA-binding protein with bound palmitoyl-CoA consists of a skewed four α -helix bundle [147]. The predicted secondary structure for the putative 100-amino acid residue palmitoyl-CoA binding region consists of four α -helices. Both ACBP and CPTI bind palmitoyl-CoA. ACBP binds long-chain fatty acyl-CoAs with high affinity, and the acyl-CoA·ACBP complex has been suggested to play a role in acyl-CoA-mediated cell signaling by interaction with, or donation of, long-chain acyl-CoA to CPTI and other proteins [144]. Palmitoyl-CoA is a substrate for L-CPTI. We suggest that this 100-amino acid residue region constitutes the putative palmitoyl-CoA-binding site of L-CPTI.

The ACBP three-dimensional structure is a shallow bowl with a rim characterized by many polar and charged groups, whereas the inside and outside surfaces are predominantly hydrophobic with patches of uncharged hydroxyl groups [145, 147]. It is predicted that the specificity of the ligand binding resides at the omega end of the acyl chain, together with strong electrostatic and hydrophobic interactions with the adenine 3'-phosphate of the CoA [145, 147]. The phosphate and hydroxyl groups of the ribose are involved in an intense network of electrostatic and polar interactions with the polar parts of the side chains of hydrophobic and positively charged hydrophilic residues [145, 147]. The large adenine ring stacks with the aromatic ring of a Tyr residue. In a separate study, it was demonstrated that the adenine ring of a CoA moiety interacts with the tryptophan residue of an enzyme that catalyzes the synthesis of acetyl-CoA, and furthermore, arginine residues electrostatically interact with the pyrophosphate moiety of CoA [238, 239].

In this study, we have investigated the functional importance of conserved arginine and tryptophan residues in L-CPTI on catalytic activity by site-directed mutagenesis. Mutations of conserved arginine and tryptophan residues affected catalytic activity, indicating the importance of electrostatic and hydrophobic contacts for the interaction of L-CPTI with the substrates. Electrostatic interactions are generally thought to play a role in the initial steps of ligand binding by guiding the productive collision of ligand and receptor. Chemical modification of mitochondria from yeast strains expressing CPTII by the histidine-specific reagent, diethylpyrocarbonate (DEPC), and site-directed mutagenesis have identified the conserved His-372 residue to be essential for catalytic activity [42, J. Shi and G. Woldegiorgis, unpublished data) However, chemical modification of mitochondria from yeast strains expressing L-CPTI and M-CPTI by DEPC had no effect on catalytic activity, suggesting that the same conserved histidine residue in L-CPTI and M-CPTI is either not important for catalysis or may be important but inaccessible for DEPC modification.

Substitution mutation of arginine residues 601 and 655 with alanine resulted in mutant proteins that had little or no detectable L-CPTI activity. Thus, the presence of both of these conserved arginine residues is probably important for maintaining the configuration of the active site of L-CPTI. The region of CPTI between amino acid residues 550 and 619 has 11 additional highly conserved residues and may contain critical residues necessary for catalysis, such as Q571, Q575, and E590, which may act as a catalytic base for the deprotonation of the hydroxyl group of carnitine.

The main purpose of the Western blot analysis was to demonstrate the expression of wild type and mutant CPTI in yeast mitochondria, but not to determine the concentration of the enzyme in the yeast mitochondria, which is extremely low and not detectable by Coomassie blue staining of SDS gels. The activity and kinetic data obtained were based on an equal amount of isolated yeast mitochondrial protein, not on the amount of CPTI, which is undetectable, an accepted procedure followed by all investigators in the field given the low protein expression levels. Future development of a quantitative procedure for determination of expression levels of these low abundance proteins may or may not have an effect on the V_{max} values obtained. At this stage, due to lack of information on the quantitative levels of expression of the different clones, the V_{max} values obtained are based solely on an equal mitochondrial protein concentration of the different clones as indicated in the table legend, a standard practice in the field. In the future, if an improved procedure indicates that expression levels of these low abundance proteins are different, the difference can be factored in the V_{max} values for comparison.

CHAPTER 3

IDENTIFICATION BY MUTAGENESIS OF CONSERVED ARGININE AND GLUTAMATE RESIDUES IN THE C-TERMINAL DOMAIN OF RAT LIVER CARNITINE PALMITOYLTRANSFERASE I THAT ARE IMPORTANT FOR CATALYTIC ACTIVITY AND MALONYL-COA SENSITIVITY*

3.1 INTRODUCTION

Carnitine palmitoyltransferase I (CPTI) catalyzes the conversion of long chain fatty acyl-CoAs to acylcarnitines in the presence of L-carnitine, the first step in the transport of long chain fatty acids from the cytoplasm to the mitochondrial matrix, a ratelimiting step in β -oxidation [21, 180]. Mammalian tissues express two isoforms of CPTI, a liver isoform (L-CPTI) and a muscle isoform (M-CPTI), that are 62% identical in amino acid sequence [40, 282, 283, 304, 305, 314]. As an enzyme that catalyzes the first rate-limiting step in β -oxidation, CPTI is regulated by its physiological inhibitor, malonyl-CoA [21, 180], the first intermediate in fatty acid synthesis, suggesting coordinated control of fatty acid oxidation and synthesis. Because of its central role in fatty acid metabolism, understanding the molecular mechanism of the regulation of the CPT system is an important first step in the development of treatments for diseases, such as myocardial ischemia and diabetes, and in human inherited CPTI deficiency diseases [9, 27, 60].

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Treber, M., Dai, J., and Woldegiorgis, G. (2003) Identification by mutagenesis of conserved arginine and glutamate residues in the C-terminal domain of rat liver carnitine palmitoyltransferase I that are important for catalytic activity and malonyl-CoA sensitivity. J. Biol. Chem. 278, 11145-11149.

Contribution: This work is an extension of previous work [64]. I designed the experiments and conducted the initial mutagenesis studies. I analyzed all the data and prepared figures for publication.

We developed a novel high level expression system for human heart M-CPTI, rat L-CPTI, and CPTII in the yeast Pichia pastoris, an organism devoid of endogenous CPT activity [72, 293, 314, 315]. Furthermore, by using this system, we have shown that CPTI and CPTII are active distinct enzymes and that L-CPTI and M-CPTI are distinct malonyl-CoA-sensitive CPTs that are reversibly inactivated by detergents. Recent site-directed mutagenesis studies from our laboratory have demonstrated that glutamic acid 3 and histidine 5 in L-CPTI are necessary for malonyl-CoA inhibition and high affinity binding but not for catalysis [241, 242]. For M-CPTI, our mutagenesis studies demonstrate that in addition to Glu-3 and His-5, Val-19, Leu-23, and Ser-24 are necessary for malonyl-CoA inhibition and high affinity binding, in agreement with the differences in malonyl-CoA sensitivity observed between M-CPTI and L-CPTI [240]. In addition, our site-directed mutagenesis studies of conserved residues in the C-terminal domain of L-CPTI demonstrated that conserved arginine and tryptophan residues are important for catalysis [64]. In this report, our mutagenesis studies demonstrate for the first time that the conserved residues Arg-601, Glu-603, and Arg-606 in L-CPTI are important for catalytic activity and malonyl-CoA sensitivity.

3.2 EXPERIMENTAL PROCEDURES

3.2.1 Construction of Rat Liver CPTI Mutants

L-CPTI mutants were constructed by the overlap extension PCR procedure using the primers shown in Table 3.1 with the wild type plasmid DNA (pGAP-L-CPTI) as template [72, 257]. For example, to construct the E603Q mutant, the primers f-GWW3·r-E603Q and r-MDR2·f-E603Q were used to generate 900 bp and 600 bp PCR products, respectively, using the wild type L-CPTI cDNA as a template. The two PCR products were purified, mixed, and used as a template for a second-round PCR with the primers f-GWW3·r-MDR2. The 1.5-kb PCR product was digested with *AvaI-SacI*, and the DNA fragment was subcloned into *AvaI-SacI*-cut wild type L-CPTI cDNA in the pGAP expression vector. Bacterial colonies obtained upon transformation of the mutagenesis reactions were screened by PCR using the primer pair f-GWW3-r-E603QCK for Gln and

Table 3.1

PCR primers used for construction of L-CPTI mutants

Primers	Sequence
fE603A	5 ' - CCGAGAAGGGAGGACA GCG ACTGTACGCTCCTGCAC - 3 '
rE603A	5 ' - GTGCAGGAGCGTACAGTCGCTGTCCTCCCTTCTCGG - 3 '
E603ACK	5'-GCAGGAGCGTACAGT CG- 3'
fE603Q	5 ' - CCGAGAAGGGAGGACA CAG ACTGTACGCTCCTGCAC - 3 '
rE603Q	5 ' - GTGCAGGAGCGTACAGT CTG TGTCCTCCCTTCTCGG-3 '
E603QCK	5 ' -GCAGGAGCGTACAGTC TG -3 '
fE603H	5 ' - CCGAGAAGGGAGGACA CAC ACTGTACGCTCCTGCAC - 3 '
rE603H	5'-GTGCAGGAGCGTACAGTGTGTGTCCTCCCTTCTCGG-3'
E603HCK*	5'-GCAGGAGCGTACAGTG-3'
fE603D	5 ' - CCGAGAAGGGAGGACAGACACTGTACGCTCCTGCAC - 3 '
rE603D	5 ' - GTGCAGGAGCGTACAGT GTC TGTCCTCCCTTCTCGG-3 '
MDR2	5 ' - GGCCGCTCGAGCTATTACTTTTTAGAATTGATGGT - 3 '
GWW3	5'-ATCACCCCAACCCATATC-3'

* E603HCK is also used to check the introduction of E603D mutation.
f-GWW3-r-E603HCK for His and Asp. The R601A and R606A mutants were constructed as described previously [64]. The mutations were confirmed by DNA sequencing.

The expression plasmids were linearized by digestion with the restriction enzyme *BspE*I and integrated into the His4 locus of *P. pastoris* GS115 by electroporation [241]. Histidine prototrophic transformants were selected on YND (yeast nitrogen base with dextrose) plates and grown on YND medium. Mitochondria were isolated by disrupting the yeast cells with glass beads [72] and used to monitor activity and malonyl-CoA sensitivity.

3.2.2 CPT Assay

CPT activity was assayed by the forward exchange method using L-[methyl-³H]carnitine [35, 72]. The K_m value for palmitoyl-CoA was determined by varying the palmitoyl-CoA concentration from 2.8 to 225 μ M at a fixed molar ratio (6.1:1) of palmitoyl-CoA to albumin as described previously [241]. The concentration of carnitine was fixed at 200 μ M. The K_m for carnitine was determined by varying the carnitine concentration from 11 to 472 μ M at a fixed 111 μ M palmitoyl-CoA.

3.2.3 Western Blot

Proteins were separated by SDS-PAGE in a 10% gel and transferred onto nitrocellulose membranes. Immunoblots were developed by incubation with the L-CPTIspecific antibodies as described previously [241]. Sources of other materials and procedures were as described in our previous publication [241].

3.3 RESULTS

Preincubation of isolated mitochondria from the yeast strain expressing rat liver L-CPTI at room temperature with dicyclohexylcarbodiimide (DCCD), a glutamatespecific modifying reagent [113], resulted in an irreversible 50% loss in catalytic activity (data not shown). These preliminary chemical modification studies with DCCD provided

	603
CPT1_RAT	KKCRTSPDAFIQLALQLAHYKDMGKFCLTYBASMTRLFREG <mark>RTETVR</mark> SCTMBSCNFVQAM
CPT1_MOUSE	KKCRTSPDAFIQLALQLAHYKDMGKFCLTYEASMTRLFREGRTETVR\$CTTESCNFVLAM
CPT1_HUMAN	KKCRTSPDTFVQLALQLAHYKDMGKFCLTYEASMTRLFREGRTETVR\$CTTESCDFVRAM
CPT1_PIG	KKCRTSPDAFVQLALQLAHYKDMGKFSLTYEASMTRLFREGRTETVR\$CTTESCNFVLAM
CPTM_HUMAN	KKCRTSPDAFVQIALQLAHFRDRGKFCLTYEASMTRMFREGRTETVR\$CTSESTAFVQAM
CPTM_RAT	KKCRTSPDAFVQIALQLAHPRDKGKPCLTYEASMTRMFREGRTETVR\$CTSESTAFVRAM
CPTM_MOUSE	KKCRTSPDAFVQIALQLAHFRDKGKFCLTYEASMTRMFREGRTETVRFCTNESAAFVQAM
CAT_PIGEON	KSEKISPDAFIQLALQLAYYRNYGHSCATYESASLRMFRLORTDTIRFTSIESHKFVQSM
CAT_MOUSE	KSEKLSPDAF1QVALQLAYYRIYGQACATYESASLRMFHLGRTDTIREASIDSLAFVKGM
CAT_HUMAN	KSEKLSPDAFIQMALQLAYYRIYGQACATYESASLRMFHLGRTDTIR FASMDSLTFVKAM
OCTC_BOVIN	KEKQLHPDTF1QLALQLAYYRLHGRPGCCYETAMTRLFYHGRTETVRPCTVEAVNWCQSM
OCTC_HUMAN	KNKMLHPDTFIQLALQLAYIRLHGHPGCCIETAPTRHFIHGRIEIMRSCTVEAVRWCQSM
OUTC_KAT	KREALHPUIFIQLALQLAIIRLINGRPGUUISIAMIRIFINGRISIVRBUTVBAVRWUUSM
CLAT POUSE	KNUNCSPIDE LUVALULAI IKLIUKLVEI LEDASIKKEULOKVUNIKDAI PAALAFVUAR VVOVOCODA FIOVALULAI IKLIUKLVEI LEDASIKKEULOKVUNIKDAI PAALAFVUAR
CLAT ROPAN	KNUNCSPUAL TO ALVEL AND
CLAT CHICKEN	KKUKIGENSTUVALUNI (MI BEVERUUPI UDTVERSTEDENERUUNI ERAN FREDEVEN KKUKIGENSTUVALUNI (MI BEVERUUPI UDTVERSTEDENERUUNI ERAN FREDEVEN
CLAT DIG	KNUKI DEDA I LUVALUUAE I KURKLIVEI LEGADI KKEDDUKVDA I KPA I ABAEKEKEVAN KAUKI DEDA I LUVALUUAE I KURKLIVEI LEGADI KKEDDUKVDA I KPA I ABAEKEKEVAN
CDRI_FIG	
CPT1 RAT:	rat carnitine o-palmitovltransferase L mitochondrial liver isoform (P32198)
CPT1 MOUSE	mouse carnitine o-palmitovitransferase L mitochondrial liver isoform (P97742)
CPT1 HUMAN	human carnitine o-palmitovltransferase L mitochondrial liver isoform (P50416)
CPT1 PIG	pig carnitine o-nalmitovitransferase I, mitochondrial liver isoform (AAK69171)
CPTM HUMAN	human carnitine o-nalmitovitransferase 1 mitochondrial muscle isoform (O92523)
CPTM RAT	rat carnitine o-nalmitovltransferase L mitochondrial muscle isoform (063704)
COTH MOLISP	mouse camitine o nalmitovitransferase L mitochondrial muscle isoform (CAC40150)
CAT PIGRON	niseon camitine o-acetyltransferase (P52826)
CAT MOUSE	mouse camitine o-acetyltransferase (P47934)
CAT HIMAN	human carnitine o-acetyltransferase (P43155)
OCTY BOVIN	hovin peroxisomal carnitine octanov(transferase (O19094)
OCTC HUMAN	human peroxisomal carnitine octanovitransferase (O9UKG9)
OCTC RAT	rat neroxisomal carnitine octanovitransferase (P11466)
CLAT MOUSE	mouse choline a-acetyltransferase (O03059)
CLAT HUMAN	human choline o-acetyltransferase (P28329)
CLAT RAT	rat choline o-acetyltransferase(A48319)
CLAT CHICKEN	chicken choline o-acetvltransferase(AAK94673)
CLAT PIG	nig choline o-acetyltransferase (P13222)
WHELL E & W	and wardeness a manual second as a subman l

Fig. 3.1 Sequence alignment of portions of the C-terminal region of various

acyltransferases.

*, identical residues; :, conserved residues.

evidence that a conserved glutamate residue(s) is important for maximal L-CPTI activity. Sequence alignment of the sequences of all carnitine and choline acyltransferases from different species showed the presence of two conserved glutamate (E) residues (Fig. 3.1), Glu-590 and Glu-603. The conserved Glu-603 residue is flanked by two highly conserved arginine residues, Arg-601 and Arg-606, that we previously demonstrated were important for L-CPTI activity and malonyl-CoA sensitivity [64].

3.3.1 Generation of Mutations and Expression in P. pastoris

Construction of plasmids carrying substitution mutations E590A, E603A, E603H, E603Q, and E603D was performed as described under "Experimental Procedures" and for R601A and R606A as described previously [64]. *P. pastoris* was chosen as an expression system for L-CPTI and the mutants, because it does not have endogenous CPT activity [72, 241, 242, 293, 314, 315]. The *P. pastoris* expression plasmids expressed L-CPTI under control of the *P. pastoris* glyceraldehyde-3-phosphate dehydrogenase gene promoter [72, 281]. Yeast transformants with the wild type L-CPTI gene and the mutants were grown in liquid medium supplemented with glucose [72].

Western blot analysis of wild type L-CPTI (88 kDa) and the mutants using a polyclonal antibody directed against a maltose-binding protein·L-CPTI fusion protein [72] is shown in Fig. 3.2. For the wild type and the mutants E603A, E603Q, E603H, and E603D, proteins of predicted sizes were synthesized with similar constitutive levels of expression. Mutants R601A and R606A were also expressed at similar constitutive levels as reported previously [72].

3.3.2 Effect of Mutations on L-CPTI Activity and Malonyl-CoA Sensitivity

Substitution mutants E603A and E603H were inactive. A change of Glu-603 to Asp resulted in only a 33% loss in L-CPTI activity, but the mutant E603D exhibited a 15fold decrease in malonyl-CoA sensitivity as shown by the IC₅₀ values in Table 3.2. Substitution of Glu-603 with glutamine resulted in a 92% loss in L-CPTI activity and a 14-fold decrease in malonyl-CoA sensitivity (Table 3.2). We previously reported that mutation of the highly conserved arginine residues Arg-601 and Arg-606 to alanine





Mitochondria (23-48 μ g) from the yeast strains expressing the wild type and each of the point mutants were separated on a 10% SDS-PAGE and blotted onto a nitrocellulose membrane. The immunoblot was developed using L-CPTI-specific antibodies as described previously [241].

Table 3.2

CPT activity and malonyl-CoA sensitivity in yeast strains expressing wild type and mutant L-CPTI

Strain	Activity	IC ₅₀
	nmol/mg·min	μΜ
Wild-type	8.7 ± 0.49	2
E603D	5.9 ± 0.51	30
E603Q	0.7 ± 0.20	27.5
E603H	Inactive	
E603A	Inactive	
R601A	0.2 ± 0.13	84
R606A	0.9 ± 0.19	82

Mitochondria (150 μ g of protein) from the yeast strains expressing wild type L-CPTI, arginine, and glutamate substitution mutations were assayed for CPT activity and malonyl-CoA sensitivity as described under "Experimental Procedures." The results are the means \pm S.D. of at least three independent experiments with different mitochondrial preparations. resulted in >98 and 93% loss in L-CPTI activity, respectively [64]. The loss in activity observed with the R601A and R606A mutants was also accompanied by decreased sensitivity to malonyl-CoA inhibition [64]. Furthermore, our site-directed mutagenesis studies demonstrate that mutants R601A and R606A have an over 40-fold decrease in malonyl-CoA sensitivity compared with the wild type L-CPTI enzyme (Table 3.2). In short, our studies identify for the first time three conserved residues in the C-terminal region of L-CPTI, Arg-601, Glu-603, and Arg-606, which are important for both catalytic activity and malonyl-CoA sensitivity.

3.3.3 Kinetic Characteristics of Mutant L-CPTIs

Mutants E603D and E603Q exhibited normal saturation kinetics when the carnitine concentration was varied relative to a constant second substrate, palmitoyl-CoA (Fig. 3.3A), a property identical to that of the wild type L-CPTI. The kinetic characteristics of mutants R601A and R606A with respect to carnitine and palmitoyl-CoA were as reported in our previous publication [64]. For mutants E603D, E603Q, and R606A, the calculated K_m values for carnitine were similar to the wild type as shown in Table 3.3 and Ref. [64]. However, the V_{max} for carnitine for the E603Q mutant was 10fold lower compared with the wild type and the E603D mutant, indicating a major effect of the mutation on catalytic activity. The catalytic efficiency as estimated by V_{max}/K_m for E603Q and E603D was decreased by 92.3 and 43.1%, respectively. With respect to the second substrate, palmitoyl-CoA, mutants E603D and E603Q exhibited normal saturation kinetics similar to the wild type (Fig. 3.3B) when the molar ratio of palmitoyl-CoA to albumin was fixed at 6.1:1. The calculated K_m values for mutants E603D and E603Q were 3- and 7-fold lower, and the V_{max} values were 62 and 93.7% lower than the wild type, respectively. For the E603Q, the catalytic efficiency was 40.7% lower than the wild type, but the E603D mutant exhibited catalytic efficiency similar to the wild type. Thus, substitution of the conserved Glu-603 residue with glutamine and the conserved Arg-601 and Arg-606 residues with alanine [64] caused a substantial loss in catalytic activity and malonyl-CoA sensitivity. In contrast, a conservative substitution of Glu-603 with



Fig. 3.3 Kinetic analysis of wild type and mutant L-CPTI activities. Isolated mitochondria (150 μ g of protein) from the yeast strains expressing the wild type (\bullet), E603Q (\blacklozenge), and E603D (\blacktriangle) mutants were assayed for CPT activity in the presence of increasing concentrations of carnitine (A) and palmitoyl-CoA (C). B and D, expanded dose-response curves for the E603Q mutant.

Table 3.3

	Carnitine		Palmitoyl-CoA	
Strain	K _m	V _{max}	K _m	V _{max}
	μΜ	nmol/mg·min	μΜ	nmol/mg·min
Wild-type	46.0	19.1	97.5	62.2
E603Q	38.8	1.2	14.0	3.7
E603D	73.0	13.2	31.9	24.2

Kinetic characteristics of yeast-expressed wild type and mutant L-CPTIs

Isolated mitochondria (150 μ g of protein) from the yeast strains expressing the wild type and glutamate substitution mutations were assayed for CPT activity in the presence of increasing concentrations of carnitine or palmitoyl-CoA. Values are averages of two independent experiments with different mitochondrial preparations.

aspartate significantly lowered malonyl-CoA sensitivity but had a minor effect on catalytic activity.

3.4 DISCUSSION

Our site-directed mutagenesis studies of conserved residues in the C-terminal region of L-CPTI demonstrated that substitution of Arg-601 and Arg-606 with alanine resulted in almost complete loss in activity and a significant decrease in malonyl-CoA sensitivity. Within the C-terminal peptide sequence -RTETVR- (Fig. 3.1), the two arginine residues are highly conserved in the family of acyltransferases, and the other four residues are also conserved. Glu-603 is a conserved residue within the family of CPT enzymes, while other acyltransferases have aspartate at this position. A change of Glu-603 to alanine or histidine resulted in complete loss in L-CPTI activity. Substitution of the highly conserved Glu-590 with alanine did not have a major effect on catalytic activity (data not shown). The site-directed mutagenesis study described here is aimed at elucidating the function of these conserved acidic and basic residues found at the proximity of the active site of L-CPTI.

To determine the role of the conserved Glu-603 on catalysis and malonyl-CoA sensitivity, we separately changed the Glu-603 residue to alanine, histidine, glutamine, and aspartate (E603A, E603H, E603Q, and E603D, respectively) and determined the effect of the mutations on L-CPTI activity and malonyl-CoA sensitivity in the yeast-expressed mutant enzyme. A change of Glu-603 to glutamine caused a significant decrease in catalytic activity. Since a conservative substitution of Glu-603 with aspartate, a negatively charged amino acid with only one methyl group less than the glutamate residue in the wild type enzyme, resulted in partial loss in CPTI activity, the presence of this negatively charged conserved residue Glu-603, is probably crucial for maintaining the configuration of the L-CPTI active site. The mutant L-CPTI with a replacement of Glu-603 with aspartate (E603D) or glutamine (E603Q) showed a 15-fold decrease in malonyl-CoA sensitivity, while a change of Arg-601 or Arg-606 to alanine resulted in an over 40-fold decrease in malonyl-CoA inhibition of the mutant L-CPTI [64]. The loss in

catalytic activity observed with the mutant L-CPTI (E603Q, E603D, R601A, and R606A) was in each case associated with a decrease in malonyl-CoA sensitivity, suggesting that these three conserved residues may be important for substrate and inhibitor binding. The effect of the mutations on activity and malonyl-CoA sensitivity suggests that this region of L-CPTI spanning the conserved C-terminal residues -RTETVR- may be important for the substrate (palmitoyl-CoA) and the inhibitor (malonyl-CoA) binding, probably through the common CoA moiety present in both compounds. Although there was a substantial decrease in V_{max} for both substrates (carnitine and palmitoyl-CoA) with the E603D, E603Q, and R606A mutants [64], all the mutants showed an increase in the affinity for palmitoyl-CoA but not carnitine, suggesting that these residues are involved in binding palmitoyl-CoA and malonyl-CoA but not carnitine. This region of the protein may thus be involved in binding site.

As a rate-limiting enzyme that transports long chain fatty acids from the cytosol to the mitochondrial matrix, L-CPTI in the presence of carnitine catalyzes the conversion of long chain acyl CoAs to acylcarnitines [21, 180]. Similar to other acyltransferases, L-CPTI contains a general acid/base, His-473, a highly conserved amino acid residue that may form a hydrogen bonding network or a salt bridge to a nearby conserved glutamate residue such as Glu-603 [17]. We hypothesize that substitution of Glu-603 with alanine or histidine may disrupt a hydrogen bonding network or a salt bridge, perhaps to the highly conserved residue His-473 that is predicted to be at the active site of L-CPTI. The significantly reduced stability of the E603Q mutant implicates Glu-603 in the maintenance of active site architecture, suggesting substitution of Glu-603 with glutamine may also disrupt a hydrogen bonding network or a salt bridge to a residue like His-473 at the active site of L-CPTI. Furthermore, the site-directed mutagenesis studies demonstrate that even a conservative substitution of Glu-603 to aspartate or glutamine resulted in partial loss of activity and malonyl-CoA sensitivity, suggesting that a change of Glu-603 to aspartate may result in the carboxylate being outside the hydrogen bond distance of His-473. Glu-603 may thus be required for L-CPTI stability and positioning of the imidazole ring of His-473 for efficient catalysis and inhibition, thus facilitating

productive interaction with the substrates and the inhibitor [17]. Mutation of the corresponding conserved residue in CPTII, Glu-500, to alanine resulted in 50% loss in activity [313].

In this report, we demonstrate that the conserved Glu-603 of L-CPTI is required for the structural stability of the enzyme. Despite its similar size and potential for hydrogen bonding formation, a glutamine residue cannot substitute for glutamate, suggesting that the negative charge of Glu-603 and/or its ability to serve as a strong hydrogen bond acceptor is needed for optimal catalysis, maintenance of active site integrity, and malonyl-CoA inhibition and binding. The reduced L-CPTI activity and malonyl-CoA sensitivity observed with the E603D mutant suggest that the loss of a methyl group may result in the carboxylate being outside the hydrogen bond distance of the conserved amino acid residue His-473, the predicted general acid/base at the active site. This suggests that the negative charge and the longer side chain of glutamate are essential for catalysis and malonyl-CoA sensitivity. Also, since Glu-603 is located adjacent to Arg-601 and Arg-606, the negatively charged Glu-603 may be positioned to form hydrogen bonds and/or a salt bridge with the positively charged residues Arg-601 and Arg-606 that form the predicted CoA binding pocket. Disruption of the hydrogen bonding network or salt bridge may also cause loss of catalytic activity and malonyl-CoA sensitivity. Since only a 15-42-fold loss in malonyl-CoA sensitivity was observed in these mutants compared with the more than 100-fold loss in inhibitor sensitivity that we reported with the N-terminal residue mutations, it is predicted that this C-terminal region may constitute the low affinity malonyl-CoA binding site in L-CPTI [241]. Characterization of the wild type and R601A, E603A, E603H, E603Q, E603D, and R606A mutant enzymes has led to the identification of conserved residues in the Cterminal region of L-CPTI that are important for catalytic activity and malonyl-CoA sensitivity. Since a mutation of any of these three conserved C-terminal residues (Arg-601, Glu-603, and Arg-606) substantially decreased catalytic activity and malonyl-CoA sensitivity, it is hypothesized that these residues are the major contact sites between L-CPTI and the CoA moiety of the substrate (palmitoyl-CoA) and the inhibitor (malonylCoA) and constitute the putative low affinity acyl-CoA/malonyl-CoA binding site in L-CPTI.

CHAPTER 4

A SINGLE AMINO ACID CHANGE (SUBSTITUTION OF THE CONSERVED GLU-590 WITH ALANINE) IN THE C-TERMINAL DOMAIN OF RAT LIVER CARNITINE PALMITOYLTRANSFERASE I INCREASES ITS MALONYL-COA SENSITIVITY CLOSE TO THAT OBSERVED WITH THE MUSCLE ISOFORM OF THE ENZYME*

4.1 INTRODUCTION

Carnitine palmitoyltransferase I (CPTI) catalyzes the conversion of long-chain fatty acyl-CoAs to acylcarnitines in the presence of L-carnitine, the first step in the transport of long-chain fatty acids from the cytoplasm to the mitochondria matrix, a ratelimiting step in β -oxidation [21, 180]. Mammalian tissues express two isoforms of CPTI, a liver isoform (L-CPTI) and a muscle isoform (M-CPTI), that are 62% identical in amino acid sequence [40, 282, 283, 304, 305, 314]. As an enzyme that catalyzes the first rate-limiting step in β -oxidation, CPTI is regulated by its physiological inhibitor, malonyl-CoA [21, 180], the first intermediate in fatty acid synthesis, suggesting a coordinated control of fatty acid oxidation and synthesis. Previous studies by our laboratory and others have demonstrated that the muscle isoform of CPTI, M-CPTI, is significantly more sensitive to malonyl-CoA inhibition than the liver isoform, but the molecular/structural basis for the differences in malonyl-CoA sensitivity between M-

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Napal, L., Dai, J., Treber, M., Haro, D., Marrero, P. F., and Woldegiorgis, G. (2003) A single amino acid change (substitution of the conserved Glu-590 with alanine) in the C-terminal domain of rat liver carnitine palmitoyltransferase I increases its malonyl-CoA sensitivity close to that observed with the muscle isoform of the enzyme. J. Biol. Chem. 278, 34084-34089.

Contribution: This work is an extension of previous work [64]. I constructed the mutants and conducted initial studies. I analyzed all the data and prepared figures for publication.

CPTI and L-CPTI remain to be established [40, 282, 283, 304, 305, 314]. Because of its central role in fatty acid metabolism, understanding the molecular mechanism of the regulation of the CPT system is an important first step in the development of treatments for diseases, such as myocardial ischemia and diabetes, and in human-inherited CPTI deficiency diseases [9, 27, 60].

We developed a novel high level expression system for human heart M-CPTI, rat L-CPTI, and CPTII in the yeast Pichia pastoris, an organism devoid of endogenous CPT activity [72, 293, 314, 315]. Furthermore, by using this system, we have shown that CPTI and CPTII are active distinct enzymes and that L-CPTI and M-CPTI are distinct malonyl-CoA-sensitive CPTs that are reversibly inactivated by detergents. Recent site-directed mutagenesis studies from our laboratory have demonstrated that glutamic acid 3 and histidine 5 in L-CPTI are necessary for malonyl-CoA inhibition and high affinity binding but not for catalysis [214, 242]. For M-CPTI, our mutagenesis studies demonstrate that in addition to Glu-3 and His-5, Val-19, Leu-23, and Ser-24 are necessary for malonyl-CoA inhibition and high affinity binding, in agreement with the differences in malonyl-CoA sensitivity observed between M-CPTI and L-CPTI [316, 240]. In addition, our sitedirected mutagenesis studies of conserved residues in the C-terminal domain of L-CPTI demonstrated that conserved arginine and tryptophan residues are important for catalysis [64]. In this report, our mutagenesis studies show for the first time that a change of the highly conserved C-terminal Glu-590 residue in L-CPTI to alanine, glutamine, or lysine significantly increased its sensitivity to malonyl-CoA inhibition.

4.2 EXPERIMENTAL PROCEDURES

4.2.1 Construction of Rat Liver CPTI Mutants

L-CPTI mutants were constructed by the overlap extension PCR procedure using the primers shown in Table 4.1 with the wild-type plasmid DNA (pGAP-L-CPTI) as template [72, 257]. For example, to construct the E590A mutant, the primers f-GWW3-r-E590A and r-MDR2-f-E590A were used to generate 1.0-kb and 500-bp PCR products, respectively, using the wild-type L-CPTI cDNA as a template. The two PCR products

Table 4.1

PCR primers used for construction of L-CPTI mutants

Mutant	Primer	Sequence
	fE590A	5'-GTTCTGCCTCACATATGCCGGC-3'
E590A	rE590A	5'-GCCGGGTCATGGAGGCCCCATATGTGAGGCAGAAC-3'
	crE590A	5'-GCCGGGTCATGGAGGCC <u>G</u> -3'
	fE590Q	5'-GTTCTGCCTCACATATCAGGCCTCCATGACCCGGC-3'
E590Q	rE590Q	5'-GCCGGGTCATGGAGGC CTG ATATGTGAGGCAGAAC-3'
	crE590Q	5'-GCCGGGTCATGGAGGCCT <u>G</u> -3'
	fE590D	5'-GTTCTGCCTCACATATGACCCGGC-3'
E590D	rE590D	5'-GCCGGGTCATGGAGGCCGTCATATGTGAGGCAGAAC-3'
	crE90D	5'-GCCGGGTCATGGAGGC <u>G</u> -3'
	fE590K	5'-GTTCTGCCTCACATATAAGGCCTCCATGACCCGGC-3'
E590K	rE590K	5'-GCCGGGTCATGGAGGC CTT ATATGTGAGGCAGAAC-3'
	cfE590K	5'-AGTTCTGCCTCACATATA-3'
	· MDR2	5'-GGCCGCTCGAGCTATTACTTTTTAGAATTGATGGT-3'
	Gww3	5'-ATCACCCCAACCCATATC-3'

were purified, mixed, and used as a template for a second-round PCR with the primer f-GWW3-r-MDR2. The 1.5-kb PCR product was digested with *AvaI-SacI*, and the 1.0-kb DNA fragment containing the desired fragment was subcloned into *AvaI-SacI*-cut wildtype L-CPTI cDNA in the pGAP expression vector. Bacterial colonies obtained upon transformation of the mutagenesis reactions were screened by PCR using the primer pairs f-GWW3-crE590A for Ala, f-GWW3-crE590Q for Gln, f-GWW3-crE590D for Asp, and f-GWW3-crE590A for Lys. The mutations were confirmed by DNA sequencing.

The expression plasmids were linearized by digestion with the restriction enzyme *BspE*I and integrated into the His-4 locus of *P. pastoris* GS115 by electroporation [241]. Histidine prototrophic transformants were selected on YND plates and grown on YND medium. Mitochondria were isolated by disrupting the yeast cells with glass beads [72] and used to monitor activity and malonyl-CoA sensitivity.

4.2.2 CPT Assay

CPT activity was assayed by the forward exchange method using L-[methyl-3H]carnitine [35, 72]. The K_m value for palmitoyl-CoA was determined by varying the palmitoyl-CoA concentration from 2.8 to 225 μ M at a fixed molar ratio (6.1:1) of palmitoyl-CoA to albumin as described previously [241]. The concentration of carnitine was fixed at 200 μ M. The K_m for carnitine was determined by varying the carnitine concentration from 11 to 472 μ M at a fixed 111 μ M palmitoyl-CoA concentration.

4.2.3 Western Blot

Proteins were separated by SDS-PAGE in a 10% gel and transferred onto nitrocellulose membranes. Immunoblots were developed by incubation with the L-CPTIspecific antibodies as described previously [241]. Sources of other materials and procedures were as described in our previous publication [241].

	590
CPT1 RAT	KKCRTSPDAFIQLALQLAHYKDMGKFCLTYBASMTRLFREGRTETVRSCTMESCNFVQAM
CPT1_MOUSE	KKCRTSPDAFIQLALQLAHYKDMGKFCLTYBASMTRLFREGRTETVRSCTTESCNFVLAM
CPT1_HUMAN	KKCRTSPDTFVQLALQLAHYKDMGKPCLTYBASMTRLPREGRTETVRSCTTESCDFVRAM
CPT1_PIG	KKCRTSPDAFVQLALQLAHYKDMGKFSLTYBASMTRLFREGRTETVRSCTTESCNFVLAM
CPTM_HUMAN	KKCRTSPDAFVQ1ALQLAHFRDRGKFCLTYBASMTRMFREGRTETVRSCTSESTAFVQAM
CPTM_RAT	KKCRTSPDAFVQIALQLAHFRDKGKFCLTYBASMTRMFREGKTETVRSCTSESTAFVKAM
CPTN MOUSE	KKCKTSPDAFVQIADQLAHFKUKGKFCLIIIKAPTKEGKIBIVKSCINESAAFVQAM
CP12 NUMAN	KUKUSPDAVAQUAPQWAPLKQIQQIVALISOCSIAAPANGKIDIIRKASVIIKKOBAP KVYALCONAVAQUAPQWAPLKQIQQIVALISOCSIAAPANGKIDIIRKASVIIKKOBAP
CPT2 MOUSE	KKKKI,SPDAVAOLAPOMAFI,ROYCOTVATYESCSTAAFKHGRTETICPASIFTKRCSEAF
CAT PIGEON	KSEKISPDAFIOLALOLAYYRMYGHSCATYESASLRMFRLGRTDTIRSTSIESHKFVOSM
CAT MOUSE	KSEKLSPDAFIOVALOLAYYRIYGOACATYESASLRMFHLGRTDTIRSASIDSLAFVKGM
CAT HUMAN	KSEKLSPDAFIQMALQLAYYRIYGQACATYESASLRMFHLGRTDTIRSASMDSLTFVKAM
OCTC BOVIN	KEKQLHPDTFIQLALQLAYYRLHGRPGCCYBTAMTRLPYHGRTETVRPCTVEAVNWCQSM
OCTC_HUMAN	KNKMLHPDTFIQLALQLAYYRLHGHPGCCY BT AMTRHFYHGRTETMRSCTVEAVRWCQSM
OCTC_RAT	KKEALHPDTFIQLALQLAYYRLHGRPGCCYBTAMTRYFYHGRTETVRSCTVEAVRWCQSM
CLAT_MOUSE	KKQKCSPDGFIQVALQLAYYRLYQRLVPTYESASIRRPQEGRVDNIRSATPEALAFVQAM
CLAT_HUMAN	KKQKCSPDAFIQVALQLAFYRLHRRLVPTYBSASIRRFQEGRVDNIRSATPEALAFVRAV
CLAT RAT	KKOKYSPDGPIQVALQLAYYRLYQRLYPTYESASIRRFQEGRVDNIRSATPEALAFVQAM
CLAT_CHICKEN	KAQKISPDAI LQVALQLAFIKUKKLVPI I SOADIKKFDEGKVDNI KOAIABAFAFVAAM
CLAI_PIG	AUUNCSPUAR IQUALAUAR I KUNUKUVPI I BOADI KKENEUKVUNI KOAI FEAUNE VAAI
CPT1_RAT:	rat carnitine o-palmitoyltransferase I, mitochondrial liver isoform (P32198)
CPT1_MOUSE	mouse carnitine o-palmitoyltransferase I, mitochondrial liver isoform (P97742)
CPT1_HUMAN	human carnitine o-palmitoyltransferase I, mitochondrial liver isoform (P50416)
CPT1_PIG	pig carnitine o-palmitoyltransferase I, mitochondrial liver isoform (AAK69171)
CPTM_HUMAN	human carnitine o-palmitoyltransferase I, mitochondrial muscle isoform (Q92523)
CPTM_RAT	rat carnitine o-palmitoyltransferase I, mitochondrial muscle isoform (Q63704)
CPTM_MOUSE	mouse carnitine o-palmitoyltransferase I, mitochondrial muscle isoform (CAC40150)
CPT2_HUMAN	human carnitine o-palmitoyltransferase II (P23786)
CPT2_RAT	rat carnitine o-palmitoyltransferase II (P18886)
CPT2_MOUSE	mouse carnitine o-palmitoyltransferase II (P52825)
CAT_PIGEON	pigeon carnitine o-acetyltransferase (P52826)
CAT_MOUSE	mouse carnitine o-acetyltransferase (P47934)
CAT_HUMAN	human carnitine o-acetyltransferase (P43155)
OCTC_BOVIN	bovin peroxisomal carnitine octanoyltransferase (O19094)
OCTC_HUMAN	human peroxisomal carnitine octanoyltransferase (Q9UKG9)
OCTC_RAT	rat peroxisomal carnitine octanoyltransferase (P11466)
CLAT_MOUSE	mouse choline o-acetyltransferase (Q03059)
CLAT_HUMAN	human choline o-acetyltransferase (P28329)
CLAT_RAT	rat choline o-acetyltransferase(A48319)
CLAT_CHICKEN	chicken choline o-acetyltransferase(AAK94673)
CLAT_PIG	pig choline o-acetyltransferase (P13222)

Fig. 4.1 Sequence alignment of portions of the C-terminal region of various

acyltransferases.

*, identical residues; :, conserved residues.

4.3 RESULTS

Preincubation of isolated mitochondria from the yeast strain expressing rat L-CPTI at room temperature with dicyclohexylcarbodiimide, a glutamate-specific modifying reagent [113], resulted in an irreversible 50% loss in catalytic activity (data not shown). These preliminary chemical modification studies with dicyclohexylcarbodiimide provided evidence that a conserved glutamate residue(s) is important for maximal L-CPTI activity.

Alignment of the sequences of all of the carnitine and choline acyltransferases from different species showed the presence of two conserved glutamate residues, Glu-590 and Glu-603 (Fig 4.1). We have previously demonstrated that the conserved Glu-603 residue together with the two adjacent highly conserved arginine residues, Arg-601 and Arg-606, are important for L-CPTI activity and malonyl-CoA sensitivity [64, 262].

4.3.1 Generation of Mutations and Expression in P. pastoris

Construction of plasmids carrying substitution mutations E590A, E590Q, E590D, and E590K was performed as described under "Experimental Procedures." *P. pastoris* was chosen as an expression system for L-CPTI and the mutants, because it does not have endogenous CPT activity [72, 241, 242, 293, 314, 315]. The *P. pastoris* expression plasmids expressed L-CPTI under control of the *P. pastoris* glyceraldehyde-3-phosphate dehydrogenase gene promoter [72, 281]. Yeast transformants with the wild-type L-CPTI gene and the mutants were grown in liquid medium supplemented with glucose [72].

Western blot analysis of wild-type L-CPTI (88 kDa) and the mutants using a polyclonal antibody directed against a maltose-binding protein, L-CPTI fusion protein [72], is shown in Fig. 4.2, A and B. For the wild type and the mutants E590A, E590Q, E590D, and E590K, proteins of predicted sizes were synthesized with similar steady-state levels of expression.



Fig. 4.2 A, Immunoblot showing expression of wild type (lane 1), control without insert (lane 2), E590A (lane 3), E590Q (lane 4), E590D (lane 5). B, wild type (lane 1), control without insert (lane 2), E590K (lane 3) mutants in *P. pastoris*.

Mitochondria (20 μ g) from the yeast strains expressing the wild type and each of the point mutants were separated on a 10% SDS-PAGE and blotted onto a nitrocellulose membrane. The immunoblot was developed using L-CPTI-specific antibodies as described previously [241].

4.3.2 Effect of Mutations on L-CPTI Activity and Malonyl-CoA Sensitivity

Substitution mutant E590D was inactive. A change of Glu-590 to Ala resulted in only a 26% loss in L-CPTI activity, but the mutant E590A exhibited a 16-fold increase in malonyl-CoA sensitivity as shown by the IC₅₀ values in Table 4.2 and Fig. 4.3, A and B. Substitution of Glu-590 with glutamine resulted in a 9-fold increase in malonyl-CoA sensitivity (Table 4.2 and Fig. 4.3B), whereas a change of Glu-590 to Lys resulted in a 53% loss in L-CPTI activity and a 14-fold increase in malonyl-CoA sensitivity (Table 4.2 and Fig. 4.3B). Thus, the partial loss in activity observed with the E590A and E590K mutants was accompanied by a significant increase in sensitivity to malonyl-CoA inhibition compared with the wild-type L-CPTI enzyme (Table 4.2). In short, our studies identify for the first time a conserved residue in the C-terminal region of L-CPTI, Glu-590, which when mutated to neutral and/or positively charged residues is important for increased sensitivity of the enzyme to malonyl-CoA inhibition.

4.3.3 Kinetic Characteristics of Mutant L-CPTIs

Mutants E590A and E590Q exhibited normal saturation kinetics when the carnitine concentration was varied relative to a constant second substrate, palmitoyl-CoA (Fig. 4.4A), a property identical to that of the wild-type L-CPTI. For mutants E590A and E590Q, the calculated K_m values for carnitine were only 26–48% higher compared with the wild type as shown in Table 4.3. However, the V_{max} value for carnitine for the E590A and E590Q mutants was only 14–34% lower compared with the wild-type L-CPTI, indicating no major effect of the mutation on catalytic activity. The catalytic efficiency as estimated by V_{max}/K_m for E590A and E590Q decreased by 39–45%. With respect to the second substrate, palmitoyl-CoA, mutants E590A and E590Q exhibited normal saturation kinetics similar to the wild type (Fig. 4.4B) when the molar ratio of palmitoyl-CoA to albumin was fixed at 6.1:1. The calculated K_m values for mutants E590A and E590Q were 2- and 2.5-fold lower, and the V_{max} values were 58 and 40% lower than the wild type, respectively. For E590A and E590Q, the catalytic efficiency was 63 and 70% lower than the wild type, respectively. Thus, the substitution of the conserved Glu-590 residue with alanine, glutamine, and/or lysine resulted in a reduction in catalytic activity and a



A





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Fig. 4.4 Kinetic analysis of wild-type and mutant L-CPTI activities. Isolated mitochondria (150 μ g protein) from the yeast strains expressing the wild type (\bullet), E590A (\blacktriangle), and E590Q (\diamond) mutants were assayed for CPT activity in the presence of increasing concentrations of carnitine (A) and palmitoyl-CoA (B).

Table 4.2

CPT activity and malonyl-CoA sensitivity in yeast strains expressing wild-type and mutant L-CPTI

Strain	Activity	IC ₅₀
	(nmol/mg.min)	(μM)
Wild-type	7.8 ± 1.3	3.39 ± 0.42
E590A	6.2 ± 1.7	0.21 ± 0.08
E590Q	7.3 ± 0.7	0.39 ± 0.83
E590D	-	
Е590К	3.7 ± 0.4	0.25

Mitochondria (150 μ g of protein) from the yeast strains expressing wild-type L-CPTI, and Glu-590 substitution mutations were assayed for CPT activity and malonyl-CoA sensitivity as described under "Experimental Procedures." The results are the means \pm S.D. of at least three independent experiments with different mitochondrial preparations.

Table 4.3

Strain	Carnitine		Palmitoyl-CoA	
	K _m	V _{max}	K _m	V _{max}
	(µM)	(nmol/mg.min)	(µM)	(nmol/mg.min)
Wild-type	59.3 ± 6.0	20.0 ± 0.5	153.5 ± 5.9	55.0 ± 0.7
E590A	87.6 ± 11.4	17.2 ± 0.6	62.2 ± 7.4	23.2 ± 0.7
E590Q	74.8 ± 3.1	13.5 ± 0.2	74.4 ± 8.1	21.8 ± 0.6

Kinetic characteristics of yeast-expressed wild type and mutant L-CPTIs

Isolated mitochondria (150 μ g of protein) from the yeast strains expressing the wildtype Glu-590 substitution mutations were assayed for CPT activity in the presence of increasing concentrations of carnitine or palmitoyl-CoA. Values are averages of two independent experiments with different mitochondrial preparations. significant increase in malonyl-CoA sensitivity. In contrast, a conservative substitution of Glu-590 with aspartate inactivated L-CPTI, suggesting that the longer side chain of glutamate is required for catalytic activity and malonyl-CoA sensitivity.

4.4 **DISCUSSION**

Our site-directed mutagenesis study of the highly conserved Glu-590 residue in the C-terminal region of L-CPTI demonstrated that substitution with alanine, glutamine, or lysine resulted in partial loss in activity and a significant increase in malonyl-CoA sensitivity. Glu-590 and Glu-603 in the C-terminal region of L-CPTI are the only two conserved glutamate residues in the family of acyltransferases (Fig. 4.1). Glu-603 is a conserved residue within the family of CPT enzymes, whereas other acyltransferases have aspartate at this position. We have recently demonstrated that a change of Glu-603 to alanine or histidine resulted in complete loss in L-CPTI activity [262], whereas a change to glutamine caused a significant loss in activity and malonyl-CoA sensitivity. In contrast with the complete loss of activity observed with the E590D mutant, substitution of Glu-603 with aspartate resulted in only partial loss in CPTI activity but a significant loss in malonyl-CoA sensitivity. A change of the highly conserved glutamate residue corresponding to Glu-590 in CPTII, Glu-487 to aspartate inactivated the enzyme [313], suggesting the importance of this highly conserved residue in CPTI and CPTII in maintaining the active site conformation of the two enzymes. Substitution of the highly conserved Glu-590 with alanine did not have a major effect on catalytic activity but caused a significant increase in L-CPTI malonyl-CoA sensitivity, indicating the opposing roles played by the conserved C-terminal glutamate residues in L-CPTI on activity and malonyl-CoA sensitivity. The site-directed mutagenesis study described here is aimed at elucidating the function of the highly conserved acidic residue, Glu-590, found in the proximity of the active site of L-CPTI.

To determine the role of the highly conserved Glu-590 on catalysis and malonyl-CoA sensitivity, we separately changed the Glu-590 residue to alanine, glutamine, aspartate, and lysine (E590A, E590Q, E603D, and E590K, respectively) and determined

the effect of the mutations on L-CPTI activity and malonyl-CoA sensitivity in the yeastexpressed mutant enzyme. A change of Glu-590 to aspartate inactivated L-CPTI. Since a conservative substitution of Glu-590 with aspartate, a negatively charged amino acid with only one methyl group less than the glutamate residue in the wild-type enzyme, resulted in total loss in CPTI activity, the presence of the extra methyl group in Glu-590 is probably crucial for maintaining the configuration of the L-CPTI active site. This finding suggests that a change of Glu-590 to aspartate may result in the carboxylate being outside the hydrogen bond distance of Asp-567, a highly conserved residue that is predicted to be at the CoA binding site of L-CPTI [135]. Glu-590 may thus be required for L-CPTI stability and positioning of the imidazole ring of His-473 for efficient catalysis and inhibition, thus facilitating productive interaction with the substrates and the inhibitor [17]. The mutant L-CPTIs with a replacement of Glu-590 with alanine (E590A), glutamine (E590Q), and lysine (E590K) showed a partial loss in activity but a significant increase in malonyl-CoA sensitivity. The partial loss in catalytic activity observed with the mutant L-CPTI (E590A, E590Q, and E590K) was in each case associated with a significant increase in malonyl-CoA sensitivity, suggesting that these highly conserved glutamate residues may be important for substrate and inhibitor binding. In contrast, mutation of the corresponding conserved Glu-603 to alanine, histidine, and glutamine resulted in an inactive enzyme or an enzyme with significantly decreased activity and malonyl-CoA sensitivity, suggesting different roles played by these conserved residues in L-CPTI activity and malonyl-CoA sensitivity. The effect of the mutations on activity and malonyl-CoA sensitivity suggest that the highly conserved Glu-590 in the C-terminal region of L-CPTI may be important for substrate (palmitoyl-CoA) and inhibitor (malonyl-CoA) binding, probably through the common CoA moiety present in both compounds. Although there was a decrease in V_{max} for both substrates (carnitine and palmitoyl-CoA) with the E590A, E590Q, and E590K mutants, all of the mutants showed an increase in the affinity for palmitoyl-CoA but not carnitine, suggesting that these residues are involved in binding palmitoyl-CoA and malonyl-CoA but not carnitine. Thus, this region may be involved in binding the CoA moiety of palmitoyl-CoA and malonyl-CoA and/or may be the low affinity acyl-CoA binding site.

As a rate-limiting enzyme that transports long-chain fatty acids from the cytosol to the mitochondrial matrix, L-CPTI in the presence of carnitine catalyzes the conversion of long-chain acyl-CoAs to acylcarnitines [21, 180]. Similar to other acyltransferases, L-CPTI contains a general acid/base, His-473, a highly conserved amino acid residue that may form a hydrogen-bonding network or a salt bridge to a nearby conserved glutamate residue such as Glu-603 [17]. We hypothesize that the substitution of Glu-590 with aspartate may disrupt a hydrogen-bonding network or a salt bridge, perhaps to the highly conserved Asp-567 residue that is predicted to be at the CoA binding pocket of L-CPTI. The hydrogen bonding between the negatively charged carboxyl groups of Glu-590 and Asp-567 may stabilize the positive charge or cation on the carnitine substrate in the wildtype enzyme, but disruption of the hydrogen-bonding network by substitution of Glu-590 with Asp, a negatively charged residue with only one methyl group less than Glu, destabilizes and inactivates L-CPTI. The complete loss in activity in the E590D mutant implicates the important role of Glu-590 in the maintenance of active site architecture, suggesting that substitution of Glu-590 with aspartate may disrupt a hydrogen-bonding network or a salt bridge to a residue like Asp-567 at the substrate binding site pocket of L-CPTI, which may stabilize the positive charge on the carnitine substrate. Disruption of the hydrogen-bonding network due to a change of Glu-590 to Asp may also result in the destabilization of the negative charge on the palmitoyl-CoA substrate due to charge repulsion, thereby inactivating L-CPTI. The substitution of Glu-590 with Gln (E590Q), a neutral residue with the same carbon chain length as Glu, with alanine, a neutral residue with a shorter chain length than Glu, or Lys, a positively charged residue with a longer carbon chain length than Glu, significantly increased the malonyl-CoA sensitivity of L-CPTI with minor change in activity. We suggest that the positive charge in the mutant E590K stabilizes the negative charge on the malonyl-CoA, thus increasing the affinity and sensitivity of the enzyme to the inhibitor, but it may also destabilize the positive charge on the carnitine substrate causing a partial decrease in activity. Because a change of Glu-590 to Gln increased the malonyl-CoA sensitivity of L-CPTI with no effect on activity, we suggest that the negatively charged acidic group of Glu-590 may be responsible for the decreased malonyl-CoA sensitivity of L-CPTI compared with M-

CPTI. The role of the negatively charged –COOH group of Glu-590 in the reduction of malonyl-CoA sensitivity of L-CPTI compared with M-CPTI is further supported by our data demonstrating that a change of Glu-590 to Ala, a neutral residue of a much shorter chain length that Glu, significantly enhanced the malonyl-CoA sensitivity of L-CPTI with a partial decrease in activity.

Recent site-directed mutagenesis studies from our laboratory have demonstrated that glutamic acid 3 and histidine 5 in L-CPTI are necessary for malonyl-CoA inhibition and high affinity binding but not for catalysis [241, 242]. For M-CPTI, our mutagenesis studies demonstrate that in addition to Glu-3 and His-5, Val-19, Leu-23, and Ser-24 are necessary for malonyl-CoA inhibition and high affinity binding, in agreement with the differences in malonyl-CoA sensitivity observed between M-CPTI and L-CPTI [240, 316]. In addition, our site-directed mutagenesis studies of conserved residues in the Cterminal domain of L-CPTI demonstrated that conserved arginine and glutamate residues are important for catalytic activity and malonyl-CoA sensitivity [64, 262]. Others have reported that the deletion of N-terminal residues 19-30 containing Ser-24 and Gln-30 or substitution of Ser-24 and Gln-30 with alanine in L-CPTI increased malonyl-CoA sensitivity but their effect was entirely dependent on the presence of Glu-3 because mutation of Glu-3 to alanine was found to override the effects of both the deletion and the combined effects of S24A and Q30A on malonyl-CoA sensitivity [131, 133], confirming our previous report that Glu-3 in the extreme N-terminal region of L-CPTI is the main determinant of malonyl-CoA sensitivity [241].

Mammalian tissues express two isoforms of CPTI, a liver isoform (L-CPTI) and a muscle isoform (M-CPTI), that are 62% identical in amino acid sequence [40, 282, 283, 304, 305, 314]. M-CPTI is specifically expressed in heart, skeletal muscle, and testis [7, 304, 314]. Previous work from our laboratory and others has shown that M-CPTI has a higher K_m for carnitine and an approximately 30-fold lower IC₅₀ for malonyl-CoA [40, 282, 283, 304, 305, 314] inhibition than L-CPTI. In adult heart mitochondria, the total carnitine level is high. Consequently, M-CPTI has a high K_m for carnitine but the molecular and/or structural basis for the high malonyl-CoA sensitivity of M-CPTI compared with L-CPTI remains to be established. In this report, we demonstrate that a

single substitution of the conserved C-terminal Glu-590 residue to Ala, Gln, and Lys in L-CPTI results in a switch in the kinetic properties of the liver to the muscle isoform of the enzyme because the malonyl-CoA sensitivity of L-CPTI increased to a level close to that observed in M-CPTI. This is the first demonstration of a change in the kinetic properties of the liver isoform of CPTI close to that observed in the muscle isoform of the enzyme by a single site mutation of a conserved negatively charged C-terminal residue to neutral or positively charged residues and provides an important clue in understanding of the differences in malonyl-CoA sensitivity between M-CPTI and L-CPTI.

In this report, we demonstrate that the highly conserved Glu-590 of L-CPTI is required for the structural stability of the enzyme and substitution of this residue with alanine increases its malonyl-CoA sensitivity close to that observed with the muscle isoform of the enzyme, M-CPTI. Despite its similar charge and potential for hydrogenbonding formation, aspartate, which has a shorter side chain, cannot substitute for glutamate, suggesting that the extra methyl group of Glu-590 and/or its ability to serve as a strong hydrogen bond acceptor is needed for optimal catalysis, maintenance of active site integrity, and malonyl-CoA inhibition and binding. For the E590D mutant, our data suggest that the loss of a methyl group may result in the carboxylate being outside the hydrogen bond distance of the conserved Asp-567 residue that is at the substrate and/or active site pocket. This finding suggests that the longer side chain of glutamate is essential for substrate binding, catalytic activity, and malonyl-CoA sensitivity. Because only a maximum 16-fold increase in malonyl-CoA sensitivity was observed in these mutants compared with >30-fold higher sensitivity to the inhibitor reported with the wildtype M-CPTI, it is predicted that this C-terminal region may constitute the low affinity malonyl-CoA binding site in L-CPTI [241]. Our site-directed mutagenesis study of the only two conserved C-terminal glutamate residues, E590A and E603, in L-CPTI has led to the identification of these residues in L-CPTI that are important for catalytic activity and malonyl-CoA sensitivity. Because a mutation of any of these two conserved Cterminal residues (Glu-590 and Glu-603) substantially decreased catalytic activity and increased (Glu-590) and/or decreased (Glu-603) malonyl-CoA sensitivity, it is hypothesized that these residues are the major contact sites between L-CPTI and the CoA

moiety of the substrate (palmitoyl-CoA) and the inhibitor (malonyl-CoA) and constitute the putative low affinity acyl-CoA/malonyl-CoA binding site in L-CPTI.

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CHAPTER 5

LEUCINE-764 NEAR THE EXTREME C-TERMINAL END OF CARNITINE PALMITOYLTRANSFERASE I IS IMPORTANT FOR ACTIVITY*

5.1 INTRODUCTION

Carnitine palmitoyltransferase I (CPTI) catalyzes the conversion of long-chain fatty acyl-CoAs to acyl-carnitines in the presence of L-carnitine, a rate-limiting step in the transport of long-chain fatty acids from the cytoplasm to the mitochondrial matrix [21, 180]. Mammalian tissues express two isoforms of CPTI—a liver isoform (L-CPTI) and a heart/skeletal muscle isoform (M-CPTI)—that are 62% identical in amino-acid sequence [6, 40, 282, 283, 304, 305, 314]. Although adult heart expresses both isoforms of CPTI, the predominant form is M-CPTI [40, 282, 283]. The IC₅₀ for malonyl-CoA inhibition of heart mitochondrial M-CPTI is ~30- to100-fold lower than that of L-CPTI, but the malonyl-CoA concentration in both tissues is similar [180, 314]. It is estimated that about 60–80% of the energy requirement of the heart is derived from fatty acid oxidation [286]. The important question of how fatty acid oxidation can proceed in heart in the presence of high tissue levels of malonyl-CoA was resolved in part by recent reports of the transcriptional regulation of M-CPTI gene expression by long-chain fatty acids via the peroxisome proliferator-activated receptor (PPAR) [33, 163, 173, 311].

We have expressed human heart M-CPTI, rat liver L-CPTI, and CPTII in the yeast *Pichia pastoris*, an organism devoid of endogenous CPT activity [72, 314, 315].

^{*} This material has been published in this or similar form in *Biochemical and Biophysical Research Communication* and is used here with permission of Academic Press:

Dai, J., Zhu, H., and Woldegiorgis, G. (2003) Leucine-764 near the extreme C-terminal end of carnitine palmitoyltransferase I is important for activity. *Biochem. Biophys. Res. Commun.* 301, 758-763.

Contribution: I designed and conducted most of the experiments except the deletion mutation analysis shown in table 1 and 2. I collected and analyzed all the data. I prepared all figures for publication.

Our recent deletion and point mutation analyses have demonstrated that glutamate-3 and histidine-5 are necessary for malonyl-CoA inhibition and high-affinity binding of L-CPTI but not for catalysis [241, 242].

In human muscle M-CPTI, unlike rat L-CPTI, deletion of the first 28 but not 18 N-terminal residues of M-CPTI abolishes malonyl-CoA inhibition and high-affinity binding [240]. Indirect studies from several laboratories have predicted that the catalytic and substrate binding sites in both L-CPTI and M-CPTI reside in the C-terminal region of the enzymes. Recent studies from our laboratory demonstrate that mutations of conserved arginine and tryptophan residues in the C-terminal region of L-CPTI abolish catalytic activity [64]. Since the major effect of the mutations was on the V_{max} , we predict that the conserved arginine and tryptophan residues stabilize the enzyme–substrate complex by charge neutralization and hydrophobic interactions [64]. In this communication, we report that deletion of a single amino-acid residue, leucine-764 (L764), from the extreme C-terminus of M-CPTI abolishes catalytic activity.

5.2 MATERIALS AND METHODS

5.2.1 Construction of Plasmids for the C-Terminal Deletion and Point Mutants of Human M-CPTI

In general, mutants of human heart M-CPTI were constructed as previously described [240]. Mutants were constructed by PCR using pGAP-M-CPTI plasmid DNA [314] as template with the forward primer F-HM883, 5'-TCATGTATCGCCGTAAAC-3', and the reverse primers (shown in Table 5.1), which carry an *Apa*I site (bold) followed by two stop codons (underlined) and substitution mutations (underlined italic). The PCR products were digested with restriction enzymes *BgI*II and *Apa*I, and the resulting DNA fragments were then ligated with *BgI*II- and *Apa*I-digested pGAP-M-CPTI. After transformation, the bacterial colonies were screened using PCR. The DNA sequences of positive colonies were then confirmed by DNA sequencing.

Table 5.1

PCR primers used for construction of L-CPTI mutants

Mutants	Sequences
Δ20 (753-772)	5 -TTAGATCTGGGCCCTCACTAAAAGCGCTGGGCGTTGGTCT-3
Δ10 (763-772)	5 [´] -TTAGATCT GGGCCC<u>CTATCA</u>AATGTCCAGCAGGGCTTTGC-3[´]
Δ9 (764-772)	5 [´] -TTAGATCT GGGCCC<u>CTATCA</u>ATCAGCAATGTCCAGCAG-3[´]
Δ8 (765-772)	5'-TTAGATCTGGGCCCCCTATCAAAGATCAGCAATGTCCAG-3'
Δ7 (766-772)	5'-TTAGATCTGGGCCCCCTATCAGAAAAGATCAGCAATGTC-3'
Δ6 (767-772)	5 TTAGATCTGGGCCCCTATCATTGGAAAAGATCAGCAATGT-3
A3 (770-772)	5 - TTAGATCTGGGCCCCCTATCACTTGGGAACTTGGAAAAGAT-3
L764A	5 - TTAGATCTGGGCCCCCTTATCAGCTGTAGGCCTTGGGAACTTGGAAAGCATCAGCAATGTCCAGCAG 3'
L764V	5'-TTAGATCTGGGCCC <u>CTATCA</u> GCTGTAGGCCTTGGGAACTTGGAA <u>AAC</u> ATCAGCAATGTCCAGCAG- 3'
L764R	5'-TTAGATCTGGGCCCCCTATCAGCTGTAGGCCTTGGGAACTTGGAAACGATCAGCAATGTCCAGCAG 3'

5.2.2 Integration of Mutant Human Heart M-CPTI cDNA into the *P. pastoris* Genome

Each plasmid was linearized by digestion with the restriction enzyme *BspE*I [314]. The linear DNA was introduced into *P. pastoris* by electroporation. Integrants were recovered as histidine prototrophic transformants after selection on YND plates and grown on YND medium containing glucose. Mitochondria were isolated from the wild-type and mutants by disrupting the yeast cells with glass beads as described previously [72, 314].

5.2.3 CPTI Activity, Malonyl-CoA Inhibition, and Kinetic Assay

M-CPTI activity was assayed in isolated mitochondria from the yeast strains expressing the wild-type and mutant CPTIs by the forward exchange method using L-[³H]carnitine as described previously [35, 72, 314]. The K_m for palmitoyl-CoA was determined by varying the palmitoyl-CoA concentration in the presence of a fixed albumin concentration (1%) or a fixed molar ratio (6.1:1) of palmitoyl-CoA to albumin [212, 221].

5.2.4 Western Blot Analysis

Proteins were separated by SDS–PAGE in a 10% gel and transferred onto nitrocellulose membranes. Immunoblots were developed by incubation with the M-CPTI-specific polyclonal antibodies as described previously [72, 314, 315]. Sources of materials and other procedures were as described in our previous publications [72, 241].

5.3 RESULTS

5.3.1 Generation of Deletion and Point Mutants and Expression in P. pastoris

Construction of plasmids carrying the C-terminal deletions and point mutation of human heart M-CPTI was performed as described in Materials and methods. The deletions and point mutations were confirmed by DNA sequencing. The deletions ranged from the smallest, 3, to the largest, 210 amino-acid residues as shown in Table 5.2 and

	754		
Sec. Struct	checceurecourcessecourcessessessessessessessessessessessessess		
CPTS_RAT	OPEQY FNELCACOCPCIPVADHCYCVEYN I ACENTRY PRYSKELSESETHAL APCORI I NQALLO I ADEPKI SETCE		
CPTN_MOUSE	DPROYPHILGAGOGPGPVADOGYGVSYNIAGENTHFFHISBKYSSSBINAGEFGRHIRGALLDIAELYKISKTOS		
CPT1_NAT	DPERIPLY'S CORPOPULATION OVSTITUENT IN PILSSET SSTATISHER GALLEGARAL LITERALIANSA.		
CPTI JUMAN	DLENNPEYVSSGGCPGPYADDGYCVSY1LVCENLINFHISSKFSCPHTOSHAFGRHLKEANTOIITLPCLSENSKK		
CAT_PIGEON	DLEXHPEYVSSGUGPGPVADDGYUVSYIIVGDHLISPXISSKFSCPETOSHRFGXHLKDANTDIIGLPSPCPNSXK		
CAT_MOUSE	STROVPARTXXVIEPGPVVPDOYGLCYNPKEAH - IMLSVBAYNSCAETNAARNAHYLEXALLINNTILONIPRAKL		
OCTC_BOVIN	et sov faktiol van polipov post gvet in preski-tik post ansele tik and an and an to exact dinamedia is praktive St SLV-Gylrvogvinv prvesky grevni i Rock-povise savksep storektik voov fir podinklikkerit.		
OCTC_HUMAN	STELV-GYLRVQGVVVPHVRNJYGJFYHIRDOR-FVVACSAMKSCFETDAEXLRQL/TFCAFHENIQL/MSTHL		
CLAT_NOUSE	ntslv-utlaluguvvyavandtupyta tala-itycleskalletlakealverlyaryndalsertall- Btsgypttaenpylygpvvyngygaltaphara-ityclesyngeretefvzyaraularlygnrdrlserdyrderpytakerangpsg		
CLAT_HUMAN	btsqvptttenfocygpvvpngygacynpopet-ilfcissthsckstssskfakaveeslidnrdlcsllppteskflatkekatkpso		
CLAT_RAT	stervy pitkerpelt topy vprotionet rppppea ~ 1 type is sprecket by by alavias ly utredk. BS repairs repairs and t Provy type subject yo pvv predy calt y popper ~ 1 lpp is sprecket is sublax a versile ir duct recess tak slakoker to las		
CLAT_PIG	etsovptthenpolygpvvpngygacynpopes-ilfclssphccketsstkfakavesspiennglcslsobcngkplatkenvtrpso		
Consensus	scaqup geven burg crup e i liss sc st a a al fur b		
CPTM_HUMAN	human carnifine o-palmitoyitransferase 1, milochondrial muscle isoform (Q92523)		
CPTM_RAT	rat carnitine o-paininoyitransierase 1, mitochondrial muscle isoform (Q03/04)		
CPTM_MOUSE	mouse carning o-painticoviransierase I, mitochonorial muscle isotorii (CAC40150)		
CPT1_MOUSE	mouse carmine o-paininoyiransicrase I, micedonariai nyer isoform (Fy/142)		
CPTI_KAT:	rat carning o-panning yuransierse L, ninochonoriai hyer isolonin (r52198)		
CPT1_HUMAN	numan carnitine o-paimitoyitransterase I, mitochondriai liver isoform (PD0416)		
CPT1_PIG	pig carnitine o-palimitoyliransterase I, mitochondrial liver isotorm (AAK69171)		
CAT_PIGEON	pigeon camitine o-acetyltransferase (P52826)		
CAT_MOUSE	mouse carnitine o-acetyltransferase (P47934)		
CAT_HUMAN	human carnitine o-acetyltransferase (P43155)		
OCTC_BOVIN	bovin peroxisomal carnitine octanoyltransferase (O19094)		
OCTC_HUMAN	human peroxisomal carnitine octanoyltransferase (Q9UKG9)		
OCTC_RAT	rat peroxisomal carnitine octanoyltransferase (P11466)		
CLAT_MOUSE	mouse choline o-acetyltransferase (Q03059)		
CLAT_HUMAN	human choline o-acetyltransferase (P28329)		
CLAT_RAT	rat choline o-acetyltransferase(A48319)		
CLAT_CHICKE	an chicken choline o-acetyltransferase(AAK94673)		
CLAT_PIG	pig choline o-acetyltransferase (P13222)		
Sec. Struct :			
Alpha hel	.ix (h)		
Extended	strand (e)		
Random co	bil (c)		
Consensus : Upper c Lower c	ase: Residues conserved for 90% or more ase: Residues conserved for more than 50% and less than 90%		
Ŧ	: NDQE conserved positions		

Fig. 5.1 Sequence alignment of the extreme C-terminal region of the CPT family of proteins





Mitochondria (40µg of protein) from the wild-type yeast strain and the strains expressing each of the deletion and point mutants were separated on a 7.5% SDS–PAGE and blotted onto a nitrocellulose membrane. The immunoblot was developed using M-CPTI-specific polyclonal antibodies as described previously [315]. 1, Wild-type. C-terminal deletions and point mutants: 2, $\Delta 9$; 3, $\Delta 10$; 4, L764R; 5, L764A; and 6, $\Delta 8$.
Table 5.2

Activity and malonyl-CoA sensitivity of mutant enzymes truncated

in the C-terminal region

Deletion mutants	Activity	
	(No malonyl-CoA)	(70nM malonyl-CoA)
Wild-type	2.02 ± 0.08	1.18 ± 0.05
$\Delta 210 (563-772)$		
Δ113 (660-772)		
∆44 (729–772)		
Δ20 (753-772)	*arterester	
∆10 (763–772)		
Δ9 (764-772)		
Δ8 (765-772)	2.67 ± 0.11	1.48 ± 0.04
Δ7 (766-772)	2.92 ± 0.13	1.57 ± 0.07
Δ6 (767-772)	2.54 ± 0.21	1.61 ± 0.07
Δ3 (770-772)	2.10 ± 0.09	1.11 ± 0.12

Fig. 5.1. *P. pastoris* was chosen as the expression system for wild-type M-CPTI and the mutants, because it does not have endogenous CPT activity [72, 242, 314, 315]. The *P. pastoris* expression plasmids expressed M-CPTI under control of the *P. pastoris* glyceraldehyde-3-phosphate dehydrogenase gene promoter [242, 281].

Western blot analysis of wild-type M-CPTI (80 kDa) and the mutants, using a Cterminal polyclonal antibody directed against a maltose binding protein-M-CPTI fusion protein [314], is shown in Fig. 5.2. For the wild-type and all the mutants, proteins of the predicted sizes were synthesized and expressed at similar steady-state levels.

5.3.2 Effect of Deletions and Point Mutations on M-CPTI Activity and Malonyl-CoA Inhibition

To determine the role of the C-terminal region of M-CPTI in activity, we first constructed an M-CPTI deletion mutant lacking the 210 C-terminal amino-acid residues. Mitochondria from the yeast strain expressing deletion mutant 210 had no CPTI activity. Subsequent deletion of the last 113, 44, 20, 10, and 9 C-terminal amino-acid residues resulted in an inactive M-CPTI. However, deletion of the last 3, 6, and 8 C-terminal amino-acid residues resulted in mutant M-CPTI that had activity similar to the wild-type, demonstrating that L764 is essential for M-CPTI activity (Table 5.2). We then constructed a series of substitution mutants L764A, L764V, and L764R, the CPT activity level was 60.4%, 100%, and 16.3%, respectively, of that observed with the wild-type (Table 5.3). Decreasing the temperature for the CPTI assay from 30 to 15 °C for the wild-type and the L764R mutant had no effect on the differences in activity observed. All of the mutants with CPT activity had a level of malonyl-CoA sensitivity similar to that of the wild-type.

5.3.3 Kinetic Properties of Wild-Type and Mutant M-CPTIs

Substitution mutant L764R exhibited normal saturation kinetics when the carnitine concentration was varied relative to a second substrate, palmitoyl-CoA (Fig. 5.3A), under standard assay conditions, a property identical to that of the wild-type M-





Table 5.3

Substitution mutants	Activity	
	(No malonyl-CoA)	(70nM malonyl-CoA)
Wild-type	2.02 ± 0.08	1.18 ± 0.05
L764V	2.2 ± 0.10	1.34 ± 0.07
L764A	1.22 ± 0.05	0.84 ± 0.03
L764R	0.33 ± 0.01	0.15 ± 0.01

Activity and malonyl-CoA sensitivity of L764 substitution mutant enzymes

CPTI. The calculated K_m for carnitine for the L764R mutant was more than 2-fold higher than the wild-type, but the V_{max} was 49.4% lower than the wild-type value (Table 5.4). The catalytic efficiency as estimated by V_{max}/K_m for the L764R mutant decreased by 78%. With respect to the second substrate, palmitoyl-CoA, both the wild-type and L764R mutant showed normal saturation kinetics when the molar ratio of palmitoyl-CoA to albumin was fixed at 6.1:1 (Fig. 5.3B). For the L764R mutant, the calculated K_m for palmitoyl-CoA was similar to that of the wild-type. However, the V_{max} for palmitoyl-CoA for this mutant was only 35.6% of the wild-type. The catalytic efficiency decreased by 79.5%.

5.4 DISCUSSION

The catalytic site pocket and the substrate binding sites in both L-CPTI and M-CPTI were predicted to be located in the C-terminal region, whereas the malonyl-CoA binding site was predicted to reside in the N-terminal region of the enzymes. Our main goal in these studies was to identify amino-acid residues in the C-terminal region of M-CPTI that are important for activity. Our results demonstrate that deletion of L764 inactivates M-CPTI. Sequence alignment of all CPTIs from different species shows that L764 is highly conserved within the CPTI family (Fig. 5.1).

Secondary structure prediction of the M-CPTI sequence by the Garnier method using PC/Gene and the coil version 2.2 software indicated the presence of a coiled-coil α helix encompassing residues 744–764 of the C-terminal region of M-CPTI [100, 111, 170]. Substitution of L764 with valine had no effect on activity and malonyl-CoA sensitivity. Replacement of L764 with alanine resulted in 40% loss in M-CPTI activity, whereas substitution of L764 with arginine, a bulky positively charged amino-acid residue, caused a significant loss in activity but not malonyl-CoA sensitivity, suggesting that the hydrophobic environment of Leu-764 promotes proper folding for optimal activity. Since a 2-fold increase in the K_m for carnitine was also observed for the L764R mutant, this domain may indirectly be involved in properly orienting the carnitine binding site for maximal activity. Although not established for the CPT system, based on

Table 5.4

Kinetic characteristics of wild-type and mutant M-CPTI

Strain	n Carnitine			Palmitoyl-CoA		
	$K_{m}(\mu M)$	$V_{\rm max}$ (nmol/mg min)	$K_{\rm in}$ (μ M)	$V_{\rm max}$ (nmol/mg min)		
Wild-type	679.1 ± 40.5	2.5 ± 0.1	46.6 ± 1.2	17.0 ± 0.4		
L764R	1588.3 ± 55.6	1.3 ± 0.1	44.8 ± 0.9	6.1 ± 0.2		

Isolated mitochondria (150µg) from the yeast strains expressing the wild-type and the L764R M-CPTI mutant were assayed for CPT activity in the presence of increasing concentrations of carnitine and palmitoyl-CoA as described in Materials and Methods.

findings with other acyltransferases, the carnitine binding site is predicted to reside within the C-terminal region of the enzyme [62].

Our results strongly suggest that L764 is an important determinant of M-CPTI activity, because deletion of L764 (Δ 9) or a single site mutation eliminates or reduces M-CPTI activity, but the extreme C-terminal deletion mutants Δ 3, Δ 6, Δ 7, Δ 8 or the substitution mutant L764V had no effect on M-CPTI activity. Mutants with deletions of 200, 113, and 44, 20, 10, or 9 extreme C-terminal amino-acid residues inactivated M-CPTI. The total loss in M-CPTI activity observed in the Δ 9 mutant compared to the Δ 8 mutant could be attributed to the deletion of the conserved residue L764, which is deleted in the first but not the second mutant. Substitution mutant L764R severely decreased but did not eliminate M-CPTI activity, while the mutant L764A moderately reduced CPT activity. Substitution of L764 with valine had no effect on CPT activity. However, substitution of L764 with arginine also decreased the *V_{max}* for both substrates by at least 2-fold, suggesting that L764 may be important in maintaining a hydrophobic environment for native enzyme folding and M-CPTI activity.

M-CPTI is an integral membrane protein located on the outer mitochondrial membrane that catalyzes the rate-limiting step in the transport of long-chain fatty acyl-CoAs from the cytosol to the mitochondrial matrix for β -oxidation. The proposed model for the membrane topology of CPTI predicts exposure of the N- and C-termini, domains crucial for activity and malonyl-CoA sensitivity (~90% of the total residues of CPTI) on the cytosolic side of the outer mitochondrial membrane [91]. In this report, we have presented evidence that the extreme C-terminal region of M-CPTI is important for activity, because deletion of L764 or substitution with arginine inactivates M-CPTI, suggesting that L764 is important for native enzyme folding and M-CPTI activity.

CHAPTER 6

SUBSTITUTION OF GLUTAMATE-3, VALINE-19, LEUCINE-23, AND SERINE 24 WITH ALANINE IN THE N-TERMINAL REGION OF HUMAN HEART MUSCLE CARNITINE PALMITOYLTRANSFERASE I ABOLISHES MALONYL COA INHIBITION AND BINDING*

6.1 INTRODUCTION

Carnitine palmitoyltransferase I (CPTI), catalyzes the conversion of long-chain fatty acyl-CoAs to acylcarnitines in the presence of L-carnitine [21, 180]. Mammalian tissues express two isoforms of CPTI—a liver isoform (L-CPTI) and a heart/skeletal muscle isoform (M-CPTI)—that are 62% identical in amino acid sequence [6, 40, 282, 283, 304, 305, 314]. Although adult heart expresses both isoforms of CPTI, the predominant form is M-CPTI [40, 282, 283]. The IC₅₀ for malonyl-CoA inhibition of M-CPTI is ~30- to100-fold lower than that of L-CPTI, but both tissues have similar malonyl-CoA concentrations [180, 314]. It is estimated that about 60 to 80% of energy requirement of heart is derived from fatty acid oxidation [286]. 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 transcriptional regulation of the M-CPTI gene expression by long-chain fatty acids via the peroxisome proliferator-activated receptor (PPAR) [33, 173, 311]. Long-chain fatty acids activate PPAR, which then heterodimerizes with the 9-cis-retinoic acid receptor, binds to the fatty acid response

Contribution: I conducted the western blot analysis.

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Zhu, H., Shi, J., Treber, M., Dai, J., Arvidson, D. N., and Woldegiorgis, G. (2003) Substitution of glutamate-3, valine-19, leucine-23, and serine-24 with alanine in the N-terminal region of human heart muscle carnitine palmitoyltransferase I abolishes malonyl CoA inhibition and binding. *Arch. Biochem. Biophys.* **413**, 67-74

element on the promoter region of the M-CPTI gene, and activates transcription of the M-CPTI gene [33]. In heart, the flux of high levels of long-chain fatty acyl-CoAs through the CPT system may transiently increase long-chain acyl-CoA levels in the microenvironment of M-CPTI, resulting in competitive displacement of malonyl-CoA from the M-CPTI binding site and a reduction in malonyl-CoA sensitivity. In addition, high levels of long-chain fatty acyl-CoAs stimulate the AMP-activated protein kinase, inhibit acetyl-CoA carboxylase, and turn off malonyl-CoA synthesis, thus decreasing M-CPTI inhibition [163]. The high rates of β -oxidation flux observed in heart despite malonyl-CoA levels exceeding the IC₅₀ for inhibition of CPTI may in part also be because CPTI is rate limiting *in vivo* due to the high concentrations of malonyl-CoA in heart and muscle [79].

We have expressed human heart M-CPTI and rat liver L-CPTI and CPTII in the yeast *Pichia pastoris*, an organism devoid of endogenous CPT activity [72, 314, 315]. Our recent deletion and substitution mutation studies have demonstrated that Glu3 and His5 are necessary for malonyl-CoA inhibition and high-affinity binding of L-CPTI but not for catalysis [241, 242]. In human muscle M-CPTI, unlike rat L-CPTI, deletion of the first 28 but not the 18 N-terminal residues of M-CPTI abolishes malonyl-CoA inhibition and high-affinity binding [240]. In this communication, we report that, in addition to Glu3, three other residues in M-CPTI (Val19, Leu23, and Ser24) are important for malonyl-CoA sensitivity and binding.

6.2 MATERIALS AND METHODS

6.2.1 Construction of Plasmids for the N-Terminal Substitution Mutants of Human M-CPTI

In general, mutants of human heart M-CPTI were constructed as previously described [240]. Mutants E3A and H5A were constructed by PCR using pGAP-M-CPTI plasmid DNA [314] as template with the reverse primer R-HM714, 5'-CCACCAGTCACTCACATA, and the *Mun*I site containing forward primers F-E3A, 5'-GAAT<u>CAATTG</u>ATGGCGGCAGCTCACCAGGCCGTGGC, or F-H5A, 5'-GAAT<u>CAATTG</u>ATGGCGGAAGCTGCCCAGGCCGTGGCCTTCCA. The *Mun*I restriction site is underlined and the mutations are shown in boldface. The 668-bp PCR product was digested with *Mun*I and *EcoR*I, and the resulting DNA fragment was then ligated with *EcoR*I-linearized expression vector pHWO10 [314] to produce plasmids pHME3AN and pHMH5AN. To complete the construction, the remaining 1950-bp *EcoR*I fragment of HHMCPTI was inserted into *EcoRI*-linearized pHME3AN and pHMH5AN to produce final constructs pHME3A and pHMH5A.

6.2.2 Construction of the Triple M-CPTI Mutant V19AL23AS24A

The plasmid was constructed using the QuickChange (Strategene) site-directed mutagenesis kit according to the manufacturer's instructions. The wild-type pGAP-M-CPTI plasmid DNA was used as a template with the complementary primers HCPTI3PM-F, forward,

5'-TCACGGTGACCCCAGACGGGGCAGACTTCCGGGCAGCACGGGAGGCCCTG A-3', and R2, reverse, 5'-TTGTCAAACCACCTGTTG-3'. A 1.4-kb *BstEII–SphI* DNA fragment containing the mutations was subcloned into a *BstEII–SphI*-cut wild-type pGAP–M-CPTI plasmid in the *P. pastoris* expression vector pHW010 to generate the V19AL23AS24A M-CPTI mutant.

6.2.3 Construction of Δ18 + V19AL23AS24A Mutant Human M-CPTI

Mutant $\Delta 18$ + V19AL23AS24A was constructed using the QuikChange (Stratagene) site-directed mutagenesis kit according to the manufacturers instructions. The template was HM $\Delta 18$ [240] and the complementary primers used were F-HCPT3M, 5'-AACTATCAAGAATTGATGGCAGACTTCCGGGCAGCACGGGAGGCCCTGAA ACACGTCTACCTGTCT, and R-HCPT3M,

5'-AGACAGGTAGACGTGTTTCAGGGCCTCCCG**TGCTGC**CCGGAAGTC**TGC**CA TCAATTCTTGATAGTT. The product of the mutagenesis reaction was digested with the restriction enzymes *Cla*I and *Xcm*I to produce a 475-bp fragment that was ligated with *Cla*I- and *Xcm*I-digested pGAP–M-CPT1.

6.2.4 Construction of the Four-residue Substitution Mutant E3AV19AL23AS24A

A 380-bp *ClaI–Bst*II DNA fragment containing the substitution mutation E3A from the mutant E3A plasmid DNA construct described above was subcloned into a *ClaI–BstE*II-cut mutant M-CPTI plasmid DNA containing the mutations V19A, L23A, and S24A described above to generate the mutant M-CPTI with the four-residue mutations.

6.2.5 Construction of the Five-residue Substitution Mutant E3AH5AV19AL23AS24A

A 1.0-kb DNA fragment containing the E3A and H5A mutations was PCRamplified using the wild-type pGAP-M-CPTI DNA as a template and the primers forward, 5'-GAATCAATTGATGGCGGCAGCTGCCCAGGCCGTGGCCTTCCA-3', and reverse R4, 5'-AGCGTCCCTTGTGGTAGA-3'. The PCR product was digested with *MunI-EcoRI*, and the 660-bp fragment was ligated into an *EcoRI*-cut *P. pastoris* expression vector pHW010. The remaining 2.0-kb *EcoRI* fragment of M-CPTI was then inserted into the *EcoRI*-cut plasmid containing the mutant E3AH5A. The mutant M-CPTI DNA containing the five-residue substitution mutation E3AH5AV19AL23AS24A was constructed by subcloning a 1.9-kb *BstEII-Bgl* II DNA fragment from the M-CPTI triple mutant V19AL23AS24A into a *BstEII-Bgl*II-cut E3AH5A mutant M-CPTI plasmid DNA in the expression vector pHW010.

6.2.6 Addition of a C-terminal His Tag to Mutant Proteins

A 923-bp DNA fragment was PCR-amplified using the plasmid pGAP-M-CPTI as a template, the forward primer F-HM1464, 5'-GCAGATGCTCCCATCATT, and the reverse primer R-HCPT-His,

5'-GGAAGATCTGAATTCCTAATGATGATGATGATGATGATGGCTGTAGGCCTTGG GAAC. The reverse primer encodes a six-histidine tag (boldface) and introduces restriction sites for *EcoRI* (underlined) and *Bgl*II (italic). The PCR product was digested with *Bgl*II to produce a 455-bp DNA fragment which was then ligated into *Bgl*IIlinearized pGAP-M-CPTI to produce plasmid pGAP-M-CPTI-His. This plasmid thus carries a 1715-bp *EcoRI* cassette that can replace the corresponding 1950-bp *EcoRI* fragment to generate proteins with C-terminal His tags.

6.2.7 Integration of Mutant Human Heart M-CPTI DNA into the *P. pastoris* Genome

Each plasmid was linearized by digestion with the restriction enzyme *BspE*I [314]. The linear DNA was introduced into *P. pastoris* by electrotransformation. Integrants were recovered as histidine prototrophic transformants after selection on YND plates and were grown on YND medium containing glucose. Mitochondria were isolated from wild-type and mutant M-CPTI cells by disruption with glass beads as described previously [72, 314].

6.2.8 CPTI Assay

CPTI activity was assayed in isolated mitochondria from the yeast strains expressing the wild-type and mutant CPTIs by the forward exchange method using L-[³H]carnitine as described previously [35, 72, 314]. The K_m for palmitoyl-CoA was determined by varying the palmitoyl-CoA concentration from 2.8 to 450 μ M at a fixed molar ratio (6.1:1) of palmitoyl-CoA to albumin as described previously [212, 221]. The concentration of carnitine was fixed at 1.0 mM. The K_m for carnitine was determined by varying the carnitine concentration from 50 μ M to 2.0 mM at a fixed 111.1 μ M palmitoyl-CoA.

6.2.9 [¹⁴C]malonyl-CoA Binding Assay

[¹⁴C]malonyl-CoA binding in isolated mitochondria from the yeast strains expressing the wild-type and mutant M-CPTIs was determined by a modified centrifugation assay as described previously [169, 241, 242]. The CPT activity and IC₅₀ values are given as a mean \pm SD for at least three independent assays with different preparations of mitochondria. The K_D values are averages of at least two independent experiments.

6.2.10 Western Blot Analysis

C-terminal His-tagged proteins were separated by SDS–PAGE in a 7.5–10% gel and transferred onto nitrocellulose membranes. Immunoblots were developed by incubations with the anti-His antibodies (Penta-His antibody; Qiagen, Valencia, CA), followed by goat anti-mouse IgG conjugated to horseradish peroxidase (Jackson Immuno Research Laboratories, West Grove, PA). The antigen–antibody complex was detected using an enhanced chemiluminescence detection kit [72, 314, 315].

Sources of materials and other procedures were as described in our previous publication [241].

6.3 RESULTS

6.3.1 Generation of Mutants and Expression in P. pastoris

Construction of plasmids carrying the N-terminal deletion and substitution mutations of human heart M-CPTI was performed as described under Materials and methods. The deletions and point mutations were confirmed by DNA sequencing. The mutations are shown in Fig. 6.1. *P. pastoris* was chosen as the expression system for the wild-type M-CPTI and the mutants, because it does not have endogenous CPT activity [241, 242, 314, 315]. The *P. pastoris* expression plasmids expressed M-CPTI under control of the *P. pastoris* glyceraldehyde-3-phosphate dehydrogenase gene promoter [72, 281].

Western blot analysis of the C-terminal His-tagged wild-type M-CPTI (80 kDa) and the mutants, using an anti-His antibody, is shown in Fig. 6.2A and B. For the wild type and all the mutants, proteins of the predicted sizes were synthesized and expressed at similar steady state levels.

6.3.2 Effect of Mutations on M-CPTI Activity and Kinetic Properties

All of the mutants showed CPT activity similar to that of the wild-type enzyme except for the E3AH5AV19AL23AS24A mutant which exhibited 40% of the wild-type M-CPTI activity as shown in Table 6.1. There was no effect of the His tag on CPTI



Fig. 6.1 Amino acid sequence of the first 30 N-terminal residues of human M-CPTI.

The position of each of the mutants is shown by an arrow





(A) Mitochondria (40 μ g of protein) from the wild-type yeast strain and the strains expressing each of the mutants were separated on a 7.5–10% SDS–PAGE and blotted onto a nitrocellulose membrane. The immunoblot was developed using anti-His antibodies as described under Materials and methods. Lanes 1, wild-type; 2, E3A; 3, H5A; 4, Δ 18 + V19AL23AS24A; 5, wild-type without His tag; 6, vector. (B) Same as A except that 30 μ g of protein was used. Lanes 1, wild-type; 2, vector; 3, E3AV19AL23AS24A (EVLS); 4, E3AH5AV19AL23AS24A (EHVLS).

Table 6.1

CPT activity, malonyl-CoA sensitivity, and binding in yeast strains expressing wildtype and mutant M-CPTI

Strain	Activity (nmol/mg min)	IC ₅₀ (µm)	K _{D1} (mm)	K _{D2} (nm)	B _{mas1} (pmol/mg)	Bmax2 (pmol/mg)
Wild type	2.5 ± 0.4	0.07 ± 0.01	13.4	97.6	23.0	76.8
$\Delta 18 + V19AL23AS24A$	3.6 ± 0.6	10.0 ± 1.0	_	-		-
E3A	2.4 ± 0.5	4.0 ± 0.5		106.9		7.9
H5A	2.2 ± 0.3	0.15 ± 0.02	26,5	-	20.1	-
EVLS	2.7 ± 0.6	10		-		-
EHVLS	1.0 ± 0.11	6.8		-		-

Mitochondria were isolated from the yeast strains separately expressing M-CPTI and the mutants and were assayed for CPT activity, malonyl-CoA sensitivity, and binding as described under Materials and methods. IC_{50} is the concentration of malonyl-CoA needed to inhibit 50% of the activity of the yeast-expressed M-CPTI, and results are mean \pm SD of at least three independent experiments with different mitochondrial preparations. The K_D and B_{max} values are averages of two independent experiments with different mitochondrial preparations. EVLS=E3AV19AL23AS24A; EHVLS= E3AH5AV19AL23AS24A. activity. Furthermore, all of the mutants showed normal saturation kinetics when the carnitine concentration was varied relative to a second substrate, palmitoyl-CoA (Fig. 6.3A). The calculated K_m and V_{max} for carnitine for all the mutants were similar to those of the wild-type M-CPTI (Table 6.2). With respect to the second substrate, palmitoyl-CoA, both the wild-type and the mutants showed normal saturation kinetics when the palmitoyl-CoA-to-albumin molar ratio was kept at 6.1 to 1 (Fig. 6.3B). Most of the mutations did not have a major effect on either the K_m or the V_{max} for palmitoyl-CoA except for the EVLS and EHVLS substitution mutants, which decreased the K_m and V_{max} , and the Δ 18 combined with the VLS substitution mutation, which increased both the K_m and V_{max} as shown in Table 6.2.

6.3.3 Malonyl-CoA Sensitivity of Mutant M-CPTIs

Separate substitution mutation of amino acid residues 19 to 28 to alanine resulted in a mutant enzyme with activity and malonyl-CoA sensitivity similar to those of the wild-type M-CPTI (data not shown). Double (V19AL28A), triple (V19AL23AL28A; V19AL23AS24A) mutants also exhibited malonyl-CoA sensitivity similar to that of the wild-type (data not shown). The IC₅₀ for malonyl-CoA inhibition for the $\Delta 18 +$ V19AL23AS24A mutant increased over 140-fold compared to that of wild-type M-CPTI (10 M versus 70 nM) as shown in Table 6.1, representing a significant loss in malonyl-CoA sensitivity compared to that of the wild-type enzyme. A change of Glu3 to Ala in M-CPTI resulted in only a 60-fold decrease in malonyl-CoA sensitivity in contrast to the more than 100-fold decrease in malonyl-CoA sensitivity observed with the corresponding L-CPTI mutant. Substitution of His5 with Ala caused a minimal 2-fold decrease in malonyl-CoA sensitivity (Table 6.1) [241]. The triple replacement mutant E3AV19AL23A and the double mutant E3AS24A showed 60-fold decreases in malonyl-CoA sensitivity (data not shown), characteristics similar to those of the single substitution mutant E3A. The four-residue substitution mutant E3AV19AL23AS24A exhibited a 140fold loss in malonyl-CoA sensitivity, which is identical to that observed with $\Delta 18 +$ V19AL23AS24A mutant M-CPTI. The mutant $\Delta 18 + V19AL23AS24A$, the single-site substitution mutant E3A, and the four-residue mutant E3AV19AL23AS24A (Fig. 6.4B)



Fig. 6.3 Kinetic analysis of wild-type and mutant M-CPTI activities Isolated mitochondria (150 µg of protein) from the yeast strains expressing the wildtype and mutant M-CPTI were assayed for CPTI activity in the presence of increasing concentrations of carnitine and palmitoyl CoA as described under Materials and methods. Shown are the resulting dose-response curves for M-CPTI with (A) carnitine and (B) palmitoyl CoA with fixed molar ratio of palmitoyl CoA:albumin (6.1:1). •, Wild type; \blacksquare , $\triangle 18 + V19AL23AS24A$; \blacktriangle , E3A; \blacklozenge , H5A; \triangle , EVLS; \diamondsuit , EHVLS.



Fig. 6.4 Effect of increasing concentrations of malonyl-CoA on the activities of yeast-expressed wild-type and mutant M-CPTIs Approximately 150 μ g of mitochondrial protein was used for the assay. (A) •, Wild type (WT); Δ , Δ 18 + V19AL23AS24A (HH_D18PM); \blacksquare , E3A; o, H5A. (B) •,

EVLS; ▲, EHVLS. HH, human heart.

Table 6.2

Kinetic characteristics of wild-type and mutant M-CPTI

Strain	Carnitine		Palmitoyl	CoA
	$K_{\rm m}$ (μ M)	$V_{\rm max}$ (nmol/mgmin)	K_{a} (μ M)	Vmax (mnol/mgmin)
Wild type	913	5,1	67.9	24.2
Δ 18+V19AL23AS24A	630	5.4	273.1	78.8
E3A	617	3.6	82.3	20.0
H5A	968	4.0	40.4	17.6
EVLS	706	4,7	7.8	6.6
EHVLS	652	1,9	6.2	3.4

Isolated mitochondria (150 µg of protein) from the yeast strains expressing the wildtype and mutant M-CPTI were assayed for CPTI activity in the presence of increasing concentrations of carnitine and palmitoyl-CoA as described under Materials and methods. EVLS=E3AV19AL23AS24A; EHVLS=E3AH5AV19AL23AS24A. showed decreased malonyl-CoA sensitivity at all levels of the inhibitor tested, while the H5A mutant showed malonyl-CoA sensitivity similar to that of the wild type as shown in Fig. 6.4.

6.3.4 [¹⁴C]malonyl-CoA Binding in Yeast-expressed Wild-type and Mutant M-CPTIs

There was no detectable malonyl-CoA binding in the yeast strain expressing the $\Delta 18 + V19AL23AS24A$ mutant or the four-residue substitution mutant E3AV19AL23AS24A. Malonyl-CoA binding to the mitochondria from the yeast strains expressing E3A and H5A was significantly lower than that observed in the mitochondria from the wild-type strain, but was saturable (Fig. 6.5). Malonyl-CoA binding clearly resolved into a high-affinity and a low-affinity site in the mitochondria from the wild-type M-CPTI as shown by the Scatchard plots in Fig. 6.6, but only very-low-affinity binding was observed in the mitochondria from E3A. The mitochondria from the yeast strain expressing the H5A mutant exhibited only the high-affinity binding. The change of Glu3 to Ala decreased the calculated B_{max2} (Table 6.1), suggesting that the observed loss in malonyl-CoA sensitivity and binding could partially be attributed to the decreased abundance or availability of the second malonyl-CoA binding entity of M-CPTI. Deletion of the first 18 N-terminal residues plus a combined change of V19, L23, and S24 to alanine significantly decreased both high- and low-affinity malonyl-CoA binding (K_{D1} , K_{D2}) (Table 6.1).

6.4 DISCUSSION

We have previously demonstrated that deletion of the first 28 but not the 18 Nterminal residues of human heart M-CPTI resulted in a mutant enzyme with a >100-fold decrease in malonyl-CoA sensitivity, complete loss in high-affinity malonyl-CoA binding, and a 20-fold reduction in low-affinity binding [240]. These results suggested to us that, in addition to the first 18 N-terminal amino acids which appeared to be partially important for malonyl-CoA inhibition and binding, there were other residues within the







Fig. 6.6 Scatchard plot for binding of [¹⁴C]malonyl-CoA to mitochondria from yeast strains expressing wild-type and mutant M-CPTIs
. •, Wild type (WT); ■, E3A; ▲, H5A.

19–28 N-terminal amino acids of M-CPTI that are essential for malonyl-CoA inhibition and binding.

To identify specific residues essential for malonyl-CoA inhibition and binding in the first 28 N-terminal amino acids of human heart M-CPTI, a series of multiple substitution mutations and a deletion mutation combined with triple substitution mutations were constructed. All of the mutants had activity similar to that of the wildtype M-CPTI. The mutant $\Delta 18 + V19AL23AS24A$ had a significantly lowered response to malonyl-CoA inhibition and binding. However, as we previously reported, $\Delta 18$ of M-CPTI showed only a 4-fold loss in malonyl-CoA sensitivity but had activity and highaffinity malonyl-CoA binding similar to those of the wild type [240]; the triple substitution mutant V19AL23AS24A had malonyl-CoA sensitivity similar to that of the wild type. Substitution of Glu3 with Ala resulted in only a partial loss in malonyl-CoA sensitivity and a complete loss in high-affinity but not low-affinity malonyl-CoA binding. This is in contrast to the corresponding L-CPTI E3A mutant, which showed a 100-fold decrease in malonyl-CoA sensitivity, a similar complete loss in high-affinity malonyl-CoA binding [241], and a significant decrease in low-affinity binding. However, the fourresidue substitution mutant E3AV19AL23AS24A exhibited a 140-fold loss in malonyl-CoA sensitivity, similar to that observed with the $\Delta 18 + V19AL23AS24A$ substitution mutant, suggesting that these four residues constitute part of the malonyl-CoA binding site in M-CPTI. We hypothesize that the E3A substitution mutation may disrupt a hydrogen-bonding network or a salt bridge, perhaps to one of these residues or to another residue at the malonyl-CoA binding site and/or active site that is hydrogen-bonded to, or forms a salt bridge to, one of these residues. There are two predicted α -helices within the first 28 N-terminal residues of M-CPTI. A change of Glu3 to Ala, located within the first predicted α -helix of M-CPTI, caused a partial loss in malonyl-CoA sensitivity. A change of amino acid residues 19 to 28, including V19, L23, and S24 which are located within the second predicted α -helix to alanine, had no effect on malonyl-CoA sensitivity. This finding is contrary to that reported for L-CPTI, but in agreement with the observed differences in malonyl-CoA sensitivity reported for L-CPTI and M-CPTI by us and others [131, 240, 314, 315]. Furthermore, the triple mutant V19AL23AS24A had no

major effect on malonyl-CoA inhibition. Our data demonstrate that only a combined mutation of the four residues within the two predicted α -helices in M-CPTI causes a major loss in malonyl-CoA sensitivity, which is confirmed by the observed similar loss in malonyl-CoA sensitivity with the $\Delta 18 + V19AL24AS24A$ mutant.

The high-affinity site (K_{D1}, B_{max1}) for binding of malonyl-CoA to M-CPTI was completely abolished in the E3A mutant, and both the high- and low-affinity sites $(K_{D1}, K_{D2}, B_{max1}, \text{ and } B_{max2})$ were not detected in the $\Delta 18$ plus the triple substitution mutant and the four-residue substitution mutant, suggesting that the decrease in malonyl-CoA sensitivity observed in these mutants was due to the significant loss of the high- and/or high- and low-affinity binding entity of the enzyme. Replacement of His5 with Ala had a much lower effect on the IC₅₀ for malonyl-CoA inhibition of M-CPTI, in contrast to that observed with L-CPTI, but resulted in complete loss of the low- but not the high-affinity malonyl-CoA binding. The corresponding L-CPTI mutation substantially decreased both the high- and the low-affinity binding but did not abolish either the high- or the lowaffinity malonyl-CoA binding [241].

The mutations did not have a significant effect on the kinetic properties of the enzyme, because there was no change in the K_m or V_{max} for carnitine; however, there were changes in the K_m and V_{max} for palmitoyl-CoA, the second substrate, a long-chain acyl-CoA that binds and/or competes for the malonyl-CoA binding site. A high concentration of palmitoyl-CoA or preincubation of mitochondria with palmitoyl-CoA has been shown to competitively displace and overcome malonyl-CoA inhibition of CPTI [35]. On immunoblotting with an anti-His-tag antibody, proteins of the expected sizes were detected in the mitochondria of the mutants and the wild-type M-CPTI. These results demonstrate clearly that Glu3, Val19, Leu23, and Ser24 are important for malonyl-CoA inhibition and binding but not for catalysis.

The V_{max} for palmitoyl-CoA suggests that the $\Delta 18$ plus VLS mutant has lost residues that interfere with catalysis, which may comprise part of the high-affinity malonyl-CoA binding site similar to that observed with $\Delta 28$ of M-CPTI [240]. Binding of malonyl-CoA to the high-affinity malonyl-CoA binding site of wild-type M-CPTI may inhibit binding of palmitoyl-CoA to the active site. Alternatively, the malonyl-CoA

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binding site may be an alternative and/or unproductive binding site, reducing its availability—a form of substrate inhibition. Since the increase in CPT activity was observed only with $\Delta 18$ combined with the three point mutations, but not with the E3A, H5A, or E3AV19AL23AS24A mutants, deletion of the first 18 residues combined with the three point mutations could induce a conformational change optimal for palmitoyl-CoA binding and catalysis. Thus, the effect of the mutations on malonyl-CoA sensitivity and on the V_{max} for palmitoyl-CoA may also be due to a conformational change affecting the relationship of the two predicted α -helices within the first 28 N-terminal amino acid residues of M-CPTI.

It is now well established that M-CPTI is 30- to 100-fold more sensitive to malonyl-CoA inhibition than L-CPTI. Our data clearly show that there are two classes of malonyl-CoA binding sites in M-CPTI, a high-affinity and a low-affinity binding site, similar to results of earlier studies with heart and skeletal muscle mitochondria [24, 131, 182, 183]. A change of Glu3 to alanine abolished the high-affinity malonyl-CoA binding and decreased malonyl-CoA sensitivity of M-CPTI. While substitution of His5 with alanine abolished low-affinity malonyl-CoA binding with minimal effect on malonyl-CoA sensitivity, deletion of the first 18 N-terminal amino acid residues combined with substitution mutations of V19A, L23A, and S24A or a four-residue substitution E3AV19AL23AS24A significantly lowered the malonyl-CoA sensitivity and the high-and low-affinity malonyl-CoA binding of the mutant M-CPTI. 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. Specifically, the N-terminal residues Glu3, Val19, Leu23, and Ser24 in M-CPTI are important for malonyl-CoA inhibition and binding, but not for catalysis.

Our Scatchard plot for the H5A mutant shows only for the initial 6 points where the binding of the ligand to the enzyme increased with increasing concentration of the added ligand. After the sixth point, increasing the concentration of ligand added decreased the amount of the ligand bound to the mutant enzyme as shown in Fig. 6.5 unlike the saturable curve obtained with the wild-type enzyme, suggesting loss in low affinity but not high affinity binding. We do not know why we lost the low affinity binding site and a scatchard plot of the data that includes the last three points was not linear and even if we drew a line the K_{D2} will be negative, which did not make sense. So, we decided to exclude the last three points from the plot and concentrate on the linear portion of the curve in Fig. 6.5, which represents the high-affinity binding site. The calculated K_{D1} and B_{max1} values shown are for the high-affinity binding site derived from the initial six points in the linear portion of the curve.

CHAPTER 7

CYSTEINE-SCANNING MUTAGENESIS OF MUSCLE CARNITINE PALMITOYLTRANSFERASE I REVEALS A SINGLE CYSTEINE RESIDUE (CYS-305) IS IMPORTANT FOR CATALYSIS*

7.1 INTRODUCTION

Carnitine palmitoyltransferase I (CPTI) catalyzes the conversion of long-chain fatty acyl-CoAs to acyl carnitines in the presence of L-carnitine, a rate-limiting step in the transport of long-chain fatty acids from the cytoplasm to the mitochondrial matrix [21, 180]. Mammalian tissues express two isoforms of CPTI—a liver isoform (L-CPTI) and a heart/skeletal muscle isoform (M-CPTI)—that are 62% identical in amino acid sequence [40, 282, 283, 304, 305, 314]. As an enzyme that catalyzes the first ratelimiting step in fatty acid oxidation, CPTI is regulated by its physiological inhibitor, malonyl-CoA, the first intermediate in fatty acid synthesis, suggesting a coordinated control of fatty acid synthesis and oxidation [21, 180]. Previous studies by our laboratory and others have established that M-CPTI is more sensitive to malonyl-CoA inhibition than L-CPTI [40, 282, 283, 304, 305, 314]. The molecular/structural basis for the differences in malonyl-CoA sensitivity between M-CPTI and L-CPTI was recently established by our demonstration that substitution of the conserved C-terminal L-CPTI residue Glu-590 with alanine increased its malonyl-CoA sensitivity close to that observed with M-CPTI [195]. Because of its central role in fatty acid metabolism, understanding

Contribution: Initial design of experiments. I prepared figure 2 and 3 for publication.

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the catalytic mechanism and regulation of the CPT system is an important first step in the development of treatments for diseases, such as myocardial ischemia, diabetes, obesity and in human-inherited CPTI deficiency diseases [5, 9, 27, 60, 195].

We have expressed human heart M-CPTI, rat liver L-CPTI, and CPTII in the yeast Pichia pastoris, an organism devoid of endogenous CPT activity [72, 293, 314, 315]. Our recent deletion and point mutation analyses have demonstrated that glutamate-3 and histidine-5 are necessary for malonyl-CoA inhibition and high-affinity binding of L-CPTI but not for catalysis [241, 242]. For M-CPTI, our site-directed mutagenesis studies demonstrate that in addition to Glu-3 and His-5, Val-19, Leu-23, and Ser-24 are necessary for malonyl-CoA inhibition and high-affinity binding but not for catalysis [240, 316]. It has been generally predicted that the catalytic and substrate binding sites in both L-CPTI and M-CPTI reside in the C-terminal region of the enzymes. Recent studies from our laboratory demonstrate that mutations of conserved arginine and tryptophan residues in the C-terminal region of L-CPTI abolish catalytic activity [64]. Since the major effect of the mutations was on the V_{max} , we predict that the conserved arginine and tryptophan residues stabilize the enzyme-substrate complex by charge neutralization and hydrophobic interactions [64]. Furthermore, our site-directed mutagenesis studies demonstrate that deletion of the conserved C-terminal M-CPTI residue leucine-764 (L764) or mutation to Arg inactivates the enzyme [65]. CPTI is an active acyltransferase that belongs to the acyltransferase family of enzymes; however, the molecular mechanism by which CPTI transfers the acyl group from the acyl-CoA to carnitine remains to be elucidated. In this communication, our cysteine-scanning mutagenesis demonstrates that a single substitution mutation of cysteine-305 to alanine abolishes M-CPTI catalytic activity.

7.2 EXPERIMENTAL PROCEDURES

7.2.1 Construction of Human Heart M-CPTI Cysteineless Mutants

Mutants were constructed using the QuickChange multisite-directed mutagenesis kit (Stratagene, La Jolla, CA) with plasmid p12E (for N-terminal mutagenesis) and p13E

(for C-terminal mutagenesis) as the templates. Plasmid p12E is a derivative of pTZ18U, containing wild-type N-terminal human M-CPTI from ATG downstream to 622 bp and a fragment of the vector pHWO10 in the extreme 5'-end, while plasmid p13E is a derivative of pTZ18U, containing the C-terminal end of M-CPTI from 623 bp to the stop codon. The mutagenesis was performed according to the manufacturer's instruction. The mutants were in the vectors p12E and p13E. Mutations were confirmed by DNA sequencing.

For construction of the N-terminal six residue cysteineless mutant, plasmid 12E containing the substitution mutants C75A, C86K, C90T, C95G, C133S, and C155M was digested with *AgeI–EcoRI* and subcloned into *AgeI–EcoRI*-cut wild-type M-CPTI cDNA in the pGAP yeast expression vector [314]. The *EcoRI*-cut 5' M-CPTI cDNA fragment containing the six-cysteine mutants was then ligated into an *EcoRI*-cut 3' M-CPTI cDNA fragment of the wild-type p-GAP–M-CPTI to generate the N-terminal cysteineless mutant M-CPTI, N6C. For construction of the C-terminal cysteineless M-CPTI, an *EcoRI* fragment of plasmid 13E containing the nine mutated C-terminal M-CPTI cysteines (C305A, C448A, C504A, C526A, C548S, C562A, C586A, C608A, and C659A) was ligated to an *EcoRI*-cut wild-type p-GAP–M-CPTI to generate C9C, the nine-residue cysteineless mutant M-CPTI in pHW010.

For construction of the cysteineless M-CPTI mutant, a 1.7-kb *EcoRI* fragment of the mutant C9C containing mutations of all the C-terminal cysteines was used to replace the corresponding *EcoRI* fragment of the plasmid N6C that contains the N-terminal six-cysteine mutants to generate the 15-residue cysteineless M-CPTI mutant in pHW010. Mutations were confirmed by DNA sequencing. All the primers used for cysteine mutagenesis are listed in Table 7.1 and the mutations are highlighted in bold. All the cysteine residues in human M-CPTI were either altered to alanine (if human L-CPTI has a cysteine residue at the corresponding position) or to the corresponding amino acid residue in human L-CPTI.

Table 7.1					
DCD Drive and for construction of M CDTI sustains mutants					
PCR Primers used for construction of M-CPTI cysteine mutants					
Primers used for the OuickChange TM multi site-directed mutagenesis					
N-terminal cystei	ne mutants:				
C75A: 5'-0	JAGATGTCCACGTT GGC GAAGGAGGAACCCAC-3´				
C133S: 5'-0	LATCCACCCATGGTAGCTGAGAAGCAGCTTCAG-3				
C155M: 5'-0	CTGGATAGAAGGCGGAT CAT CATAGCCCAGATCCTGGTC-3´				
CKTG(C86K,	C90T,C95G):				
5 [°] - TGCGGGG	ICTGGTAGGGGCCACCCCCTGAGGGAGGGTTCTCTGGATCTTACTGACCAGCCCCA-3´				
C-terminal cystei	ne mutation:				
C305A: 5'-C	TCCATCTGGTAGGAGGCCATAGGCACTATGCC-3				
C448A: 5'-0	LAAACCACCTGTTGTA GGC GTTGCCATGTAGCAG-3				
C504A: 5'-0	JTTCGGTTTGCCCAG GGC GTGCCCGGTCTCCGT-3				
C526A: 5'-0	TCGATGACCGCCTGGGCCTGTTTTGGAATGTC-3				
C548S: 5'-0	BGCAGGAACTGGAA GGA GTACAACTCCACGTC-3				
C562A: 5'-C	DATCAGGGCTGGTCCG GGC CTTCTTCTTGATGAGGCC-3				
C586A: 5'-G	GCCTCATAGGTCAGGGCGAACTTACCCCTGTC-3				
C608A: 5'-G	TGGACTCGCTGGT AGC GGAACGCACAGTCTCAG-3				
C659A: 5'-C	GAGACCAAGTAAAG GGC GAAGAGGTGCCTGTC-3´				
Primers used for	the single overlap mutations				
C-terminal single	cysteine mutation:				
C305AF:	5'-GGCATAGTGCCTATGGCCTCCTACCAGATGGAG-3'				
C305AR:	5'-CTCCATCTGGTAGGAGGCCATAGGCACTATGCC-3'				
C305ACK:	5 [°] -GGCATAGTGCCTATG GC -3 [°]				
C448AR:	5'-CAAACCACCTGTTGTAGGCGTTGCCATGTAGCAG-3'				
C448ACK:	5' - CTGCTACATGGCAACG-3'				
C504AF:	5'-ACGGAGACCGGGCACGCCCTGGGCAAACCGAAC-3				
C504AR:	5 - GTTCGGTTTGCCCAGGGCGTGCCCGGTCTCCGT-3				
C504ACK:	5 - ACGGAGACCGGGCACGC-3				
C526AF:	5 -GACATTCCAAAACAGGCCCAGGCGGTCATCGAG-3				
C526AR:	5 - CTCGATGACCGCCTGGGCCTGTTTTTGGAATGTC-3				
C526ACK:	5 - GACATTUCAAAACAGGC-3				
C548SF:	5 -GACGTGGAGTTGTACTCCTTTCCAGTTCCTGCCC-3				
C548SR:	5 - GGGLAGGAACTGGAAGGAGTALAACTULAUGTC~5				
C548SCK:	5 - GACGTGGAGTTGTACTC-3				
C562AF:	5 - GGCCTCATCAAGAAGGCCCGGACCAGCCCTGATG-5				
C562AR:	5 -CATCAGGGCTGGTCCGGGCCTTCTTGATGAGGCC-5				
CS62ACK:					
CS86AF:	5 - GACAGGGGTAAGTICGCCTGACCTATGAGGCC-5				
CS86AR:	5 - GGUUILAIAGGIUAGGGGAAUIIAUUUUIGIU~5				
CS86ACK:					
C608AF:	5 - CTGAGACTGTGCGTTCCGCTACCAGCGAGTCCAC-5				
COUSAR:	0 - GIGGACICGCIGGIAGGGAACGCACAGICICAG-0 51 - GIGGACICGCIGGIAGGGAACGCACAGICICAG-0				
COUSACK:	D - CIGAGACIGIGGGIICCGC-D 5 (
COSYAK:	J - GUAGACAAGIAAAGUGAGAGGIGUUIGICIGIC-J				
LOSYACK:					
к з: то-	0 - ACLAAGIAAAGUCAGAAG- 3 5 1 - TCATGTATGCCCCCTA & & C = 3 1				
0ver EF2.	5 - FORIGIAIOGOUGIARAC-3 51 - GCCCGCTCGAGATGGCGGAAGCTCACCAGGC-31				
GVGL BFZ.					
Primers used for	the QuickChange R XL site-directed mutagenesis kit				
A305C revertant m	utation:				
A305CF2:	5 - CTGGGCATAGTGCCTATG TGCT CCTACCAGATGGAGAGG-3				
A305CR2:	5 - CCTCTCCATCTGGTAGGAGCACATAGGCACTATGCCCAG-3				
A305CCK:	5´-CTGGGCATAGTGCCTATGTG-3´				

7.2.2 Construction of the Single C-Terminal M-CPTI Cysteine Mutants C504A, C526A, C548S, C562A, C586A, and C608A

Mutants were constructed by the overlap extension PCR method using the primers shown in Table 7.1 with the wild-type plasmid DNA (pGAP M-CPTI) as template [257]. For example, to construct the C562A mutant, the primers F2-C562AR and C562AF-R3 were used to generate 840-bp and 300-bp PCR products, respectively, using the wild-type M-CPTI cDNA as template. The two PCR products were purified, mixed, and used as template for a second-round PCR with the primer F2-R3. The 1.13-kb PCR product was digested with *SphI–Bgl*II, and ligated into *SphI–Bgl*II-cut wild-type M-CPTI cDNA in the pGAP expression vector. The construction of C504A, C526A, C548S, C586A and C608A mutants was similar to that of C562A using the primers listed in Table 7.1, F2 and R3.

The C305A mutant was constructed by the overlap extension PCR method using the primers F2-C305AR and C305AF-C608AR with the wild-type M-CPTI plasmid DNA as template. The 926-bp and 940-bp PCR products were purified as above, mixed, and used as a template for a second-round PCR with the primer F2-C608AR. The 1860bp PCR product was digested with *AfI*III–*Sph*I and ligated to *AfI*II–*Sph*I-cut wild-type pGAP–M-CPTI.

Mutants C448A and C659A were constructed using the QuickChange multisitedirected mutagenesis kit with plasmid p13E (containing wild-type C-terminal human M-CPTI cDNA in pTZ18U vector) as the template. Primers C448AR and C659AR were each used to separately generate the C448A and C659A mutants. The mutated fragments in p13E were cut with *EcoRI* and used to replace the corresponding *EcoRI* fragment in the wild-type plasmid pGAP–M-CPTI.

7.2.3 Construction of the Revertant Mutant A305C

The Quick Change®XL site-directed mutagenesis kit was used with plasmid 13E9C41 (containing the C-terminal of human M-CPTI with nine mutated cysteines in the vector pTZ18U). The primers used were PAGE-purified A305CF2 and A305CR2.

The mutants in the plasmid 13E9C41 were *EcoRI*-cut and used to replace the corresponding *EcoRI* fragment in the wild-type plasmid pGAP–M-CPTI.

Bacterial colonies obtained upon transformation of the mutagenesis reactions were screened for the their ability to productively serve as templates for PCR using forward primers with 3' end specific to each of the mutants. For example, the C305A mutant was screened with the primer C305ACK. The mutations were confirmed by DNA sequencing. The plasmids were linearized by digestion with the restriction enzyme *BspE*I and integrated into the His4 locus of *P. pastoris* GS115 by electroporation [241]. Histidine prototrophic transformants were selected on YND plates and grown on YND medium. Mitochondria were isolated by disrupting the yeast cells with glass beads (14) and used to monitor activity and malonyl-CoA sensitivity.

7.2.4 CPT Assay

CPTI activity was assayed in isolated mitochondria from the yeast strains expressing the wild-type and mutant CPTIs by the forward exchange method using L-[methyl-³H] carnitine as described previously [35, 314]. The K_m for palmitoyl-CoA was determined by varying the palmitoyl-CoA concentration from 12.5 to 400 µM at a fixed molar ratio (6.1:1) of palmitoyl-CoA to albumin as described previously [212, 241, 316]. The concentration of carnitine was fixed at 1.0 mM. The K_m for carnitine was determined by varying the carnitine concentration from 100 µM to 4.0 mM at a fixed 100 µM palmitoyl-CoA. 150 µg of mitochondrial protein were used, and all incubations were performed at 30 °C for 3 min. The malonyl-CoA inhibition was determined by varying the concentration of malonyl-CoA from 50 nM to 10 µM at fixed 1.0 mM carnitine and 100µM palmitoyl-CoA. The incubations were performed at 30 °C for 5 min.

7.2.5 Labeling of Wild-type and Mutant M-CPTI with [U-¹⁴C]palmitic Acid

Isolated mitochondria (100 μ g protein) from the yeast strains expressing the wildtype and mutant M-CPTI were incubated with 50 μ M [U-¹⁴C]palmitic acid (specific activity 824 mCi/mmol; Amersham Biosciences, Inc.) and 250 μ M CoA and 5mM ATP for 3 h at 30°C [158]. At the end of the incubation, the samples were divided into two fractions of equal volume, and one fraction was treated with 2.0 M hydroxylamine (adjusted to pH 7.5 with NaOH) at 30°C for 3 h. Hydroxylamine treated and untreated samples were heat denatured and subjected to SDS-PAGE followed by electrophoretic transfer onto nitrocellulose membranes. The blots were dried and exposed to screens of Molecular Dynamics phosphorimaging plates for 1–7 days, and signals were analyzed in a Molecular Dynamics PhosphorImager SI.

7.2.6 Western Blot

Proteins were separated by SDS-PAGE in a 10% gel and transferred onto nitrocellulose membranes. Immunoblots were developed by incubation with the M-CPTI-specific polyclonal antibodies as described previously [72, 314, 315]. Sources of other materials and procedures were as described in our previous publications [65, 240, 262].

7.3 RESULTS

7.3.1 Chemical Modification with DTNB

Preincubation of isolated mitochondria from the yeast strain expressing human heart M-CPTI with 250 μ M DTNB (5,5'-dithiobis-(2-nitrobenzoic acid)), an –SHspecific modifying reagent, at 4 °C for 30 min caused a 75% loss in CPTI activity, indicating that there are cysteine residues in M-CPTI that are important for catalysis and/or substrate binding. These preliminary chemical modification studies with DTNB by us and others provided evidence that CPTI may contain a cysteine residue that is important for catalysis [233].

7.3.2 Generation of Mutations and Expression in *P. pastoris*

Construction of plasmids carrying the N-terminal cysteineless (N6C), the Cterminal cysteineless (C9C), the cysteineless (NC15C) M-CPTI and all the single substitution mutations was performed as described in Experimental Procedures. *P. pastoris* was chosen as an expression system for M-CPTI and the mutants, because it does not have endogenous CPT activity [72, 241, 242, 293, 314, 315]. The *P. pastoris* expression plasmids expressed M-CPTI under the control of the *P. pastoris* glyceraldehyde-3-phosphate dehydrogenase gene promoter [281]. Yeast transformants with the wild-type M-CPTI gene and the mutants were grown in liquid medium supplemented with glucose [72].

Western blot analysis of wild-type and M-CPTI (80 KDa) and the mutants using a polyclonal antibody directed against a maltose-binding protein–M-CPTI fusion protein [240, 314] showed proteins of predicted sizes with similar steady-state levels of expression.

7.3.3 Effect of Mutations on M-CPTI Activity and Malonyl-CoA Sensitivity

The C-terminal nine-residue cysteineless and the complete 15-residue cysteineless M-CPTI were inactive despite the high level of protein expression observed for the mutants on the western blot. The N-terminal six-residue cysteineless mutant M-CPTI had activity and malonyl-CoA sensitivity similar to the wild type as shown in Table 7.2, suggesting that one or more of the nine-cysteine residues in the C-terminal region of M-CPTI may be important for activity. Separate substitution mutation of C305A, C448A, C504A, C526A, C548S, C562A, C586A, C608A, and C659A showed that of the ninecysteine mutants only the substitution mutant C305A was inactive, demonstrating that a single change of Cys-305 to Ala [or serine (unpublished)] resulted in complete loss in M-CPTI activity. As shown in Table 7.2, a change of cysteine to either alanine or serine of the other eight C-terminal cysteines had no effect on activity and malonyl-CoA sensitivity. A single change of Ala-305 to Cys, A305C, in the nine-residue C-terminal cysteineless mutant resulted in a mutant enzyme with similar activity and malonyl-CoA sensitivity (Table 7.2) as the wild-type M-CPTI, demonstrating that only Cys-305 is essential for M-CPTI activity, but not the other eight C-terminal cysteine residues. In short, our studies identify for the first time that Cys-305 in the C-terminal region of M-CPTI is essential for catalytic activity, because mutation of this residue to Ala inactivated M-CPTI.

Table 7.2

Strain	Activity	IC ₅₀
Strain	(nmol/mg·min)	(μM)
WT	2.90 ± 0.10	0.50 ± 0.04
C305A	Inactive	
A305C	2.80 ± 0.03	0.52 ± 0.05
N6C	2.82 ± 0.14	0.50 ± 0.04
C9C	Inactive	
Cys-less	Inactive	
C448A	2.83 ± 0.20	0.55 ± 0.05
C504A	2.75 ± 0.19	0.50 ± 0.02
C526A	2.90 ± 0.15	0.20 ± 0.03
C548S	2.80 ± 0.13	0.50 ± 0.04
C562A	3.75 ± 0.14	0.20 ± 0.02
C586A	3.75 ± 0.03	0.70 ± 0.04
C608A	2.61 ± 0.20	0.52 ± 0.01
C659A	3.50 ± 0.37	0.24 ± 0.03

CPTI activity and malonyl-CoA sensitivity of wild-type and mutant M-CPTI

Mitochondria (150 µg of protein) from the yeast strains expressing wild-type and human heart M-CPTI mutants were assayed for CPT activity and malonyl-CoA sensitivity as described under "Experimental Procedures." The results are the means ± SD of at least two independent experiments with different mitochondrial preparations. N6C, six-residue N-terminal cysteineless mutant; C9C, nine-residue C-terminal cysteineless mutant; Cys-less, cysteineless M-CPTI mutant.
7.3.4 Kinetic Characteristics of Wild-Type and Mutant M-CPTIs

Mutant A305C exhibited normal saturation kinetics when the carnitine concentration was varied relative to a constant second substrate, palmitoyl-CoA, a property identical to that of the wild-type M-CPTI. For the mutant A305C, the calculated K_m value for carnitine was only 12% higher and the V_{max} was 15% lower compared with the wild-type M-CPTI as shown in Table 7.3, indicating almost complete restoration of catalytic activity and no major effect of substitution of the eight C-terminal cysteine residues with Ala or Ser on M-CPTI activity. The catalytic efficiency as estimated by V_{max}/K_m for the A305C decreased by 24% compared to the wild type. With respect to the second substrate, palmitoyl-CoA, mutant A305C exhibited normal saturation kinetics similar to the wild type when the molar ratio of palmitoyl-CoA to albumin was fixed at 6.1:1. The calculated K_m and V_{max} values for the A305C mutant were only 10% lower than the wild type, and the catalytic efficiency was similar to that of the wild type. Thus, substitution of Ala-305 with cysteine in the inactive nine-residue C-terminal cysteineless M-CPTI resulted in a mutant, A305C, that had activity, malonyl-CoA sensitivity, and kinetic properties similar to the wild-type M-CPTI, demonstrating for the first time that of the nine C-terminal cysteine residues present in M-CPTI only Cys-305 is essential for catalytic activity and malonyl-CoA sensitivity.

7.3.5 Acylation of Wild-Type and Mutant M-CPTI

The loss in CPTI activity in the C305A mutant and the recovery of activity in the A305C revertant suggested that an acyl-enzyme intermediate may be involved in the catalytic mechanism of M-CPTI. We therefore investigated whether the loss in activity in the C305A mutant was due to the failure of an acyl-enzyme intermediate formation in the catalytic mechanism of M-CPTI by incubation of the wild-type and mutant M-CPTI with [U-¹⁴C]palmitic acid in the presence of CoA and ATP, but in the absence of carnitine and measurement of covalent incorporation of labeled palmitate into the enzyme. Both the wild-type and A305C revertant were strongly labeled with radioactive palmitate (Fig. 7.1) but not the C305A mutant. Furthermore, the [U-¹⁴C]palmitate was covalently bound, as the labeling could be removed by treatment with neutral



Fig. 7.1 Labeling of wild-type and mutant M-CPTI with $[U^{-14}C]$ palmitic Acid. Mitochondria (100 µg protein) from the yeast strains expressing the wild-type and mutant M-CPTI were incubated with $[U^{-14}C]$ palmitic acid in the presence of CoA and ATP, but in the absence of carnitine, and aliquots of the acylated enzyme were treated with neutral hydroxylamine. The hydroxylamine treated and untreated labeled proteins were subjected to SDS-PAGE and blotted onto nitrocellulose membranes and incorporation of $[U^{-14}C]$ palmitate was analyzed using a Molecular Dynamics PhosphorImager. 1, wild type; 2, C305A; 3, A305C; 4, 5, and 6, neutral hydroxylamine-treated wild type, C305A and A305C, respectively.

Table 7.3

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	Carnitine		Palmitoyl-CoA	
Strain	K _m μM	V _{max} nmol/mg∙min	K _m μM	V _{max} nmol/mg·min
Wild-type	824.2 ± 8.2	3.4 ± 0.3	73.0 ± 3.1	17.5 ± 0.5
A305C	920.0 ± 10.5	2.9 ± 0.2	66.0 ± 1.9	15.8 ± 0.3

Kinetic characteristics of yeast-expressed wild-type and mutant human M-CPTIs

hydroxylamine (Fig. 7.1). These results provide strong support for an acyl-enzyme intermediate in the catalytic mechanism of M-CPTI.

7.4 DISCUSSION

Our cysteine-scanning mutagenesis of M-CPTI demonstrated that both the nineresidue C-terminal and the complete 15-residue cysteineless mutant enzyme are inactive, but the six-residue N-terminal cysteineless mutant enzyme had activity and malonyl-CoA sensitivity similar to the wild-type M-CPTI. These results suggested that one or more of the nine C-terminal cysteine residues was important for catalysis. Separate substitution mutation of each of the nine C-terminal cysteines to alanine or serine identified a single residue, Cys-305, to be essential for catalysis. Substitution of Cys-305 with Ala in the wild-type enzyme inactivated M-CPTI, and a single change of Ala-305 to Cys in the nine-residue C-terminal cysteineless mutant resulted in an eight-residue C-terminal cysteineless mutant enzyme that had activity and malonyl-CoA sensitivity similar to the wild type. Cys-305 is a conserved residue within the family of enzymes that includes CPTI, CAT, and CLAT from different species, but not in CPTII or COT whereby the corresponding Cys-305 is replaced by Asp as shown in Fig. 7.2. Of the remaining eight C-terminal cysteine residues in M-CPTI, seven are conserved in both M-CPTI and L-CPTI but not in the other acyltransferase family of enzymes. The eighth residue, Cys-548 is only present in M-CPTI, and Ser replaces Cys in the wild-type L-CPTI. The M-CPTI mutant C548S had activity and malonyl-CoA sensitivity similar to the wild type. Human heart M-CPTI has six N-terminal cysteine residues that are absent in L-CPTI. Since M-CPTI is much more sensitive to malonyl-CoA inhibition than L-CPTI, we hypothesized that the differences in malonyl-CoA sensitivity observed between the two isoforms could be due to the presence of the six additional N-terminal cysteine residues in M-CPTI compared to L-CPTI. However, our cysteine-scanning mutagenesis demonstrated that the N-terminal cysteineless M-CPTI had activity and malonyl-CoA sensitivity similar to the wild-type enzyme, suggesting that these residues do not play a role in M-CPTI activity and inhibitor sensitivity.

	305	454	473	
CPTM_HUMAN	VPMCSYQMERMFNTTRIPGKDT	DNRWF D KSFTLISFKNG	OLGLNAEHAWADA	
CPTM_RAT	VPMCSYQMERMFNTTRIPGKETI	DNRWFDKSFTLISCKNG	DLGLNTEHSWADA	
CPTM_MOUSE	VPMCSYQMERMFNTTRIPGKET	DNRWFDKSFTLISCKNGI	LLGLNTE H SWADA	
CPT1 RAT	IPLCSAQWERLFNTSRIPGEETI	DDRWFDKSITFVVFKNSH	KIGINAE H SWADA	
CPT1 HUMAN	IPLCSAQWERMFNTSRIPGEETI	DDRWFDKSFTFVVFKNGH	MGLNAEHSWADA	
CPT1_MOUSE	IPLCSAQWERLFNTSRIPGEETI	DDRWF D KSITFVVFKNSF	KIGINAE H SWADA	
CPT1 PIG	VPLCSAQWERMFNTSRIPGEETI	DDRWFDKSFTFIVFKNGH	CMGMNAEHSWADA	
CPT2 HUMAN	NAYPLDMSQYFRLFNSTRLPKPSRI	NRWFDKSFNLIIAKDGS	STAVHFE H SWGDG	
CPT2 RAT	NAYPLDMSQYFRLFNSTRIPRPNRI)NRWFDKSFNLIVAEDG1	TAAVHFE H SWGDG	
CPT2 MOUSE	NAYPLDMSQYFRLFNSTRIPKPSRI	NRWFDKSFNLIVAKDG	TAAVHFEHAWGDG	
OCTC BOVIN	TPLDMNQFRMLFSTCKIPGITRI	VRWGDKSYNLIAFSNG	FGSNCDHAPFDA	
OCTC HUMAN	TPLDMNQFRMLFSTCKVPGITRI	VRWGDKSYNLISFSNGV	FGCNCDHAPFDA	
OCTC RAT	TPLDMNQFRMLFSTCKVPGITRI)VRWGDKSYNLISFANGI	FGCSCDHAPYDA	
CAT PIGEON	GKPLCMNQYYQILSSCRIPGPKRI	NRWFDKTLQFIIAEDGS	SCGLVYEHAPAEG	
CAT MOUSE	GOPLCMNQYYQILSSCREPGPKQI	NRWFDKTLQFIVAEDGS	SCGMVYEHAAAEG	
CATHUMAN	GKPLCMNQYYQILSSCRVPGPKQI	NRWFDKTLOFIVAEDGS	CGLVYEHAAAEG	
CLAT MOUSE	GOPLCMKQYYRLFSSYRLPGHTQI	NRWYDKSLOFVVGRDGT	CGVVCEHSPFDG	
CLAT HUMAN	GOPLCMKQYYGLFSSYRLPGHTQI	NRWYDKSLOFVVGRDAT	CGVVCEHSPFDG	
CLAT RAT	GOPLCMKOYYRLFSSYRLPGHTO)NRWYDKSLOFVVGRDGT	CGVVCEHSPFDG	
CLAT CHICKEN	GOPLCMKOYYGLFSSYRLPGHTKI)NRWYDKPMOFVVGRDGV	CGTVCEHSPFDG	
CLAT PIG	GOPLCMKOYYGLFSSYRLPGHTO)NRWYDKSLOFVVGRDGI	CGVVCEHSPFDG	
-	*: * ::.: * *	** **. :: :.	. :*: :.	
CPTM HIMAN	human carnitine o-nalmitovltransferase L mitoc	handrial muscle isoform (A92523)		
CPTM RAT	rat carnitine o-palmitovltransferase I, mitochon	trial muscle isoform (063704)		
CPTM MOUSE	mouse carnitine o-palmitovltransferase I, mitoc	hondrial muscle isoform (CAC40150)		
CPTI RAT:	rat carnitine o-palmitoyltransferase I, mitochondrial liver isoform (P32198)			
CPT1 HUMAN	human carnitine o-palmitoyltransferase I, mitochondrial liver isoform (P50416)			
CPT1_MOUSE	mouse carnitine o-palmitoyltransferase I, mitochondrial liver isoform (P97742)			
CPT1_PIG	pig carnitine o-palmitoyltransferase I, mitochon	drial liver isoform (AAK69171)		
CPT2_HUMAN	human carnitine o-palmitoyltransferase II (P237	'86)		

Fig. 7	.2	Sequence alignment of portions of the C-terminal region of various
a	icyli	transferases

rat carnitine o-palmitoyltransferase II (P18886) mouse carnitine o-palmitoyltransferase II (P52825)

mouse carnitine o-acetyltransferase (P47934)

human carnitine o-acetyltransferase (P43155) mouse choline o-acetyltransferase (Q03059) human choline o-acetyltransferase (P28329) rat choline o-acetyltransferase(A48319)

chicken choline o-acetyltransferase(AAK94673)

pig choline o-acetyltransferase (P13222)

bovin peroxisomal carnitine octanoyltransferase (O19094)

human peroxisomal carnitine octanoyltransferase (Q9UKG9) rat peroxisomal carnitine octanoyltransferase (P11466) pigeon carnitine o-acetyltransferase (P52826)

*, identical residues; :, conserved residues.

CPT2_RAT CPT2_MOUSE

OCTC_BOVIN

OCTC_HUMAN OCTC_RAT CAT_PIGEON

CAT_MOUSE

CAT_HUMAN CLAT_MOUSE CLAT_HUMAN

CLAT_RAT CLAT_CHICKEN

CLAT_PIG

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More recently, in a patient with CPTI deficiency disease, a complete loss in L-CPTI activity was reported, which was due to substitution of Cys-305 with Trp, the conserved cysteine residue corresponding to that of human L-CPTI and M-CPTI that we demonstrated to be essential for human heart M-CPTI activity by our cysteine scanning mutagenesis [39]. The acyltransferase family of enzymes, including M-CPTI and L-CPTI, contain a highly conserved His residue (Fig. 7.2) at the active site pocket, His-473, a general acid/base which when mutated to Ala in CPTI was shown to cause complete loss in activity [188]. As a general acid/base, His-473 may form a hydrogen-bonding network or a salt bridge to a nearby conserved aspartate residue. There are also three highly conserved Asp residues within the family of acyltransferases, namely Asp-323, Asp-454, and Asp-567, which are present in both CPTI and CPTII as shown in Fig. 7.2. In a patient with hepatic CPTI deficiency disease that was homozygous for a D454G missense mutation, the yeast-expressed mutant L-CPTI exhibited only 2% of the wildtype L-CPTI activity, demonstrating that a change of D454 to Gly in CPTI was the cause for the disease [126]. Since the conserved Asp-454 is closer to the conserved CPTI active site residue, His-473, that may form a hydrogen-bonding network or a salt bridge to Asp-454, the loss in activity observed in the D454G mutant may be due to disruption of the hydrogen-bonding network or salt bridge to His-473. Asp-323 and Asp-567 may be too far from the conserved His-473 at the active site pocket and may thus not be ideally positioned for such an interaction due to their location. Furthermore, the recent 3D-structure of CAT, a membrane-associated enzyme that belongs to the acyltransferase family of enzymes, suggests that Asp-567 may hydrogen bond with Glu-590 [135], and more recent data from our lab (unpublished) shows that a change of Asp567 to Ala or His but not Glu resulted in a significant loss in CPTI activity. Our cysteine-scanning mutagenesis and the His-473 and Asp-454 mutation studies with CPTI suggest that there are at least two conserved residues, namely Cys-305 and His-473, at the active site pocket of CPTI that are essential for catalysis, because separate mutation of these residues to Ala inactivates CPTI. In addition, Asp-454 may interact with His-473 and indirectly facilitate catalysis, because a mutation of this conserved residue that is located close to the active site His-473 caused a significant loss in CPTI activity [123, 184, 201, 213, 237, 291].

As a rate-limiting enzyme that transports long-chain fatty acids from the cytosol to the mitochondrial matrix, CPTI in the presence of carnitine catalyzes the conversion of long-chain acyl-CoAs to acylcarnitines. However, the molecular mechanism by which CPTI transfers the acyl group from the acyl-CoA to carnitine remains to be elucidated. We have previously hypothesized that the reaction catalyzed by CPTI at the active site, conversion of palmitoyl-CoA to palmitoylcarnitine in the presence of L-carnitine involves deprotonation of the hydroxyl group of carnitine by the catalytic base, His-473, and attack by the resultant oxyanion at the carbonyl of the thioester of palmitoyl-CoA to generate palmitoylcarnitine and free CoA [157]. Based on our cysteine-scanning mutagenesis and acylation studies with CPTI and studies by others, we now propose a mechanism for the acyltransferase activity of CPTI that uses a catalytic triad composed of residues Cys-305, His-473, and Asp-454 as shown in Fig. 7.3. The cysteine residue forms a more stable acyl-enzyme intermediate which may allow the acceptor molecule, carnitine, to act as a second nucleophile and complete the acyl transfer reaction. We propose a mechanism for the catalysis whereby the active site catalytic base His-473 aided by Asp-454 abstracts a proton from the -SH group of Cys-305 to generate a reactive thiolate anion that carries out a nucleophilic attack on the substrate acyl-CoA yielding a tetrahedral intermediate which is subsequently converted to a thioacyl-enzyme covalent intermediate (acyl-S-enzyme). It is envisaged that the negative charge from the carboxyl ion of Asp-454 is transferred to His-473 and then to Cys-305 to enhance the power of the nucleophile (33-38). Deacylation and transfer of the acyl group to carnitine occurs by a nucleophilic attack of the thioester bond of the acyl-S-enzyme intermediate with the reactive carnitine oxyanion generated by abstraction of a proton from the hydroxyl group of carnitine by the general base catalyst at the active site, His-473, assisted by Asp-454, resulting in the formation of a second tetrahedral intermediate which breaks down to yield the product acylcarnitine and regenerate the enzyme as shown in Fig. 7.3.

CPTII and COT, membrane-associated enzymes located on the matrix face of the inner mitochondrial membrane, contain Asp instead of Cys at the corresponding position for Cys-305 as shown in Fig. 7.2. This is probably due to the differences in the reaction



Fig. 7.3 Proposed catalytic mechanism of CPTI.

The reaction catalyzed by CPTI has five steps: (1) nucleophilic attack by the reactive thiolate anion of Cys-305 on the carbonyl carbon of the acyl-CoA substrate to form a tetrahedral intermediate; (2) hydrolysis of the tetrahedral intermediate to form an acyl-S-enzyme complex and free CoA; (3) nucleophilic attack by the reactive oxyanion of carnitine on the carbonyl carbon of the acyl-S-enzyme intermediate; (4) formation of a second tetrahydral acyl-carnitine-E-complex; and (5) hydrolysis of the tetrahydral intermediate to form acyl-carnitine and the regenerated active enzyme.

mechanisms catalyzed by CPTI and CPTII, generally referred to as the forward and reverse reactions, respectively. In vivo, CPTII catalyzes the transfer of an acyl group from acylcarnitine to CoA–SH, which is the reverse of the reaction catalyzed by CPTI resulting in the regeneration of acyl-CoA on the matrix side, thus completing the transport of long-chain acyl-CoAs from the cytosol to the mitochondrial matrix [42, 313]. The conserved active site His-373 in CPTII shown in Fig. 7.3 probably abstracts a proton from the CoA–SH to generate a nucleophile that attacks the acylcarnitine resulting in the transfer of the acyl group to CoA and formation of the acyl-CoA.

Sequence alignments of CPTI with the acyltransferase family of enzymes in the data bank led to the identification of a putative catalytic triad in CPTI consisting of residues Cys-305, Asp-454, and His-473. This catalytic triad was found to be conserved in human, rat and mouse CPTI and other acyltransferases except CPTII and COT. Mutation of Cys-305, Asp-454, and His-473 results in loss of CPTI activity, which strongly suggests that CPTI is an enzyme containing a Cys-His-Asp catalytic triad, providing a possible mechanism for the acyltransferase activity. Site-directed mutagenesis of His-473 and Asp-454 to alanine resulted in inactivation of CPTI, suggesting that these two residues are critical for CPTI activity. Since the Asp and His residues are responsible for providing the nucleophile negative charge and relaying it to the cysteine residue, their mutation would be expected to destroy the charge relay system, thereby inactivating the enzyme. While Cys-305 is important for CPTI activity, it is clear that none of the remaining eight C-terminal cysteine residues are, and the disulfide bond formation involving these residues must also not be important for catalytic activity, although they may be involved in mediating the interaction of CPTI with other proteins. In short, our studies demonstrate that CPTI is a thiol acyltransferase, and Cys-305 is the essential nucleophilic residue critical for catalysis. In the presence of both substrates, carnitine and palmitoyl-CoA, CPTI primarily acts as an acyltransferase, but in the absence of either carnitine or CoA, CPTI may exhibit low hydrolase activity resulting in the breakdown of palmitoyl-CoA or palmitoylcarnitine by a mechanism similar to that of the cysteine proteases and hydrolases which utilize a Cys-His-Asp catalytic triad [123, 184, 201, 213, 237, 291].

CHAPTER 8

IDENTIFICATION BY MUTAGENESIS OF A CONSERVED GLUTAMATE (GLU487) RESIDUE IMPORTANT FOR CATALYTIC ACTIVITY IN RAT LIVER CARNITINE PALMITOYLTRANSFERASE II*

8.1 INTRODUCTION

Carnitine palmitoyltransferase (CPT) I and CPTII, in conjunction with carnitine translocase, transport long chain fatty acids from the cytoplasm to the mitochondrial matrix for β -oxidation [21, 180]. Mammalian mitochondrial membranes express two active but distinct carnitine palmitoyltransferases (CPTI and CPTII), a malonyl-CoA-sensitive, detergent-labile CPTI, and a malonyl-CoA-insensitive, detergent-stable CPTII. CPTI is an integral membrane enzyme located on the outer mitochondrial membrane, and CPTIII is a membrane-associated enzyme loosely bound to the matrix side of the inner mitochondrial membrane. 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 [91]. 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 [21, 180]. This is an important regulatory mechanism in fatty acid metabolism and suggests coordinated control of fatty acid oxidation and synthesis. Mammalian tissues express two

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Zheng, G., Dai, J., and Woldegiorgis, G. (2002) Identification by mutagenesis of a conserved glutamate (Glu487) residue important for catalytic activity in rat liver carnitine palmitoyltransferase II. J. Biol. Chem. 277, 42219-42223.

Contribution: I constructed active expression system for the wild-type CPTII in E. coli.

isoforms of CPTI, a liver isoform of CPTI and a heart/skeletal muscle isoform of CPTI, that are 62% identical in amino acid sequence [40, 283, 304, 305, 314].

Rat and human CPTII cDNAs have been cloned and sequenced [90, 292]. The cDNA sequences predicted proteins of 658 amino acid residues (71 kDa) that had 82 and 85% identity at the amino acid and nucleotide levels, respectively. Because the size of the mRNA in different rat tissues was identical, CPTII appears to be the product of a single gene that is expressed uniformly in every tissue examined thus far [180]. CPTII is a distinct, catalytically active, malonyl-CoA-insensitive enzyme, because a rat liver cDNA encoding CPTII synthesizes an active protein when expressed in *Escherichia* coli, in the yeasts Saccharomyces cerevisiae and Pichia pastoris, or in baculovirus or COS cells [42, 43, 72, 136, 292]. Detergent-solubilized yeast-expressed CPTII showed normal saturation kinetics with both substrates, carnitine and palmitoyl-CoA, and the calculated K_m and V_{max} were similar to that observed with the rat liver mitochondrial CPTII [72. With the S. cerevisiae-expressed CPTII, mutations of the conserved residues His372, Asp376, and Asp464 to alanine resulted in complete loss of CPTII activity, suggesting that these residues may be required for catalysis [42]. We and others hypothesize that the reaction catalyzed by CPTI and CPTII in the direction of palmitoylcarnitine formation at the active site pocket involves deprotonation of the hydroxyl group of carnitine by a catalytic base (α -proton abstraction by His, Glu, or Gln) and attack by the resultant oxyanion at the carbonyl of the thioester of palmitoyl-CoA to generate palmitoylcarnitine and free CoA [42, 66, 64].

Carnitine palmitoyltransferase deficiencies are common disorders of mitochondrial fatty acid oxidation. Rare human genetic defects in fatty acid oxidation specifically ascribed to CPTI, CPTII, and the carnitine translocase have been reported [28, 210]. CPTII deficiency, the most common inherited disorder of lipid metabolism affecting skeletal muscle, is an autosomal recessive disorder with three distinct clinical phenotypes [28]. The human CPTII gene is 20 kb in length, contains five exons, and is located at chromosome Ip32 [90]. More than 25 different mutations and three polymorphisms have been identified in the CPTII gene [64, 271, 280]. One missense mutation, S113L, accounts for ~60% of the mutant alleles responsible for the adult

myopathic form of the disease [258, 276]. Recently, an E487K missense mutation in conjunction with S113L was reported in a child with CPTII deficiency characterized by recurrent episodes of myalgia and myoglobinuria triggered by fever [45].

A comprehensive structure-function study of the CPTII enzyme is necessary for a better understanding of the pathogenesis of human CPTII deficiency diseases and for diagnosis and therapy. In this report, site-directed mutagenesis studies of conserved glutamate residues in the C-terminal domain of a liver isoform of CPTII demonstrate for the first time that the conserved glutamate residue Glu487 is important for catalysis.

8.2 MATERIALS AND METHODS

8.2.1 Construction of Rat Liver CPTII Mutants

Mutants were constructed using the overlap extension PCR procedure [257] with the pPROEX-CPTII plasmid DNA as the template. Plasmid pPROEX-CPTII is a derivative of pPROEX-1, a protein expression vector in *E. coli* (Invitrogen) and the plasmid pYGW6 [72]. To construct pPROEX-CPTII, the *EcoR*I fragment of pYGW6 [72], containing the full-length rat liver CPTII cDNA, was released and ligated to *EcoR*Icut pPROEX-1. Mutants pE487A, pE487D, pE487K, pE500A, and pE500D were made using the outer pair primers PEX-F (5'-GTGGAATTGTGAGCGGATAACAA-3') and PEX-R(5'-GGCTGAAAATCTTCTCTCATCCGCCAAA-3') and the corresponding internal pairs of primers containing mutations as shown in Table 8.1.

The PCR product obtained by this overlap extension PCR was then purified, digested with *SfiI-Hind*III, and ligated into *SfiI-Hind*III-cut wild-type CPTII cDNA in pPROEX-1 to get the mutant CPTII cDNA. Transformants obtained upon transformation of the competent *E. coli* BL21 cells by the mutant DNAs were screened for the ability to productively serve as templates for PCR using forward primers with 3' ends specific to each of the above mutations. For example, the E500A mutant was screened for with the following primer: E500ACK 5'-CAAGCACGGCCGCACAGC-3'. The mutation-specific 3' nucleotides are indicated in bold above and in Table 8.1. The other primers used for screening were E500DCK, E487ACK, E487KCK, and E487DCK. The mutations were

Table 8.1

PCR primers used for construction of CPTII mutants

Mutant	Primer
E500AF	5 ' - CAAGCACGGCCGCACAGCAACTATCCGCCCAGCTC - 3 '
E500AR	5 ' - GAGGCTGGGCGGATAGT TGC TGTGCGGCCGTGCTTG - 3
E500ACK	5 ' - CAAGCACGGCCGCACA GC - 3 '
E500DF	5'-CAAGCACGGCCGCACAGACACTATCCGCCCAGCTC-3'
E500DR	5 ' - GAGGCTGGGCGGATAGTGTCTGTGCGGCCGTGCTTG-3 '
E500DCK	5 ' - CAAGCACGGCCGCACAGA - 3 '
E487AF	5 ' - GACGGTGGCTACCTAT GCA TCCTGCAGCACTGCAGC-3 '
E487AR	5 ' - GCTGCAGTGCTGCAGGA TGC ATAGGTAGCCACCGTC - 3 '
E487ACK	5 ' - GACGGTGGCTACCTAT GC - 3 '
E487KF	5'-GACGGTGGCTACCTATAAGTCCTGCAGCACTGCAGC-3'
E487KR	5 ' ~GCTGCAGTGCTGCAGGAC TTA TAGGTAGCCACCGTC-3 '
E487KCK	5 ' - GACGGTGGCTACCTAT GC - 3 '
E487DF	5 ' - GACGGTGGCTACCTAT GAC TCCTGCAGCACTGCAGC-3 '
E487DR	5 ' - GCTGCAGTGCTGCAGGA GTC ATAGGTAGCCACCGTC - 3 '
E487DCK	5 ' - GACGGTGGCTACCTAT GA ~ 3 '

confirmed by DNA sequencing. The transformants are designated as EC-CPTII, EC-PPROEX, EC-E487A, EC-E487D, EC-E487K, EC-E500A, and EC-E500D.

8.2.2 Rat Liver CPTII Expression

A single colony of EC-CPTII, EC-PPROEX, EC-E500A, EC-E500D, EC-E487A, EC-E487D, or EC-E487K was inoculated into a 2-ml LB medium containing 100μ g/ml ampicillin and incubated at 37 °C overnight with shaking (280 rpm). The culture was then transferred to 200 ml of the 2-ml LB medium containing 100μ g/ml ampicillin and incubated at 37 °C with shaking until the A600 reached 0.5-0.8 unit. 200 µl of 1 M isopropyl-1-thio--D-galactopyranoside was then added to each culture, and the incubation was continued at 37 °C with shaking for 5 h. The cells were harvested by centrifugation at 12,000 × g for 20 min, washed by resuspension in 20 ml of PBS, and finally suspended in 4 ml of PBS. The cells were then lysed by French press at 1500 p.s.i. Whole-cell extracts were obtained after centrifugation of the cell lysates at 12,000 × g for 20 min. Protein was determined using the protein assay kit (Bio-Rad), and the cell extracts were stored at 80 °C.

8.2.3 CPT Assay

CPT activity was assayed by the forward exchange method using L-[³H]carnitine [35, 241], and the K_m for palmitoyl-CoA was determined by varying the palmitoyl-CoA concentration from 2.8 to 222.2 μ M at a fixed molar ratio (6.1:1) of palmitoyl-CoA to albumin as described previously [241]. The concentration of carnitine was fixed at 200 μ M. The K_m for carnitine was determined by varying the carnitine concentration from 11.1 to 471.7 μ M at a fixed 111.1 μ M palmitoyl-CoA.

8.2.4 Western Blot

Proteins in whole-cell extracts were separated by SDS-polyacrylamide gel electrophoresis in a 10% gel and transferred onto nitrocellulose membranes. Immunoblots were developed by incubation with the anti-His tag antibodies (Qiagen, Valencia, CA) as described previously [241]. For wild-type and mutant CPTII protein determination in the whole-cell extracts, a quantitative Western blot analysis was employed with a His6 protein ladder (Qiagen). The fluorescence intensity was measured using the Scion Image software (Scion Corp.).

Sources of other materials and procedures were as described in our previous publication [241].

8.3 RESULTS

8.3.1 Expression of Wild-type and Mutant CPTII in E. coli

We have previously cloned the full-length rat liver CPTI and CPTII cDNA, separately expressed the genes in the yeast *P. pastoris*, and demonstrated for the first time that CPTI and CPTII are distinct catalytically active enzymes [72]. Construction of plasmids carrying the wild-type and mutant CPTIIs was performed as described under " MATERIALS AND METHODS". Mutations were confirmed by DNA sequencing (Table 8.1). *E. coli* was chosen as an expression system for the wild-type and mutant CPTIIs because it has no endogenous CPTII activity, and the *E. coli*-expressed CPTII is catalytically active [43]. We expressed the full-length rat liver CPTII cDNA in *E. coli* and demonstrated that, unlike CPTI, the expressed CPTII is catalytically active (Table 8.2). Furthermore, no CPTII activity was found in the control *E. coli* strain with the vector but without the CPTII cDNA insert.

8.3.2 Effect of Mutations on CPTII Activity

Separate substitution of the highly conserved glutamate residue at position 487 (Glu487) (Fig. 8.1) with alanine (E487A), lysine (E487K), or aspartate (E487D) resulted in 96, 99, and 97% losses in CPTII activity, respectively, compared with the activity observed in the wild-type CPTII. A change of the conserved glutamate 500 (Glu500) to alanine (E500A) or aspartate (E500D) resulted in losses of 42 and 75% in CPTII activity, respectively, compared with the wild-type CPTII activity.

Western blot analysis of the extract from the *E. coli* strain expressing the wildtype and mutant CPTIIs using an anti-His tag antibody showed the presence of a 71-kDa

	487 500
RCPTII	VAQLAFQMAFLRQYGQTVATYESCSTAAFKHGRTETIRPASIF
MCPTII	VAQLAFQMAFLRQYGQTVATYESCSTAAFKHGRTETICPASIF
HCPTII	VAQLAFQMAFLRQYGQTVATYESCSTAAFKHGRTETIRPASVY
RLCPTI	FIQLALQLAHYKDMGKFCLTYEASMTRLFREGRTETVRSCTME
MLCPTI	FIQLALQLAHYKDMGKFCLTYEASMTRLFREGRTETVRSCTTE
HLCPTI	FVQLALQLAHYKDMGKFCLTYEASMTRLFREGRTETVRSCTTE
CATHMOUSE	FIQVALQLAYYRIYGQACATYESASLRMFHLGRTDTIRSASID
CATHUM	FIQMALQLAYYRIYGQACATYESXSLRMFHLGRTDTIRSASMD
CHATPIG	FIQVALQLAFYRLHGRLVPTYESASIRRFHEGRVDNIRSATPE
CHATDMEL	YIQLALQLAHYKLYGRLVATYESASTRRFLHGRVDCIRAASTE
COTRAT	FIQLALQLAYYRLHGRPGCCYETAMTRYFYHGRTETVRSCTVE
CATYEAST	FVQQVFQVAYFALYGRFETVYEPAMTKAFQNGRTEAIRSVTGQ
	* * . * . * * * *

Fig. 8.1 Sequence alignment of portions of the C-terminal region of various acyltransferases.

*, identical conserved residues; ·, conserved residues.



Fig. 8.2 A, immunoblots showing expression of wild type (lane 1), E. coli bl21(pPROEX-1) (lane 2), EC-E500A (lane 3), EC-E500D (lane 4), EC-E487A (lane 5), and EC-E487K (lane 6), EC-487D (lane 7), and wild type (lane 8). Proteins (20 µg) of whole-cell extract from each strain expressing the wild-type and mutant CPTIIs were separated on a 10% SDS-PAGE and blotted onto a nitrocellulose membrane. The immunoblots were developed using anti-His tag antibodies as described under " MATERIALS AND METHODS" B, immunoblots of the His6 protein at different concentrations. C, a plot of the band intensity (densitometry units) versus the His6 protein at different concentrations.

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Fig. 8.3 A, Kinetic analysis of wild-type and mutant CPTII activities. Proteins (150 μ g) of whole-cell extract from wild type (squares), EC500A (circles), and EC500D (triangles) were assayed for CPT activity in the presence of increasing concentration of carnitine. B, same as A, except CPT activity was measured in the presence of an increasing concentration of palmitoyl-CoA.

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Table 8.2

Straina	Activi	ty
Strains	nmol/mg·min	%
Wild-type	122.4 ± 0.48	100
EC-E487A	4.9 ± 0.09	4.0
EC-E487K	1.2 ± 0.05	1.0
EC-E487D	4.0 ± 0.07	3.3
EC-E500A	70.6 ± 0.33	57.6
EC-E500D	30.6 ± 0.05	25.0

CPT activity in E. coli strains expressing wild-type and mutant CPTIIs

Whole-cell extract containing 3.6 μ g of CPTIIs of each strain were assayed for CPT activity as described under "MATERIALS AND METHODS" The results are the means ± S.D. of at least three independent experiments with different protein preparations. Numbers in parentheses represent percent of CPT activity in the mutants compared with the wild type (100%).

protein corresponding to CPTII (Fig. 8.2). For the wild-type and mutant CPTIIs, proteins of the predicted size were expressed at similar steady-state levels as determined by quantitative immunoblot using a pure His6 protein as the standard. The quantitative Western blot and the densitometry analysis for the His6 protein are shown in Fig. 8.2, B and C. Based on the quantitative immunoblot of the standard protein, the wild-type and mutant CPTII proteins were estimated to contain 7 pmol (0.49 μ g) of CPTII in 20 μ g of whole-cell extract protein.

8.3.3 Kinetic Properties of Wild-type and Mutant CPTIIs

Because of the extremely low activity in mutants E487A, E487D, and E487K, it was not possible to determine the K_m or V_{max} values for the carnitine or palmitoyl-CoA substrates. Mutants E500A and E500D exhibited normal saturation kinetics when the carnitine concentration was varied relative to a constant second substrate, palmitoyl-CoA (Fig. 8.3A), a property identical to the wild-type CPTII. For the E500A and E500D, the calculated K_m values for carnitine were ~2-fold higher than the value for the wild-type CPTII, as shown in Table 8.3, and the V_{max} values were 26 and 60% lower than the wild type, respectively, indicating a major effect of the E500D mutation on catalytic activity. The catalytic efficiency as calculated by the V_{max}/K_m was decreased by 55 and 76% for the E500A and E500D, respectively. With respect to the second substrate, palmitoyl-CoA, mutants E500A and E500D exhibited normal saturation kinetics similar to the wild type (Fig. 8.3B) when the molar ratio of palmitoyl-CoA to albumin was fixed at 6.1:1. The calculated K_m values for palmitoyl-CoA for mutants E500A and E500D were 76 and 89% lower, respectively, than the wild type. The V_{max} values for mutants E500A and E500D were 13 and 29% of the wild-type values (Table 8.3), and the catalytic efficiency was similar to the wild type for both mutants E500A and E500D. Thus, substitution of the conserved Glu500 residue with either alanine or aspartate resulted in substantial loss in catalytic activity, but substitution of the highly conserved Glu487 with alanine, lysine, or aspartate resulted in nearly complete loss in CPTII activity (Table 8.2).

Table 8.3

	Carnitine		Palmitoyl-CoA	
Strains	K _m	V _{max} nmol/mg · min	K_m μM	V _{max} nmol/mg · min
	μΜ			
Wild-type CPTII	63.7	187.6	81.9	395.7
EC-E500A	105.6	138.7	19.9	114.2
EC-E500D	111.4	77.5	8.8	53.0

Kinetic characteristics of E. coli-expressed wild-type and mutant CPTIIs

Whole-cell extract containing 3.6 μ g of CPTIIs of wild-type and mutant CPTIIs were assayed for CPT activity in the presence of increasing concentrations of carnitine or palmitoyl-CoA. Values are the average of at least two independent experiments with different protein preparations.

8.4 **DISCUSSION**

The site-directed mutagenesis study described here is aimed at elucidating the function of highly conserved acidic residues found at the proximity of the active site of CPTII. Two glutamate residues, Glu487 and Glu500 in rat liver CPTII, and the corresponding residues Glu590 and Glu603 in a liver isoform of CPTI, are conserved throughout the family of acyltransferases with known primary sequences. Specifically, Glu487 in CPTII and Glu590 in CPTI are highly conserved within the family of acyltransferases, but Glu500 in CPTI are highly conserved within the family of acyltransferases, but Glu500 in CPTII and Glu603 in CPTI are conserved residues within the family of CPT enzymes, whereas other acyltransferases have aspartate at this position (Fig. 8.1).

To determine the role of the conserved Glu487 and Glu500 residues in CPTII in catalysis, we separately changed the highly conserved glutamate residue (Glu487) to alanine (E487A), aspartate (E487D), and lysine (E487K), and the second conserved glutamate residue (Glu500) in CPTII to alanine (E500A) and aspartate (E500D), and we determined the effect of the mutations on CPTII activity in the E. coli-expressed mutant enzyme. Substitution of Glu487 with alanine, lysine, or aspartate resulted in loss of >96% CPTII activity. Thus, the presence of this highly conserved Glu487 residue is probably crucial for maintaining the configuration of the CPTII active site. Mutation of the corresponding highly conserved residue in a liver isoform of CPTI, Glu590, to alanine resulted in a 50% loss in CPTI activity (data not shown). The substantial loss in CPTII activity (99%) observed with our E487K mutant along with the loss observed in the child with the CPTII deficiency disease [45] establishes that Glu487 is probably crucial for maintaining the configuration of the liver isoform of CPTII active site. All of the Glu487 mutants had insufficient activity to allow measurement of K_m or V_{max} for either carnitine or palmitoyl-CoA. Because a conservative substitution of Glu487 with aspartate (E467D), a negatively charged amino acid that has only one less methyl group than the glutamate residue in the wild-type enzyme, resulted in 97% loss in activity, we suggest that Glu487 is at the active-site pocket of CPTII.

As the terminal enzyme that transports long chain fatty acids from the cytosol to the mitochondrial matrix, CPTII in the presence of CoA-SH reversibly catalyzes the conversion of long chain acylcarnitines to long chain acyl-CoAs. Similar to other acyltransferases, CPTII contains a general acid/base, His372, a highly conserved residue that may form a hydrogen-bonding network or a salt bridge to a nearby conserved glutamate residue such as Glu487 or Glu500. Substitution of Glu487 with alanine, lysine, or aspartate resulted in an inactive enzyme. Glu487 may thus be involved in facilitating catalysis by orienting the imidazole ring of His372 for optimum productive interaction with the substrate [17]. We hypothesize that the Glu487 to alanine or lysine substitution may disrupt a hydrogen-bonding network or a salt bridge, perhaps to a residue like His372 at the active site of CPTII. However, a change of Glu487 to aspartate may result in the carboxylate being outside the hydrogen bond distance of His372, the predicted general acid/base at the active site [17].

The characterization of E487K, E487A, and E487D described here is consistent with previously reported studies of a child with a novel CPTII deficiency disease that exhibited clinical episodes of myalgia and myoglobinuria induced by intercurrent febrile illnesses [45]. The patient was heterozygous for a G-to-A substitution at codon 487, changing the highly conserved Glu487 to lysine (E487K), whereas the other allele carried the common benign S113L missense mutation. However, for the rat liver CPTII, a single substitution mutation of Glu487 to lysine resulted in almost complete loss (99%) in activity, and even a single conservative change of Glu487 to aspartate (E487D) or alanine (E487A) caused >96% loss in catalytic activity, further supporting a pathogenic role for a mutation of this residue. Thus, substitution mutation of the highly conserved negatively charged Glu487 residue located in the C-terminal region inactivates CPTII, suggesting a critical role of this residue in catalysis. CPTII deficiency, inherited as an autosomal recessive trait, is the most common disorder of lipid metabolism affecting muscle and is the most frequent cause of hereditary myoglobinuria in adults that is triggered by prolonged exercise or fasting (or both) and by cold, stress, or fever, conditions associated with increased dependence of muscle on lipid metabolism [28, 258, 276]. A hepatic form

of CPTII deficiency associated with hypoketotic hypoglycemia, hepatopathy, cardiopathy, and sudden death has also been reported in infants [28].

To investigate the role of the second conserved glutamate residue in CPTII (Glu500) on catalytic function, Glu500 was changed to alanine and aspartate and the mutant enzyme was characterized. Substitution of the conserved Glu500 with alanine and aspartate resulted in partial loss of activity and a decrease in the V_{max} for both substrates. In addition, a change of the conserved Glu500 to aspartate caused a significant decrease in the V_{max} for both substrates with a 2-fold increase in the K_m for carnitine. For both substrates, a lower but similar decrease in the V_{max} and a 2-fold increase in the K_m for carnitine was observed with the E500A mutant, suggesting that the main effect of the mutations was to decrease the stability of the enzyme-substrate complex. However, the mutations could lead to misfolding in 90% of the molecules, producing a lower kcat and unchanged K_m values. Because the E500A and E500D mutations caused a substantial decrease in the V_{max} , this could be related to the alteration of intrinsic CPTII stability. Furthermore, Glu500 appears to be critical for the structural stability of CPTII. A conservative substitution of Glu500 to aspartate resulted in >7- and 2.5-fold decreases in the V_{max} for the substrates palmitoyl-CoA and carnitine, respectively, with a relatively minor change in carnitine binding but a significant increase in the affinity for palmitoyl-CoA. It is suggested that the Glu500 mutation exists within a region containing a possible adenine-binding loop, because the affinity of the enzyme for palmitoyl-CoA that has an adenine group is greatly increased, but additional experimental evidence is needed to substantiate this hypothesis. Hence, Glu500 may help orient the loop containing the active site residue by hydrogen bonding of a backbone residue and may play a role in substrate binding and catalysis [17].

Sequence alignment of various acyltransferases shows the presence of highly conserved histidine and glutamate residues in CPTII. Mutation of the conserved His372 residue in CPTII and the corresponding residue in CPTI, His473 to alanine, inactivates both enzymes [42, 188]. In this report, we have demonstrated that the conserved Glu487 of rat liver CPTII is required for catalysis. Despite its similar negative charge and potential for hydrogen bonding, an aspartate residue cannot fulfill this requirement, suggesting that the extra methyl group in glutamate is needed for optimal catalysis and maintenance of active site integrity. A change of Glu487 to lysine or alanine also resulted in an inactive enzyme. The loss in activity observed with the E487K is consistent with previously reported studies of a child with a novel CPTII deficiency disease [45]. Substitution of the conserved Glu500 in CPTII with alanine or aspartate reduced the catalytic efficiency, suggesting that this residue may be important in stabilization of the enzyme-substrate complex.

CHAPTER 9 SUMMARY AND FUTURE DIRECTIONS

9.1 SUMMARY

9.1.1 CPTI

Mutations of the highly conserved arginine and tryptophan residues in the Cterminal 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 [64]. Mutation of the highly conserved Arg601, Arg606 and Arg655 to Ala resulted in a major loss in catalytic activity [64]. Therefore, the presence of these conserved arginine residues is probably crucial for maintaining the configuration of the L-CPTI active site. We suggested that the conserved Arg655 in carnitine acyltransferases contributes to substrate binding by forming a salt bridge with the carboxylate moiety of carnitine [134]. 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 [65]. 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 [162]. 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 [42, 188].

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 [262]. 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

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Fig. 9.1 L-CPTI homology model

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 [262]. 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. 9.1). The highly conserved C-terminal acidic residue, Glu590 was found to be a negative determinant of L-CPTI malonyl-CoA sensitivity, because mutation of this residue to Ala increased the malonyl-CoA sensitivity of the enzyme to that of the wild-type muscle, M-CPTI [195].

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, the model for L-CPTI is less ideal than the 3D model for CPTII. However, the catalytic core in the 3D model is still highly similar to that of CAT. It contains both N and C domains. The active residue H473 is located at the interface of N and C domains. We were able to map the residues we mutated into the 3D model for L-CPTI. Some of the residues are located in the N domain (R388, W391, R451, W452), while others are located in the C domain (E590, R601, E603, R606, R655, L764) (Fig. 9.1).

9.1.2 CPTII

E487 in CPTII is essential for catalysis because substitution of E487 with alanine, aspartate, or lysine resulted in almost complete loss in CPTII activity [313]. Because a conservative substitution mutation of this residue, E487D, resulted in almost complete loss in activity, we predicted that E487 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 [45] establishes that E487 is crucial for maintaining the configuration of the liver CPTII active site (Fig. 9.2).

The 3D model of CPTII based on the published 3D structure of mouse carnitine acetyltransferase (CAT) [103] exhibits very high similarity to that of CAT (Fig. 9.2).



Fig. 9.2 CPTII homology model

More than 80% of the atoms are in stable state as shown by the blue color. The model also contains both N and C domains. The active site residue, H340 is located at the interface of N and C domains. E487 forms a hydrogen bond with D464 at the interface of the C domain.

9.2 FUTURE DIRECTIONS

9.2.1 Large Scale Purification and Structural Characterization of CPT I and CPTII

The lack of X-ray crystal structures of CPTs has been a barrier to understanding the structure-function relationship and regulation of the CPTs. The first step to resolve this problem is to obtain milligram quantities of the purified CPTs. CPT II, a membrane associated protein, is not toxic to host bacteria. Large quantities of CPT II have been purified by chromatography on a Ni-NTA affinity column for determination of the 3-D structure by X-ray crystallography. Both M-CPTI and L-CPTI are inactivated by detergents, but the inactive enzymes can be reactivated by reconstitution [314]. It is important to purify both the wild-type and mutant proteins for structure analysis.

9.2.2 Determine the Role of the Signature Motif in Rat L-CPTI on Carnitine Binding

Replacement of VDN in the signature motif, RT(V)E(D)T(N)I(V)R, in choline acetyltransferase (ChAT) to TET together with a change of N655 to arginine, which is conserved in all the acytransferase, conferred ChAT the ability to accept carnitine rather than choline as a substrate [62]. We would like to determine whether we can engineer CPTI to ChPTI (choline palmitoyltransferase I). This study could provide direct evidence that this signature motif is the core of the carnitine binding site if the replacement changes the substrate specificity of CPT I.

9.2.3 Characterization of Some Important Residues in Rat L-CPTI Corresponding to those Identified in Carnitine Acetyltransferase (CAT)

The crystal structure of carnitine acetyltransferase (CAT) reveals several new residues, which are important for the substrate binding and catalysis [186]. Some of them are hydrogen-bonded to the carboxylic oxygen atom of carnitine through their hydroxyl groups (Tyr452, Ser454, Thr465). Others form tetrahedral coordination with a water molecule, which is hydrogen-bonded with the carboxylic oxygen atom (Trp102, Tyr107, Glu347) and shield the carnitine from solvent, while some are very close to the carnitine (Phe566, Val569, Ser552) [186]. It is important to mutate the corresponding residues in rat liver L-CPTI and determine their effect in activity and malonyl-CoA sensitivity.

9.2.4 Determination of the Role of the Extreme C-terminal Region of CPTII on Enzyme Activity

Using the deletion and point mutation analysis, L764 was identified to be essential for native folding and activity of M-CPTI [65]. This residue is conserved through all the members of the acyltransferase family including CPTII. It is thus important to determine the effect of the corresponding leucine residue on CPTII activity.

9.3 EXPERIMENT ENHANCEMENTS

9.3.1 Protein Expression Levels

The main purpose of the Western blot analysis was to demonstrate the expression of wild type and mutant CPTI in yeast mitochondria, but not to determine the amount of the enzyme in the yeast mitochondria, which is extremely low and not detectable by Coomassie blue staining of SDS gels. The activity and kinetic data obtained were based on an equal amount of isolated yeast mitochondrial protein, not on the amount of CPTI, which is undetectable. The activity and kinetic data can be normalized as follows. First, separate Western blot analysis of the standard protein (pure His6 protein from Qiagen) with different concentrations and CPTI will be carried out. From the immunoblots, the fluorescence intensity of the bands (in densitometer units) will be measured for the standard protein and CPTI. From a plot of band intensity (densitometry units) versus the different protein concentrations for the standard protein (standard curve), the CPTI protein concentration can then be estimated. Then, based on the estimated concentration of wild-type and mutant CPTI proteins by quantitative immunoblot, the concentration of yeast mitochondrial protein to be used for the kinetic assay can be determined.

9.3.2 Gene Dosage

Rare multiple gene insertion events at a single locus in a cell during electroporation can be detected by Southern blot analysis. A blot of digested, gelseparated genomic DNA can be generated and probed with the appropriate fragment from the expression vector. The intensity of a single copy gene can be used as a control. If the clones have multiple insertions, the blots should show higher intensity. Gene dosage can also be estimated by densitometry.

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