CLONING, EXPRESSION AND FUNCTIONAL CHARACTERIZATION OF THE MITOCHONDRIAL CARNITINE PALMITOYLTRANSFERASES

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ABBREVIATIONS

ACC	acetyl-CoA carboxylase
AMPK	AMP-dependent protein kinase
ATP	adenosine triphosphate
CoA	coenzyme A
CPT	carnitine palmitoyltransferase
FA	fatty acid
FISH	fluorescence *in situ* hybridization
K_{ATP}	ATP-sensitive potassium channel
LCFA	long-chain fatty acid
L-CPTI	liver isoform of carnitine palmitoyltransferase I
MBP	maltose binding protein
M-CPTI	heart/skeletal muscle isoform of carnitine palmitoyltransferase I
ORF	open reading frame
PPAR	peroxisome proliferator-activated receptor
PPREs	PPAR response elements
RHM	rat heart mitochondria
RLM	rat liver mitochondria
TDG-CoA	tetradecylglycidyl-CoA
UTR	untranslated region

ABSTRACT

Cloning, Expression, and Functional Characterization of the Mitochondrial Carnitine Palmitoyltransferases

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Carnitine palmitoyltransferase I (CPTI) catalyzes the first reaction in the transport of long-chain fatty acids from the cytoplasm to the mitochondrial matrix, converting long-chain acyl-CoA to acylcarnitine. The acylcarnitine is then translocated across the inner mitochondrial membrane by the carnitine/acylcarnitine carrier and converted back to acyl-CoA by CPTII. CPTI is regulated by malonyl-CoA, the first intermediate in fatty acid synthesis.

In this study, we report the cloning and functional expression of rat liver CPTI (L-CPTI) and human heart/skeletal muscle CPTI (M-CPTI) in the yeast *Pichia pastoris*, an organism with no endogenous CPT activity. The yeast-expressed CPTIs are catalytically active, malonyl-CoA sensitive, and detergent labile. The two isoforms of CPTI are 62% identical in amino acid sequence, and the first 18 amino acids are conserved. M-CPTI has a 30-fold lower IC_{50} for malonyl-CoA inhibition and a 20-fold higher K_m for carnitine compared to L-CPTI. Reconstitution studies demonstrated for the first time that detergent inactivation of L-CPTI and M-CPTI is reversible.

To assess the role of the N-terminal region of CPTI on malonyl-CoA sensitivity and binding, and the molecular basis for the differences in malonyl-CoA sensitivity between L-CPTI and M-CPTI, a series of deletion and substitution mutants of the first 130 N-terminal amino acid residues in both L-CPTI and M-CPTI were constructed. The mutants were expressed in the yeast *P. pastoris*. The effects of

these mutations on CPTI activity, malonyl-CoA sensitivity, and binding were determined in isolated mitochondria. Rat L-CPTI mutants that lacked the first 18 N-terminal amino acid residues, Δ18, had activity and kinetic properties similar to wild-type L-CPTI, but were insensitive to malonyl-CoA inhibition and lost malonyl-CoA binding. Site-directed mutagenesis studies identified glutamic acid 3 and histidine 5 as necessary for malonyl-CoA binding and inhibition of L-CPTI, but not required for catalysis.

Deletion of the first 28 N-terminal amino acid residues of M-CPTI caused loss of malonyl-CoA sensitivity and high affinity binding. Replacement of the N-terminal domain of L-CPTI with the N-terminal domain of M-CPTI had no effect on the activity or malonyl-CoA sensitivity of the chimeric L-CPTI.

CHAPTER 1
INTRODUCTION

1.1 INTRODUCTION TO THE CARNITINE PALMITOYLTRANSFERASE SYSTEM

A major source for energy production in the cell is the mitochondrial β-oxidation of long-chain fatty acids (LCFA). The first conceptualized mechanism for the mitochondrial transport of LCFA emerged in the early 1960s [30,90,91]. Interest in the carnitine palmitoyltransferase (CPT) system started in the mid-1970s following two important developments. First was the discovery of the inherited mitochondrial CPT deficiency that formed the basis of serious human disease [71], and second was the recognition of the pivotal role of CPT in the regulation of fatty acid (FA) oxidation [20,178]. With the newly developed molecular biology tools, the recent cloning and expression of the CPT gene has provided considerable insight into the structure, function, regulation, and physiological role of the CPT system.

LCFAs must first enter the mitochondria by a carnitine-dependent process to be available for β-oxidation. Since its operation was first hypothesized by Fritz and Yue [91], this process has been shown to be composed of several steps (Fig. 1.1). First, LCFAs are activated to their CoA ester in the extramitochondrial space by long-chain acyl-CoA synthetases localized in the mitochondrial outer membrane as well as the peroxisomal and endoplasmic reticulum membrane. Second, the fatty acyl moiety is transferred from CoA to carnitine by carnitine acyltransferase I located on the outer mitochondrial membrane. Third, acylcarnitine is translocated from the cytosol into the mitochondrial matrix by carnitine acylcarnitine translocase, which belongs to the mitochondrial carrier family. Fourth, once inside the mitochondrial lumen, CPTII reconverts the carnitine ester to the CoA ester which can then serve as

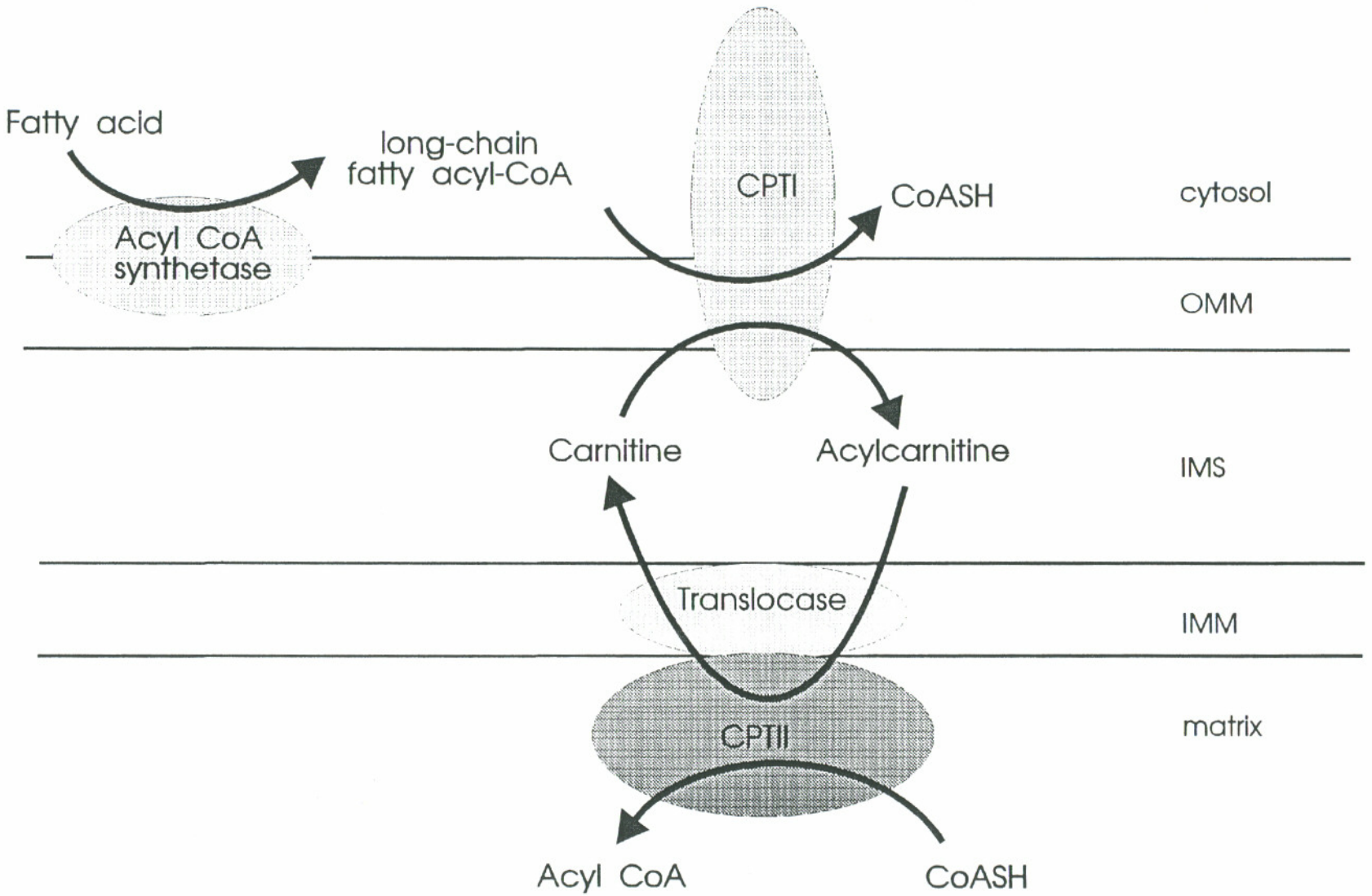

Figure 1.1 Reactions catalyzed by CPTI and CPTII.

a substrate for β-oxidation. CPTII is loosely bound on the matrix side of the mitochondrial inner membrane [18,100,115,124,182,194]. The overall effect of the CPT system is to transport long-chain acyl-CoA esters across the mitochondrial inner membrane into the matrix compartment where β-oxidation takes place.

Carnitine palmitoyltransferase I (CPTI) activity is strictly regulated by malonyl-CoA, the first intermediate in FA synthesis. Therefore, CPTI is considered as the first rate-limiting enzyme in FA oxidation. CPTI and CPTII are distinct catalytically active enzymes. There is only one CPTII isoform, whereas two isoforms of CPTI, encoded by distinct genes, have been described: the liver isoform (L-CPTI) and the heart/skeletal muscle isoform (M-CPTI) [172].

Because of its central role in the regulation of FA oxidation and FA synthesis, it is imperative that we understand the biochemical and molecular characteristics of the CPT system.

1.2 PHYSIOLOGICAL AND BIOCHEMICAL FUNCTIONS OF THE CPT SYSTEM

The mitochondrial β-oxidation of LCFA is a major source of energy. FAs are ubiquitous biological molecules that are used as metabolic fuels, as covalent regulators of signaling molecules, and as essential components of cellular membranes. It is thus logical that FA levels should be closely regulated [206]. Indeed, some of the most common disorders (cardiovascular disease, hyperlipidemia, obesity, and insulin resistance) are characterized by altered levels of FAs or their metabolites [73,215].

CPTI is the rate-limiting enzyme regulating FA oxidation in mitochondria [180]. CPTI is inhibited by malonyl-CoA, and this inhibition is overcome by high levels of long-chain acyl-CoA [183]. The malonyl-CoA/CPTI interaction has emerged as a key component of the metabolic signaling system in a number of mammalian tissues, including liver [182], heart [159], skeletal muscle [226], and pancreatic β-cells [8].

Because of its central role in lipid metabolism, CPTI has attracted much attention as a site for pharmacological intervention in diseases such as diabetes [5,58] and myocardial ischemia [65,212], and in human inherited CPT-deficiency diseases.

1.2.1 Regulation of Fatty Acid Synthesis and Oxidation in Liver

In the late 1970s, McGarry and Foster and their coworkers established that CPTI in rat liver mitochondria is inhibited by physiological concentrations of malonyl-CoA [177,178,181]. It was concluded that this interaction was an important locus for control of hepatic FA oxidation and ketogenesis [174]. Furthermore, it was found that the hepatic rate of FA synthesis and oxidation was inversely regulated such that, when the latter is active, the former is suppressed and vice-versa [174,175,181]. Control over this key metabolic branch point is vested largely in the concentration of malonyl-CoA, the first committed intermediate in FA synthesis, which proved to be a potent inhibitor of CPTI, the first step in FA oxidation [175,182].

Earlier studies of the role of CPTI in regulating FA oxidation concentrated on the rat liver enzyme, where the rate of FA oxidation changes dramatically with the physiological state and is accompanied by changes in CPTI activity and malonyl-CoA sensitivity. Starvation, diabetes, and hyperthyroidism increase hepatic mitochondrial outer membrane CPT activity [59,62,63,100,140,223,238,275]. When FA oxidation increases, the sensitivity of the hepatic enzyme to malonyl-CoA inhibition decreases due to a $\sim$10-fold increase in the K_i for malonyl-CoA [57,62,100,238], but there is no change in the K_m for the substrates [57]. In contrast, increased malonyl-CoA sensitivity of the enzyme has been reported in genetic obesity [49], after estrogen treatment [265] or ethanol feeding [110], and during pregnancy or lactation [216]. Under these physiological conditions, the activity of L-CPTI decreases, FA oxidation decreases, but CPTII activity is not altered. Reports of only slight change in inner membrane CPT activity in diabetes or hypothyroidism [100] may be due to an incomplete separation of the membranes.

In the mid-1970s, it became clear that the process of hepatic ketogenesis is governed largely by the relative concentration of two hormones, insulin and glucagon. Available evidence at that time indicated that the ketogenic process is triggered by a

fall in the absolute level of circulating insulin, coupled with elevation of the glucagon/insulin ratio. In the well-fed state (high insulin, low glucagon), carbon flow through the lipogenic pathway is efficient, malonyl-CoA concentration is high, and CPTI activity is suppressed. This ensures a unidirectional carbon flow from glucose to fatty acyl-CoA to triacylglycerol and to very-low-density lipoprotein, which is then exported from the liver for use in adipose and muscle tissues. Conversely, in the ketotic states like fasting (low insulin, high glucagon), malonyl-CoA concentrations decrease, FA biosynthesis ceases, CPTI becomes derepressed, and FAs delivered to the liver from fat depots are efficiently oxidized with the production of ketone bodies [100,182,206]. Up-regulation of L-CPTI in diabetes was also observed with an increased glucagon/insulin ratio [27,31,58,106,140,146]. This may be due to the increased abundance of CPTI in mitochondria. It has been proposed that hepatic CPT activity is regulated by glucagon through protein phosphorylation [117,118], but there is no direct support for such a mechanism.

The physiological control of CPTI activity involves inhibition by malonyl-CoA and is referred to as a malonyl-CoA-dependent control. In recent years, however, a novel control mechanism of hepatic CPTI activity has been proposed. Studies using permeabilized hepatocytes have shown that various agents cause short-term changes in CPTI activity in parallel with changes in the rate of LCFA oxidation [111,112]. These short-term changes in hepatic CPTI activity are presumed to be mediated by a malonyl-CoA-independent mechanism [113]. More recently, a number of reports have described the existence of specific interactions between mitochondrial outer membrane and cytoskeletal elements [13,131,153,254,255]. CPTI activity is stimulated directly by cytoskeletal components which are phosphorylated by Ca^{2+}/calmodulin-dependent protein kinase II [255–258] or by AMP-dependent protein kinase (AMPK) [254]. Results thus far support a novel mechanism of short-term control of hepatic CPTI activity. It is presumed that malonyl-CoA-dependent and malonyl-CoA-independent acute control of hepatic CPTI activity operate in concert [257].

Recent studies indicate that CPT activity and its malonyl-CoA sensitivity can be greatly altered by the membrane environment [36]. The physiological changes in

malonyl-CoA sensitivity are mediated via changes in membrane lipid composition [197], lipid molecular order in the membrane core [292], and membrane fluidity [137,198].

1.2.2 Regulation of Fatty Acid Synthesis and Oxidation in Heart and Skeletal Muscle

In general, changes in physiological state do not appreciably alter the activity and malonyl-CoA sensitivity of CPTI in extrahepatic tissues such as heart [57,176,199,253], skeletal muscle [253], brown fat [224], or kidney cortex [225]. These non-lipogenic tissues also contain appreciable levels of malonyl-CoA, generated by a distinct isoform of acetyl-CoA carboxylase (ACC) [1,114,249,269]. Heart and skeletal muscle contain a CPTI isoform (M-CPTI) distinct from liver CPTI (L-CPTI) [172] that is more sensitive to inhibition by malonyl-CoA than the liver enzyme [157,187]. In heart and skeletal muscle, FA synthase activity is absent [191,273]. However, several studies suggest that malonyl-CoA still plays a key role in the regulation of FA oxidation in these tissues [171]. Regulation of FA oxidation in the adult heart apparently responds to large changes in malonyl-CoA concentrations [159,206]. A decrease in the concentration of malonyl-CoA in both heart and skeletal muscle has been reported during starvation [179], which is responsible for increased FA oxidation. Furthermore, in newborn rabbit heart [162], both CPTI activity and the sensitivity of CPTI to malonyl-CoA inhibition do not change [163]. Rather, a decrease in malonyl-CoA levels appears to be primarily responsible for the dramatic increase in FA oxidation in the newborn heart. During exercise or contraction, there is an increase in FA oxidation and a decrease in malonyl-CoA content in rat skeletal muscle [129,202,236,252,270,271]. This seems to result from the inactivation of the β isoform of ACC through protein phosphorylation by AMPK [120,129,219,252,271].

Although nutritional states such as fasting have no effect on M-CPTI activity and sensitivity to malonyl-CoA, recent studies indicate that the FA composition of the diet may be involved in the regulation of mitochondrial CPTI activity in heart and skeletal muscle. Rats fed a high-fat diet had significantly greater M-CPTI-specific

activity and lower sensitivity of M-CPTI to malonyl-CoA inhibition compared with rats fed a low-fat diet [211].

A major unanswered question in this field is why M-CPTI has higher malonyl-CoA sensitivity than L-CPTI [10,163,179,220]. The total tissue content of malonyl-CoA in heart and skeletal muscle is similar to that of liver (i.e., 2–8 μmol/liter) [220] which is 50–100-fold higher than the estimated K_i value [185]. With such a high malonyl-CoA concentration in the cell and high malonyl-CoA sensitivity of M-CPTI, FA β-oxidation would be expected to be completely suppressed at all times [170], but we know that is not the case. One explanation is that most of the malonyl-CoA in heart and skeletal muscle is in a bound form and inaccessible to CPTI [185]. Awan and Saggerson [10] proposed that the malonyl-CoA within cardiac myocytes may be bound by an enzyme of a FA elongation system that incorporates malonyl-CoA into FAs. The capacity of this enzyme to bind malonyl-CoA and act as a "sink" for the metabolite would be significantly high, thus presumably reducing the "free" concentration that can interact with CPTI. Another possibility is that, even if the free concentration of malonyl-CoA were to reach an inhibitory level for M-CPTI, the presence of some L-CPTI (IC_{50} for malonyl-CoA $\sim$2–3 μM) might allow a basal rate of acylcarnitine formation and thus of β-oxidation [185]. Clearly, this issue requires further study.

1.2.3 Fatty Acid Oxidation in the Ischemic/Reperfused Heart

FAs are the major fuel sources of the heart, with FA oxidation normally providing 60–70% of the heart's energy requirements [22,43,148,221]. During ischemia, when the supply of O_2 becomes limiting for oxidative phosphorylation, both FA and carbohydrate oxidation decrease and ATP (adenosine triphosphate) production is impaired. Reperfusion of ischemic hearts results in high levels of FAs and a high rate of FA oxidation [102,148,154,161]. Perfusion with FAs can cause *in situ* membrane damage and cardiac dysfunction. Clinically, high circulating levels of FAs are also commonly seen following a myocardial infarction [192,203,204] or during and following cardiac surgery [159,241].

One potential site where FA oxidation may be altered is at CPTI, a key enzyme that controls mitochondrial uptake of LCFA [177,182]. FA-induced injury to the ischemic myocardium can be reduced by inhibition of CPTI. This raises the possibility that either a decrease in malonyl-CoA levels, an increase in CPTI activity, or a decrease in the sensitivity of CPTI to malonyl-CoA inhibition may contribute to the high FA oxidation rates seen during reperfusion of the ischemic heart. However, neither the activity of CPTI nor the IC_{50} value for malonyl-CoA inhibition of CPTI was altered in mitochondria isolated from aerobic, ischemic, or reperfused ischemic hearts, suggesting that the primary control of cardiac CPTI activity by malonyl-CoA is unaffected by ischemia and reperfusion. In contrast, an extremely low level of malonyl-CoA was found as well as a decreased activity of ACC, the enzyme which produces malonyl-CoA [148]. A decrease in ACC activity in conjunction with basal levels of malonyl-CoA decarboxylase activity could explain the dramatic drop in malonyl-CoA levels seen during reperfusion of ischemic hearts. The observation that ACC activity could be increased *in vitro* by addition of 10 mM citrate suggests that the enzyme was in the phosphorylated inhibited state during reperfusion. It is hypothesized that activation of AMPK during ischemia and reperfusion is primarily responsible for the phosphorylation and inhibition of ACC during reperfusion [148].

A hypothetical scheme outlining the mechanism of FA oxidation in the ischemic/reperfused heart was proposed [254]. Myocardial ischemia results in an increase in 5′-AMP levels. This can either directly activate AMPK or enhance the phosphorylation of AMPK by an AMPK kinase. AMPK remains activated throughout the reperfusion period, resulting in a phosphorylation and inhibition of ACC during reperfusion. The decrease in ACC activity, together with sustained malonyl-CoA degradation (possibly by malonyl-CoA decarboxylase), will result in a decrease in malonyl-CoA levels during reperfusion. This will relieve malonyl-CoA inhibition of CPTI, resulting in an increase in FA oxidation. High FA oxidation rates will decrease cardiac efficiency and contribute to ischemic injury during reperfusion of ischemic hearts [148,149,158,219,220].

1.2.4 Role of ACC and Malonyl-CoA Decarboxylase in Fatty Acid Oxidation

Malonyl-CoA is synthesized by ACC, which catalyzes the carboxylation of acetyl-CoA to malonyl-CoA, the rate-limiting step in FA synthesis [119,143,260]. Although the importance of malonyl-CoA in regulating CPTI activity has long been recognized, the importance of ACC as the source of malonyl-CoA and thus regulating CPTI and FA oxidation has only been characterized recently [160]. It is also becoming apparent that cytoplasmic ACC has an important role in regulating the initial transport of FAs into the mitochondria [10,160,163,220]. A rise in acetyl-CoA activity is associated with an increase in malonyl-CoA levels [220]. This results in a decrease in FA β-oxidation and acetyl-CoA production.

There are two isoforms of ACC, a 265-kDa isoform (ACC 265 or ACC1 or ACC-α) and a 280-kDa isoform (ACC 280, or ACC2 or ACC-β). Both isoforms have been cloned and sequenced [1,2,4,114,164,244]. The two enzymes are different, although they catalyze the same reaction and are 70% homologous in their sequences. These differences are reinforced by the fact that, while ACC1 is mainly expressed in lipogenic tissues, ACC2 is present at relatively high levels in skeletal muscle [17,133,220]. Moreover, ACC2 is also found in rat [272] and human [1] liver.

The physiological role of ACC2 is not clear at this time. Nevertheless, the presence of ACC2 in heart and muscle as the predominant form of acetyl carboxylase has led many investigators to propose that ACC2 is involved in the regulation of FA oxidation, rather than FA biosynthesis [10,17,148,160,163,220,247]. ACC2 is thought to control FA oxidation by its ability to synthesize malonyl-CoA that inhibits CPTI. ACC2 contains 200 amino acids at the N-terminus which are not present in ACC1 and may be uniquely related to the role of ACC2 in controlling CPTI activity and FA oxidation in mitochondria [1].

While ACC appears to be the primary source of malonyl-CoA, the fate of malonyl-CoA in heart is not clear. Unlike liver, FA synthesis does not appear to be a major fate of malonyl-CoA in heart. An alternate fate of malonyl-CoA is decarboxylation to acetyl-CoA. Although the synthesis of malonyl-CoA in heart by ACC has been well characterized, no information is available as to how malonyl-CoA

is degraded. Malonyl-CoA decarboxylase activity has been reported in heart [74,148], which suggests that heart does have an active malonyl-CoA decarboxylase that plays an important role in regulating FA oxidation.

1.2.5 Role of the Malonyl-CoA and CPTI Interaction in the Pancreatic β-Cell

A third extrahepatic site in which CPTI regulation by malonyl-CoA appears to play an important physiological role is the pancreatic β-cell [45]. It has been proposed that malonyl-CoA, via its inhibitory action on CPTI, causes a rise in cytosolic long-chain acyl-CoA, which, by its action on K_{ATP} (ATP-sensitive potassium channel), has been implicated in the transduction mechanisms whereby nutrients induce the insulin secretory process [64,200,212]. Understanding the regulation of pancreatic β-cell metabolism might be important in the treatment of β-cell dysfunction characteristic of insulin-resistant type 2 diabetes.

1.2.6 Fatty Acid Oxidation and CPT Activity in Tumor Cells

Tumor cells have been reported to oxidize FAs at low rates despite the presence of CPT activity [51–56,230,240]. Alterations in the control of FA oxidation within tumor cells could potentially cause disturbances in other FA-utilizing pathways such as triacylglycerol and cholesterol synthesis, as well as the biosynthesis of membranes which is necessary at high rates in rapidly dividing cells. Indeed, evidence exists for the deleterious effects of the inhibition of FA synthase. Studies in this area could lead to selective inhibition of tumor cell proliferation [150,210].

1.2.7 CPTI in Other Tissues

Studies to date indicate that mitochondrial membrane systems possess two isoforms of CPT (CPTI and CPTII), with only CPTI being sensitive to malonyl-CoA inhibition [16,35,47,155,195,196,205]. There is considerable evidence for the presence of additional malonyl-CoA-sensitive CPT isoforms in liver peroxisomes and microsomes [46,60,69,155,195,196], but the presence of these isoforms has not been demonstrated in heart. Tritiated etomoxir labeled a ~47-kDa protein in these organelles [16]. Further studies indicated that the N-terminal domain of the

microsomal protein differs from that in the mitochondrial or peroxisomal protein. Mitochondria accounted for 65% of total cellular CPT activity, with the microsomal and peroxisomal contributions accounting for the remaining 25% and 10%, respectively [86]. Peroxisomal and microsomal CPT also showed decreased malonyl-CoA sensitivity during starvation [207].

At present, the function of the microsomal and peroxisomal enzymes can only be speculated. In peroxisomes, carnitine acyltransferase may be involved in transfer of the chain-shortened products of the oxidation of LCFA out of the organelle [214]. In microsomes, carnitine-dependent transfer of acyl moieties across the microsomal membrane may also take place [35,196].

1.3 PRIMARY STRUCTURE AND PROPERTIES OF CPT IN MITOCHONDRIA

1.3.1 Membrane Topology of the CPT System

As early as 1963, Fritz and Yue postulated that carnitine palmitoyltransferase was likely to have a dual localization, with the same activity residing on either side of the mitochondrial membrane [91]. Subsequent work on submitochondrial localization of CPT led to the conclusion that this activity resided exclusively in the inner membrane, with the overt (CPTI) and the latent (CPTII) activities residing on the outer and inner side of the inner membrane, respectively [37,126]. This view was widely assumed to be correct [19,124] until 1987, when Murthy and Pande [193] offered an alternative membrane localization based on a swelling method for separating the mitochondrial membranes. They suggested that CPTII is on the matrix side of the inner membrane, whereas the malonyl-CoA-sensitive CPTI is localized on the mitochondrial outer membrane [146,193,194]. Recently, Fraser [89] and Hoppel [125] reported that in rat liver, both CPTI and CPTII are enriched at the mitochondrial contact sites, thus raising the possibility of facilitated acylcarnitine transfer into the mitochondrial matrix.

Although the amino acid sequences of the two isoforms of CPTI are only 62.7% identical, they both predict two relatively highly conserved hydrophobic

domains (residues 48–75 and 103–133) within the N-terminal region. These domains are the only ones within the protein that are sufficiently long to span the mitochondrial outer membrane and have, therefore, been considered to be putative transmembrane domains [147].

A model for the membrane topology of CPTI has been proposed by Fraser [88] (Fig. 1.2). In this model, the protein contains a cytosolic N-terminal region of about 50 residues, two transmembrane domains connected by a loop of about 30 amino acids facing the intermembrane space, and a larger extramitochondrial C-terminal domain of about 650 residues. Thus, it appears that most of the protein residues (90%) are located in the cytosol.

So far, the 3D structure of any of the CPT enzymes is unknown. Cloning and expression of CPT should facilitate the determination of the 3D structure of the enzymes by X-crystallography.

1.3.2 Kinetic Properties of CPT

In addition to their separate membrane locations, CPTI and CPTII differ in their kinetic properties. First, CPTI is potently inhibited by malonyl-CoA, in sharp contrast to CPTII which is insensitive to malonyl-CoA. Second, exposure of mitochondria to detergents (Triton X-100, octyl glucoside, and Tween 20) solubilizes CPTII in its active form but causes complete loss of CPTI activity, most likely due to the disruption of its membrane environment [31,177,222]. Third, the two enzymes display very different properties which are reflected in the apparent K_m values for palmitoyl-CoA and L-carnitine. CPTI (measured in intact rat liver mitochondria) had apparent K_m values of 48 μM for palmitoyl-CoA and 26 μM for L-carnitine, and CPTII (measured in rat liver mitochondrial inner membranes in the presence of Triton X-100) had much higher apparent K_m values of 99 μM for palmitoyl-CoA and 153 μM for L-carnitine [38]. These kinetic differences suggest that CPTI and CPTII are distinct proteins. The K_m difference was also reported in yeast-expressed rat L-CPTI and CPTII [70].

Biochemical studies on mitochondria isolated from liver, heart, and skeletal muscle reveal significant differences in the properties of liver and muscle CPTI.

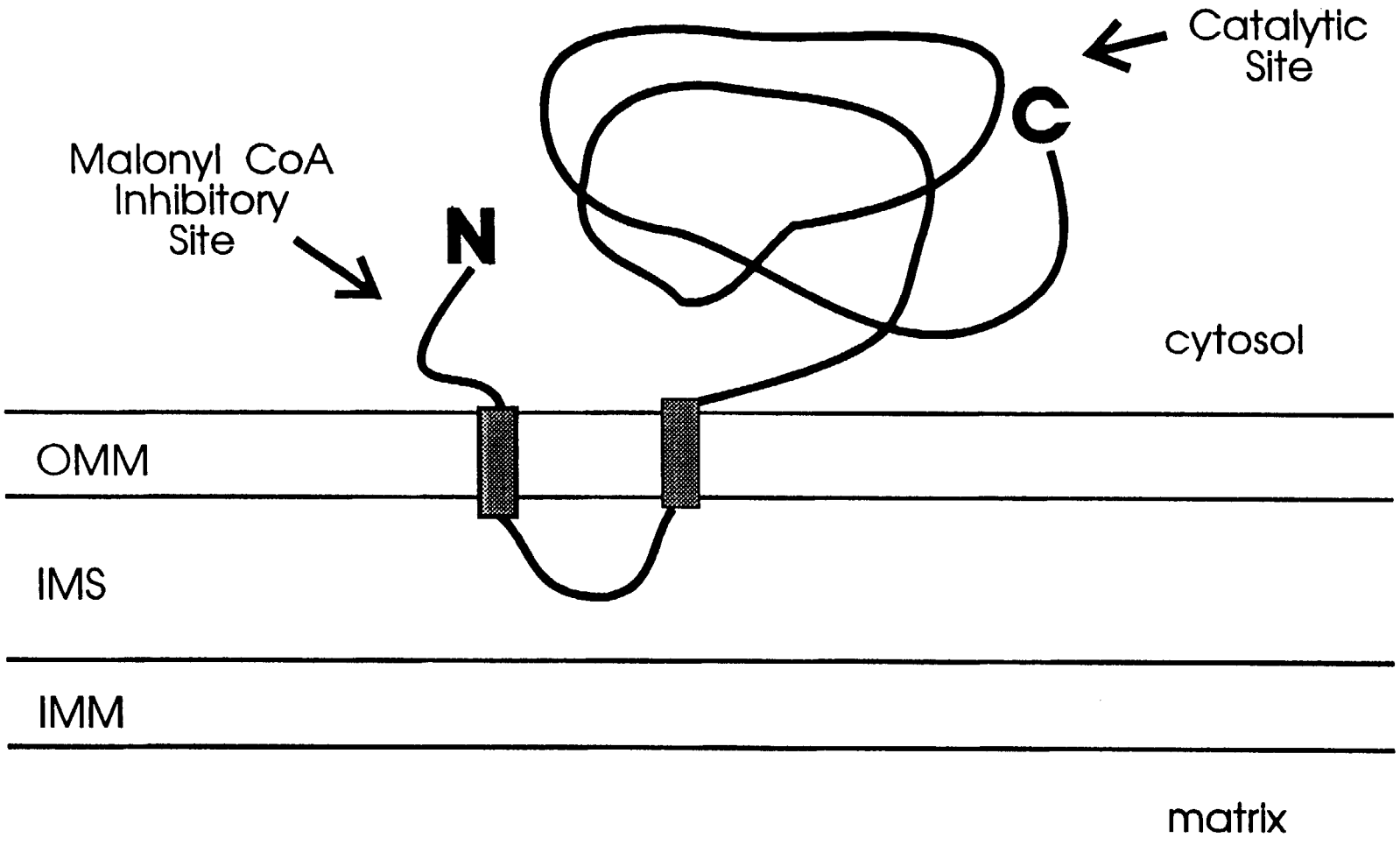

Figure 1.2 Model for CPTI membrane topology.

These differences included a higher K_m for carnitine in heart and a lower IC_{50} for malonyl-CoA [75,188]. In intact mitochondria, the K_m for carnitine of L-CPTI is 30–50 μM, for muscle is 500 μM, and for heart is 200 μM. The IC_{50} for malonyl-CoA inhibition is 2.7 μM for liver, 0.06 μM for muscle, and 0.1 μM for heart [267]. There are also no long-term adaptive changes in CPTI-specific activity or in malonyl-CoA sensitivity in heart comparable to what is observed in liver [57,223]. These findings suggest the presence of tissue-specific isoforms of CPTI. This possibility has recently been confirmed by the molecular cloning of two isoforms of L-CPTI and M-CPTI. In mitochondria from the yeast *Pichia pastoris* strains expressing L-CPTI and M-CPTI, L-CPTI and M-CPTI exhibited a 30-fold difference in sensitivity to malonyl-CoA (IC_{50} value 2.0 μM for L-CPTI and 0.07 μM for M-CPTI). They also displayed a 20-fold difference in K_m values for carnitine (32 μM for L-CPTI and 660 μM for M-CPTI). These differences were also reported in COS cells expressed L-CPTI and M-CPTI [78,242].

1.3.3 Albumin Effect on CPT Activity

Determination of CPT activity, which involves long-chain acyl-CoA esters as substrates, presents certain difficulties. These esters are amphipathic molecules which form micelles in solution above the critical micelle concentration [233]. Furthermore, at levels above the critical micelle concentration, they act as detergents and inhibit the activities of CPT and a number of other membrane and soluble proteins [141,190,278]. To lower the detergent effect of long-chain acyl-CoA, FA-free bovine serum albumin is generally added to bind palmitoyl-CoA in CPT assays [58,177,224,290].

For measuring CPTI activity, radiolabled carnitine is generally the tracer of choice [124], because it is easier to separate from the reaction mixture than palmitoyl-CoA. Palmitoyl-CoA is delivered from an excess palmitoyl-CoA pool, usually bound to albumin. This pool prevents substrate depletion over the time course of the measurement and allows sufficient palmitoyl-[^{3}H]carnitine to be produced for detection. Albumin is included to reduce the detergent properties of the substrate and

the effective concentration of palmitoyl-CoA for CPT activity, so that submaximal velocities can be measured.

Bovine serum albumin is generally employed as a substrate depot for the delivery of acyl units to lipid-metabolizing enzymes *in vitro*. In fact, albumin does alter the availability of substrate to mitochondrial CPT and thereby its apparent kinetics [208]. The albumin concentration in the CPT assay affects the IC_{50} and the V_{max} [169]. CPTI activity is low at the low palmitoyl-CoA/albumin ratio of 0.51, and is high at the upper ratio of 6.1. Studies by our group [231] and others [213] found that, in the presence of a fixed concentration of albumin (1%), the kinetic curve was in fact sigmoidal, which is in agreement with previous observations [58,208,223]. Under these conditions, it is not possible to determine the K_m or V_{max} for palmitoyl-CoA. Artifactual sigmoidicity has been attributed to substrate binding to albumin [11,208]. At low palmitoyl-CoA concentrations, the tight binding sites of albumin may compete very effectively for palmitoyl-CoA, resulting in an underestimation of the actual CPTI activity for the total concentration of palmitoyl-CoA in the assay medium [235]. The problem could be solved by using a 6.1:1 molar ratio of palmitoyl-CoA/albumin [208]. At higher ratios, when these sites are saturated, the substrate could become available to CPTI [11,14]. When the CPT assay was performed under these conditions, yeast-expressed L-CPTI exhibited a normal hyperbolic saturation kinetic curve for palmitoyl-CoA [231]. To interpret these data, the modulation of palmitoyl-CoA availability by albumin binding may be important. In the presence of albumin, the actual concentration of palmitoyl-CoA available to the enzyme is not known [11,57].

1.3.4 Malonyl-CoA Inhibition of CPTI and Malonyl-CoA Binding Sites

The inhibitory interaction between malonyl-CoA and CPTI exists in its natural membrane environment. First described for liver [177,178,181], this inhibitor has since been reported for a wide variety of mammalian tissues [179,187,223]. The key observations are:

(1) Inhibition of CPTI by a fixed concentration of malonyl-CoA can be overcome by raising the concentration of palmitoyl-CoA (a substrate for CPTI), but not in a classically competitive manner [177,181].

(2) When released from the mitochondrial membrane with detergents, CPTI loses its sensitivity to malonyl-CoA inhibition [31,84,177,181,222].

(3) Malonyl-CoA inhibition of CPTI was found to be pH and temperature dependent [21,239]. Lowering the intracellular pH, such as might occur in ketoacidosis, may attenuate hepatic FA oxidation by increasing malonyl-CoA sensitivity of CPTI. In rat liver, heart and skeletal muscle, raising the pH from 6.8 to 7.4 resulted in a fall in K_m for carnitine, no change of K_m for palmitoyl-CoA, and a marked decrease in the malonyl-CoA inhibition, suggesting that the affinity of the active site for its acyl-CoA substrate is not pH-sensitive over this range [188]. In hepatic mitochondria, increasing the temperature caused a decrease in K_d for malonyl-CoA binding and an increase K_i for inhibition of CPTI by malonyl-CoA [135].

(4) Malonyl-CoA inhibition is sensitive to changes in membrane composition. Analyzing the major lipid components of the membranes during different physiological states using fluorescence probes, Zammit et al. [292] concluded that although desensitization of mitochondrial overt CPT (CPTI) to malonyl-CoA inhibition existed as reported before, CPTI kinetic characteristics are sensitive to changes in lipid composition that are localized to specific membrane microdomains. Similar phenomena have been reported before, such as alteration in malonyl-CoA sensitivity with changes in membrane fluidity [146] and membrane phospholipids [197,198]. The mechanism underlying these sensitivity changes is still not clear. Theoretically, these could be brought about by alterations in the parameters of malonyl-CoA binding or by alterations in the transmission of conformational information between the malonyl-CoA-binding entity and the CPT catalytic entity. Such changes might be envisaged as rising directly from modification of these proteins or indirectly as a result of changes in the composition/fluidity of the mitochondria outer membrane.

(5) Malonyl-CoA sensitivity of CPTI is subject to changes with the different physiological and nutritional states (discussed in Chapter 2).

(6) Treatment of rat liver mitochondria with Nagarse, chymotrypsin, papain or proteinase K result in a marked loss of malonyl-CoA sensitivity of CPTI before catalytic activity is entirely destroyed [87,136,193,194]. Trypsin destroys CPTI catalytic activity while having little or no effect [136] on malonyl-CoA sensitivity when the mitochondria are not damaged. This indicates that the desensitization phenomenon is probably the result of protease specificity and availability of cleavage sites and is not simply due to the active site being protected [139,193,194]. Proteolysis of CPTI can be prevented by preincubating mitochondria with malonyl-CoA [138] and hydroxyphenylglyoxylate but not with active-site-directed inhibitors, such as DL-2-bromopalmitoyl-CoA and N-benzyladriamycin 14-valerate.

Proteases have a similar desensitizing effect on L-CPTI with regard to inhibition by succinyl-CoA, methylmalonyl-CoA and Ro-25-0187 (a malonyl-CoA analog), whereas the potency of CoASH, acetyl-CoA, and propionyl-CoA is unaffected [139]. This suggests that the coenzyme A moiety of malonyl-CoA is not absolutely necessary for the binding of malonyl-CoA to its regulatory site. The primary requirement for inhibition of CPTI by malonyl-CoA is the presence of the dicarboxylic acid esters. Proteolytic treatment does not appear to diminish sensitivity to the presumed active-site-directed inhibitors, such as DL-2-bromopalmitoyl-CoA [136], hemipalmitoylcarnitinium [139], or etomoxir-CoA [173].

Taken together, all these observations suggest that CPTI has two distinct inhibitor binding sites [61]. One site is located on the cytoplasmic side of the mitochondrial outer membrane. Malonyl-CoA, the most important physiologic inhibitor of CPTI, binds primarily to this site, but it can also bind to another site. A second inhibitory site is located at the active site of CPTI. The coenzyme A moiety of malonyl-CoA is not essential for the binding of malonyl-CoA to its regulatory site, but the dicarbonyl function (present in malonyl-CoA, hydroxyphenylglyoxylate, and Ro 25-0187) is absolutely essential [136,139].

One critical question that remains unanswered is whether malonyl-CoA acts as a simple, competitive inhibitor by binding at the active site or whether it acts at a separate allosteric locus. Several authors have suggested a competitive-like fashion with malonyl-CoA reacting at the active site of CPTI [31,75,177,181,187,188,223].

Alternatively, malonyl-CoA could bind to a separate regulatory component or to an allosteric site on CPTI, a hypothesis that is supported by proteolytic studies [61,139], reconstitution studies [277], and by detailed kinetic analysis of the inhibition of CPTI by malonyl-CoA in rat liver mitochondria [61,105,290].

Another important question is, if the malonyl-CoA binding site is separate from the substrate binding site, where are the two different binding sites located relative to the membrane? Data presented by Murthy and Pande [193,194], who used proteinase treatment of intact mitochondrial-outer-membranes, suggested that CPTI has its substrate binding site facing the intermembrane space and malonyl-CoA binding site facing the cytosol. Recently, using outer mitochondrial membrane vesicles, Fraser et al. [87,88] studied the effect of proteolytic enzymes on the regulation of malonyl-CoA-sensitive CPT activity and concluded that both the regulatory and catalytic sites of rat L-CPTI are located on the cytosolic face of the outer membrane.

The binding characteristics of malonyl-CoA to CPTI are commonly studied using [^{14}C]-malonyl-CoA. However, analysis of these binding data is complicated mostly by the difficulty of preparing pure outer membrane containing CPTI and without inner membrane CPTII. Although [^{14}C]-malonyl-CoA binding to rat liver mitochondrial outer membrane (CPTI) [146] has suggested only one high-affinity binding site for malonyl-CoA, binding studies by many other groups [23,61,100] clearly indicate two classes of binding sites—a high-affinity malonyl-CoA binding site and a low-affinity malonyl-CoA binding site—with over 100-fold difference in K_d values [23,100]. The high-affinity binding site is the functional site mediating inhibition of CPTI by malonyl-CoA and is unlikely to be the catalytic unit of CPTI. The physiological importance of the low-affinity binding sites for malonyl-CoA inhibition is unknown. Palmitoyl-CoA could displace [^{14}C]-malonyl-CoA binding at the low-affinity site [105,291], suggesting that the low-affinity malonyl-CoA-binding site may be a second allosteric acyl-CoA-binding site on CPTI. Malonyl-CoA binding is pH dependent, consisting of the IC_{50} for malonyl-CoA inhibition of CPTI, which is also pH dependent. K_{D1} values measured at pH 6.8 [100] were 15–50-fold lower than those reported for an outer membrane binding site at pH 7.4 [23,146].

Fasting, diabetes, and hypothyroidism changed the K_{D1} for binding of malonyl-CoA at the high-affinity site in the outer mitochondrial membranes in a manner that correlated closely with changes in the IC_{50} for malonyl-CoA inhibition of outer-membrane CPTI. Fasting and diabetes increased the abundance (B_{max1}) of the high-affinity malonyl-CoA-binding site, whereas hypothyroidism decreased its abundance. The increased B_{max1} for binding to outer mitochondrial membranes in fasting and diabetes suggests that the decreased sensitivity of CPTI to malonyl-CoA observed in these two states is not due to decreased abundance of the malonyl-CoA binding entity of the enzyme. K_{D2} and B_{max2} decreased in diabetes, whereas the K_{D2} increased in hypothyroidism [100]. At present the physiological significance of these changes in malonyl-CoA binding are still unknown.

In liver, the IC_{50} value (μM range) is substantially greater than the K_{D1} value (nM range). This apparent discrepancy is reconciled by the fact that palmitoyl-CoA has, of necessity, to be included in the CPT assay. This metabolite increases the K_D for binding of malonyl-CoA [23,146,188].

Binding studies of [^{14}C]-malonyl-CoA to M-CPTI are very limited. One problem is that mitochondria in cardiac tissues are usually isolated by either mechanical rupture or proteinase digestion, or both [234], which can cause mitochondrial damage. Another problem is the potential presence of cytoplasmic carnitine acyltransferase activities in the peroxisomes [69] and microsomes [47,184] of the cardiac myocyte. However, current data suggest that heart and skeletal muscle also contain two malonyl-CoA binding sites similar to liver with a high-affinity binding site and a low-affinity binding site [23,105]. In heart and skeletal muscle, pH changes do not have as much effect on K_{D1} as they do in liver [188].

1.3.5 Reconstitution Studies of the CPT System

Early reconstitution studies were done by Woldegiorgis et al. [277] and Chung et al. [48]. They demonstrated restoration of malonyl-CoA-sensitive CPT activity by reconstitution of purified rat liver mitochondria (RLM) and rat heart mitochondria malonyl-CoA binding proteins with detergent-solubilized malonyl-CoA-insensitive CPTII in the presence of phospholipids. Using a modified reconstitution procedure,

they succeeded in restoring malonyl-CoA-sensitive CPT activity to Triton X-100-solubilized RLM by removal of the detergent in the presence of phospholipids, indicating reactivation of detergent-inactivated L-CPTI.

Ghadiminejad and Saggerson [97–99] claimed to have conferred malonyl-CoA-sensitivity to CPTII in reconstitution experiments with extracts of inner membranes (CPTII). Their CPT assay conditions were originally designed for the measurement of CPTI, not CPTII, and their inner membranes were contaminated by marker enzymes for mitochondrial outer membranes, peroxisomes, and microsomes. However, in reconstitution experiments using chlorate extracts of RLM outer and inner membranes, Mynatt [198] obtained results identical to those of Ghadiminejad and Saggerson.

Until recently, it was presumed that CPTI was irreversibly inactivated by detergents, but our reconstitution studies with yeast-expressed L-CPTI and M-CPTI demonstrated that detergent inactivation of CPTI is partially reversible [70,293]. Yeast-expressed, detergent-inactivated rat liver L-CPTI and human heart M-CPTI can be reactivated by detergent removal in the presence of phospholipids. The reconstituted yeast-expressed M-CPTI is more malonyl-CoA sensitive than the reconstituted rat heart mitochondria CPTI, mostly likely due to the fact that, unlike mammalian mitochondria that contain a mixture of both CPTs, the yeast mitochondria contain only M-CPTI. Thus, removal of M-CPTI from its membrane lipid environment clearly inactivates this enzyme, and reconstitution via detergent removal in the presence of phospholipids reactivates a portion of the inactivated M-CPTI, suggesting that M-CPTI is active only in a membrane environment.

1.4 MOLECULAR CLONING AND EXPRESSION

The relationship between CPTI and CPTII and the characteristic differences between liver CPTI and heart/skeletal muscle CPTI have long remained an enigma. Although biochemical studies shed some light on the CPT system, most of the evidence was not conclusive. With the newly developed molecular cloning and gene expression technology, it is clear that CPTI and CPTII are catalytically distinct

enzymes encoded by different genes. There is only a single form of CPTII expressed in all oxidative tissues. On the other hand, two different isoforms of CPTI have been described with distinct tissue distributions. These two forms are called the liver form of CPTI (L-CPTI) and muscle form of CPTI (M-CPTI) and are encoded by different genes localized on chromosome 11q13.1-13.5 and 22q13.31-13.32, respectively [3,33,34,250].

The cDNAs for CPTII have been cloned and sequenced from human [83] and rat liver [274]. The cDNA has an open reading frame (ORF) of 1974 bp encoding 658 amino acids. Human and rat CPTII are highly homologous with 87% identity at the DNA level and 82% identity at the amino acid level. However, the human mRNA ($\cong$3 kb) is approximately 0.5 kb larger than its rat equivalent. CPTII contains a 25 amino acid N-terminal leader sequence that is cleaved upon mitochondrial import to yield a mature protein with a molecular size of approximately 71 kDa [40,274]. The CPTII gene from rat has been expressed *in vitro* in several expression systems such as *Escherichia coli* [41], COS cells [274], baculovirus [134], *Saccharomyces cerevisiae* [39], and *P. pastoris* [70]. The kinetic properties of expressed CPTII are similar to those of the intact inner mitochondrial protein.

Unlike CPTII, a single gene product expressed body-wide, CPTI has two structural genes (L-CPTI and M-CPTI) that are differentially expressed among tissues. The cDNAs for CPTI from rat and human liver, heart, skeletal muscle and brown adipose tissue have been cloned and sequenced [77,78,267,281,282]. Both rat and human M-CPTI have an ORF of 2316 bp, while both rat and human L-CPTI have an ORF of 2319 bp. Unlike CPTII, no N-terminal signal peptide is removed from the nascent CPTI during its mitochondrial import, a characteristic of outer-membrane proteins. The L-CPTI protein has 773 amino acids. The size of mRNA is 4.7 kb due largely to a long 2-kb 3′ untranslated region in the CPTI mRNA. M-CPTI protein has 772 amino acids, with an mRNA size of 3kb, which is much smaller than that of rat and human L-CPTI. The difference reflects the much shorter 3′ untranslated region of M-CPTI mRNA. The two proteins migrated on SDS-PAGE with apparent monomeric size of approximately 88 kDa (L) and 82 kDa (M). The differences in migration may be due to the different electrophoretic mobility of the two proteins.

Rat and human CPTI proteins of the same isoform have very high homology (86% identity), while different isoforms of the same species only have 63% identity [70,294]. The first 18 N-terminal amino acids are identical in the two isoforms in both rat and human species.

Northern analysis of RNA from tissues and isolated cells has led to the discovery that L-CPTI is mainly expressed in liver, brain, pancreatic β-cells, kidney and fibroblasts, and to a lesser extent in heart [34,77,206,267,282,294]. M-CPTI is expressed in skeletal muscle and heart, as well as both white and brown adipocytes [78,281,282,294]. The non-adipocytes of brown adipose tissue contain an abundance of the liver isoform [78]. Heart expresses two isoforms of CPTI. The liver isoform of CPTI contributes only 2–3% of the total CPTI in the adult heart, with a contribution in the newborn of $\sim$20% [266,279].

L-CPTI from rat was expressed in COS cells [77] and the yeasts *S. cerevisiae* [39] and *P. pastoris* [70]. M-CPTI from rat adipose tissue has been expressed in COS cell [78]. M-CPTI from human heart has only been expressed in *P. pastoris* [294].

P. pastoris is by far the best expression system for functional studies of CPT, because *P. pastoris* does not have endogenous CPT activity. Expression of rat L-CPTI and CPTII in *P. pastoris* gave very high CPT activity. The level of expression of CPTI in *P. pastoris* at 7.8 nmol/mg $\cdot$ min is $\sim$2–3 times that of the malonyl-CoA-sensitive CPT activity observed in RLM [32,77] or in COS cell-expressed CPTI [77] and is at least 1000-fold higher than the activity reported for the expression of RLM in *S. cerevisiae* [39]. So far, human M-CPTI is the only human CPT gene that has been expressed. The *P. pastoris*-expressed human M-CPTI has enzymatic properties distinct from L-CPTI. The successful expression of human M-CPTI in *P. pastoris* provides a useful tool for structure/function studies of the human CPT enzyme [293,294]. The high-level expression of M-CPTI using a multicopy *P. pastoris* expression strain and the reconstitution of the enzyme are critical steps toward the purification and determination of 3D structure by X-crystallography of the human CPTI enzyme.

1.5 GENOMIC STRUCTURE AND REGULATION OF CPT GENE EXPRESSION

1.5.1 Genomic Sequence and Chromosome Localization

The CPTII gene has been mapped to chromosome 1p32 by Gellera et al. [95] and confirmed by Britton [34] and Verderio [259] using the fluorescence *in situ* hybridization (FISH) technique. The human CPTII gene has been cloned and sequenced. It is approximately 20 kb in size and is composed of five exons ranging from 81 to 1,305 bp in length [259]. The exon–intron boundaries conform to the consensus splice junction sequences. The 5′ and 3′ untranslated region (UTR) of exons 1 and 5, respectively, also have been determined, including the polyadenylation signal and the polyadenylation site [259]. The mouse CPTII gene has been isolated, and its genomic structure has been determined. Like the human CPTII gene, the mouse CPTII gene contains five exons, including an unusually long 1305-nucleotide exon 4 [94].

In 1995, Britton et al. localized the human L-CPTI gene to chromosome 11q [34]. In later experiments using the FISH method, they were able to further map human L-CPTI to chromosome 11q13 [33,34]. Human muscle M-CPTI was first mapped by Van der Leij et al. and confirmed by Britton et al. to chromosome 22q13.3 [3,33,250].

Human heart M-CPTI is composed of 18 exons interrupted by 17 introns spanning more than 8576 bp with an additional 1.0 kb 5′ noncoding region and a 10-kb 3′-noncoding region [3,283]. The rat M-CPTI gene has been cloned from a Lambda Fix II rat kidney genomic library and mapped to chromosome 7 at band q34 [261]. The rat M-CPTI gene contains 18 introns and 19 exons, the latter 18 exons showing 85% homology to the human M-CPTI cDNA [33]. A partial clone of human L-CPTI has been reported by Britton et al. [33,34]. The mRNA size of human L-CPTI is the same as that of rat liver L-CPTI.

In conclusion, genomic sequencing and chromosomal localization have established unequivocally that the enzymes CPTI and CPTII are distinct proteins

encoded by separate genes. The two isoforms of CPTI (L-CPTI and M-CPTI) are not the result of alternative splicing but encoded by different genes.

1.5.2 Gene Regulation

1.5.2.1 Transcriptional regulation and transcription start site identification. Only the CPTII gene has been studied in detail. The promoter region of the CPTII gene has been isolated and sequenced from rat, mouse, and human. The 5′ UTR region of the mouse CPTII gene precedes exon I and is nearly identical to the 5′ UTR of the rat liver CPTII cDNA [94]. This 5′ promoter region is the major control point for CPTII gene expression in heart and may also be active in liver [94]. In 1994, Montermini et al. identified regulatory elements in the promoter region of the human CPTII gene [189]. The transcription start site of human CPTII was identified at 34 nucleotides downstream from a consensus sequence for the TATA box. The identification of this transcription start site adds 0.5 kb of 5′ UTR to the 1974 bp of the ORF and to the 0.6 kb of the 3′ untranslated sequence, thus fitting the estimated size of human CPTII mRNA of 3.1 kb [83].

The promoter regions of the L-CPTI and M-CPTI genes have been identified in rats and humans. Cook et al. recently isolated and sequenced the upstream region of the rat L-CPTI gene [206]. In the L-CPTI gene, there are two exons 5′ to the exon containing the ATG initiation codon. There is an alternatively spliced form of the L-CPTI mRNA in which exon 2 is skipped. The proximal promoter of the L-CPTI gene is extremely GC rich and does not contain a TATA box [206].

The rat heart M-CPTI gene contains 19 exons, with the transcription start site located in a short first exon. A putative promoter region was defined to within 391 bp of the transcription start site. The 5′ flanking region of M-CPTI was verified by comparison of luciferase expression to that of β-galactosidase in cardiac myocyte and in HepG2 cells [261]. Yu et al. recently described the coexpression of two novel M-CPTI mRNA splicing forms in humans [288] and rats [289]. The relative levels of alternative human M-CPTI transcripts that arise from two closely approximated promoters that have tissue specific or preferred activities were estimated. One transcript is present in heart and muscle (M promoter), whereas the other is

ubiquitously (U promoter) expressed in tissues with high FA oxidation rates. Both M-CPTI transcripts are alternatively spliced, giving rise to β1, β2 and β3 isoforms. β1 mRNA was 80–90% of total M-CPTI mRNA in both heart and muscle tissues, whereas β2 and β3 each contributed 5–10% to the total mRNA pool [287]. β3 mRNA results from exon 5 skipping and represents a minor tissue component of human and rat total M-CPTI mRNA. β2 is derived from using a cryptic exon 3 splice donor in combination with exon 4 skipping. Both the CPTI U and M promoters are TATA-less. The location of the major U transcript TSS at 702 bp differs from that previously reported by Yamazaki et al. [283], who suggested that it was located at 745 bp based on 5′ rapid amplification of cDNA ends. The existence of numerous proximal U promoter regulatory elements in the absence of a TATA box suggests that regulated activities of cognate factors may influence the precise locus of polymerase II binding as well as its activity.

1.5.2.2 CPT gene regulation by FAs. FAs have been reported to induce several genes of hepatic FA metabolism, including those encoding FA binding proteins [108] and enzymes of peroxisomal [107] and mitochondrial [107,109,217] β-oxidation. CPTI is the rate-limiting enzyme regulating FA oxidation in mitochondria [180]. The transcription of CPTI gene has been reported to be regulated by FAs.

In 1996, Chatelain et al. reported cyclic AMP and FAs increase CPTI gene transcription in cultured fetal rat hepatocytes [44]. The concentration of CPTI mRNA increased dramatically after exposure of the cells to dibutyryl cAMP (Bt2cAMP) and LCFA. In contrast, CPTII gene expression, which was already high in fetal hepatocytes, was unaffected by any of the above manipulations. In 1997, Assimacopoulos-Jeannet et al. [8] showed that LCFA, at concentrations within the physiological range (0.1–0.6 mM), are the major regulators of the CPTI gene in clonal pancreatic (INS-1) cells. Recently, Brandt et al. [29] studied the expression of M-CPTI in primary cardiac myocytes in culture following exposure to the long-chain monounsaturated FA. Oleate induced steady-state levels of M-CPTI mRNA 4.5-fold. The transcription of a plasmid construct containing the human M-CPTI gene promoter region fused to a luciferase gene reporter transfected into cardiac myocytes was

myocytes was induced over 20-fold by LCFA in a concentration-dependent and fatty acyl chain length-specific manner.

1.5.2.3 FAs regulate CPTI gene expression through PPARα activation. FAs and their derivatives are essential cellular metabolites and are important in the regulation of CPTI gene expression; thus, FA concentrations must be closely regulated. This implies that regulatory circuits exist which can sense changes in FA levels. It has been reported that CPTI gene expression is regulated by FAs and peroxisome proliferators [7,44,109]. Recently, it became clear that FAs regulate CPTI gene expression through peroxisome proliferator-activated receptor (PPAR) α.

The PPAR is highly expressed in liver and was originally identified by Issemann and Green [132] as a molecule that mediates the transcriptional effects of drugs that induce peroxisome proliferation in rodents [152]. The PPAR is a member of the nuclear receptor superfamily that includes receptors for steroid, thyroid, and retinoid hormones [167]. Like other members of this superfamily, the PPAR contains a central DNA-binding domain that recognizes response elements in the promoters of their target genes. The PPAR response elements (PPREs) are composed of a directly repeating core site separated by 1 nucleotide [145]. To bind to a PPRE, PPARs must heterodimerize with the 9-cis-retinoic acid receptor. Three PPAR-related genes (PPARα, PPARγ and PPARβ/δ) have been identified in mammals. PPARγ is highly enriched in adipocytes while the β/δ isoform is ubiquitously expressed [229].

In contrast to γ and β/δ isoforms of PPAR, PPARα appears to regulate FA oxidation [109], suggesting that PPARα ligands may act as endogenous signals for FA degradation [229]. Issemann and Green [132] originally demonstrated that PPARα is activated by fibrates, a group of drugs that induce peroxisome proliferation and FA oxidation in rodents. Gottlicher et al. [104] examined the ability of FAs to activate PPARα. Those studies have led to the suggestion that these compounds alter FA metabolism, which indirectly leads to the accumulation of an endogenous PPARα ligand [103]. This indicates that FAs simultaneously serve as intermediary metabolites and as primary regulators of transcriptional networks. Forman [85] developed a novel conformation-based assay that screens activators for their ability to bind to PPARα and induce DNA binding, and showed that specific FAs, eicosanoids,

and hypolipidemic drugs are ligands for PPARα. Because altered FA levels are associated with obesity, atherosclerosis, hypertension, and diabetes, PPARs may serve as molecular sensors that will be central to the development and treatment of these metabolic disorders.

Several groups [29,168,287] have recently studied the transcriptional regulation of the human M-CPTI gene. All confirmed that FAs regulate CPTI gene expression through PPARα activation and showed that FAs increase M-CPTI gene promoter activity in a PPARα-dependent fashion. The M-CPTI gene promoter FA response element is a PPRE and is localized to a hexameric repeat sequence located between 775 and 763 bp upstream of the initiator codon. When free FA levels increase in the cell, the PPARα expression level is induced; PPARα heterodimerizes with the 9-cis-retinoic acid receptor and binds to the FA response element on the M-CPTI promoter, thus activating the transcription of the M-CPTI gene. The physiological significance of this regulation is reflected in a robust increase in CPTI mRNA levels in tissues under catabolic conditions, when elevated circulating free FAs are presented as fuel and are available to activate gene expression.

1.5.2.4 Hormonal regulation of gene expression. While little is known about hormonal regulation of the M-CPTI gene, a few direct studies have been conducted on the regulation of the L-CPTI gene by hormones. L-CPTI mRNA abundance was found to increase 40-fold in response to thyroid hormone, which suggests regulation at the level of transcription [197]. Park et al. [207] reported that insulin exerts its effect on L-CPTI primarily by decreasing gene transcription, thus demonstrating that insulin plays a major role in regulating CPTI mRNA abundance in liver.

Thyroid hormone differs from insulin and other hormones in the coordinate regulation of FA oxidation and synthesis. These two pathways are regulated in a reciprocal manner by insulin [175]. However, the activity of both pathways is increased by thyroid hormone, and both pathways decrease in hypothyroidism [100,237,238]. The change mechanisms in CPTI activity probably differ from the mechanisms altering sensitivity to inhibition by malonyl-CoA. The change in sensitivity is rapid and easily reversible and appears to be mediated through an

insulin-dependent mechanism [58,92], whereas the change in CPTI activity is much slower and may indicate a change in protein synthesis [237,238].

Manipulations that enhance the capacity of FA oxidation, such as fasting [28] and induction of diabetes [26], all cause an increase in the mRNA level and CPT activity. The hormone response of this change is still unclear.

Very little has been published on the L-CPTI genes in tissues other than liver, but two papers describing expression in the small intestine and heart are notable [6,127]. CPTI (probably L-CPTI) and CPTII activities have been reported to increase in neonatal rat cardiac myocytes in response to insulin. This response to insulin is opposite to that in liver and is thought to be cell growth related [127]. These observations raise the possibility that the mRNA abundance of the L-CPTI isoform may be differentially regulated by hormones or diet in a tissue-specific manner.

1.5.2.5 Developmental regulation of the CPTI gene in liver and heart. Rat hepatic CPTI mRNA increases at birth, remains elevated during the suckling period, and decreases after weaning on a high-carbohydrate diet [248]. The CPTII gene is expressed in liver early in fetal life and remains constant throughout. Dietary manipulations do not affect the abundance of the CPTII gene [248]. Hormonal mechanisms responsible for these changes in mRNA during development are not known. It has been speculated that high plasma glucagon or low plasma insulin might be responsible [248].

The heart is unique in that it expresses both muscle and liver isoforms of CPTI. During the perinatal developmental period, a switching of isoforms occurs in the heart. L-CPTI is highly expressed in the fetal heart, but decreases very slowly from birth until weaning and decreases more rapidly after weaning on a high-carbohydrate diet [42]. The level of M-CPTI, which is very low at birth, increases from birth to weaning, and remains highly expressed thereafter, becoming the predominant enzyme during postnatal development [42].

1.5.3 CPT Deficiency Disease

First reported in 1970 by Engel et al. [76] and DiMauro [71], CPTII deficiency is the most common inherited disorder of lipid metabolism. There are two

distinct clinical phenotypes, the adult (muscular) form and infantile (hypatocardiomuscular) form. The adult form typically presents in young adulthood with recurrent episodes of exercise-induced myoglobinuria. More than 60 patients with these clinical presentations and CPTII deficiency have been reported [295]. The infantile form deficiency in newborns is a generalized lethal disease with reduced CPTII activity in multiple organs, reduced concentrations of total and free carnitine, and increased concentrations of lipids and long-chain acylcarnitines. Typical phenotypes of the infantile form include hypoketotic hypoglycemia, cardiomyopathy and sudden death in newborns and children, liver failure, and peripheral myopathy [68,96,128,209,245,246]. A severe and usually fatal infantile form of CPTII deficiency had been recognized in 6 patients, according to Roe and Coates [218].

Taroni et al. [245] demonstrated that the most frequent molecular defect in CPTII deficiency is a C-to-T transition at nucleotide 439, resulting in a ser133-to-leu substitution. The S113L mutation accounts for approximately 60% of the mutant CPTII alleles. The reported mutations that cause CPTII deficiency are listed below:

ser113leu:	adult form mutation [245,284,295]
arg631c:	infantile form and adult form mutation [245,246]
pro50his:	adult form, [259]
asp553asn:	adult form, [259]
tyr628ser:	infantile form [24]
glu174lys:	infantile form [262,280]
phe383tyr:	infantile form [262,280]
Arg124Stop:	adult form [285]

CPTI deficiency is one of several life-threatening disorders of long-chain FA oxidation and energy metabolism which appear to be treatable with medium-chain triglycerides; medium chain FAs bypass the CPT-mediated, carnitine-dependent mitochondrial transport system [80,81,121,122]. Compared to CPTII deficiency, CPTI deficiency appears to be very rare, or possibly to be under-diagnosed. After the first report on CPTI deficiency in 1981 [25], at least 11 cases of CPTI deficiency have been described (for overview, see reference 15). Recessively inherited deficiency of CPTI has two distinct phenotypes. In adults, CPTI deficiency causes a muscle disease with weakness, cramps, and myoglobinuria [71]. In infants, CPTI deficiency is characterized by hyperammonemia, increased levels of serum

transaminases and plasma-free FAs, hepatomegaly, nonketotic hypoglycemia, and coma [25].

There are two isoforms of CPTI. Most of the case reports of CPTI deficiency are of the liver isoform. To date, no cases have been reported of muscle CPTI deficiency, which may be due to the lethal character of M-CPTI deficiency. Loss of M-CPTI might be incompatible with life, given the importance of the enzyme for heart function. If M-CPTI deficiency exists, it might be expected to show a clinical phenotype similar to CPTII deficiency.

A "hepatic" type of CPT deficiency due to defect in the CPTI gene may be called type I. A rare hepatic form of CPTII deficiency [280] also occurs, although it is much rarer than the classical myopathic form of CPTII deficiency. The CPTII-related hepatic disorder might be called CPT deficiency type II. Both of the hepatic forms are associated with coma, seizures, and hypoketotic hypoglycemia, but type II has always been fatal early in life, whereas most of the infants with type I have survived. Patients with type I do not show the cardiomegaly and other cardiac manifestations that are a striking feature of type II.

The molecular basis of CPTI deficiency is still unknown. In a patient with L-CPTI deficiency, Ijlst et al. [130] demonstrated a homozygosity for a missense mutation, asp454gly. Cloning of the human L-CPTI and M-CPTI genes has provided a tool for molecular characterization of the CPTI deficiency, and more information will be forthcoming.

Carnitine palmitoyltransferase deficiency and carnitine deficiency are separate genetic abnormalities of FA metabolism leading to myopathy.

1.6 STRUCTURE/FUNCTION STUDIES OF CPT

Previous structure/function studies of CPT were conducted by proteolysis and other biochemical techniques. The results from those studies were inconclusive and sometimes controversial. The cloning and yeast expression of the CPT enzymes made it possible to study the structure/function of CPT at the molecular level. Several

groups are currently conducting mutagenesis studies to map the malonyl-CoA binding and catalytic sites.

1.6.1 Mapping of the Malonyl-CoA Binding Site of CPTI

Amino acid sequence alignment of human and rat L-CPTI, M-CPTI, and CPTII sequences reveals a conserved N-terminal sequence of 124 residues with two putative transmembrane domains that are present in all known CPTI sequences but absent from CPTII. Because CPTII is malonyl-CoA insensitive, this suggests that the malonyl-CoA binding site may reside in this region. Limited proteolysis studies of intact rat liver mitochondria and activity studies with immobilized impermeable substrate and inhibitor also indicated that the malonyl-CoA sensitivity of CPTI may reside in these 124 N-terminal amino acid residues.

Serial deletions and point mutations of rat L-CPTI have clearly demonstrated that glutamine 3 and histidine 5 are critical amino acid residues for malonyl-CoA binding of L-CPTI [231,232]. Swanson et al. [242] also reported the involvement of histidine 5 and histidine 140 in malonyl-CoA inhibition by expressing L-CPTI in COS cells.

M-CPTI is much more sensitive to malonyl-CoA than L-CPTI. Little is known about the structural basis for the high malonyl-CoA sensitivity of M-CPTI. Serial deletions of human M-CPTI were also carried out by our group, and the results indicate that the malonyl-CoA binding site resides between amino acid residues 18 and 28 in human M-CPTI (see Chapter 6).

The most important question is why liver and heart react differently to malonyl-CoA inhibition. What is the molecular basis for these differences in malonyl-CoA sensitivity? Although the first 18 amino acids are conserved in L-CPTI and M-CPTI, they are of critical importance for L-CPTI malonyl-CoA binding but not essential for M-CPTI malonyl-CoA inhibition [232]. Deletion of the first 83 amino acid residues of human M-CPTI resulted in loss of catalytic activity, while deletion of the first 83 amino acids of rat L-CPTI had minimal effect. All these indicated that the differences in malonyl-CoA sensitivity observed in liver and heart are caused by the differences in malonyl-CoA binding sites between L-CPTI and M-CPTI.

To further elucidate the differences in malonyl-CoA sensitivity of L-CPTI and M-CPTI, several chimeras were constructed in which the L-CPTI N-terminal region was exchanged with the corresponding M-CPTI N-terminal region. If the malonyl-CoA binding sites are localized at the N-terminal region of both liver and heart CPTI, these chimeras should display an M-CPTI malonyl-CoA sensitivity. All the chimeras showed the same malonyl-CoA sensitivity as L-CPTI, except CH78, which was about 4 times less sensitive than the liver enzyme. Chimeras similar to ours were constructed by Swanson [242] and had identical outcomes as ours. These studies suggest that the N-terminal regions of L-CPTI and M-CPTI are not the only regions required for malonyl-CoA inhibition. 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.

1.6.2 Mapping of Substrate Binding Sites

Catalytically important residues of CPTII were first mapped by Brown et al. in 1994 [38] by site-directed mutagenesis and chemical modifications using yeast-expressed CPTII. Chemical modification of *S. cerevisiae*-expressed CPTII with diethyl pyrocarbonate, a reagent that modifies histidine residues, abolished CPT activity. Alignment of the amino acid sequences of CPTII, acyltransferase, and citrate synthase in rats and humans showed 8 conserved residues. Point mutations of the conserved residues were conducted, leading to the finding that His 372 as well as Asp 376 and 464 are essential for CPTII catalytic activity [38]. Chemical modification of the same conserved His residues of rat liver and human heart CPTI had no effect on the catalytic activity of CPTI [Shi, unpublished data], suggesting that the catalytically important domain may be different in CPTI.

Multiple sequence alignment of the choline/carnitine acyltransferase superfamily members indicates that the catalytic domains of CPTI reside in the C-termini of the proteins. Alignment of CPTI and CPTII and also some other carnitine acetyltransferase found 10 amino acids that are conserved and may be the catalytically important sites. These conserved residues include W238, R388, R451, W452, D454, K455, Q464, Q468, R601, and R606. Chemical modifications of arginine residues of

rat L-CPTI, CPTII and human heart M-CPTI by phenylglyoxal resulted in 70% loss of CPT activity but no effect on the malonyl-CoA sensitivity, indicating that conserved arginine residues in CPTI play an important role in catalysis.

Site-directed mutagenesis studies of the malonyl-CoA binding and substrate binding sites suggest that malonyl-CoA and substrate binding sites are separate. Although they are separate binding sites, they may interact with each other and cooperatively regulate CPTI activity and malonyl-CoA sensitivity, thus regulating FA oxidation in mitochondria. Malonyl-CoA binds at different regions of L-CPTI and M-CPTI, thus forming the basis of the different malonyl-CoA sensitivity of L-CPTI and M-CPTI.

CHAPTER 2

FUNCTIONAL STUDIES OF YEAST-EXPRESSED HUMAN HEART MUSCLE CARNITINE PALMITOYLTRANSFERASE I*

2.1 INTRODUCTION

Transport of long-chain fatty acids from the cytoplasm to the mitochondrion involves the conversion of their acyl-CoA derivatives to acyl carnitines, translocation across the inner mitochondrial membrane, and reconversion to acyl-CoA [18,268]. These transferase reactions are rate-limiting steps in β-oxidation and are catalyzed by two distinct enzymes, carnitine palmitoyltransferases I and II (CPTI and CPTII) that are located on the outer and inner mitochondrial membranes, respectively [97,182,193]. Because of its central role in fatty acid metabolism, CPTI has attracted attention as a potential site for pharmacological intervention in the ischemic heart where elevated levels of acylcarnitines have been associated with arrhythmias [65], in diabetes mellitus where fatty acid oxidation is excessive and interferes with glucose metabolism [212], and in human inherited CPT deficiency diseases [24]. Membrane-bound CPTI but not CPTII is inhibited by malonyl-CoA, a metabolite that exerts a major control over β-oxidation [66,67,79,144,275]. Detergent solubilization of the mitochondrial membranes inactivates malonyl-CoA-sensitive CPTI [31,32,182]. TDG-CoA/etomoxiryl-CoA binding proteins have been identified as 90–94-kDa proteins in liver and 82-kDa proteins in heart and skeletal muscle

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Zhu, H., Shi, J., de Vries, Y., Arvidson, D. N., Cregg, J. M., and Woldegiorgis, G. (1997) Functional studies of yeast-expressed human heart muscle carnitine palmitoyltransferase I. *Arch. Biochem. Biophys.* **347,** 53–61.

[31,32,48,66,67,78,79,144,166,225,275–277]; however, it could not be demonstrated that these proteins have CPT activity. Both rat and human liver CPTI cDNAs have been cloned and sequenced [34,77], and rat liver cDNAs have been expressed [39,77].

We have developed a novel high-level expression system for rat liver CPTI in the yeast *Pichia pastoris*, an organism devoid of endogenous CPT activity. Using this system, we have demonstrated conclusively that this protein is an active malonyl-CoA-sensitive CPTI [70]. Thus, mammalian mitochondrial membranes contain two active but distinct CPTs, a malonyl-CoA-sensitive detergent-labile CPTI and a catalytically active malonyl-CoA-insensitive detergent-stable CPTII.

Adult rat heart expresses two isoforms of mitochondrial CPTI: a liver form (L-CPTI) that comprises 2–3% of the total CPTI activity in the heart and a skeletal muscle isoform (M-CPTI) that is the predominant heart form [42,266,267]. In the developing rat heart, a switch occurs in CPTI isoforms [42]. In the neonatal heart, L-CPTI contributes ~25% of total CPTI activity, but its contribution falls during growth to the adult level of 2–3% [42]. Recently, a cDNA clone for a CPTI-like protein was isolated from rat brown adipose tissue [281]. This cDNA encodes a protein of 88 kDa which is 62.6% identical to that of rat L-CPTI. The rat brown adipose tissue was the first M-CPTI gene to be cloned. The rat M-CPTI cDNA was expressed in COS cells, but results were inconclusive. Due to the presence of endogenous CPTI activity (L-CPTI isoform) in the COS cells [78], it was not possible to determine the IC_{50} for malonyl-CoA inhibition of M-CPTI and the K_ms for the substrates. In this communication, we report the functional characterization of a human heart M-CPTI cDNA expressed in *P. pastoris*.

2.2 EXPERIMENTAL PROCEDURES

2.2.1 Screening of a Human Heart cDNA Library for M-CPTI cDNAs

Pairs of PCR primers were designed based on the published cDNA sequence of rat brown adipose tissue CPTI [281] and used to amplify a 472-bp 5′ cDNA fragment and a 398-bp 3′ fragment from a rat heart cDNA library in λgt11 (Clontec,

Palo Alto, CA). PCR primers used were 5′-AGCACACCAGGCAGTAGC-3′ and 5′-GGACAGGAGACGAACACA-3′ for the 5′ fragment, and 5′-ACCACATCCGTCAA-GCACTG-3′ and 5′-GTCGCCACCACCCTAACCTG-3′ for the 3′ fragment. The PCR-amplified fragments were radioactively labeled using the random-labeling method and used to screen a rat heart 5′ stretch plus cDNA library in λgt11. A 700-bp clone encoding a portion of the 5′ end and another clone encoding a 1200-bp portion at the 3′ end of the rat M-CPTI gene were isolated.

A human heart cDNA library in λgt10 (Clontec) was also screened by the plaque hybridization method using the same primers described above [201,227]. The resulting PCR-amplified fragments were labeled [82] and used together to screen a human heart cDNA library in λgt10. Plaques were transferred to nitrocellulose membranes and prehybridized for 4 h at 42°C in 5× SSPE, 25% formamide, 5× Denhart's solution, 0.1% SDS, and 100 μg/ml of denatured salmon sperm DNA. Hybridization was done overnight in the same solution at 42°C with both cDNA probes at 10^6 cpm/ml. The filters were then washed in 1× SSPE, 0.1% SDS at 55°C for 1 h. One positive clone was obtained out of a total of 2×10^5 plaques that were screened. The size of the cDNA insert in the potential human heart M-CPTI clone was 2.6 kb. The cDNA insert was removed from the λgt10 vector by *Eco*RI digestion, and inserted into pUC18 as two fragments, one of which was a 700-bp 5′ fragment and the other a 2.0-kb 3′ fragment [228]. The sequence of both fragments was determined in both directions on an automatic DNA sequencer using synthetic primers. The sequences were analyzed using the PC/Gene program, release 6.85 (Intelligenetics, Mountain View, CA).

2.2.2 Northern Blot Analysis

A nylon membrane containing Poly(A)$^+$ RNA (~2 μg/lane) from eight different human tissues (heart, brain, placenta, lung, liver, skeletal muscle, kidney, and pancreas) was obtained from Clontech. Prehybridization of the nylon membrane was performed in ExpressHyb solution [286] at 68°C for 1 h according to the supplier's instructions (Clontech). Hybridization was first performed with the ^{32}P-labeled random-primed 2.0-kb *Eco*RI fragment from the human heart CPTI cDNA

(nucleotides 666–2610) at 68°C for 1 h. The membrane was washed in 0.1× SSC, 0.1% SDS at 50°C for 40 min as recommended by the supplier; the labeled blot was exposed to X-ray film at –70°C and the autoradiogram was developed. The same nylon membrane was then reprobed with a ^{32}P-labeled human L-CPTI cDNA. As probe for L-CPTI mRNA, a fragment of ~700 bp (nucleotides 168–875) was amplified from a human heart cDNA library in λgt10 using PCR primers that were designed based on the published human L-CPTI sequence [34]. PCR primer sequences were 5′-TCTATCTCTCTGGACTTCAT-3′ and 5′-TGTGAGTTGGAAGGATATAC-3′. The northern blot was finally stripped of the L-CPTI probe and reprobed with a human β-actin cDNA control probe supplied by Clontech.

2.2.3 Generation of Anti-Human Heart M-CPTI Antibodies

Rabbit polyclonal antibodies were raised against bacterially expressed human heart M-CPTI using the maltose binding protein (MBP) system (New England Biolabs, Beverly, MA). A MBP–CPTI fusion construct encoding the C-terminus of human M-CPTI (amino acid residues 574–772) was made by ligation of a 700-bp *Pst*I fragment into pMAL-c2 cut with *Pst*I. The plasmid was transformed into *Escherichia coli* and the fusion protein was induced with IPTG and purified by affinity chromatography on an amylose column following the instructions of the supplier. The purified fusion protein was used to immunize rabbits following standard procedures (Josman Laboratories, Napa, CA).

2.2.4 Construction of Plasmids for Human Heart M-CPTI Expression in *P. pastoris*

A *Mun*I site was introduced by PCR immediately 5′ of the ATG start codon of the human heart M-CPTI cDNA to enable cloning into the unique *Eco*RI site located just 3′ of the glyceraldehyde 3-phosphate dehydrogenase promoter (GAP_p) in plasmid pHWO10 [264]. PCR primers were designed to generate a 700-bp fragment that included the *Mun*I restriction site immediately 5′ of the ATG start codon in the CPTI

cDNA and an internal *Eco*RI site in the M-CPTI cDNA. The sequences of these primers were 5′-AAGACAATTGATGGCGGAAGCTCACCAG-3′ and 5′-CCACCA-GTCACTCACATA-3′. The PCR product was digested with *Eco*RI and *Mun*I and ligated into *Eco*RI-cut pHWO10. The remainder of the CPTI gene in pUC18 was released as a 2.0-kb *Eco*RI fragment and ligated into the pHWO10 derivative containing the *Mun*I–*Eco*RI 5′ PCR fragment to generate the final human M-CPTI expression vector pGAP–M-CPTI.

2.2.5 *P. pastoris* Transformation

The expression plasmid was linearized in the *HIS4* gene by digestion with *BspE*I and integrated into the *HIS4* locus of *P. pastoris* strain GS115 (*his4*) by electrotransformation [12]. Histidine prototrophic transformants were selected on YND plates and grown in liquid YND medium (*GAPp*) [0.17% yeast nitrogen base without amino acids and ammonium sulfate (Difco Laboratories Inc., Detroit, MI)] supplemented with 0.5% ammonium sulfate and 0.5% (w/v) glucose and harvested during the exponential growth phase [156]. As a control, a yeast strain containing the vector, but without a cDNA insert, was grown in parallel cultures.

2.2.6 Isolation of Yeast Mitochondria

The cell wall was enzymatically lysed; spheroplasts were homogenized and subjected to differential centrifugation by a modification of the procedure by Liu et al. [156] as described by de Vries et al. [70]. The final mitochondrial pellet was rinsed with 1 ml of homogenization buffer and resuspended in 0.5 ml of homogenization buffer per 500 OD_{600} units of culture. Crude organelle preparations were immediately assayed for malonyl-CoA-sensitive CPTI activity. To determine the subcellular localization of CPTI, the crude organelle preparation was loaded on top of a sucrose density gradient [70,263] and centrifuged at 4°C for 6 h at 27,000 rpm in a Beckman SW27Ti rotor. Fractions of ~1.2 ml were collected from the bottom of the tube and assayed for CPT [32] and cytochrome *c* oxidase activities [72].

2.2.7 CPT Assay

CPT activity was assayed by the forward exchange method using L-[^{3}H]carnitine as previously described [32]. In a total volume of 0.5 ml, the standard enzyme assay mixture contained: 1.0 mM L-[^{3}H]carnitine (~10,000 dpm/nmol), 50 μM palmitoyl-CoA, 20 mM HEPES (pH 7.0), 1% fatty acid-free albumin, and 40–75 mM KCl with or without 10–100 μM malonyl-CoA. Reactions were initiated by addition of mitochondria, membranes containing expressed proteins, or detergent extracts. The reaction was linear up to 12 min, and all incubations were performed at 30°C for 5 min. Reactions were stopped by addition of 6% perchloric acid and centrifuged at 2000 rpm for 7 min. The resulting pellet was suspended in water, and the product, [^{3}H]-palmitoylcarnitine, was extracted with butanol at low pH. After centrifugation at 2000 rpm for 2 min, an aliquot of the butanol phase was transferred to a vial for radioactive counting.

2.2.8 Mitochondrial Membrane Solubilization

Yeast mitochondria containing CPTI were sedimented by centrifugation at 16,000 × *g* for 30 min at 4°C, and the pellet was solubilized in 5% Triton X-100 (10 mg protein/ml) containing 500 mM KCl and 10 mM HEPES (pH 7.0) at 0°C for 30 min. The insoluble membrane residue was sedimented by centrifugation at 16,000 × *g* for 30 min, and the supernatant was used for CPT assay.

2.2.9 Western Blot Analysis

Proteins were separated by SDS–PAGE [151] in a 7.5% gel and transferred onto nitrocellulose membranes using a Mini Trans-Blot electrophoretic transfer cell according to the manufacturer's instructions (Bio-Rad, Hercules, CA). Immunoblots were developed by incubation with the CPTI-specific polyclonal antibodies, followed by an anti-rabbit IgG conjugated to alkaline phosphatase. The antigen–antibody complex was detected using a Tropix Western Light chemiluminescence kit (Bedford, MA).

2.2.10 Miscellaneous Materials and Methods

DNA sequencing was performed at the Oregon Regional Primate Research Center Core Facility using an automatic DNA sequencer [228]. Protein was determined by the Lowry procedure [165]. All restriction enzymes were from New England Biolabs (Beverly, MA); the *E. coli* strain used was $DH5_{\alpha}$. L-[^{3}H]-carnitine was from Amersham (Arlington Heights, IL); nucleotides and palmitoyl-CoA were from Pharmacia (Piscataway, NJ); octylglucoside was from Sigma (St. Louis, MO).

2.3 RESULTS

2.3.1 Cloning of a cDNA Encoding the Human Heart/Skeletal Muscle Isoform of CPTI

Screening of a human heart cDNA library in λgt10 (Clontec) using PCR-amplified cDNA fragments from a rat heart CPTI cDNA as probes resulted in isolation of a single cDNA clone that encoded a full-length human heart (skeletal muscle) CPTI (M-CPTI). The complete cDNA sequence is shown in Fig. 2.1. The sequence revealed an open reading frame of 2316 bp with a 5′ untranslated region of 38 bp and a 256-bp 3′ untranslated region with the poly(A)$^+$ addition sequence AATAAA. The predicted protein contains 772 amino acids, the same number of residues as the rat heart CPTI, and encodes a protein predicted to be 88 kDa. The nucleotide and amino acid sequences of the human and rat brown adipose tissue/heart M-CPTIs were similar with 83% homology at the DNA level and 86% identity at the amino acid level [282]. Comparison of the predicted amino acid sequence of the human heart M-CPTI with that of human and rat liver CPTI gave an overall identity of 61% for each [34,77]. Rat liver CPTII was found to be only 15% identical with human M-CPTI [274].

2.3.2 Tissue Distribution of Human M-CPTI and L-CPTI mRNAs

Northern blot analysis of mRNA from different human tissues using a fragment encoding the human heart M-CPTI revealed an abundant transcript of ~3.1 kb that was only present in human heart and skeletal muscle tissues (Fig. 2.2). Other

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-38                                                                    GCAATTCCCACCGCGGAAGGTGCCGACCAACCCCCAGG

  1 ATGGCGGAAGCTCACCAGGCCGTGGCCTTCCAGTTCACGGTGACCCCAGACGGGGTCGACTTCCGGCTCAGTCGGGAGGCCCTGAAACAC
    MetAlaGluAlaHisGlnAlaValAlaPheGlnPheThrValThrProAspGlyValAspPheArgLeuSerArgGluAlaLeuLysHis  30

 91 GTCTACCTGTCTGGGATCAACTCCTGGAAGAAACGCCTGATCCGCATCAAGAATGGCATCCTCAGGGGCGTGTACCCTGGCAGCCCCACC
    ValTyrLeuSerGlyIleAsnSerTrpLysLysArgLeuIleArgIleLysAsnGlyIleLeuArgGlyValTyrProGlySerProThr  60

181 AGCTGGCTGGTCGTCATCATGGCAACAGTGGGTTCCTCCTTCTGCAACGTGGACATCTCCTTGGGGCTGGTCAGTTGCATCCAGAGATGC
    SerTrpLeuValValIleMetAlaThrValGlySerSerPheCysAsnValAspIleSerLeuGlyLeuValSerCysIleGlnArgCys  90

271 CTCCCTCAGGGGTGTGGCCCCTACCAGACCCCGCAGACCCGGGCACTTCTCAGCATGGCCATCTTCTCCACGGGCGTCTGGGTGACGGGC
    LeuProGlnGlyCysGlyProTyrGlnThrProGlnThrArgAlaLeuLeuSerMetAlaIlePheSerThrGlyValTrpValThrGly 120

361 ATCTTCTTCTTCCGCCAAACCCTGAAGCTGCTTCTCTGCTACCATGGGTGGATGTTTGAGATGCATGGCAAGACCAGCAACTTGACCAGG
    IlePhePhePheArgGlnThrLeuLysLeuLeuLeuCysTyrHisGlyTrpMetPheGluMetHisGlyLysThrSerAsnLeuThrArg 150

451 ATCTGGGCTATGTGTATCCGCCTTCTATCCAGCCGGCACCCTATGCTCTACAGCTTCCAGACATCTCTGCCCAAGCTTCCTGTGCCCAGG
    IleTrpAlaMetCysIleArgLeuLeuSerSerArgHisProMetLeuTyrSerPheGlnThrSerLeuProLysLeuProValProArg 180

541 GTGTCAGCCACAATTCAGCGGTACCTAGAGTCTGTGCGCCCCTTGTTGGATGATGAGGAATATTACCGCATGGAGTTGCTGGCCAAAGAA
    ValSerAlaThrIleGlnArgTyrLeuGluSerValArgProLeuLeuAspAspGluGluTyrTyrArgMetGluLeuLeuAlaLysGlu 210

631 TTCCAGGACAAGACTGCCCCCAGGCTGCAGAAATACCTGGTGCTCAAGTCATGGTGGGCAAGTAACTATGTGAGTGACTGGTGGGAAGAG
    PheGlnAspLysThrAlaProArgLeuGlnLysTyrLeuValLeuLysSerTrpTrpAlaSerAsnTyrValSerAspTrpTrpGluGlu 240

721 TACATCTACCTTCGAGGCAGGAGCCCTCTCATGGTGAACAGCAACTATTATGTCATGGACCTTGTGCTCATCAAGAATACAGACGTGCAG
    TyrIleTyrLeuArgGlyArgSerProLeuMetValAsnSerAsnTyrTyrValMetAspLeuValLeuIleLysAsnThrAspValGln 270

811 GCAGCCCGCCTGGGAAACATCATCCACGCCATGATCATGTATCGCCGTAAACTGGACCGTGAAGAAATCAAGCCTGTGATGGCACTGGGC
    AlaAlaArgLeuGlyAsnIleIleHisAlaMetIleMetTyrArgArgLysLeuAspArgGluGluIleLysProValMetAlaLeuGly 300

901 ATAGTGCCTATGTGCTCCTACCAGATGGAGAGGATGTTCAACACCACTCGGATCCCGGGCAAGGACACAGATGTGCTACAGCACCTCTCA
    IleValProMetCysSerTyrGlnMetGluArgMetPheAsnThrThrArgIleProGlyLysAspThrAspValLeuGlnHisLeuSer 330

991 GACAGCCGGCACGTGGCTGTCTACCACAAGGGACGCTTCTTCAAGCTGTGGCTCTATGAGGGCGCCCGTCTGCTCAAGCCTCAGGATCTG
    AspSerArgHisValAlaValTyrHisLysGlyArgPheHeLysLeuTrpLeuTyrGluGlyAlaArgLeuLeuLysProGlnAspLeu 360

1081 GAGATGCAGTTCCAGAGGATCCTGGACGACCCCTCCCCACCTCAGCCTGGGGAGGAGAAGCTGGCAGCCCTCACTGCAGGAGGAAGGGTG
     GluMetGlnPheGlnArgIleLeuAspAspProSerProProGlnProGlyGluGluLysLeuAlaAlaLeuThrAlaGlyGlyArgVal 390

1171 GAGTGGGCGCAGGCACGCCAGGCCTTCTTTAGCTCTGGAAAGAATAAGGCTGCCTTGGAGGCCATCGAGCGTGCCCCTTTCTTCGTGGCC
     GluTrpAlaGlnAlaArgGlnAlaPhePheSerSerGlyLysAsnLysAlaAlaLeuGluAlaIleGluArgAlaAlaPhePheValAla 420

1261 CTGGATGAGGAATCCTACTCCTATGACCCCGAAGATGAGGCCAGCCTCAGCCTCTATGGCAAGGCCCTGCTACATGGCAACTGCTACAAC
     LeuAspGluGluSerTyrSerTyrAspProGluAspGluAlaSerLeuSerLeuTyrGlyLysAlaLeuLeuHisGlyAsnCysTyrAsn 450

1351 AGGTGGTTTGACAAATCCTTCACTCTCATTTCCTTCAAGAATGGCCAGTTGGGTCTCAATGCAGAGCATGCGTGGGCAGATGCTCCCATC
     ArgTrpPheAspLysSerPheThrLeuIleSerPheLysAsnGlyGlnLeuGlyLeuAsnAlaGluHisAlaTrpAlaAspAlaProIle 480

1441 ATTGGGCACCTCTGGGAGTTTGTCCTGGGCACAGACAGCTTCCACCTGGGCTACACGGAGACCGGGCACTGCCTGGGCAAACCGAACCCT
     IleGlyHisLeuTrpGluPheValLeuGlyThrAspSerPheHisLeuGlyTyrThrGluThrGlyHisCysLeuGlyLysProAsnPro 510

1531 GCGCTCGCACCTCCTACACGGCTGCAGTGGGACATTCCAAAACAGTGCCAGGCGGTCATCGAGAGTTCCTACCAGGTGGCCAAGGCGTTG
     AlaLeuAlaProProThrArgLeuGlnTrpAspIleProLysGlnCysGlnAlaValIleGluSerSerTyrGlnValAlaLysAlaLeu 540

1621 GCAGACGACGTGGAGTTGTACTGCTTCCAGTTCCTGCCCTTTGGCAAAGGCCTCATCAAGAAGTGCCGGACCAGCCCTGATGCCTTTGTG
     AlaAspAspValGluLeuTyrCysPheGlnPheLeuProPheGlyLysGlyLeuIleLysLysCysArgThrSerProAspAlaPheVal 570

1711 CAGATCGCGCTGCAGCTGGCTCACTTCCGGGACAGGGGTAAGTTCTGCCTGACCTATGAGGCCTCAATGACCAGAATGTTCCGGGAGGGA
     GlnIleAlaLeuGlnLeuAlaHisPheArgAspArgGlyLysPheCysLeuThrTyrGluAlaSerMetThrArgMetPheArgGluGly 600

1801 CGGACTGAGACTGTGCGTTCCTGTACCAGCGAGTCCACAGCCTTTGTGCAGGCCATGATGGAGGGGTCCCACACAAAAGCAGACCTGCGA
     ArgThrGluThrValArgSerCysThrSerGluSerThrAlaPheValGlnAlaMetMetGluGlySerHisThrLysAlaAspLeuArg 630

1891 GATCTCTTCCAGAAGGCTGCTAAGAAGCACCAGAATATGTACCGCCTGGCCATGACCGGGGCAGGGATCGACAGGCACCTCTTCTGCCTT
     AspLeuPheGlnLysAlaAlaLysLysHisGlnAsnMetTyrArgLeuAlaMetThrGlyAlaGlyIleAspArgHisLeuPheCysLeu 660

1981 TACTTGGTCTCCAAGTACCTAGGAGTCAGCTCTCCTTTCCTTGCTGAGGTGCTCTCGGAACCCTGGCGTCTCTCCACCAGCCAGATCCCC
     TyrLeuValSerLysTyrLeuGlyValSerSerProPheLeuAlaGluValLeuSerGluProTrpArgLeuSerThrSerGlnIlePro 690

2071 CAATCCCAGATCCGCATGTTCGACCCAGAGCAGCACCCCAATCACCTGGGCGCTGGAGGTGGCTTTGGCCCTGTAGCAGATGATGGCTAT
     GlnSerGlnIleArgMetPheAspProGluGlnHisProAsnHisLeuGlyAlaGlyGlyGlyPheGlyProValAlaAspAspGlyTyr 720

2161 GGAGTTTCCTACATGATTGCAGGCGAGAACACGATCTTCTTCCACATCTCCAGCAAGTTCTCAAGCTCAGAGACGAACGCCCAGCGCTTT
     GlyValSerTyrMetIleAlaGlyGluAsnThrIlePhePheHisIleSerSerLysPheSerSerSerGluThrAsnAlaGlnArgPhe 750

2251 GGAAACCACATCCGCAAAGCCCTGCTGGACATTGCTGATCTTTTCCAAGTTCCCAAGGCCTACAGCTGAAGGTTGGAGAAATGCCAGCTG
     GlyAsnHisIleArgLysAlaLeuLeuAspIleAlaAspLeuPheGlnValProLysAlaTyrSer***                        772

2341 CCCTTTCGTCCCCACACTGTGGAGGAAGGGACCTGTGGCAGCTCACAGGCATGAGGGGTGGCCGTGCACAGGTGCCCAGGCTCCAAGGAC
2521 AGCTCCGGCAGCAGGTCCTCGCTGGGCAGATGCTGCTCCCTGAGGGCCCAGGTGGTGGAGGTGGGGTTGGAGCAGGAAGGGAATTTTGAT
2611 TTTTTTTTTTTCTTGATAGATACTAATAAAAATAAGGCTGTGTAATTTTCTC
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Figure 2.1 A complete nucleotide sequence of the human heart/skeletal muscle isoform CPTI cDNA. Nucleotide numbers are in the left column and amino acid numbers are in the right column. The deduced amino acid sequence of the protein is shown under the nucleotide sequence.

human tissues, such as brain, placenta, lung, liver, kidney and pancreas, did not contain the transcript. Thus, it appears that our human CPTI cDNA encodes a gene that is transcribed only in heart and skeletal muscle, a result that supports the notion that the cDNA encodes the skeletal muscle isoform of CPTI. Northern blot analysis of the same tissues, using a DNA fragment encoding human liver CPTI (L-CPTI) as probe, showed a predominant transcript of ~4.7 kb in heart, placenta, liver, and pancreas (Fig. 2.2). Other tissues, such as lung, skeletal muscle, and kidney, have less but significant amounts of the L-CPTI transcript, except perhaps the brain, an indication that L-CPTI, unlike the M-CPTI, is present in a wide variety of tissues. In the human pancreatic tissue, the level of mRNA for L-CPTI was especially high.

2.3.3 Expression of Human Heart M-CPTI in the Yeast *P. pastoris*

P. pastoris was chosen as an expression system for CPTI, because it does not have endogenous CPT activity. A *P. pastoris* expression plasmid was constructed to express CPTI under control of the *P. pastoris* glyceraldehyde-3-phosphate dehydrogenase gene promoter *GAPp* [264]. *P. pastoris* strains transformed with the CPTI expression vector were grown in liquid media supplemented with glucose. Mitochondria were isolated and assayed for CPT activity. Strains transformed with the CPTI-containing vector showed substantial amounts of CPT activity, while no activity was found in a non-CPT-containing control strain [data not shown].

2.3.4 Subcellular Location of Human M-CPTI

To determine the subcellular location of the CPTI enzyme in *P. pastoris*, homogenized spheroplasts derived from a M-CPTI expression strain were fractionated into mitochondrial (pellet) and cytoplasmic (supernatant) fractions, which were then assayed for cytochrome *c* oxidase (an inner mitochondrial membrane marker enzyme) and CPT activities. CPTI activity was found mainly in the pellet fraction [data not shown]. Sucrose density gradient profiles of the mitochondria-enriched pellets from CPTI (Fig. 2.3A) showed a distribution pattern for CPT activity that was nearly identical to that for cytochrome *c* oxidase, indicating that CPTI was localized to the

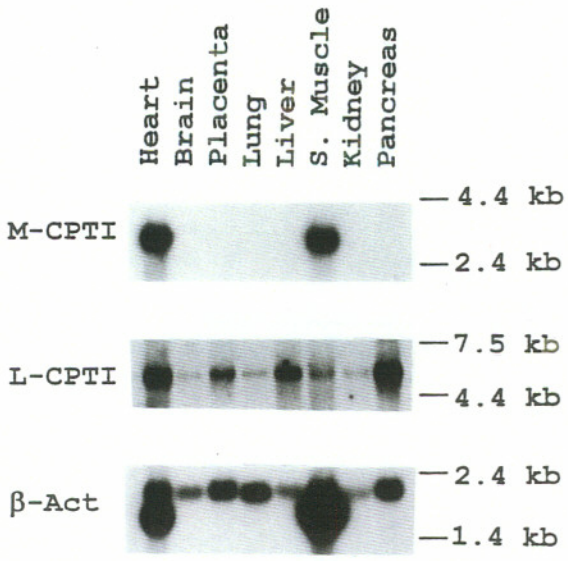

Figure 2.2 Northern blot analysis of poly(A)$^+$ RNA from human tissues. Approximately 2 μg of poly(A)$^+$ RNA was loaded in each lane. The probe for M-CPTI was a 2.0-kb 3′-end *Eco*RI fragment of the human heart/skeletal muscle isoform CPTI cDNA. L-CPTI, the probe, was a 700-bp 5′-end human liver CPTI cDNA fragment. β-Act, the probe was a human β-actin used to check levels of RNA between lanes. The position of the standards are shown to the right.

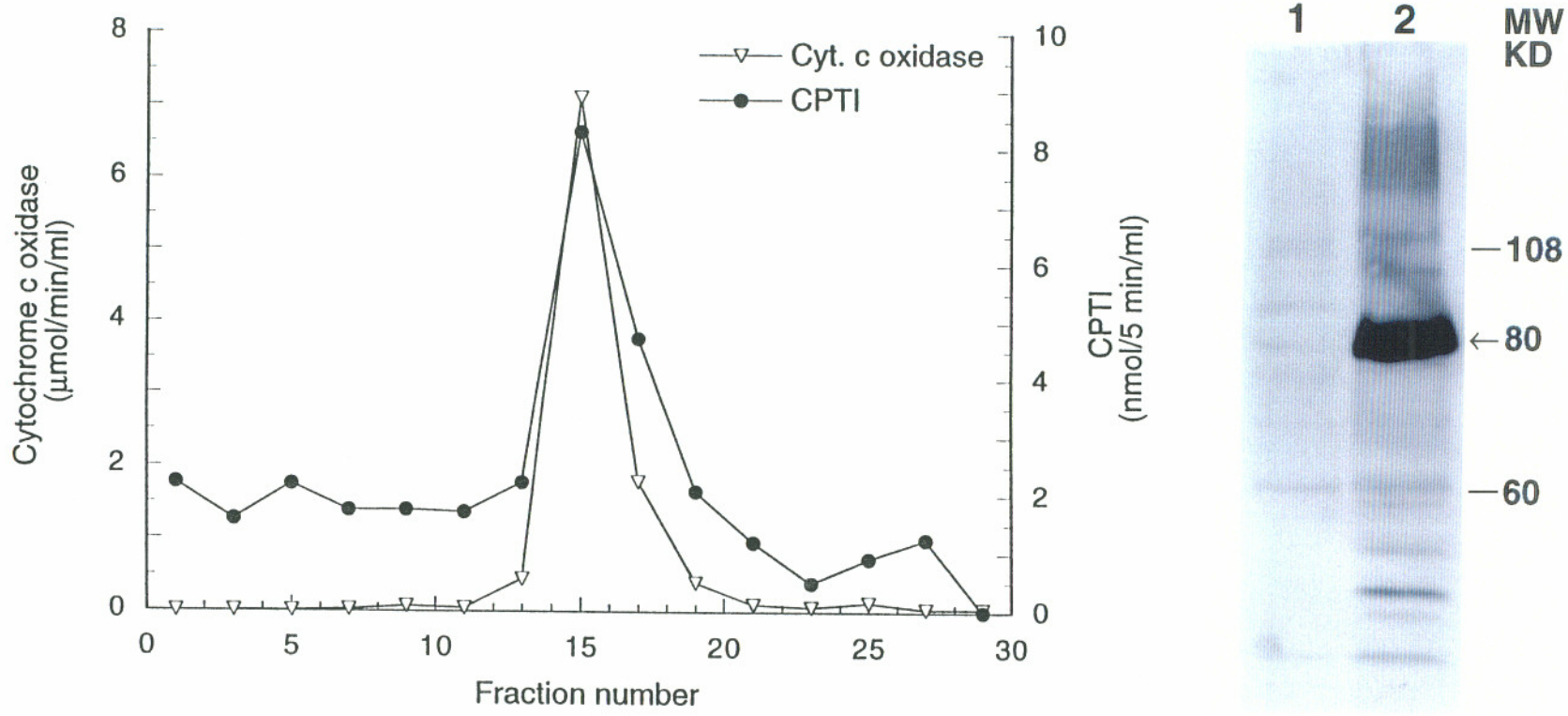

Figure 2.3 **(A)** Distribution of the human heart/skeletal muscle isoform CPTI and cytochrome *c* oxidase activities after centrifugation of crude yeast mitochondrial preparation through a sucrose gradient. Mitochondria-enriched pellet was prepared from glucose-grown yeast strain expressing M-CPTI and subjected to sucrose density gradient centrifugation as described in Section 2.2. Fractions were then assayed for CPTI and cytochrome *c* oxidase activities. **(B)** Immunoblot showing the expression of the human M-CPTI protein in the mitochondrial fraction of the *P. pastoris* strain containing multiple copies of M-CPTI cDNA. Approximately 50 μg of protein were applied in each lane. *1*, control strain without insert; *2*, multicopy yeast strain expressing M-CPTI. The arrow indicates the protein species recognized, 80 kDa for CPTI.

yeast mitochondria. There was no M-CPTI activity in the control yeast strain with the vector but without M-CPTI cDNA.

Immunoblots with the CPTI-specific polyclonal antibodies showed the presence of an 80-kDa protein species corresponding to CPTI (Fig. 2.3B) in the mitochondrial fraction of the strain that expressed M-CPTI, but not in a non-CPT-expressing control strain.

2.3.5 Biochemical and Kinetic Properties of Yeast-Expressed Human M-CPTI

Isolated mitochondria from the CPTI expression strain exhibited malonyl-CoA-sensitive CPT activity. The level of CPTI activity observed was 7.5 nmol/5 min/mg protein. Malonyl-CoA-sensitive M-CPTI activity was only observed in cells transformed with M-CPTI cDNA, but not in control cells lacking the M-CPTI cDNA. CPTI activity in intact mitochondria was completely inhibited by 100 μM malonyl-CoA [data not shown] with an IC_{50} of 69 nM (Fig. 2.4), and solubilization of the mitochondria in 5% Triton X-100 completely abolished CPT activity [data not shown]. These results demonstrate that both CPT catalytic activity and sensitivity to malonyl-CoA reside as part of the same M-CPTI polypeptide and that membrane-bound human heart M-CPTI is detergent labile.

M-CPTI in yeast mitochondria exhibited normal saturation kinetics when the carnitine concentration was varied relative to palmitoyl-CoA, as shown in Fig. 2.5A. The calculated K_m for carnitine was 666 μM. The V_{max} was 6.5 nmol/5 min/mg protein. However, the yeast-expressed M-CPTI demonstrated an abnormal response to increasing concentrations of palmitoyl-CoA. The curve was biphasic (Fig. 2.5B), showing saturable kinetics up to 75 μM, with a K_m of 42 μM and a V_{max} of 7.2 nmol/5 min/mg protein (Fig. 2.5C) and non-saturable kinetics in the concentration range of 100–300 μM palmitoyl-CoA (Fig. 2.5B). Comparison of the substrate vs. velocity plots of the data for carnitine and palmitoyl-CoA clearly demonstrates the nonideal behavior of the expressed enzyme with respect to palmitoyl-CoA. This abnormal behavior is also exhibited by yeast-expressed rat liver CPTI and CPTII [70] and to a lesser degree in rat liver mitochondria (RLM) and may be due to the presence of albumin in the reaction mixture, the detergent properties of palmitoyl-

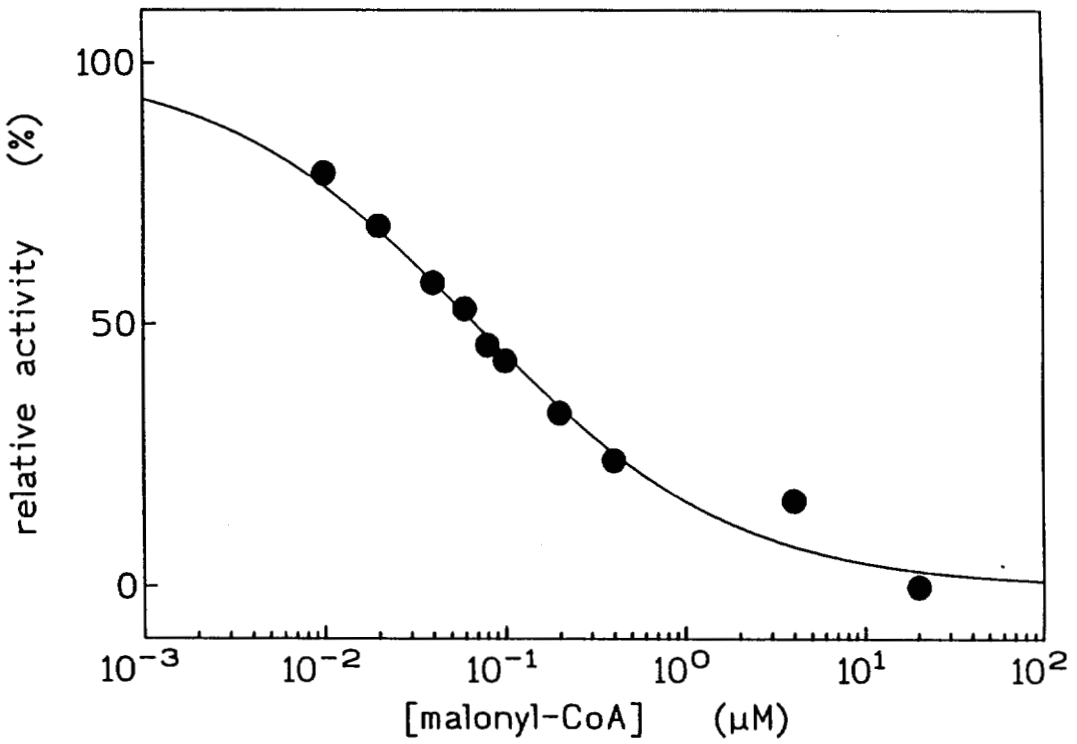

Figure 2.4 Effect of malonyl-CoA concentration on yeast-expressed CPTI activity. Isolated mitochondria (310 μg) from the yeast strain expressing human heart/skeletal muscle isoform CPTI were assayed for CPT activity in the presence of increasing concentrations of malonyl-CoA as described in Section 2.2.

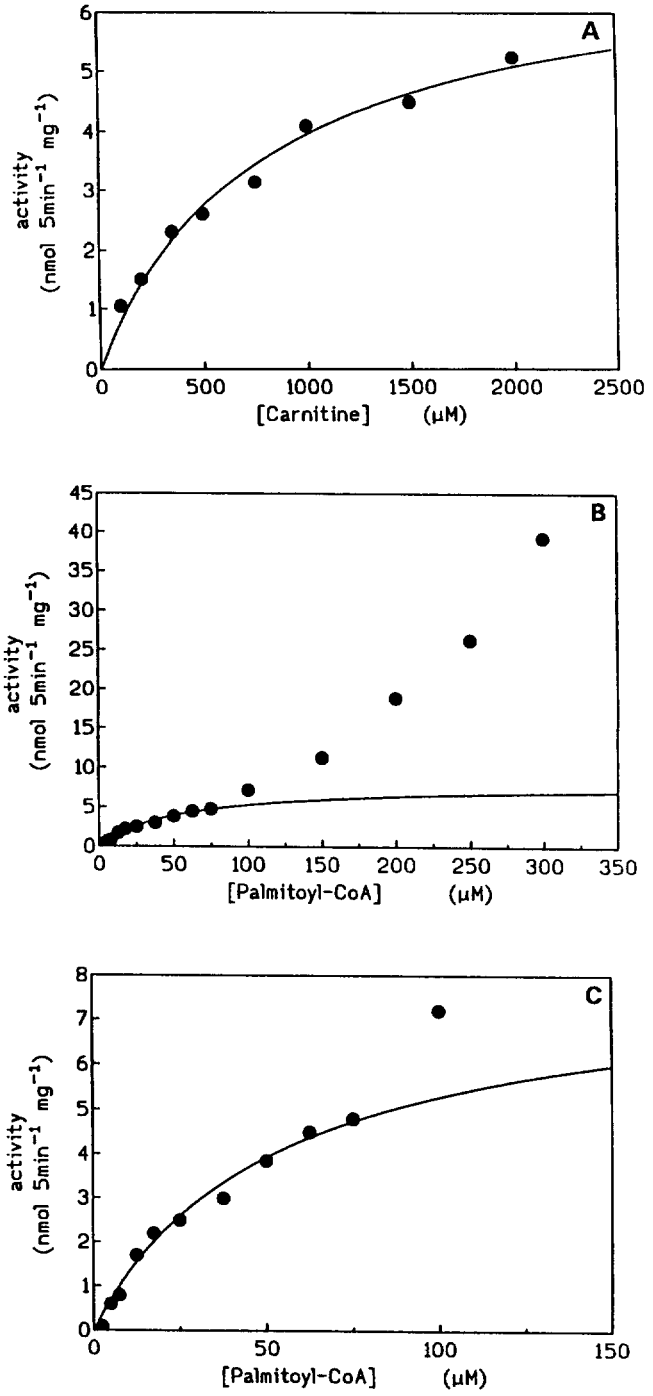

Figure 2.5 Effect of increasing concentrations of carnitine and palmitoyl-CoA on yeast-expressed human M-CPTI activity. Isolated mitochondria (239 μg) from the yeast strain expressing M-CPTI were assayed for CPT activity in the presence of increasing concentrations of carnitine and palmitoyl-CoA, as described in Section 2.2. **A**, carnitine, dose-response curve; **B**, palmitoyl-CoA, dose-response curve, 0-300 μM; **C**, palmitoyl-CoA, dose-response curve, 0-100 μM.

CoA, and/or to differences in the membrane environment of the enzymes in yeast mitochondria compared to heart.

2.4 DISCUSSION

Previous biochemical studies have indicated that mammals contain two isoforms of CPTI, one that is the predominant form in liver tissue (L-CPTI) and another that is the predominant form in skeletal muscle and heart tissues (M-CPTI) [42,266,267]. Our group and others have cloned and expressed cDNAs encoding L-CPTI [39,70,77]. Here we report the cloning and characterization of a human heart M-CPTI cDNA and its product. Evidence that our clone in fact encodes M-CPTI is provided from the following results. First, the amino acid sequence predicted by the cDNA shares similarity with those of known L-CPTIs, but at 61% identity to the L-CPTIs it is clearly a distinct gene. Second, a *P. pastoris* strain expressing the cDNA contained activity for CPT while a control strain that lacked the cDNA did not. Thus, the clone must encode an active CPT enzyme. Third, as discussed below, the yeast-expressed CPTI has the enzymatic properties expected of M-CPTI and not L-CPTI. The IC_{50} for malonyl-CoA inhibition of the heart enzyme is 30 times lower than that of the yeast-expressed liver CPTI, and the K_m for carnitine is more than 20 times higher than that of liver CPTI [70]. Fourth, northern blot studies showed that skeletal muscle and heart tissues contain high levels of transcript for the cDNA, whereas other human tissues do not. Our laboratory and others have independently cloned and sequenced the cDNA [282] and the gene [3] for human heart M-CPTI. The predicted amino acid sequence of our cDNA is identical to the sequences in the data bank. Thus, this cDNA most likely encodes human heart muscle M-CPTI. Expression of the cDNA from rat brown adipose tissue that encodes a CPTI-like protein in COS cells resulted in the induction of CPT activity, although it was possible that the activity was the result of the induction of the endogenous liver form of CPTI which is present at high levels in COS cells [78]. It was thus not possible to determine the IC_{50} for malonyl-CoA inhibition of rat heart M-CPTI and the K_ms for the substrates.

A search of the human genomic databases identified a BAC clone of ~140 kb that includes an exact match to our M-CPTI cDNA sequence [3]. Comparison of the genomic and cDNA sequences showed that the human M-CPTI gene is ~10 kb in length. The genomic clone (and hence the M-CPTI gene) is located on chromosome 22 at coordinates 22q13. The gene for human liver CPTI is located on chromosome 11q [34]. Thus, human liver and skeletal muscle CPTI are different proteins encoded by separate genes. Heart expresses both isoforms of CPTI. The structural gene for M-CPTI is composed of 18 exons interrupted by 17 introns spanning more than 8576 bp [3].

We report expression of active CPTI from human heart in the yeast *P. pastoris*. The yeast-expressed CPTI is sensitive to inhibition by malonyl-CoA and detergent solubilization, unique properties predicted for rat heart and liver mitochondrial CPTIs [31,32,170]. This is the first report of (i) high-level synthesis of active human heart CPTI of the skeletal muscle isoform in an expression system devoid of endogenous CPT activities, and (ii) the kinetic characteristics of a human heart M-CPTI in the absence of either L-CPTI or CPTII or both. Our human heart M-CPTI cDNA encoded a protein of 88 kDa, but on western blot analysis the polyclonal antibodies cross-reacted with an 80-kDa protein species from mitochondria isolated from the yeast M-CPTI expression strain. This is in agreement with the abnormal migration behavior on SDS–PAGE reported for the rat heart M-CPTI [78]. Thus, *P. pastoris* appears to be an excellent model system to investigate human heart CPTI.

With respect to the second substrate, palmitoyl-CoA, yeast-expressed M-CPTI showed non-Michaelis–Menton saturation kinetics. This unusual kinetic behavior is also seen with the CPTs from RLM [38,179], heart and skeletal muscle mitochondria [38,84,222], but to a lesser degree, and *P. pastoris*-expressed RLM CPTI and II [70]. The reason for this unusual kinetic behavior is not known. Since the expressed enzyme exhibited saturable kinetics with respect to carnitine and the immunoblots indicate that the molecular masses of the yeast-expressed human heart M-CPTI is identical to the molecular species detected in rat heart mitochondria (RHM) [42,78,266,267], the nonsaturable kinetic behavior does not appear to be due to

specific or nonspecific processing of the enzymes in the yeast expression system. It is possible that this could be due to the differences in the membrane lipid environment between the human heart and yeast mitochondria or that one or more protein–protein interactions that occur in heart do not occur in yeast. The expressed mitochondrial M-CPTI is kinetically similar to the native enzyme [38,84,222].

Isolated mitochondria from the yeast strain expressing human heart M-CPTI cDNA exhibited a high K_m for carnitine and a low IC_{50} for malonyl-CoA, characteristics similar to those of RHM CPTI. In adult RHM, the total carnitine level is high, consequently M-CPTI has a high K_m for carnitine [78,179]. Although rat heart has a measurable level of malonyl-CoA, it is much more lower than that of rat liver [182]. Since the IC_{50} for malonyl-CoA inhibition of human heart M-CPTI is at least 30-fold lower than that of the rat L-CPTI, the level of malonyl-CoA reported in heart [182] is sufficient to significantly inhibit fatty acid oxidation. It is estimated that about 60–80% of the energy requirement of the heart is derived from fatty acid oxidation [268]. The important question in cardiac tissue metabolism then is how fatty acid oxidation can proceed in heart in the presence of such high tissue levels of malonyl-CoA. It has been suggested but not proven that (i) most of the cellular malonyl-CoA may be in a bound state and unavailable for interaction with CPTI, and/or (ii) the presence of L-CPTI in heart with a higher IC_{50} for malonyl-CoA may permit fatty acid oxidation to occur at a basal level when the free malonyl-CoA concentration rises toward an inhibitory level for M-CPTI [42].

In a normal nonlipogenic tissue like heart, which is primarily dependent on fatty acids as a fuel energy source, high rates of fatty acid oxidation should increase cellular long-chain fatty acyl-CoA levels, which should in turn decrease malonyl-CoA affinity to CPTI. The result is expected to be the activation of CPTI and stimulation of fatty acid oxidation. In the ischemic, fatty acid reperfused heart, high levels of long-chain fatty acyl-CoAs may (i) stimulate AMP-activated protein kinase and inhibit acetyl-CoA carboxylase, thereby decreasing malonyl-CoA synthesis [148]; and (ii) decrease the affinity of malonyl-CoA for CPTI. Both of these events would result in activation of M-CPTI and stimulate fatty acid oxidation. In the ischemic myocardium, elevated levels of acylcarnitines have been associated with arrhythmias

[212], and inhibition of CPTI in the ischemic heart prevents increase in long-chain acylcarnitines and significantly reduces the incidence of malignant arrhythmias. Long-chain acylcarnitines, products of the reaction catalyzed by CPTI, are surface-active metabolites that are more powerful detergents than their corresponding CoA derivatives [101]. It may be that heart M-CPTI is extremely sensitive to malonyl-CoA inhibition to prevent accumulation of damaging levels of long-chain acylcarnitines such as palmitoylcarnitine, a surface-active amphipathic metabolite. Since inhibition of CPTI prevents accumulation of long-chain acylcarnitines and has a significant antiarrhythmic effect, development of a specific CPTI inhibitor could be of considerable therapeutic benefit for prevention of sudden cardiac death in patients with ischemic heart disease [212]. The ability to express human heart M-CPTI in yeast is critical to its purification for determination of its three-dimensional structure and for structure–function studies of this important enzyme.

CHAPTER 3

RECONSTITUTION OF HIGHLY EXPRESSED HUMAN HEART MUSCLE CARNITINE PALMITOYLTRANSFERASE I*

3.1 INTRODUCTION

Carnitine palmitoyltransferase I (CPTI) catalyzes the first reaction in the transport of long-chain fatty acids from the cytoplasm to the mitochondrion, a rate-limiting step in β-oxidation [18,182]. Mammalian mitochondrial membranes contain two distinct CPTs, an outer malonyl-CoA-sensitive CPTI and an inner malonyl-CoA-insensitive CPTII. Furthermore, mammalian tissues express two isoforms of CPTI—a liver form, L-CPTI, and a heart/skeletal muscle form, M-CPTI—that are 62.6% identical in amino acid sequence [42,266,267]. Adult heart expresses both isoforms of M-CPTI, but the predominant isoform is M-CPTI (97–98%). Because of its central role in fatty acid metabolism, a thorough understanding of CPTI is an important first step in the development of treatments for diseases such as diabetes and myocardial ischemia, and in human inherited CPT-deficiency diseases.

CPTI, but not CPTII, is inhibited by malonyl-CoA [66,67,79,144,275]. Detergent solubilization of the mitochondrial membranes inactivates malonyl-CoA-sensitive CPTI, and, until recently, detergent inactivation of CPTI was presumed to be irreversible [31,32,48,70,78,142,166,182,225,276,277]. We have successfully expressed both human heart M-CPTI and rat liver L-CPTI in the yeast *P. pastoris* and

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Zhu, H., Shi, J., Cregg, J. M., and Woldegiorgis, G. (1997) Reconstitution of highly expressed human heart muscle carnitine palmitoyltransferase I. *Biochem. Biophys. Res. Commun.* **239,** 498–502.

have demonstrated the presence of malonyl-CoA-sensitive CPT activity that is detergent labile [70,294]. We have also determined the kinetic characteristics of the yeast-expressed CPTs, the first such report for a CPTI enzyme in the absence of CPTII and of M-CPTI in the absence of L-CPTI [70,294]. Mitochondria from the yeast strain containing M-CPTI had a high K_m for carnitine and were more sensitive to inhibition by malonyl-CoA than the mitochondria from the yeast strain with L-CPTI [70,294]. In this communication, we report an improved high-level expression of human heart M-CPTI using a multicopy *P. pastoris* expression strain, and reconstitution and functional characterization of the yeast-expressed detergent-inactivated M-CPTI.

3.2 MATERIALS AND METHODS

3.2.1 Construction of Plasmids for Human Heart M-CPTI Expression in the *P. pastoris* Multicopy Vector pPICZ-B and Selection of Multicopy Integrants by Dot Blot

The vector pPICZ-B containing the zeocin-resistant gene was obtained from Invitrogen (Carlsbad, CA). The full-length human heart M-CPTI cDNA was subcloned into the *Eco*RI site of the multicopy vector as described previously [294]. The expression plasmid was linearized at the MunI site of the alcohol oxidase (AOX) promoter and integrated into the AOX locus of *P. pastoris* GS115 by electrotransformation [70,294]. The cells were plated onto YPD (1% yeast extract, 2% peptone, 1% glucose) plates (Difco Laboratories Inc., Detroit, MI) containing 1–2 mg/ml zeocin (Invitrogen) and incubated at 30°C for 3 days. The yeast cell containing the vector alone was used as a control and grown under the same conditions.

Twenty-six positive transformants were picked, grown in liquid YPD medium, and harvested during the exponential growth stage. Yeast genomic DNA was isolated after breaking the cells with glass beads [9]. For dot blot, 100 ng of the DNA was denatured in 0.4 M NaOH and 10 mM EDTA, heated to 100°C for 10 min, and neutralized with an equal volume of cold 2 M ammonium acetate, pH 7.0. The

denatured DNA was immobilized on a nitrocellulose membrane under gentle vacuum (Bio-Dot-SF Microfiltration Apparatus, Bio-Rad, Hercules, CA), and dried at 80°C for 2 h. The DNA was hybridized with the labeled 2.0-kb *Eco*RI fragment of human heart M-CPTI probe (666–2610 bp; cDNA sequence) prepared by the random primer labeling method [82]. *P. pastoris* formadehylde dehydrogenase partial cDNA was used as a control probe for hybridization. The dot blots were quantitated by PhosphorImage SI analysis (Molecular Dynamics, Sunnyvale, CA).

3.2.2 Yeast Culture and Mitochondrial Isolation

Yeast cells with one–two (low), eight (intermediate), and 24 (high) copies of CPTI cDNA were selected and cultured in YPD medium overnight [70]. The culture was refreshed once with YPD medium to $OD_{600} = 1$. The cells were pelleted down and transferred to fresh YNB medium (0.17% yeast nitrogen without amino acids and ammonium sulfate, 0.25% ammonium sulfate, 0.005% His) supplemented with 0.5% (v/v) methanol. Cells were harvested by centrifugation during the exponential growth phase (24 h after methanol induction), and stored at -70°C [70]. For mitochondrial isolation, cell pellets were resuspended in ice-cold buffer (10 mM HEPES, pH 7.8, 1 mM EDTA, 10% glycerol, 1 mM PMSF, 1 μg/ml leupeptin), mixed with an equal volume of acid-washed glass beads (450–600 μm), and lysed by six 1-min cycles of vigorous vortex mixing separated by intermittent 30-s periods of cooling on ice [243]. The sample was subjected to low-speed centrifugation to remove unbroken cells and then centrifuged at 25,000 $\times$ *g* for 30 min at 4°C. Mitochondria were resuspended in sample buffer and protein was determined by the Lowry method 165]. Yeast mitochondria containing the expressed M-CPTI were solubilized in 5% Triton X-100 as described previously [70].

3.2.3 Reconstitution of Malonyl-CoA-Sensitive M-CPTI Activity

A modified reconstitution procedure [93] described by de Vries et al. [70] was used. Briefly, to a suspension of dried phospholipids (50 mg) composed of asolectin and cardiolipin at a ratio of 9:1, in 700 μl HEPES, pH 7.0, containing 100 μl of octylPOE was added 200 μl of Triton X-100 extracts of yeast mitochondria from the

M-CPTI expression strain. The detergent–protein–lipid mixture was applied to a Biobeads SM-2 column (3 ml) and incubated for 90 min at 4°C to remove the detergent. The proteoliposomes were recovered by low-speed centrifugation. Malonyl-CoA-sensitive CPT activity in the proteoliposomes was measured as described previously [70].

3.2.4 CPT Assay

CPT activity was assayed by the forward exchange method using 1.0 mM L-[^{3}H]carnitine ($\sim$10,000 dpm/nmol) as previously described [32].

3.2.5 Western Blot Analysis

Mitochondria were isolated after 0, 13, 24, and 36 h of methanol induction; proteins were separated by SDS-PAGE [151] in an 8% gel and transferred onto nitrocellulose membranes. Immunoblots were developed by incubation with human heart M-CPTI-specific polyclonal antibodies (1:2000), followed by an anti-rabbit IgG conjugated to horseradish peroxidase. The antigen–antibody complex was detected using an ECL-enhanced chemiluminescence detection kit (Amersham, Arlington Heights, IL).

3.3 RESULTS

3.3.1 High-Level Expression of Human Heart M-CPTI in the Yeast *P. pastoris*

We have successfully expressed the human heart/skeletal muscle isoform of CPTI (M-CPTI) in *P. pastoris*, a yeast that does not have endogenous M-CPTI activity [294]. Isolated mitochondria from the yeast strain expressing the human heart cDNA was malonyl-CoA sensitive and detergent labile, exhibiting a high K_m for carnitine and a low IC_{50} for malonyl-CoA, characteristics similar to those observed with rat heart mitochondria. However, the level of expression of human heart M-CPTI in *P. pastoris* was at least five-fold lower than that observed with rat liver L-CPTI or CPTII in the same expression system. To increase human heart M-CPTI levels in *P. pastoris*, the M-CPTI cDNA was inserted under control of the *P. pastoris*

alcohol oxidase I gene promoter (*AOX1p*) in vector pPICZ-B [123]. This vector contains the antibiotic zeocin-resistant gene which allows for the selection of multiple copy transformants by using a high concentration of zeocin. At 1 mg/ml zeocin, most transformants containing a single copy of the vector die, and only transformants containing additional copies of the vector survive. By dot blot hybridization, one *P. pastoris* strain containing ~24 copies of the human heart M-CPTI expression vector was obtained from 26 zeocin-resistant transformants (Fig. 3.1). The majority of the remaining transformants had between one and three cDNA copy numbers. *P. pastoris* transformants containing one, eight, and 24 copies of the M-CPTI expression vector were grown in liquid medium supplemented with methanol. Mitochondria were prepared by disrupting the cells with glass beads and assaying for CPT activity.

Mitochondria from the strain containing ~24 copies of the vector exhibited a greater than ten-fold increase in CPT activity compared to the activity in the single-copy strain and about 25% higher activity than the medium-copy strain (Fig. 3.2). Immunoblots with the M-CPTI-specific polyclonal antibodies showed the presence of an 80-kDa protein species corresponding to M-CPTI in the mitochondria of the multicopy transformant strain that expressed M-CPTI, but not in the non-CPTI cDNA containing the control strain. The immunoblots demonstrated that the amount of the 80-kDa protein species synthesized increased with the induction time (Fig. 3.3). M-CPTI activity in the multicopy strain was over 93% inhibited by 100 μM malonyl-CoA in isolated mitochondria. Solubilization of the mitochondria with 5% Triton X-100 abolished malonyl-CoA-sensitive M-CPTI activity, a demonstration that the expressed enzyme was CPTI (Fig. 3.4).

3.3.2 Reconstitution of Yeast-Expressed Human Heart M-CPTI

The detergent-inactivated M-CPTI in 5% Triton X-100 was added to a detergent–phospholipid mixture, and the detergent–proteolipid mixture was passed through a Biobeads SM-2 column to remove the detergent and generate proteoliposomes. The reconstituted proteoliposomes exhibited a malonyl-CoA-sensitive M-CPTI activity of 2.4 nmol palmitoylcarnitine formed/mg protein/min (Fig. 3.4), a recovery of about 23% of the activity present in the starting mitochondrial

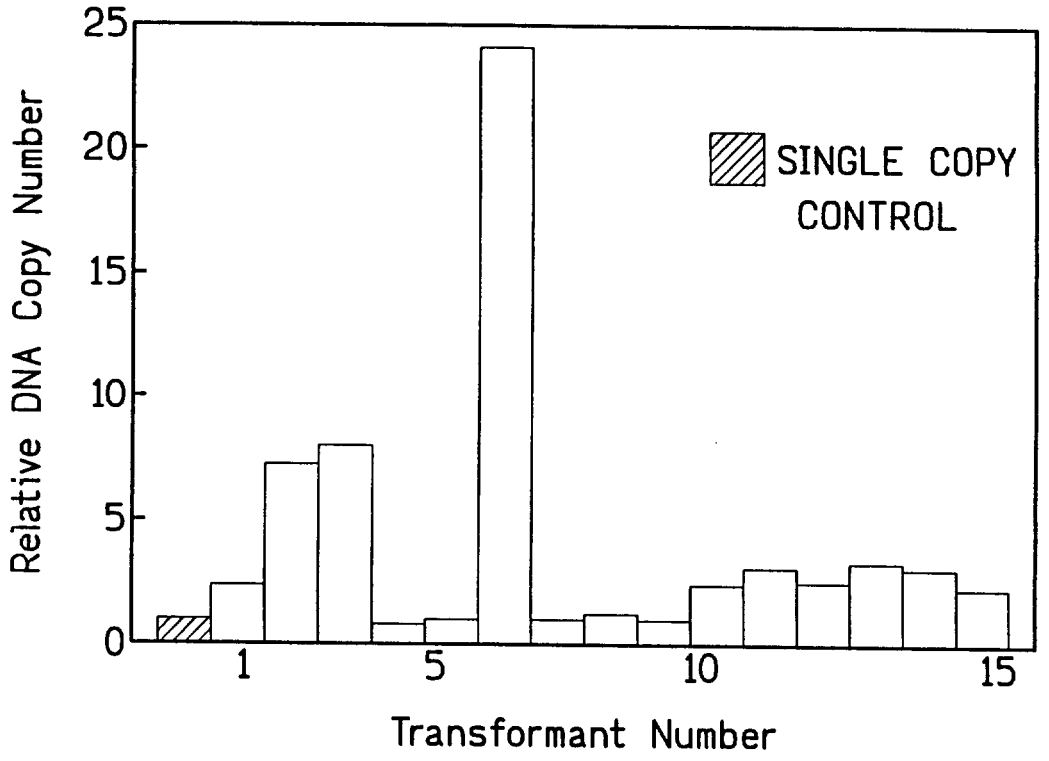

Figure 3.1 Isolation and identification of yeast transformants containing multiple copies of human heart M-CPTI cDNA. Approximately 100 ng of denatured DNA from 15 transformants were blotted onto a nitrocellulose membrane. The DNA was hybridized with the labeled human heart M-CPTI cDNA probe as described in Section 3.2. The dot blots were quantified by PhosphorImage analysis, and the units were divided by the value for the single-copy control to obtain the relative copy number. The DNA of each transformant was normalized to the yeast FDH gene that is not methanol inducible.

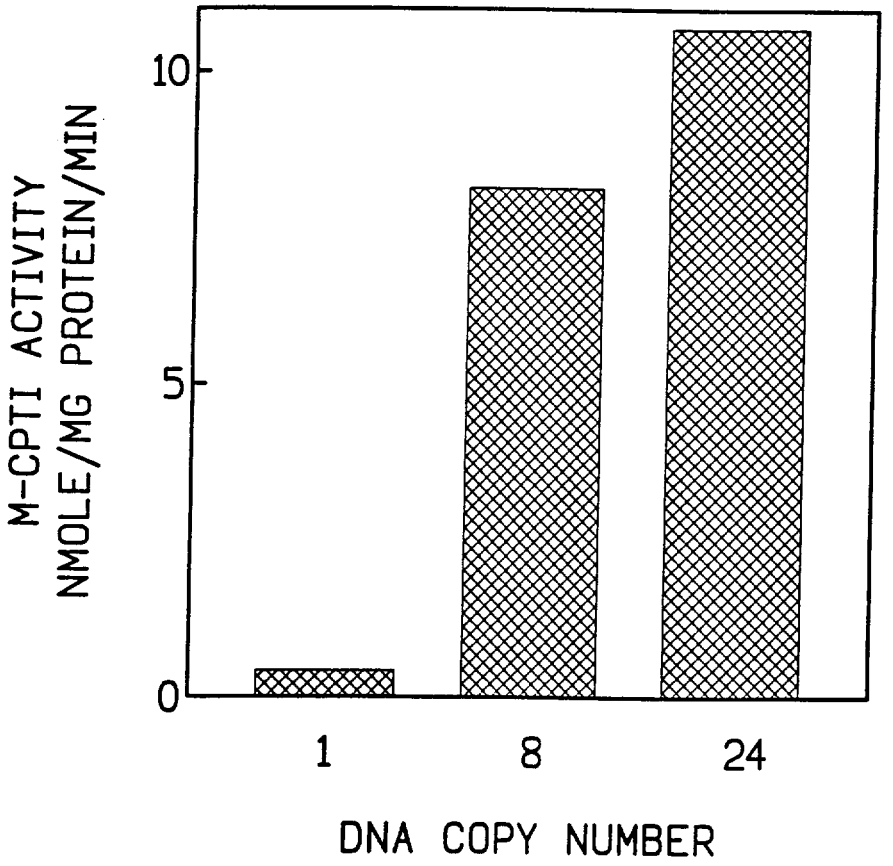

Figure 3.2 High-level expression of human heart M-CPTI in *P. pastoris* with an increase in the M-CPTI cDNA copy number. Yeast transformants with one, eight, and 24 copies of M-CPTI cDNA were cultured in YNB medium plus His supplemented with 0.5% methanol for 24 h as described in Section 3.2. Approximately 350 μg of mitochondrial protein were used for the CPT assay.

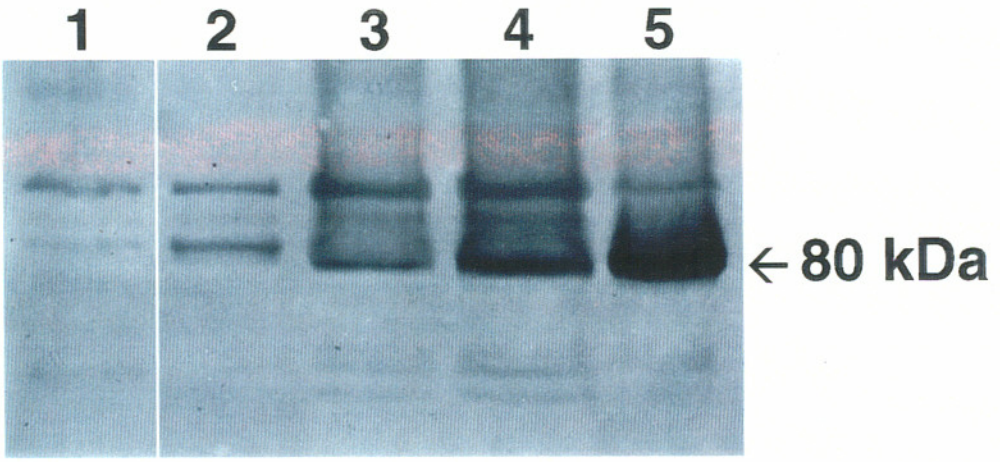

Figure 3.3 Immunoblot showing an increase in the level of expression of human heart M-CPTI with induction time in the yeast *P. pastoris* under control of the *AOXp* gene. Mitochondria (50 μg protein) from the multicopy yeast strain and the control strain with the vector, but without insert, were separated on an 8% SDS-PAGE and blotted onto a nitrocellulose membrane; the immunoblot was developed as described in Section 3.2. *Lane 1*, yeast cell with vector but no insert; *lanes 2–5*, yeast transformant with multicopy M-CPTI cDNA after 5, 13, 24, and 36 h culture, respectively. Arrow indicates the position of the 80-kDa yeast-expressed M-CPTI.

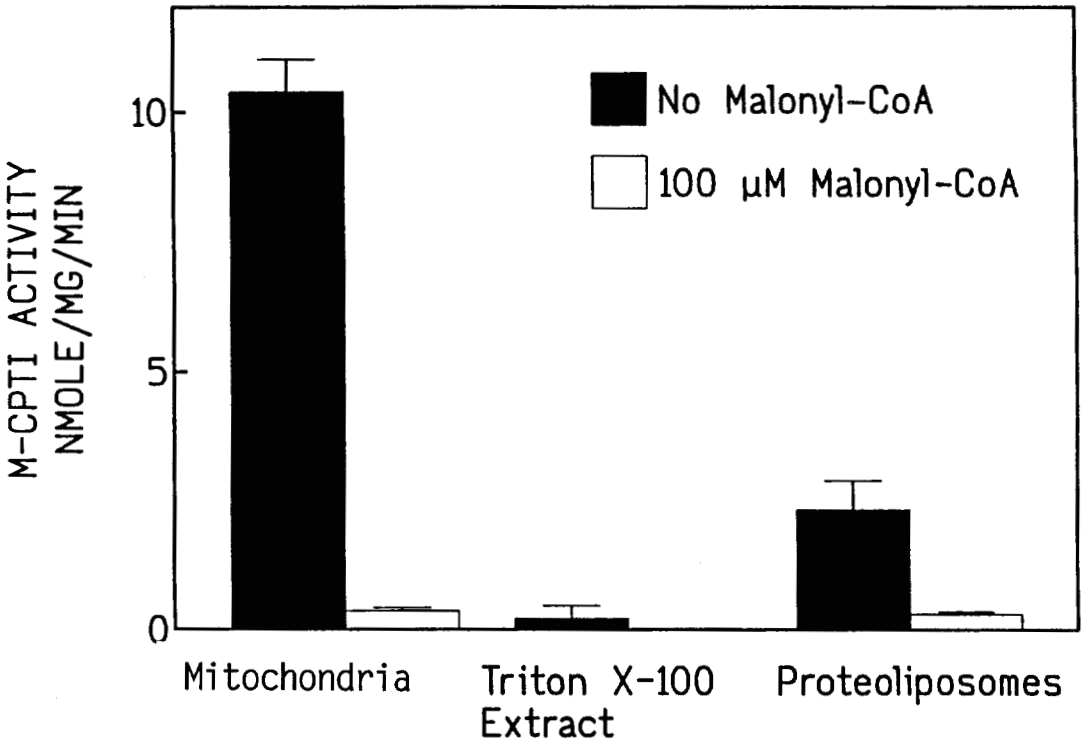

Figure 3.4 Reactivation of detergent-inactivated human heart M-CPTI by reconstitution. Mitochondria were solubilized in 5% Triton X-100 and reconstituted into artificial liposomes by removal of the detergent in the presence of phospholipids on a Biobeads SM-2 column as described in Section 3.2. Mitochondria (170 μg), Triton X-100-solubilized mitochondria (200 μg), and proteoliposomes (100 μg) were assayed for malonyl-CoA-sensitive M-CPTI activity.

preparation and several-fold higher in activity over that observed with the detergent extract. The malonyl-CoA sensitivity of the reconstituted reactivated M-CPTI was 88%, a value close to that observed with M-CPTI in isolated mitochondria prior to solubilization. These results strongly suggest that detergent inactivation of M-CPTI was at least partially reversible.

3.4 DISCUSSION

We report a high level of expression of human heart/skeletal muscle isoform CPTI in the yeast *P. pastoris* under control of the *AOX1p*. Mitochondria from the *P. pastoris* transformant with ~24 copies of the M-CPTI cDNA had CPTI activity that was malonyl-CoA sensitive and detergent labile. We previously expressed human heart M-CPTI in the yeast *P. pastoris* under control of the glyceraldehyde-3-phosphate dehydrogenase gene promoter and demonstrated the presence of a malonyl-CoA-sensitive M-CPTI that was detergent labile. The yeast-expressed M-CPTI had a high K_m for carnitine and a low IC_{50} for malonyl-CoA, characteristics similar to those observed with rat heart mitochondrial CPTI, but different from those of yeast-expressed L-CPTI. However, the level of expression of human heart M-CPTI was lower than that obtained with L-CPTI [70]. As a result, we were unable to demonstrate restoration of malonyl-CoA-sensitive CPT activity in detergent-inactivated M-CPTI by reconstitution.

This is the first report of a high-level synthesis of active human heart M-CPTI in a yeast system that does not have endogenous CPTI activity and reactivation of detergent-inactivated M-CPTI by reconstitution. The kinetic characteristics of the *P. pastoris*-expressed M-CPTI under control of the *AOXp* gene was identical to that expressed under control of the *GAPp* gene, despite the large difference in the level of expression between the two strains [data not shown].

Detergent solubilization of rat liver mitochondria (RLM), rat heart mitochondria (RHM), and yeast-expressed L-CPTI and M-CPTI, but not CPTII, abolishes malonyl-CoA-sensitive CPT activity [31,32,78]. Until recently, it was presumed that CPTI was irreversibly inactivated by detergents, but our reconstitution

studies with the yeast-expressed L-CPTI demonstrate that detergent inactivation is partially reversible [70].

Previous studies from our laboratory and others have demonstrated that solubilization of rat liver, heart, and skeletal muscle mitochondria results in loss of malonyl-CoA-sensitive CPT activity, but not malonyl-CoA binding [32,48,70,166, 225,276,277]. Thus, detergent-solubilized RLM and RHM contain a protein that binds malonyl-CoA and related compounds such as tetradecylglycidyl-CoA and etomoxiryl-CoA that is distinct from the major CPT activity fraction. This binding protein, identified as a 90–94-kDa protein in liver and an 80–82-kDa protein in heart and skeletal muscle, had no CPT activity [31,32,48,78,79,142,166,225,275–277]. Previously, we demonstrated restoration of malonyl-CoA-sensitive CPT activity by reconstitution of purified RLM or RHM malonyl-CoA-binding proteins with detergent-solubilized malonyl-CoA-insensitive CPTII in the presence of phospholipids [48,277]. Using a modified reconstitution procedure, we succeeded in restoring malonyl-CoA-sensitive CPT activity to Triton X-100-solubilized RLM by removal of the detergent in the presence of phospholipids, indicating reactivation of detergent-inactivated L-CPTI (unpublished data). Here, we show that yeast-expressed, detergent-inactivated human heart M-CPTI is also reactivated by detergent removal in the presence of phospholipids similar to that observed with RLM and yeast-expressed L-CPTI [70]. The reconstituted yeast-expressed M-CPTI is more malonyl-CoA-sensitive than the reconstituted RHM CPTI, most likely due to the fact that, unlike mammalian mitochondria that contain a mixture of both CPTs, the yeast mitochondria contain only M-CPTI. Thus, removal of M-CPTI from its membrane lipid environment clearly inactivates this enzyme, and reconstitution via detergent removal in the presence of phospholipids reactivates a portion of the inactive M-CPTI, suggesting that M-CPTI is active only in a membrane environment. Until recently, CPTI (M-CPTI and L-CPTI) were presumed to be irreversibly inactivated by detergents and hence not recoverable as active enzymes [182]. This report establishes for the first time that detergent inactivation of M-CPTI is reversible. Our human heart M-CPTI cDNA encoded a protein of 88 kDa, but on western blot analysis, the polyclonal antibodies cross-reacted with an 80-kDa protein species in mitochondria

isolated from the yeast M-CPTI expression strain. This is in agreement with the abnormal migration behavior on SDS-PAGE reported for heart M-CPTI [78].

Here we have demonstrated for the first time that the yeast-expressed human heart M-CPTI is malonyl-CoA sensitive, detergent labile, and reconstitutable. The ability to reconstitute yeast-expressed M-CPTI is critical to its purification and further studies on the structure and function of this important enzyme. Thus, *P. pastoris* appears to be an excellent model system to investigate human heart M-CPTI.

CHAPTER 4

DELETION OF THE CONSERVED FIRST 18 N-TERMINAL AMINO ACID RESIDUES IN RAT LIVER CARNITINE PALMITOYLTRANSFERASE I ABOLISHES MALONYL-COA SENSITIVITY AND BINDING*

4.1 INTRODUCTION

Mammalian mitochondrial membranes contain two active but distinct carnitine palmitoyltransferases (CPTs), a malonyl-CoA-sensitive, detergent-labile CPTI and a malonyl-CoA-insensitive, detergent-stable CPTII [18,70,182]. CPTI, a rate-limiting enzyme in β-oxidation, catalyzes the conversion of long-chain acyl-CoAs to acylcarnitines in the presence of L-carnitine. As an enzyme that catalyzes the first rate-limiting step in fatty acid oxidation, CPTI is tightly regulated by its physiological inhibitor malonyl-CoA [18,182]. This is an important regulatory mechanism in fatty acid oxidation, because malonyl-CoA is the first intermediate in fatty acid synthesis and suggests coordinated control of fatty acid oxidation and synthesis. Regulation of CPTI by malonyl-CoA provides a mechanism for cellular fuel sensing based on the availability of fatty acids and glucose [212]. Understanding the molecular mechanism of the regulation of CPTI by malonyl-CoA is important in the design of drugs for control of excessive fatty acid oxidation in diabetes mellitus [212] and in myocardial ischemia, where accumulation of acylcarnitines has been associated with arrhythmias [65].

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Shi, J., Zhu, H., Arvidson, D. N., Cregg, J. M., and Woldegiorgis, G. (1998) Deletion of the conserved first 18 N-terminal amino acid residues in rat liver carnitine palmitoyltransferase I abolishes malonyl-CoA sensitivity and binding. *Biochemistry* **37**, 11033-11038.

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 [3,42,266,267,281,282,294]. We have developed a novel high-level expression system for rat L-CPTI and human heart M-CPTI in the yeast *Pichia pastoris*, an organism devoid of endogenous CPT activity [70,293,294]. Using this system, we have demonstrated conclusively that L-CPTI and M-CPTI are active, distinct, malonyl-CoA-sensitive CPTIs that are detergent labile. Previously, L-CPTI and M-CPTI were presumed to be irreversibly inactivated by detergents [182]. However, our reconstitution studies with yeast-expressed L-CPTI and M-CPTI demonstrate that both enzymes are reversibly inactivated by detergents [48,277,293,294]. Our development of a good expression system for CPTI in *P. pastoris* has enabled us to begin to map the malonyl-CoA binding site by mutational analysis as described below.

Amino acid sequence alignment of human and rat heart M-CPTI, human and rat L-CPTI, and human and rat liver CPTII sequences [3,34,77,83,172,274,281,282, 294] reveals a conserved N-terminal sequence of 124 residues with two putative transmembrane domains (Fig. 4.1, shaded area) that is present in all known CPTI sequences, but is absent from CPTII. Based on limited proteolysis studies of intact rat liver mitochondria and activity studies with immobilized impermeable substrate and inhibitor, a model for the membrane topology of L-CPTI has been proposed that predicts exposure of the N- and C-termini, domains crucial for activity and malonyl-CoA sensitivity of L-CPTI, on the cytosolic side of the outer mitochondrial membrane [88]. Furthermore, it is predicted that the two transmembrane domains of L-CPTI are separated by a 30 amino acid linker (residues 76–103) that is located within the mitochondrial intermembrane space (Fig. 4.1). It is hypothesized that malonyl-CoA sensitivity of CPTI may reside in these 124 N-terminal amino acid residues, thus forming the structural basis for the malonyl-CoA sensitivity of CPTI. To test this hypothesis, we constructed six deletion mutants of the first 130 N-terminal amino acid residues of rat L-CPTI, ranging in size from 18 to 130 residues.

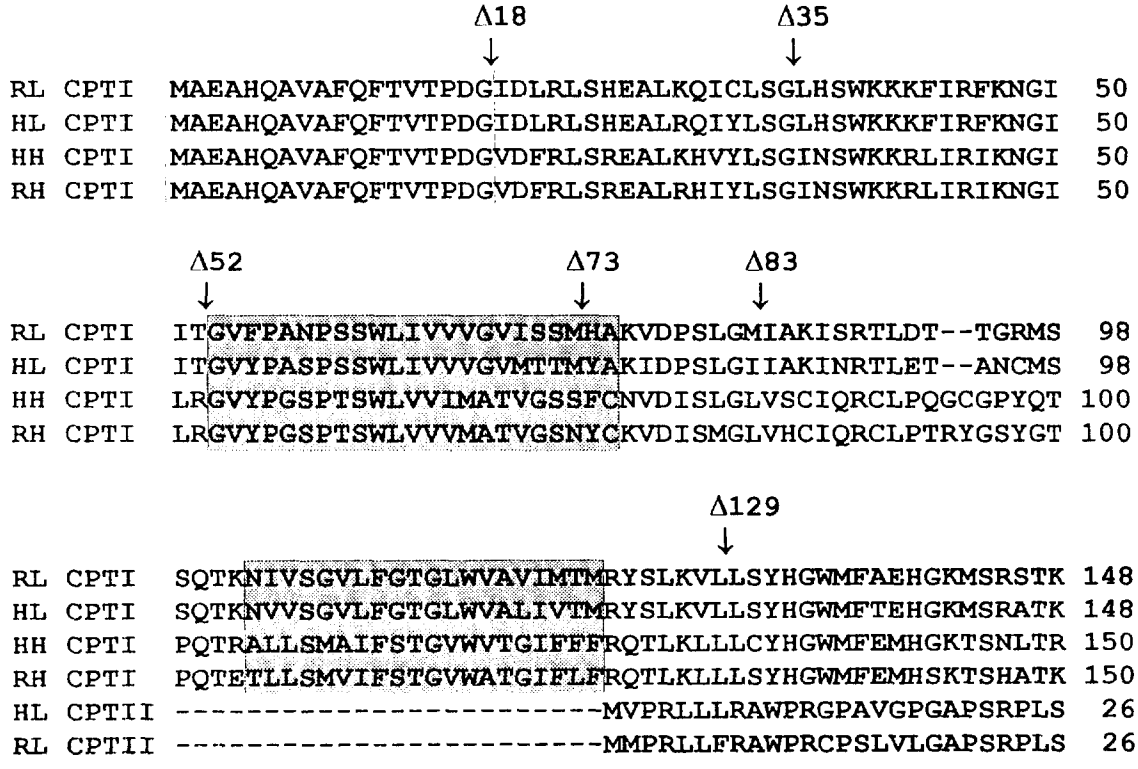

Figure 4.1 N-terminal amino acid sequence lineup of human and rat CPTs. The shaded areas represent the position of the two predicted membrane-spanning domains of all known CPTIs. The position of the conserved 18 N-terminal amino acid residues within the CPTIs is boxed. The position of each of the deletion mutants is shown by an arrow. RL, HL—rat, human liver; HH, RH—human, rat heart. Sources of the sequences from the data bank were from references 3, 172, 281, 282, and 294 as indicated in the text.

4.2 EXPERIMENTAL PROCEDURES

4.2.1 Construction of Plasmids for the N-terminal Deletion Mutants of Rat L-CPTI

The cDNA used and the construction of the plasmid (wild type) for rat liver CPTI expression in *P. pastoris* is described in our previous publication [70]. A *Hin*dIII–*Kpn*I fragment (1–566 bp; cDNA sequence [70,77]) was excised from pYGW9, a plasmid containing the full-length rat L-CPTI in pUC119 to generate the plasmid pGYW12. A 637-bp *Hin*dIII–*Eco*RI fragment was polymerase chain reaction (PCR) amplified using the plasmid pYGW9 as a template with the primers: RL655, 5′-CCACCAGGATTTTAGCT-3′, and RLD18, 5′-CTTCACAAGCTTGAATTC-**ATG**ATTGACCTCCGCCTGAGC-3′. An ATG start codon (shown in bold) was added immediately after the *Eco*RI site. The PCR product was digested with *Hin*dIII and *Kpn*I, then ligated into pYGW12 to generate plasmid pYGW13. An *Eco*RI fragment of pYGW13 containing the deletion mutant RLD18 (Δ18) was then ligated into the *Eco*RI-cut *P. pastoris* expression vector pHW10 [70,264]. The DNA sequences of the deletions were confirmed by sequencing.

Deletion mutants RLD35 (Δ35), RLD52 (Δ52), RLD72 (Δ72), RLD83 (Δ83) and RLD130 (Δ129) (Fig. 4.1) were constructed in a manner similar to Δ18, using primer RL655 above and the following primers for each deletion mutant:
Δ35, 5′-AAACAGAAGCTTGAATTCATGCTGCACTCCTGGAAGAAG-3′;
Δ52, 5′-CAAGAAGCTTGAATTCATGGGTGTGTTCCCCGCGAA-3′;
Δ72, 5′-GTGGGTAAGCTTGAATTCATGCATGCCAAAGTGGAC-3′;
Δ83, 5′-GGACAAGCTTGAATTCATGATCGCAAAGATCA; and
Δ129 5′-TGCGCAAGCTTGAATTCATGCTGCTCTCCTACCACGGCT-3′,
respectively, with plasmid pYGW9 as a template to generate 585-, 533-, 474-, 439-, and 299-bp *Hin*dIII–*Kpn*I PCR fragments. All subsequent procedures were identical to those used for construction of Δ18.

The expression plasmids were linearized and integrated into the HIS4 locus of *P. pastoris* strain GS115 by electrotransformation [12]. Histidine prototrophic

transformants were selected on YND plates and grown on YND medium, and mitochondria were isolated from both the wild-type and deletion mutant L-CPTIs as described previously [70].

4.2.2 CPT Assay

CPT activity was assayed by the forward exchange method using L-[^{3}H]carnitine as previously described [32]. The standard assay reaction mixture contained in a total volume of 0.5 ml: 0.2 mM L-[^{3}H]carnitine (~10,000 dpm/nmol), 50 μM palmitoyl-CoA, 20 mM HEPES (pH 7.0), 1% fatty acid-free albumin, and 40–75 mM KCl, with or without malonyl-CoA as indicated in the figure legends and tables. Reactions were initiated by addition of mitochondria. The reaction was linear up to 6 min, and all incubations were performed at 30°C for 5 min. Reactions were stopped by addition of 6% perchloric acid and centrifuged at 2000 rpm for 7 min. The resulting pellet was suspended in water, and the product, [^{3}H]palmitoylcarnitine, was extracted with butanol at low pH. After centrifugation at 2000 rpm for 2 min, an aliquot of the butanol phase was transferred to a vial for radioactive counting.

4.2.3 ^{14}C-Malonyl-CoA Binding Assay

^{14}C-Malonyl-CoA binding was determined by a modified centrifugation assay as described previously [166]. Isolated mitochondria from wild-type and deletion mutants were suspended in 0.5 ml of ice cold medium composed of 72 mM sorbitol, 25 mM KCl, and 10 mM HEPES (pH 7.0). This was followed by addition of 0.1–1000 nM [2-^{14}C]malonyl-CoA and the suspension was incubated at 4°C for 30 min with periodic vortexing. After 30 min, bound and free malonyl-CoA were separated by centrifugation at 14,000 rpm for 30 min at 4°C. The mitochondrial pellet was superficially washed twice (without resuspension by slow addition of ice-cold buffer to the wall of the tube) and centrifuged as described above. The pellet was then solubilized in 300 μl of 2% SDS and quantitatively transferred to a vial for radioactive counting. The final malonyl-CoA binding values for the wild-type and deletion mutants were corrected for background malonyl-CoA binding by the yeast

control strain which carried the vector, but without the CPTI insert. No correction for non-specific binding was made, as suggested by Mendel and Mendel [186]. Binding analysis was performed using Graph Pad software and a nonlinear regression procedure. The CPT activity and IC_{50} values are given as a mean $\pm$ SD for at least three independent assays with different preparations of mitochondria.

4.2.4 Western Blot Analysis

Proteins were separated by SDS-PAGE in a 7.5% gel and transferred onto nitrocellulose membranes. Immunoblots were developed by incubation with the CPTI-specific polyclonal antibody (1:2500 dilution) followed by an anti-rabbit IgG conjugated to horseradish peroxidase as described previously [70]. The antigen–antibody complex was detected using an ECL-enhanced chemiluminescence detection kit (Amersham, Arlington Heights, IL).

4.2.5 Other Materials and Procedures

DNA sequencing was performed at the Oregon Regional Primate Research Center core facility using an automatic DNA sequencer [228]. Protein was determined by the Lowry procedure [165]. All restriction enzymes were from New England Biolabs (Beverly, MA). L-[^{3}H]Carnitine and [2-^{14}C]malonyl-coenzyme A were from Amersham. Nucleotides were from Pharmacia (Piscataway, NJ), palmitoyl-CoA was from Boehringer Mannheim (Indianapolis, IN) and malonyl-CoA was from Sigma (St. Louis, MO).

4.3 RESULTS

4.3.1 Generation of Deletion Mutants and Expression of Wild-type and Deletion Mutants in *P. pastoris*

Construction of plasmids carrying the N-terminal deletion mutants of rat L-CPTI was performed as described in Section 4.2, and the deletions were confirmed by DNA sequencing. The positions of the N-termini of the L-CPTI deletion mutants are indicated in Fig. 4.1. The deletions range from the smallest, 18, to the largest,

129 amino acid residues. *P. pastoris* was chosen as an expression system for L-CPTI and the deletion mutants, because it does not have endogenous CPT activity [70,293,294]. The *P. pastoris* expression plasmids expressed L-CPTI under control of the *P. pastoris* glyceraldehyde-3-phosphate dehydrogenase gene promoter [70,264]. *P. pastoris* strains transformed with the wild-type L-CPTI gene and the deletion mutants were grown in liquid medium supplemented with glucose. As previously reported [70], no CPT activity was found in the control yeast strain with the vector, but without the CPT cDNA insert.

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

All of the deletion mutants except Δ129 retained significant CPT activity, which was 64–69% of that observed with the wild-type yeast strain expressing L-CPTI (Table 4.1). Δ129 had no CPT activity. In agreement with our previous report [70], the IC_{50} for malonyl-CoA inhibition of the wild-type strain expressing L-CPTI was 2.0 μM, while the IC_{50} for the minimal deletion mutant 18 was 380 μM, representing a 190-fold decrease in malonyl-CoA sensitivity compared to the wild-type strain (Fig. 4.2). Deleting 35, 52, 73 and 83 amino acid residues from the N-terminus increased the IC_{50} for malonyl-CoA sensitivity of L-CPTI in each of the mutants from 2.0 μM in the wild-type strain to 170–300 μM in the deletion mutants, thus decreasing the malonyl-CoA sensitivity by 85–150-fold (Table 4.1). Δ18 showed decreased malonyl-CoA sensitivity at all levels of the inhibitor tested compared to the wild type, as shown in Fig. 4.2. Except for Δ129 which had no CPT activity [data not shown], this was also true for Δ35, Δ52, Δ73, and Δ83. Δ18 exhibited normal saturation kinetics when the carnitine concentration was varied relative to a constant second substrate, palmitoyl-CoA, a property identical to that of wild-type L-CPTI previously reported from our laboratory [70]. For Δ18, the calculated K_m for carnitine was 31 μM and the V_{max} was 6.5 nmol min^{-1} mg^{-1} of protein which is similar to the wild-type L-CPTI [70]. With respect to the second substrate, palmitoyl-CoA, Δ18 showed non-Michaelis–Menten saturation kinetics, characteristics similar to yeast-expressed wild-type L-CPTI [70]. Thus, deletion of the first 18 N-terminal

Table 4.1

Activity, Malonyl-CoA Sensitivity, and Malonyl-CoA Binding in Yeast Strains Expressing Wild-type L-CPTI and N-terminal Deletion Mutants[a]

Strain	Activity (nmol/mg · min)	IC_{50} (μM)	K_D (nM)	B_{max} (pmol/mg)
Wild type	7.8 ± 0.5[b]	2.0 ± 0.2	1.1	1.9
Δ18	5.3 ± 0.6	380 ± 30	70	2.2
Δ35	5.0 ± 0.2	200 ± 20	240	0.4
Δ52	5.4 ± 0.6	170 ± 20	420	2.6
Δ73	5.1 ± 0.4	180 ± 20	880	3.1
Δ83	5.2 ± 0.8	300 ± 30	no binding	—
Δ129	no activity	—	no binding	—

[a]Mitochondria were isolated from the yeast strains separately expressing L-CPTI, and the deletion mutants and were assayed for CPT activity, malonyl-CoA sensitivity, and binding as described in Section 4.2. The IC_{50} is the concentration of malonyl-CoA needed to inhibit 50% of the activity of the yeast-expressed L-CPTI, and results are mean ± SD of at least four independent experiments with different mitochondrial preparations. The K_D and B_{max} values are averages of two independent experiments with different mitochondrial preparations.

[b]In intact rat liver mitochondria, values for CPTI activity under similar assay conditions are 1.0–3.0 nm min^{-1} mg^{-1} of protein [32,166].

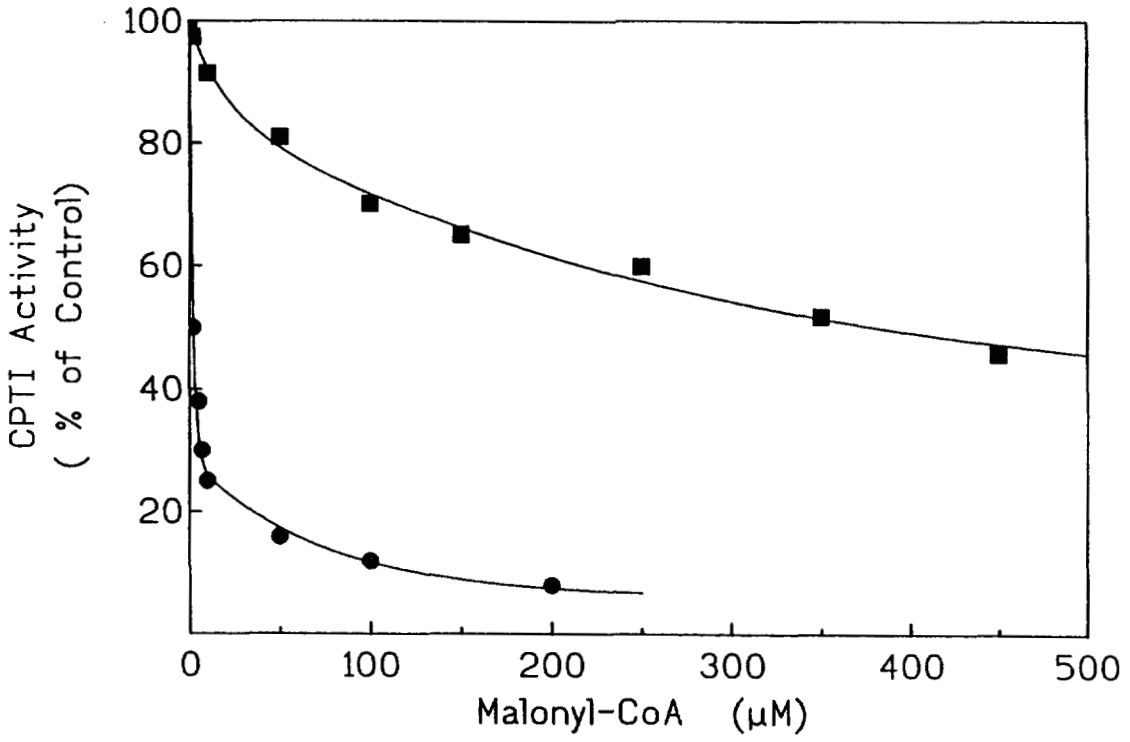

Figure 4.2 Effect of increasing concentrations of malonyl-CoA on the activities of yeast-expressed wild-type L-CPTI and Δ18. ● = wild type; ■ = Δ18. Approximately 150 μg of mitochondrial protein were used for the assay.

residues from L-CPTI appears to abolish malonyl-CoA sensitivity of L-CPTI with minimal effect on the catalytic activity of the enzyme.

4.3.3 ^{14}C-Malonyl-CoA Binding in Yeast-Expressed L-CPTI and Deletion Mutants

A significant increase in K_D values was observed for all of the deletion mutants compared to the wild-type strain when [^{14}C]malonyl-CoA binding was determined in isolated mitochondria from the yeast strains expressing wild-type and mutant L-CPTI. The K_D value for Δ18 was 70-fold higher (1.1 nM versus 70 nM) than that of the wild type (Table 4.1). Δ35, Δ52, Δ73, Δ83, and Δ129 had K_D values of 240–1000-fold higher than that of the wild type (Table 4.1). The stepwise increase in K_D values for the observed deletion mutants suggests a direct effect of the deletions on malonyl-CoA binding.

Malonyl-CoA binding to the mitochondria from the yeast strains expressing the wild-type and deletion mutants clearly resolved into a high affinity site (wild-type) and a low affinity site (deletion mutants) as shown in Figs. 4.3 and 4.4, and in both cases was saturable. Our studies also show small increases in B_{max} with an increase in the number of residues deleted, as shown in Table 4.1, except for Δ35 where a decrease in B_{max} was observed.

4.3.4 Effect of Deletions on Protein Expression

Western blot analysis of wild-type L-CPTI (88 kDa) and the deletion mutants using a polyclonal antibody directed against a maltose-binding protein–L-CPTI fusion protein [70] is shown in Fig. 4.5. For Δ18, Δ51, Δ72, and Δ83, proteins of predicted sizes were synthesized, but they had 30% lower activity than the wild type. Δ35 was barely detectable on the immunoblots, but it had 64% of the activity of the wild type and was insensitive to malonyl-CoA inhibition. Δ129 was completely inactive, but was synthesized with the predicted size protein, as shown by the immunoblot.

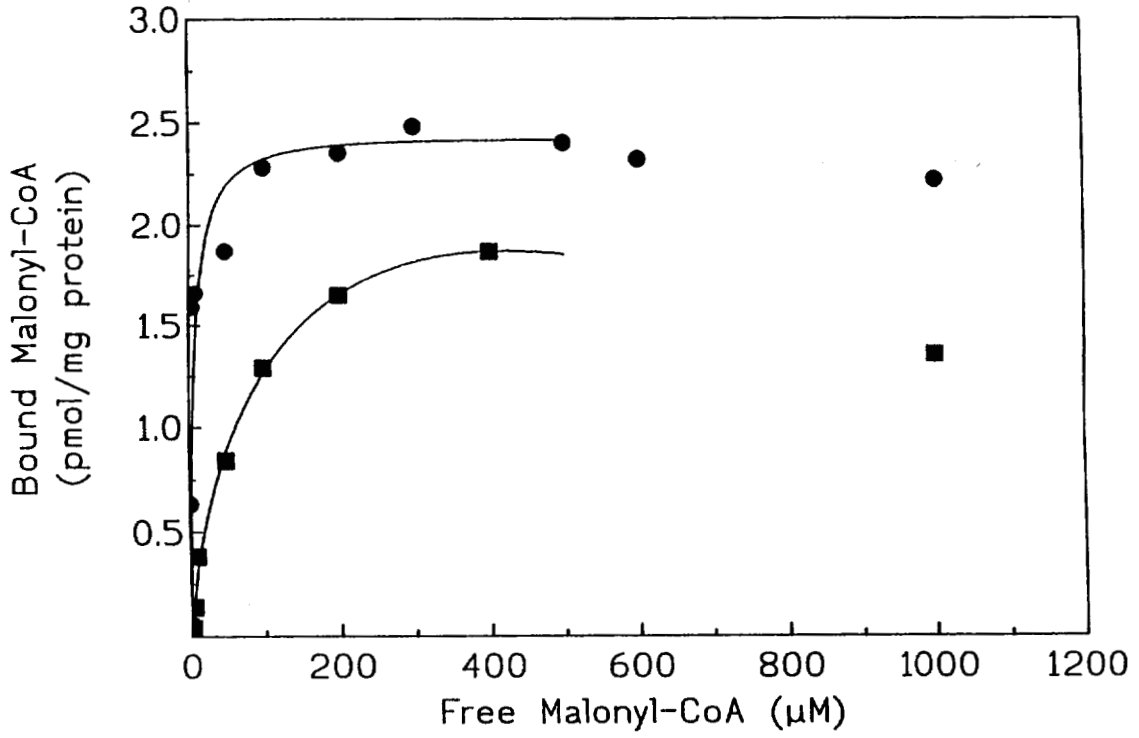

Figure 4.3 Binding of ^{14}C-malonyl-CoA to mitochondria isolated from the yeast strain expressing the wild type and Δ18. Approximately 240 μg of protein were used for the binding assay. ● = wild type; ■ = Δ18. Malonyl-CoA binding values for the wild-type and deletion mutants were corrected for malonyl-CoA binding to the mitochondria from the yeast strain with the vector but no insert.

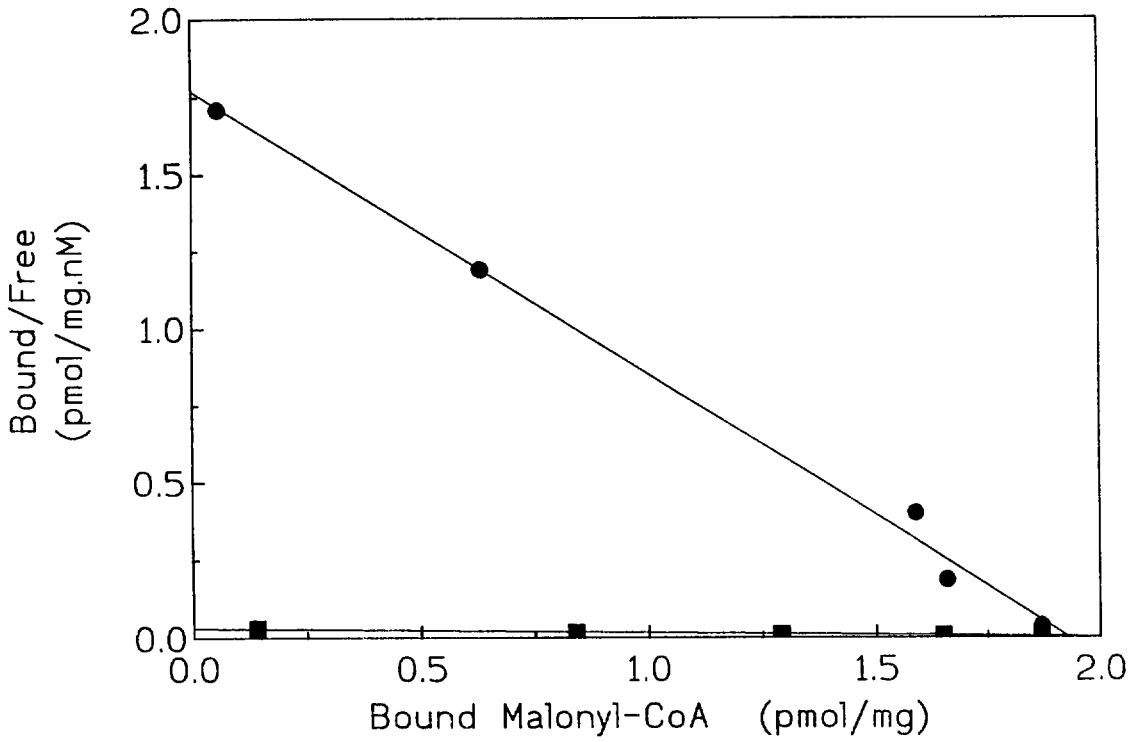

Figure 4.4 Scatchard plots for binding of ^{14}C-malonyl-CoA to mitochondria from yeast strains expressing wild-type (●) and Δ18 (■) for L-CPTI.

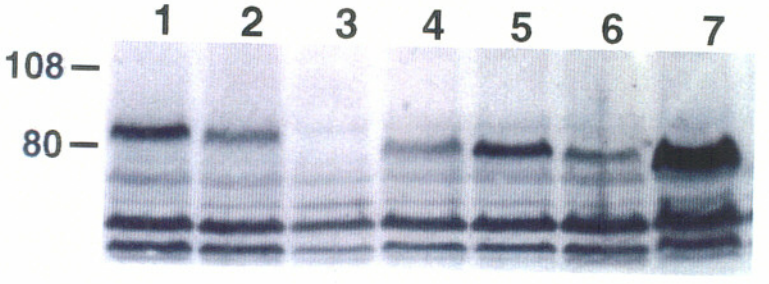

Figure 4.5 Immunoblot showing expression of wild-type and deletion mutant L-CPTIs in the yeast *P. pastoris*. Mitochondria (100 μg of protein) from the wild-type yeast strain and the strains expressing each of the deletion mutants were separated on an 8% SDS-PAGE and blotted onto a nitrocellulose membrane. The immunoblot was developed as described in the experimental procedures. Lane 1, wild-type L-CPTI; lanes 2, 3, 4, 5, 6, and 7 represent Δ18, Δ35, Δ52, Δ73, Δ83, and Δ129, respectively.

4.4 DISCUSSION

Mitochondria from the yeast strain expressing Δ18 had 64% of the activity of the wild-type L-CPTI, but were insensitive to malonyl-CoA inhibition and had very low affinity for malonyl-CoA. Furthermore, the decrease in malonyl-CoA sensitivity (increase in IC_{50}) was accompanied by a decrease in the affinity for malonyl-CoA (increase in K_D). No additional loss of malonyl-CoA sensitivity was detectable with increasing deletion size. However, increased loss of binding energy was observed with increasing deletion size. The 70-fold lower affinity seen in Δ18 corresponds to 2.3 kcal/mol in lost binding energy. The increasingly larger deletions exhibited additional, albeit smaller, losses of binding energy (Δ35, +0.7 kcal/mol; Δ52, +0.3 kcal/mol; Δ73, +0.4 kcal/mol). It should be noted that Δ73, when compared to Δ18, shows only 1.4 kcal/mol further reduction in binding energy. These results suggest that, while Δ18 abolishes malonyl-CoA sensitivity, significant affinity (K_D = 70 nM) for malonyl-CoA binding remains. Since the affinity for malonyl-CoA binding decreased incrementally with the number of residues deleted, we suggest that amino acid residues between 19 and 129 may make weak contacts important for malonyl-CoA binding.

Earlier malonyl-CoA binding studies in isolated liver and heart mitochondria suggested the presence of two classes of malonyl-CoA binding sites, i.e., high- and low-affinity sites in each tissue [23,188]. Furthermore, inhibition of CPTI by malonyl-CoA was proposed to be mediated by the high-affinity binding sites in both tissues, which were suggested to be separate from the catalytic sites. The possibility of multiple classes of malonyl-CoA binding sites or cooperativity between sites was also raised. A previous attempt to express a mutant L-CPTI that lacked the first 82 N-terminal residues was described by Brown et al. [39], but results were inconclusive due to low expression levels [70]. Interestingly, the residual malonyl-CoA sensitivity shown by the deletion mutants is similar to that observed with yeast-expressed CPTII [70], suggesting that, for these mutants, malonyl-CoA inhibits via direct interaction with the active site.

The yeast strains expressing the deletion mutants synthesized proteins of the predicted size as shown by the immunoblot, except for Δ35 which was barely detectable on immunoblots. Δ35 had 64% of the wild-type activity, but the synthesized mutant protein may be unstable and quickly degraded due to improper folding. Δ129 had no measurable CPT activity and had extremely low malonyl-CoA binding, but the predicted size protein was synthesized as indicated by the strong signal on the immunoblot. The synthesized protein was located in the mitochondria of the yeast strain expressing each deletion mutant.

All of the deletion mutants except Δ129 had 65–70% of the activity of the wild type, indicating that deletion of up to 83 residues from the N-terminus of L-CPTI had minimal effect on palmitoyl-CoA binding as a substrate, thus suggesting the existence of two separate acyl-CoA binding sites, a malonyl-CoA and palmitoyl-CoA binding site. The observed 30–35% loss in activity is likely due to changes in the tertiary structure of the enzyme, caused by lack of interaction of the N-terminal domain with the catalytic C-terminal domain as a result of N-terminal residue deletions or a reduction in the expression level.

The complete loss of CPT activity observed with Δ129 could be due to loss of the palmitoyl-CoA binding site or synthesis of an unstable, improperly folded mutant enzyme that is catalytically inactive. Alternatively, it could be due to change in the interaction of CPTI with the outer mitochondrial membrane as a result of the large deletion that includes the two predicted transmembrane domains [39,88].

The B_{max} for binding of malonyl-CoA to the mitochondria from the yeast strains expressing the deletion mutants showed a slight increase with the number of residues deleted, a pattern similar to that observed in liver outer mitochondrial membranes from fasted and diabetic rats [97]. We suggest that the small increase in B_{max} observed with the loss of the high-affinity site for malonyl-CoA in the deletion mutants may be due to an increase in the abundance of the low-affinity site. Of the mutants studied, only Δ35 had a lower B_{max} than wild-type L-CPTI. The decrease in B_{max} observed with Δ35 could be due to the extremely low level of synthesis of this mutant protein which was barely detectable on immunoblotting. Our studies suggest that the malonyl-CoA binding site is separate from the catalytic site and that the first

conserved 18 N-terminal amino acid residues are critical for malonyl-CoA binding and inhibition.

Deletion of the first 18 N-terminal amino acid residues decreases binding at the high-affinity inhibitor binding site, but has no effect on the catalytic site. Further deletions decrease affinity for malonyl-CoA at the high-affinity malonyl-CoA binding site without further affecting the active site. Thus, deletion of the conserved 18 amino acid residues uncouples the high-affinity malonyl-CoA binding site from the catalytic site. Deletion of the conserved first 18 N-terminal residues of heart M-CPTI, which are identical to those of L-CPTI, also decreased the malonyl-CoA sensitivity of M-CPTI, indicating the same residues play a critical role in sensitizing heart and liver enzymes to malonyl-CoA [unpublished observation]. However, the magnitude of the loss in malonyl-CoA sensitivity of M-CPTI, as a result of the 18 residue deletion, was lower than that of liver L-CPTI, suggesting the involvement of unknown additional residues in the high malonyl-CoA sensitivity of heart M-CPTI. Previous studies have demonstrated that mammals contain two isoforms of CPTI, referred to as liver L-CPTI and muscle M-CPTI, that are 62% identical in amino acid sequence [3,281,282,294]. Isolated mitochondria from rat heart and yeast strains expressing human heart muscle M-CPTI exhibited a high K_m for carnitine and a low IC_{50} for malonyl-CoA [172,294]. In adult rat heart, the total carnitine level is high; consequently, M-CPTI has a high K_m for carnitine [42]. Although rat heart has a measurable level of malonyl-CoA, it is much lower than that of rat liver [182]. Since the IC_{50} for malonyl-CoA inhibition of heart mitochondrial M-CPTI is at least 30-fold lower than that of rat liver L-CPTI, the level of malonyl-CoA reported in heart [182] is sufficient to significantly inhibit fatty acid oxidation. It is estimated that about 60–80% of the energy requirement of the heart is derived from fatty acid oxidation [268]. The important question in cardiac tissue metabolism then is how can fatty acid oxidation proceed in heart in the presence of such high tissue levels of malonyl-CoA?

CHAPTER 5

A SINGLE AMINO ACID CHANGE (SUBSTITUTION OF GLUTAMATE-3 WITH ALANINE) IN THE N-TERMINAL REGION OF RAT LIVER CARNITINE PALMITOYLTRANSFERASE I ABOLISHES MALONYL-COA INHIBITION AND HIGH AFFINITY BINDING*

5.1 INTRODUCTION

Carnitine palmitoyltransferase I (CPTI) catalyzes the conversion of long-chain acyl-CoA to acylcarnitines in the presence of L-carnitine, the first reaction in the transport of long-chain fatty acids from the cytoplasm to the mitochondria, a rate-limiting step in β-oxidation [18,182]. 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 [3,42,266,267,281,282,294]. As an enzyme that catalyzes the first rate-limiting step in fatty acid oxidation, CPTI is regulated by its physiological inhibitor malonyl-CoA [18,182], the first intermediate in fatty acid synthesis, suggesting coordinated control of fatty acid oxidation and synthesis. Understanding the molecular mechanism of the regulation of CPTI by malonyl-CoA is important in the design of drugs for control of excessive fatty acid

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Shi, J., Zhu, H., Arvidson, D. N., and Woldegiorgis, G. (1999) A single amino acid change (substitution of glutamate-3 with alanine) in the N-terminal region of rat liver carnitine palmitoyltransferase I abolishes malonyl-CoA inhibition and high-affinity binding. *J. Biol. Chem.* **274**, 9421–9426.

oxidation in diabetes mellitus [212] and in myocardial ischemia where accumulation of acylcarnitines has been associated with arrhythmias [65].

We developed a novel high-level expression system for rat L-CPTI and human heart M-CPTI in the yeast *Pichia pastoris*, an organism devoid of endogenous CPT activity [70,293,294]. Using this system, we demonstrated conclusively that L-CPTI and M-CPTI are active, distinct, malonyl-CoA-sensitive CPTIs that are reversibly inactivated by detergents. We recently showed that deletion of the conserved first 18 N-terminal amino acid residues of rat L-CPTI abolishes malonyl-CoA inhibition and high affinity malonyl-CoA binding [231]. In this study, we have constructed and characterized rat L-CPTI deletion mutants of the first 12 and 6 N-terminal amino acid residues. To identify specific residue(s) involved in malonyl-CoA binding and inhibition of L-CPTI, we also constructed three substitution mutations within the conserved first 6 N-terminal amino acid residues ($Glu^3 \rightarrow Ala$, $His^5 \rightarrow Ala$, and $Gln^6 \rightarrow Ala$).

5.2 EXPERIMENTAL PROCEDURES

5.2.1 Construction of Plasmids for the N-terminal Point and Deletion Mutants of Rat L-CPTI

The cDNA used and the construction of the plasmids for the wild-type, deletion, and point mutants for rat L-CPTI expression in *P. pastoris* was as described in our previous publications [70,293]. A *Hin*dIII–*Kpn*I fragment (1–566 bp; cDNA sequence [70,77]) was excised from pYGW9, a plasmid containing the full-length rat L-CPTI in pUC119 to generate the plasmid pGYW12. The Δ12 mutant was constructed by polymerase chain reaction (PCR) amplification of a 662-bp *Hin*dIII–*Eco*RI fragment using the plasmid pYGW9 as a template with the primers RL665 (5′-CCACCAGGATTTTAGCT-3′) and RLD12 (5′-CAAGCTAAGCTTGAA-TTC**ATG**ACTGTCACCCCCGATGGCAT-3′). *Hin*dIII and *Eco*RI enzyme restriction sites were introduced in primer RLD12. An ATG start codon (shown in bold type) was added immediately after the *Eco*RI site and before the fourth amino acid alanine. The PCR product was digested with *Hin*dIII and *Kpn*I and then ligated into pYGW12

to generate plasmid pYGWD12. An *Eco*RI fragment of pYGWD12 containing the mutant Δ12 was then ligated into the *Eco*RI-cut *P. pastoris* expression vector pHW10 [70,264]. The DNA sequences of the deletion and point mutants were confirmed by sequencing.

Mutants Δ6 and Δ18 and point mutants $Glu^3 \rightarrow Ala$, $His^5 \rightarrow Ala$, and $Gln^6 \rightarrow Ala$ were constructed in a similar manner as Δ12, using primer RL665 above and the following primers for each deletion and point mutant. The new translation start site or mutated amino acid codon is shown in bold: RLD6, 5-GCGATGAAGCTTGAAT-TC**ATG**GCTGTGGCCTTCCAGTTC3′; RLD18, 5′-CTTCACAAGCTTGAATTC-**ATG**ATTGACCTCCGCCTGAGC-3′; RLE3A, 5′CCCAAGCTTGAATTCATGGCA-**GCG**GCTCACCAAGCTGTGGC-3′; RLH5A, 5′-CCCAAGCTTGAATTCATGGCA-GAGGCT**GCC**CAAGCTGTGGCC-TTC-3; and RLQ6A, 5′CCCAAGCTTGAATT-CATGGCAGAGGCTCAC**GCA**GC-TGTGGCCTTCCAGTT-3′. All subsequent procedures were identical to those used for construction of Δ12.

The expression plasmids were linearized and integrated into the HIS4 locus of *P. pastoris* strain GS115 by electrotransformation [12]. Histidine prototrophic transformants were selected on YND plates and grown on YND medium. Mitochondria were isolated from the wild-type and mutant L-CPTIs as described previously [70].

5.2.2 CPT Assay

CPT activity was assayed by the forward exchange method using L-[^{3}H]carnitine as previously described [32,70]. 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/albumin [208,213]. The K_i for malonyl-CoA inhibition of the yeast-expressed wild-type and mutant (E3A) L-CPTIs was determined by assaying CPT activity with varying concentrations of palmitoyl-CoA (12.5, 25, 50, 75, and 100 μM) in the presence of 0, 1, 2, and 5 μM malonyl-CoA (wild type), or 0, 2, 25, and 200 μM malonyl-CoA (E3A), respectively. The concentration of the second substrate, carnitine, was fixed

at 200 μM in all the assays. 106 μg (wild type) and 118 μg (E3A) of mitochondrial protein were used, and all incubations were performed at 30°C for 3 min.

5.2.3 [^{14}C]-Malonyl-CoA Binding Assay

[^{14}C]-Malonyl-CoA binding was determined by a modified centrifugation assay as described previously [166,231]. Isolated mitochondria from wild type and mutants were suspended in 0.5 ml of ice-cold medium composed of 72 mM sorbitol, 60 mM KCl, 25 mM Tris/HCl (pH 6.8), 1.0 mM EDTA, 1.0 mM dithiothreitol, 1.3 mg/ml fatty acid-free bovine serum albumin [7]. This was followed by addition of 0.1–1000 nM [2-^{14}C]malonyl-CoA, and the suspension was incubated at 4°C for 30 min with periodic vortexing. All subsequent procedures were as described previously [231]. The CPT activity and IC_{50} values are given as a mean $\pm$ S.D. for at least three independent assays with different preparations of mitochondria. The K_D values are averages of at least two independent experiments.

5.2.4 Western Blot Analysis

Proteins were separated by SDS-PAGE in a 7.5% gel and transferred onto nitrocellulose membranes. Immunoblots were developed by incubation with the CPTI-specific polyclonal antibody (1:4000 dilution) followed by an anti-rabbit IgG conjugated to horseradish peroxidase (1:10,000 dilution) as described previously [70]. The antigen–antibody complex was detected using an ECL-enhanced chemiluminescence detection kit (Amersham Pharmacia Biotech, Arlington Heights, IL).

5.2.5 Other Materials and Procedures

DNA sequencing was performed at the Oregon Regional Primate Research Center core facility using an automatic DNA sequencer [228]. Protein was determined by the Lowry procedure [165]. All restriction enzymes were from New England Biolabs (Beverly, MA). L-[^{3}H]Carnitine and [2-^{14}C]malonyl-coenzyme A were from Amersham Pharmacia Biotech. Nucleotides were from Pharmacia

(Piscataway, NJ), palmitoyl-CoA was from Boehringer Mannheim (Indianapolis, IN) and malonyl-CoA was from Sigma (St. Louis, MO).

5.3 RESULTS

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

Construction of plasmids carrying the N-terminal deletions Δ6 and Δ12 and point mutants $Glu^3 \rightarrow Ala$, $His^5 \rightarrow Ala$, and $Gln^6 \rightarrow Ala$ of rat L-CPTI (Fig. 5.1) was performed as described in Section 5.2. 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 [70,231,293,294]. The *P. pastoris* expression plasmids expressed L-CPTI under control of the *P. pastoris* glyceraldehyde-3-phosphate dehydrogenase gene promoter [70,264]. Yeast transformants with the wild-type L-CPTI gene and the mutants were grown in liquid medium supplemented with glucose. As previously reported [70], no CPT activity was found in the control yeast strain with the vector but without the CPTI cDNA insert.

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 [70] is shown in Fig. 5.2. For the wild-type and all the deletion and point mutants (Δ18, Δ12, Δ6, $Glu^3 \rightarrow Ala$, $His^5 \rightarrow Ala$, and $Gln^6 \rightarrow Ala$), proteins of predicted sizes were synthesized and were expressed at similar steady-state levels.

5.3.2 Effect of Mutations on L-CPTI Activity and Malonyl-CoA Inhibition

All of the mutants retained significant CPT activity, which was 60–80% of that observed with the wild-type yeast strain expressing L-CPTI (Table 5.1). The IC_{50} for malonyl-CoA inhibition of the wild-type strain expressing L-CPTI was 2.0 μM, in agreement with our previous report [70], while the IC_{50} for Δ6, Δ12, and the point mutant $Glu^3 \rightarrow Ala$ was 200 μM, representing a 100-fold decrease in malonyl-CoA sensitivity compared to the wild-type strain (Table 5.1). The K_i for malonyl-CoA inhibition of the $Glu^3 \rightarrow Ala$ mutant L-CPTI was approximately 10-fold higher than that

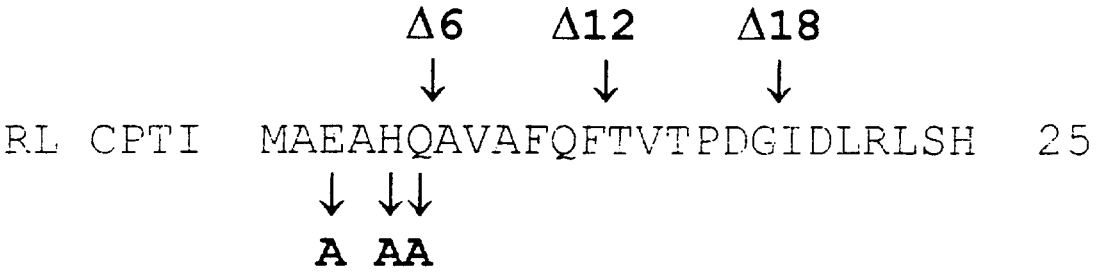

Figure 5.1 The amino acid sequence of the first 25 N-terminal residues of rat L-CPTI. The position of each of the deletion and point mutants is shown by an arrow. Sources of the sequences from the data bank were from refs. 70 and 77 as indicated in the text.

Table 5.1

Activity, Malonyl-CoA Sensitivity, and Malonyl-CoA Binding in Yeast Strains Expressing Wild-Type L-CPTI, N-Terminal Deletion, and Point Mutants

Strain	Activity* (nmol/ mg · min)	IC_{50} (μM)	K_{D1} (nM)	K_{D2} (nM)	B_{max1} (pmol/mg)	B_{max2} (pmol/mg)
wild-type L-CPTI	7.8±0.5	2.0±0.2	1.0	33	10	110
Mutants						
Q6A	5.5±0.5	2.0±0.2	-	-	-	-
H5A	5.1±0.5	25±3	102	500	15.9	33.6
E3A	5.5±0.5	200±20	-	3.7×10^3	-	103
Δ6	4.7±0.7	197±20	-	6.0×10^3	-	113
Δ12	4.7±0.7	210±20	-	-	-	-

Mitochondria were isolated from the yeast strains separately expressing L-CPTI, and the deletion and point mutants were assayed for CPT activity, malonyl-CoA sensitivity, and binding, as described in Section 5.2. The IC_{50} is the concentration of malonyl-CoA needed to inhibit 50% of the activity of the yeast-expressed L-CPTI, and results are mean ± S.D. 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.

* In intact rat liver mitochondria, values for CPTI activity under similar assay conditions are 1.0–3.0 nm/min/mg protein [32,166].

of the wild type (32 μM versus 2.7 μM), a trend similar to the increase in IC_{50} for malonyl-CoA inhibition observed in the Glu^3→Ala mutant. Mutation of histidine residue 5 to alanine increased the IC_{50} for malonyl-CoA inhibition to 25 μM compared to the 2.0 μM value of the wild-type strain, representing a mild 12.5-fold decrease in malonyl-CoA sensitivity, whereas mutation of glutamine residue 6 to alanine had no effect on malonyl-CoA sensitivity (Table 5.1). Δ6 and point mutant Glu^3→Ala showed decreased malonyl-CoA sensitivity at all levels of the inhibitor tested compared to the wild type (Fig. 5.3), whereas only minor reduction in malonyl-CoA sensitivity was observed in the point mutant His^5→Ala compared to the control.

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

The Glu^3→Ala mutant exhibited normal saturation kinetics when the carnitine concentration was varied relative to a constant second substrate, palmitoyl-CoA, a property identical to that of wild-type L-CPTI (Fig. 5.4A) and similar to that previously reported from our laboratory [70]. For the Glu^3→Ala mutant, the calculated K_m for carnitine was 74.5 μM and the V_{max} was 8.3 nmol/min/mg protein, which is similar to the 45 μM and 12.6 nmol/min/mg for wild-type L-CPTI [70]. With respect to the second substrate, palmitoyl-CoA, the wild-type, but not the Glu^3→Ala mutant, showed non-Michaelis–Menten saturation kinetics at a fixed concentration of albumin (1% W/V) (Fig. 5.4B), characteristics similar to our previous report [70]. However, both the wild-type and the Glu^3→Ala mutant exhibited normal saturation kinetics when the molar ratio of palmitoyl-CoA:albumin was fixed at 6.1:1 (Fig. 5.4C). The calculated K_m for palmitoyl-CoA for the point mutant Glu^3→Ala was 69.4 μM and the V_{max} was 23.3 nmol/min/mg, which is similar to the wild-type values of 104 μM and 41.5 nmol/min/mg. Thus, deletion of the first 6 N-terminal amino acid residues or a substitution mutation of Glu residue 3 to Ala in L-CPTI abolishes malonyl-CoA sensitivity, but not catalytic activity. Mutation of glutamine residue 6 to alanine had no effect on malonyl-CoA inhibition or catalytic activity.

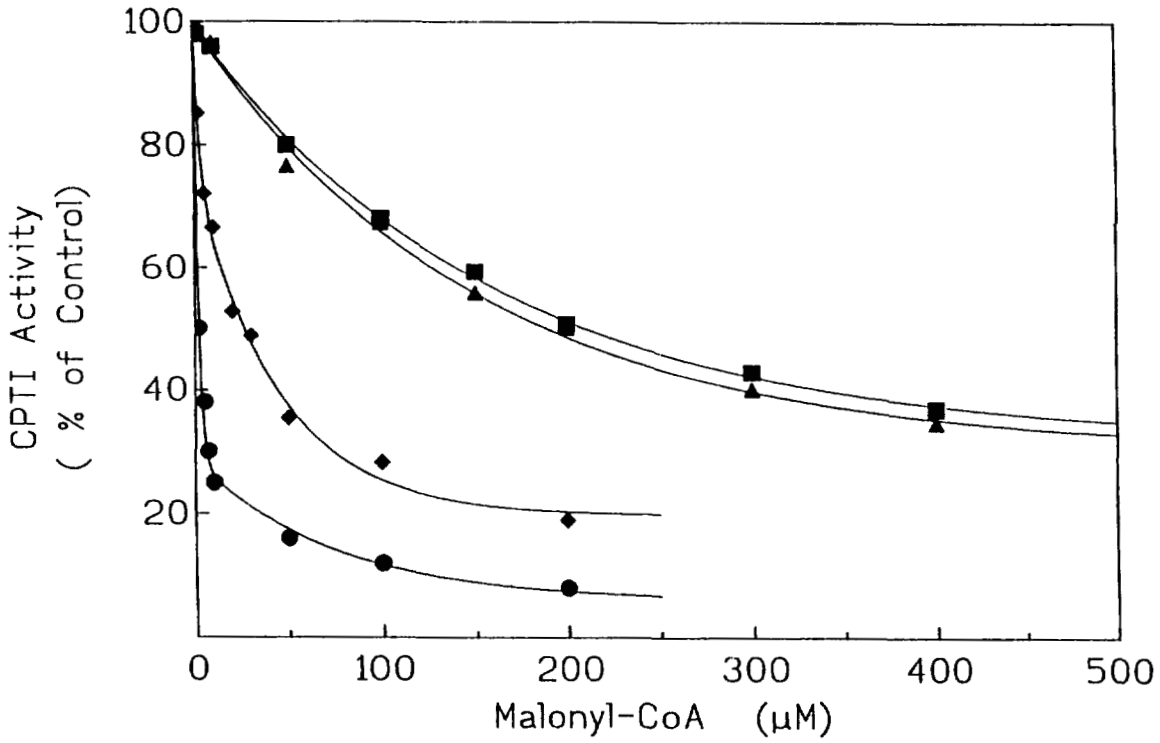

Figure 5.3 Effect of increasing concentrations of malonyl-CoA on the activities of yeast-expressed wild-type and mutant L-CPTI. Approximately 150 μg of mitochondrial protein were used for the assay. • = wild type; ■ = Δ6; ▲ = Glu^3→Ala; ◆ = and His^5→Ala.

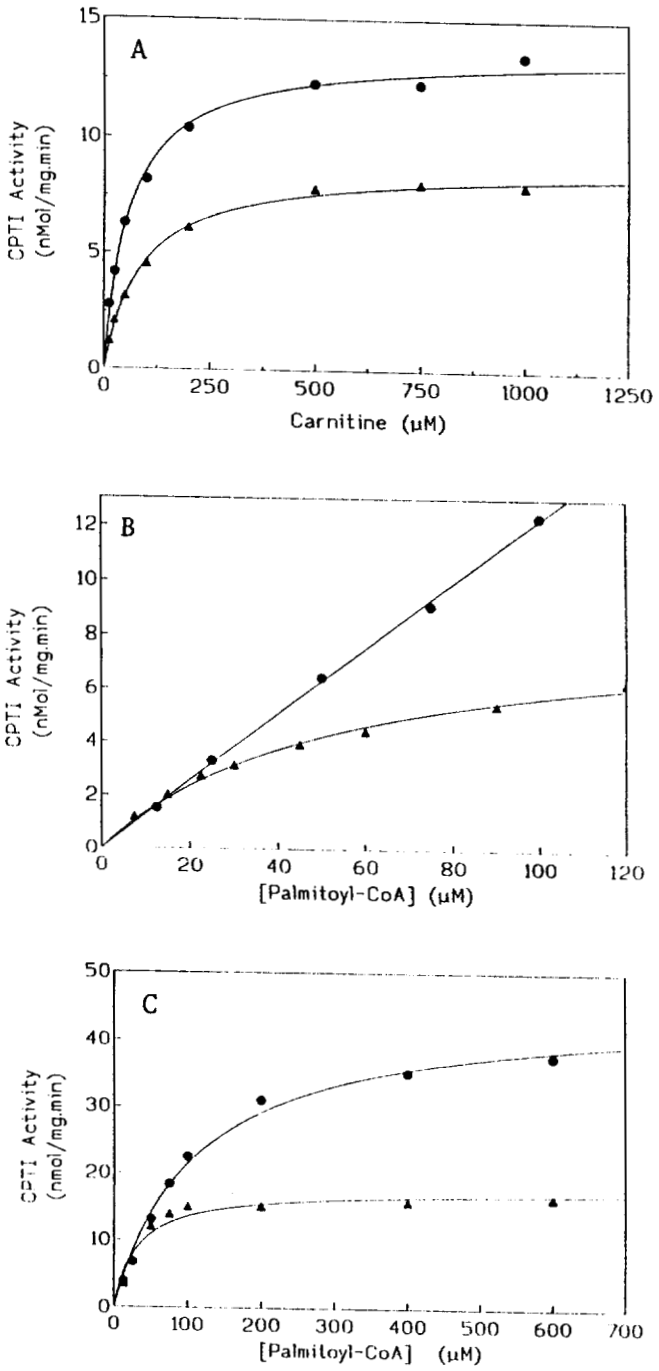

Figure 5.4 Kinetic analysis of wild-type and Glu^3→Ala mutant L-CPTI activities. Isolated mitochondria (150 μg protein) from the yeast strains expressing the wild-type (•), and the Glu^3→Ala (▲) mutant L-CPTI were assayed for CPTI activity in the presence of increasing concentrations of carnitine and palmitoyl-CoA as described in Section 5.2. The figure shows the resulting dose–response curves for L-CPTI. (A) carnitine; (B) palmitoyl-CoA with fixed albumin concentration (1% W/V); (C) palmitoyl-CoA with fixed molar ratio of palmitoyl-CoA/albumin (6.1:1).

5.3.4 [^{14}C]-Malonyl-CoA Binding in Yeast-Expressed Mutant L-CPTIs

Malonyl-CoA binding to the mitochondria from the yeast strains expressing Δ6 and point mutants Glu^3→Ala and His^5→Ala was significantly lower compared with that observed in the mitochondria from the wild-type strain but was saturable (Fig. 5.5). Malonyl-CoA binding clearly resolved into a high affinity and a low affinity site in the mitochondria from the wild-type and point mutant His^5→Ala as shown by the Scatchard plots in Figs. 5.6A and B, but only very-low affinity binding was observed in the mitochondria from point mutant Glu^3→Ala and Δ6 (Fig. 5.6B). Deletion of the first 6 N-terminal residues or substitution of glutamic acid 3 with alanine completely abolished high affinity malonyl-CoA binding (K_{D1}), and further decreased the low affinity malonyl-CoA binding (K_{D2}) by 100-fold (Table 5.1). Furthermore, the loss of high affinity malonyl-CoA binding observed with the Glu^3→Ala mutant correlates with the increase in IC_{50} and K_i for inhibition of L-CPTI by malonyl-CoA. Although the mutations increased the K_{D2} for the low affinity malonyl-CoA binding site (Δ6 and Glu^3→Ala), there was no change in the calculated B_{max2} (which was 10-fold higher than the B_{max1} of the wild type), suggesting that the observed loss in malonyl-CoA sensitivity and binding observed is not due to decreased abundance of the second malonyl-CoA-binding entity of L-CPTI.

Substitution of histidine 5 with alanine showed a moderate loss in malonyl-CoA sensitivity of L-CPTI (12.5-fold), resulting in loss of the high affinity and a significant decrease in low affinity malonyl-CoA binding (Table 5.1). The low affinity binding site in mutant His^5→Ala resolved into two classes of binding sites (Fig. 5.6B), with the K_{D1} and K_{D2} for the mutant being 100- and 16-fold higher, respectively, than the corresponding wild-type values (Table 5.1). The calculated B_{max1} for mutant His^5→Ala was close to the wild-type value, but B_{max2} was three-fold lower, indicating a decrease in the malonyl-CoA binding entity of L-CPTI. These studies suggest that histidine 5 in L-CPTI is one of the residues involved in malonyl-CoA binding and inhibition of the enzyme.

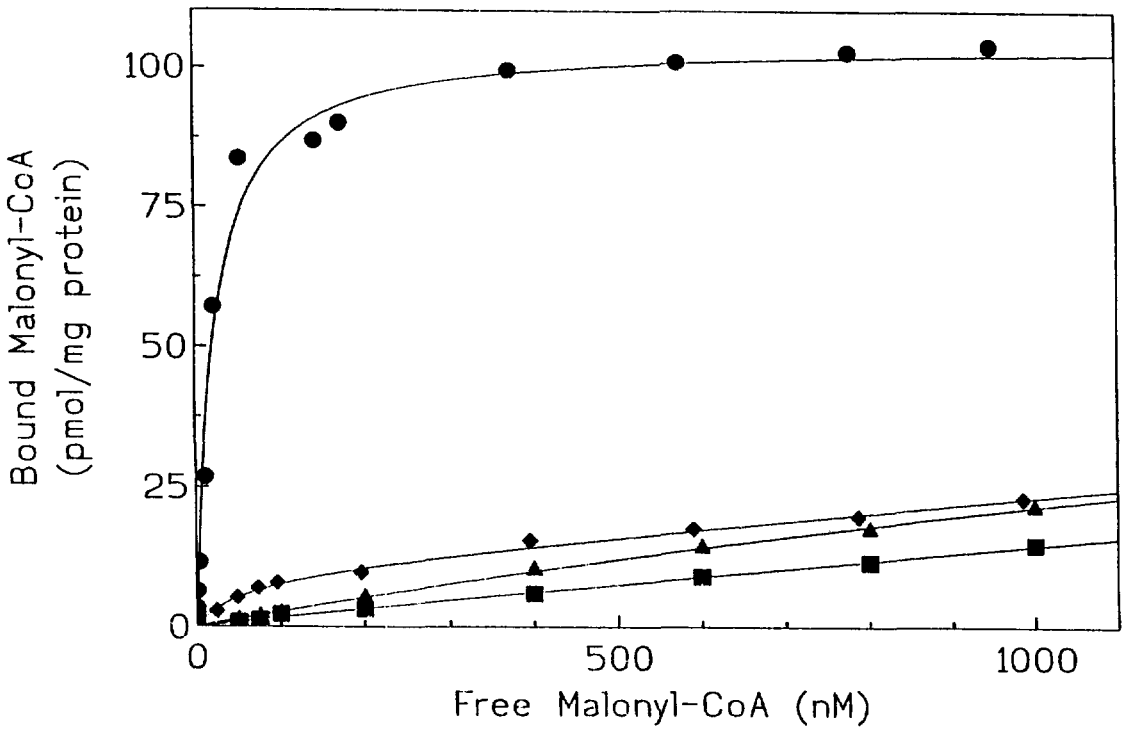

Figure 5.5 Binding of [^{14}C]-malonyl-CoA to mitochondria isolated from the yeast strain expressing the wild-type L-CPTI, Δ6, and point mutants Glu^{3}→Ala and His^{5}→Ala. Approximately 200 μg of protein were used for the binding assay. Malonyl-CoA binding values for the wild-type and deletion mutants were corrected for malonyl-CoA binding to the mitochondria from the yeast strain with the vector but no insert. • = wild type; ■ = Δ6; ▲ = Glu^{3}→Ala; and ◆ = His^{5}→Ala.

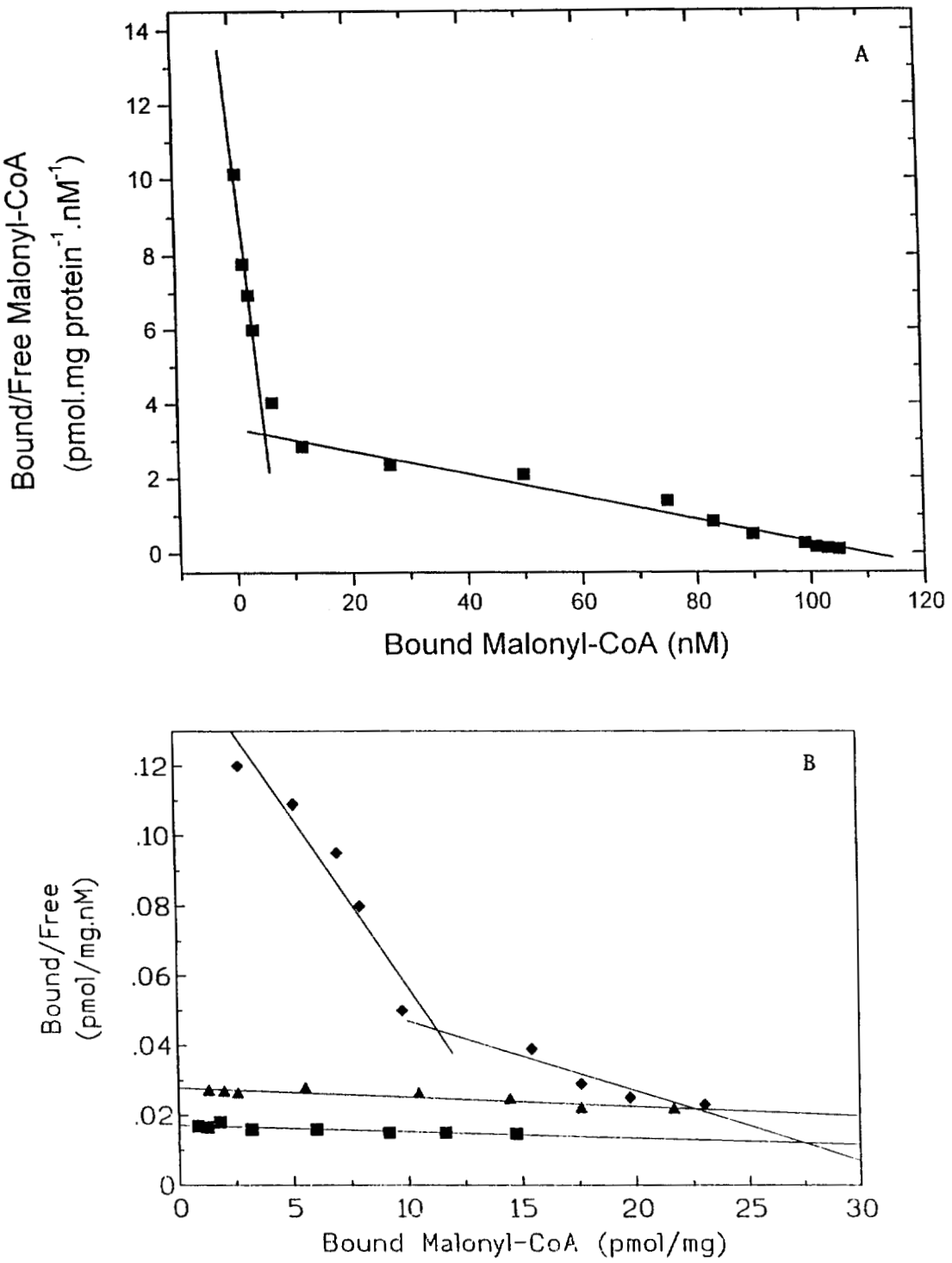

Figure 5.6 **(A)** Scatchard plot for binding of [^{14}C]-malonyl-CoA to mitochondria from yeast strains expressing wild-type L-CPTI. **(B)** Scatchard plot for binding of [^{14}C]-malonyl-CoA to mitochondria from yeast strains expressing mutant L-CPTIs Δ6 (■), Glu^{3}→Ala (▲), and His^{5}→Ala (◆).

5.4 DISCUSSION

To determine the role of the first 130 N-terminal amino acid residues of rat L-CPTI in malonyl-CoA sensitivity and binding, we previously constructed a series of deletion mutants and demonstrated that a mutant lacking the first conserved 18 N-terminal amino acid residues had activity and kinetic properties similar to those of wild-type L-CPTI but had completely lost malonyl-CoA sensitivity and high affinity binding [231]. Based on these previous studies [231], we report here on deletion mutations of the conserved first 12 and 6 N-terminal residues of L-CPTI. Like Δ18, Δ12 and Δ6 had 60–70% of the wild-type activity and showed loss of both malonyl-CoA sensitivity and high affinity malonyl-CoA binding, indicating that residue(s) essential for malonyl-CoA binding and sensitivity reside within the conserved first 6 N-terminal amino acids. Of these conserved first 6 N-terminal amino acids, including the start codon Met, residues 2 and 4 are Ala, residue 3 is Glu, residue 5 is His, and residue 6 is Gln. Therefore, we constructed mutants with substitutions of Glu^3 with Ala, His^5 with Ala, and Gln^6 with Ala of L-CPTI.

The mutant L-CPTI with a replacement of Glu^3 with Ala had a phenotype similar to the N-terminal deletion mutants. The mutation resulted in complete loss of malonyl-CoA sensitivity and high affinity malonyl-CoA binding, and a decrease in the low affinity malonyl-CoA binding. In contrast, substitution of Glu^3 with Ala did not have a significant effect on the kinetic properties of the enzyme, because there was no change in the K_m value for palmitoyl-CoA and only a slight increase in the K_m value for carnitine. The 29–40% loss in catalytic activity observed with the deletion and point mutants compared with the wild type could be due to a reduction in the expression level or lack of interaction of the N-terminal domain with the catalytic domain as a result of the N-terminal mutations. A protein of the expected size (88 kDa) was detected in the mitochondria of the $Glu^3 \rightarrow$Ala mutant strain on immunoblotting with L-CPTI specific antibodies. These results demonstrate clearly that Glu^3 in the wild-type L-CPTI is essential for malonyl-CoA inhibition and binding but not for catalysis, because the kinetic properties of the mutant enzyme are virtually

indistinguishable from those of the wild type. This is the first report to demonstrate the critical role of Glu^3 residue of L-CPTI for malonyl-CoA sensitivity and binding.

The high affinity site (K_{D1}, B_{max1}) for binding of malonyl-CoA to L-CPTI was completely abolished in the Glu^3→Ala, Δ6, and Δ18 mutants, suggesting that the > 100-fold decrease in malonyl-CoA sensitivity observed in these mutants was due to the loss of the high affinity binding entity of the enzyme. Although low affinity malonyl-CoA binding was weakened, there was no change in the B_{max2} value between wild-type L-CPTI and mutants Glu^3→Ala, Δ6, and Δ18, suggesting that the residual malonyl-CoA sensitivity observed in the mutants was due to the low affinity malonyl-CoA binding entity of the enzyme. The results of this study provide strong evidence implicating Glu^3 as one of the residues involved in high affinity malonyl-CoA binding. We hypothesize that the Glu^3→Ala substitution may disrupt a hydrogen bonding network or a salt bridge, perhaps to a residue near the active site of CPTI. As the high affinity site is abolished and binding to the low affinity site is weakened, the two sites may partially overlap. Alternatively, the possible loss of a salt bridge may weaken K_{D2} indirectly.

Replacement of His^5 with Ala had a much less drastic effect on the IC_{50} for malonyl-CoA inhibition of L-CPTI but severely diminished both high and low affinity malonyl-CoA binding. The B_{max1} for this mutant showed a slight increase, but B_{max2} showed a significant decrease compared to the wild-type value, suggesting that the 10-fold lower IC_{50} for malonyl-CoA inhibition observed with this mutant, compared with mutants Glu^3→Ala, Δ6, and Δ18, may be due to a slight increase in abundance of the high affinity binding entity with a lowered (100-fold) affinity for malonyl-CoA. The decrease in low affinity malonyl-CoA binding observed for the His^5→Ala mutant (~ 15-fold increase in K_{D2}) may be due, in part, to the decreased abundance of the low affinity binding entity of the enzyme (~ 3-fold decrease in B_{max2}). Because mutation of His^5→Ala reduced the malonyl-CoA sensitivity and binding, L-CPTI may be affected by pH. A pH-induced shift in malonyl-CoA sensitivity has been reported for CPTI [188,239].

Our data clearly demonstrate that there are two classes of malonyl-CoA binding sites in L-CPTI, namely, a high affinity and a low affinity binding site,

similar to earlier studies in isolated rat liver and heart mitochondria [23,188]. A previous attempt to express a mutant L-CPTI that lacked the first 82 N-terminal residues was described by Brown et al. [39], but results were inconclusive due to extremely low expression levels [70]. The residual malonyl-CoA sensitivity shown by the deletion mutants is similar to that observed with yeast-expressed CPTII [70], suggesting that for these mutants malonyl-CoA may inhibit via direct interaction with the active site. Additional studies are needed to determine whether the active site acts as a low affinity malonyl-CoA binding site, but our data suggest that there may be some overlap between the malonyl-CoA and palmitoyl-CoA binding sites. In the absence of malonyl-CoA, free CoA (50 μM) and acetyl-CoA (500 μM) inhibited the activities of both the wild-type and the $Glu^3 \rightarrow Ala$ mutant L-CPTI by 50% (unpublished observation). Since a total loss of the high affinity malonyl-CoA binding site was observed in the $Glu^3 \rightarrow Ala$ mutant, the results suggest that CoA and acetyl-CoA inhibit by binding to the active site or the low affinity malonyl-CoA binding site. At high concentrations, both CoA and the substrate palmitoyl-CoA reduce the inhibition of L-CPTI by malonyl-CoA [32,61], suggesting partial overlap between the malonyl-CoA and the substrate binding sites.

Based on limited proteolysis studies of intact and outer membrane rat liver mitochondria, a model for the membrane topology of L-CPTI has been proposed that predicts exposure of 90% of L-CPTI, including N and C termini domains crucial for activity and malonyl-CoA sensitivity of the enzyme on the cytosolic side of the outer mitochondrial membrane [88]. A more recent detailed deletion mutation analysis study of the 129 N-terminal amino acid residues of the yeast-expressed L-CPTI from our laboratory clearly demonstrated that residues critical for malonyl-CoA inhibition and binding of L-CPTI are located within the conserved first 18 N-terminal amino acid residues of the enzyme [231]. In this study, we demonstrate that glutamic acid residue 3 and histidine 5 are essential for malonyl-CoA binding and inhibition.

Limited proteolysis of intact and outer membrane preparations of rat liver mitochondria result in a marked loss in L-CPTI activity and malonyl-CoA sensitivity [88,139], accompanied by the cleavage of the extreme N terminus (<1 kDa) of L-CPTI [88]. Mitochondria isolated from fasted and diabetic rat livers, metabolic

conditions with increased fatty acid oxidation, exhibit increased L-CPTI activity and decreased malonyl-CoA sensitivity [58]. Furthermore, insulin reverses the effects of diabetes on L-CPTI activity and malonyl-CoA sensitivity [58]. Thus, fasting and diabetes, metabolic conditions that enhance protein degradation, reduce the sensitivity of CPTI to malonyl-CoA inhibition [58,135,207]. The L-CPTI gene in INS-1 cells may be an early response gene like *c-fos* [8], suggesting that the enzyme may be subject to metabolic regulation by proteolysis [251], employing the cytosolic ubiquitin–proteasome system [251]. Diabetes is a pathophysiologic condition associated with increased protein degradation, fatty acid oxidation, CPTI activity, and decreased malonyl-CoA inhibition of CPTI [207]. Thus limited *in vivo* proteolysis of L-CPTI induced by diabetes, such as cleavage of the first 6 N-terminal residues, may decrease malonyl-CoA sensitivity and alter the normal control of hepatic fatty acid oxidation. Insulin inhibits proteasome activity resulting in decreased cellular protein degradation and controlled fatty acid oxidation [116]. We are currently conducting *in vitro* partial proteolysis studies with yeast-expressed L-CPTI to determine the role of protein degradation on malonyl-CoA sensitivity.

CHAPTER 6

THE FIRST 28 N-TERMINAL AMINO ACID RESIDUES OF HUMAN HEART MUSCLE CARNITINE PALMITOYLTRANSFERASE I ARE ESSENTIAL FOR MALONYL-COA SENSITIVITY AND HIGH AFFINITY BINDING*

6.1 INTRODUCTION

Carnitine palmitoyltransferase I (CPTI), catalyzes the conversion of long-chain fatty acyl-CoAs to acyl carnitines in the presence of L-carnitine [18,172]. As an enzyme that catalyzes the first rate-limiting step in fatty acid 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 [18,172]. Understanding the regulation of CPTI by malonyl-CoA is important in the design of drugs for control of excessive fatty acid oxidation in diabetes mellitus [212], and in myocardial ischemia where accumulation of long-chain acylcarnitines has been associated with arrhythmias [65].

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 [3,42,266,267,281,282,294]. Although adult heart expresses both isoforms of CPTI, the predominant form is M-CPTI [42,266,267]. The IC_{50} for malonyl-CoA inhibition of heart mitochondrial M-CPTI is ~30–100-fold lower than that of

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Shi, J., Zhu, H., Arvidson, D. N., and Woldegiorgis, G. (2000) The first 28 N-terminal amino acid residues of human heart muscle carnitine palmitoyltransferase I are essential for malonyl-CoA sensitivity and high affinity binding. *Biochemistry*, in press.

L-CPTI, but both tissues have similar malonyl-CoA concentration [172,294]. It is estimated that about 60–80% of the energy requirement of the heart is derived from fatty acid oxidation [268]. 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 recent reports of the transcriptional regulation of M-CPTI gene expression by long-chain fatty acids via the peroxisome proliferator-activated receptor α (PPARα) [29,168,287]. Long-chain fatty acids activate PPARα, which then heterodimerizes with the 9-cis-retinoic acid receptor, binds to the fatty acid response element on the promoter region of the M-CPTI gene, and in turn activates M-CPTI gene transcription [29]. In heart, the flux of high levels of long-chain fatty acyl-CoAs through the CPT system directly compete for the malonyl-CoA binding site on M-CPTI to overcome inhibition by malonyl-CoA. 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 [158].

We have expressed both human heart M-CPTI, L-CPTI and CPTII in the yeast *Pichia pastoris*, an organism devoid of endogenous CPT activity [70,293,294]. Recently, we showed that deletion of the conserved first 18 N-terminal amino acid residues of rat L-CPTI abolishes malonyl-CoA inhibition and high affinity binding [231]. We further demonstrated that substitution of glutamate-3 in the N-terminal region of L-CPTI abolishes malonyl-CoA inhibition and binding, while a mutant L-CPTI with a change of histidine-5 to alanine causes partial loss in malonyl-CoA inhibition [232]. These results demonstrate that glutamate-3 and histidine-5 are necessary for malonyl-CoA inhibition and high affinity binding, but not for catalysis [232]. In this communication, we report that, unlike the 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.

6.2 EXPERIMENTAL PROCEDURES

6.2.1 Construction of Plasmids for the N-terminal Mutants of Human Heart M-CPTI

Deletion mutants of M-CPTI were constructed by PCR using pGAP–M-CPTI plasmid DNA as template, as previously demonstrated [294]. For example, to construct HMΔ18, a 668-bp fragment, was obtained after using primer H18D, 5′-AAGACAATTGATGGTCGACTTCCGGCTCAGT, as the forward PCR primer, and HR1700, 5′-CCACCAGTCACTCACATA, as the reverse primer, followed by restriction digestion of the PCR product with the restriction enzymes *Mun*I and *Eco*RI. Note that for each forward primer the introduced *Mun*I site is shown underlined and is followed by the new start codon. The resulting fragment was then ligated with *Eco*RI-linearized pHWO10, and the product, pHZ18N, with the proper orientation was confirmed by restriction analysis with *Eco*RI. To complete the HMΔ18 mutant CPT gene, the 2.0-kb *Eco*RI fragment from pHZ01 was then ligated to the *Eco*RI site of pHZ18N as described [294]. Deletion mutants HMΔ28, HMΔ39, HMΔ51, HMΔ72, and HMΔ83 were constructed as above but with the following forward primers: H28D, 5′-AAGACAATTGATGAAACACGTCTACCTGTC; H39D, 5′-AAGACAATTGATGAAGAAACGCCTGATCC; H51D, 5′-AAGACAATTGATGAGGGGCGTGTACCCTGGCA; H72D, 5′-AAGACAATTGATGTCCTTCTGCAACGTGGA; and H83D, 5′-AAGACAATTGATGGTCAGTTGCATCCAGAGAT.

6.2.2 Construction of Plasmids Carrying Chimeric CPTI Genes

A plasmid carrying the N-terminal portion of human M-CPTI was prepared by PCR using pGAP–M-CPTI as a template with the forward primer HHRI, 5′-GAGGAATTCATATGGCGGAAGCTCACCAG, which introduced an *Eco*RI site (underlined) 2 bp upstream of the start codon and the reverse primer HR1700; the product was cut with *Eco*RI, and the 670-bp fragment was ligated to *Eco*RI-cut pUC119 to produce pJS200. To construct a CPTI chimera in which 41 N-terminal amino acids of human M-CPTI are fused to the C-terminal portion of rat L-CPTI, we

took advantage of the *Xmn*I present in this position for rat L-CPTI and introduced a translationally silent matching site into human M-CPTI. pJS200 was used as a template for PCR with forward primer HHRI and reverse primer CHX41, 5′-CTTGAAGCTTATGAACCTTTTCTTCCAGGAGTTG, which introduced the *Xmn*I site (underlined). The 130-bp PCR fragment was blunt-end ligated to *Srf*I-cut pCR-Script (Stratagene) to produce pJS109. pJS109 was linearized with *Xmn*I and ligated to *Xmn*I-cut pM1R [70] to produce pJS301. The final step in the construction was to subclone the chimeric CPTI to the *P. pastoris* expression vector pHW010. pJS301 was digested with *Eco*RI to release the full-length chimeric CPTI which was then ligated into *Eco*RI-cut pHW010 to produce pCHH41RL.

A CPTI chimera in the which 79 N-terminal amino acids of human M-CPTI are fused to the C-terminal portion of rat L-CPTI was constructed as follows: The initial construct introduced a translationally silent *Hin*dIII restriction site into the N-terminal coding region of RLCPTI. A 320-bp *Hin*dIII–*Kpn*I fragment was produced by restriction enzyme digestion of the PCR product prepared using pYGW9 as a template with the forward primer RLC79, 5′-GACCCAAGCTTGGGCATGATCGC-AAA, and the vector-specific reverse primer RL655, 5′-CAGGAAACAGCTATGAC, and subcloned into *Hin*dIII–*Kpn*I-cut pYGW9 to produce pJS102. A fragment encoding the N-terminal portion of HMCPTI was prepared by PCR using the plasmid pJS200 as a template, the forward primer RIHH and the reverse primer HHC79, 5′-GCCCCAAGCTTATGTCCACGTTGCAGA, which introduced a translationally silent *Hin*dIII restriction site (underlined) into the N-terminal coding region of HMCPTI. The 250 bp PCR product was blunt-end ligated into the pCR-Script (Stratagene) vector to produce pJS106. Next, the heart/liver CPTI chimeras were ligated together. pJS106 was digested with *Hin*dIII, and the resulting fragment was ligated into the *Hin*dIII site of pJS102 to produce pJS302. Note that pJS302 carries the 79 N-terminal amino acids of HMCPTI fused to RLCPTI at the introduced *Hin*dIII restriction site. PJS302 was digested with *Eco*RI to release the full-length chimeric CPTI which was then ligated into the *Eco*RI-cut pHW010 to produce pCHH79RL.

A CPTI chimera, in which 130 N-terminal amino acids of human M-CPTI are fused to the C-terminal portion of rat L-CPTI, was constructed in a similar manner to pCHH79RL. A 170 bp *Hin*dIII–*Kpn*I fragment was produced by restriction enzyme digestion of the PCR product prepared using pYGW9 as a template with the forward primer RLC130, 5′-GCTGAAGCTTCTGCTCTCCTACCACGGCTGGAT, and the reverse primer RL655 and ligated with *Hin*dIII–*Kpn*I-cut pYGW9 to produce pJS115. A fragment encoding the N-terminal portion of HMCPTI was prepared by PCR using as a template the plasmid pJS200, the forward primer RIHH and the reverse primer HCH130, 5′-CTTAAGCTTCAGGGTTTGGCGGAAGAA, which introduces a translationally silent *Hin*dIII site (underlined). The 390-bp PCR product was blunt-end ligated into *Sur*I-cut pCR-Script (Stratagene) to produce pJS113. pJS113 was digested with *Hin*dIII, and the resulting fragment was ligated into the *Hin*dIII site of pJS115 to produce pJS303. pJS303 was digested with *Eco*RI to release the full-length CPTI chimera which was then ligated into the *Eco*RI-cut pHW010 to produce pCHH130RL.

A CPTI chimera in which the 197 N-terminal amino acids of human M-CPTI are fused to the C-terminal portion of rat L-CPTI was constructed by cutting pJS200 with *Kpn*I and *Hin*dIII and ligating the 563-bp fragment to *Kpn*I and *Hin*dIII-cut pYGW9 to form pJS316. pJS316 was then cut with *Eco*RI and the fragment ligated to pHW010 to form CHH197RL. The DNA sequences of all mutants were confirmed by sequencing.

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

Each plasmid was linearized by digestion with the restriction enzyme *Bsp*EI [294]. The linear DNA was introduced into *P. pastoris* by electrotransformation. 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 mutant M-CPTIs and L-CPTIs, as described previously [70,294].

6.2.4 CPTI Assay

CPTI and L-CPTI activity were assayed in isolated mitochondria from the yeast strains expressing the wild-type and mutant CPTIs by the forward exchange method using L-[^{3}H]carnitine, as described previously [32,70,294]. 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 [208,213].

6.2.5 ^{14}C-Malonyl-CoA Binding Assay

^{14}C-Malonyl-CoA binding in isolated mitochondria from the yeast strains expressing the wild-type and mutant CPTIs was determined by a modified centrifugation assay as described previously [166,231,232]. The CPT activity and IC_{50} 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.

6.2.6 Western Blot Analysis

Proteins were separated by SDS-PAGE in a 7.5% gel and transferred onto nitrocellulose membranes. Immunoblots were developed by incubation with either the M-CPTI- or L-CPTI-specific polyclonal antibodies as described previously [70,293,294].

Sources of materials and other procedures were as described in our previous publication [232].

6.3 RESULTS

6.3.1 Generation of Deletion Mutants and Chimeras and Expression in *P. pastoris*

Construction of plasmids carrying the N-terminal deletions of human heart M-CPTI and chimeras was performed as described in Section 6.2. The deletions and chimeras were confirmed by DNA sequencing. The deletions ranged from the

smallest 18, to the largest, 83 amino acid residues as shown in Fig. 6.1. *P. pastoris* was chosen as the expression system for M-CPTI, the deletion mutants and the chimeras, because it does not have endogenous CPT activity [70,231,293,294]. The *P. pastoris* expression plasmids expressed M-CPTI and L-CPTI under control of the *P. pastoris* glyceraldehyde-3-phosphate dehydrogenase gene promoter [70,264].

Western blot analysis of wild-type M-CPTI (80 kDa) and the mutants, using a C-terminal polyclonal antibody directed against a maltose binding protein-M-CPTI fusion protein [294], is shown in (Fig. 6.2). For the wild type and all the deletion mutants, proteins of the predicted sizes were synthesized and were expressed at similar steady-state levels.

6.3.2 Effect of Deletions on L-CPTI Activity and Malonyl-CoA Inhibition

All of the deletion mutants except Δ83 retained significant CPT activity. For deletion mutants Δ18, Δ51, and Δ72, the CPT activity level was 84%, 56%, and 20%, respectively, of that observed with the wild-type yeast strain expressing M-CPTI (Table 6.1). In contrast, Δ28 and Δ39 showed over 2.5-fold higher CPT activity compared to the wild-type strain expressing M-CPTI. Δ83 had no CPT activity. The IC_{50} for malonyl-CoA inhibition of the wild-type strain expressing M-CPTI was 70 nM [294], while the IC_{50} for the minimal deletion mutant Δ18 was 300 nM, representing only a 4-fold decrease in malonyl-CoA sensitivity compared to the 190-fold decrease in sensitivity observed with the corresponding L-CPTI Δ18 [231]. Deleting 28, 39, 51, and 72 amino acid residues from the N-terminus of M-CPTI increased the IC_{50} for malonyl-CoA inhibition in each of the mutants from 70 nM in the wild-type strain to 3.5–7.5 μM in the deletion mutants, thus decreasing the malonyl-CoA sensitivity by 50–100-fold (Table 6.1). Δ28 showed decreased malonyl-CoA sensitivity at all levels of the inhibitor tested compared to the wild type, as shown in Fig. 6.3.

6.3.4 Kinetic Properties of Wild-Type and Mutant M-CPTs

Δ28 exhibited normal saturation kinetics when the carnitine concentration was varied relative to a second substrate, palmitoyl-CoA, but showed a much higher

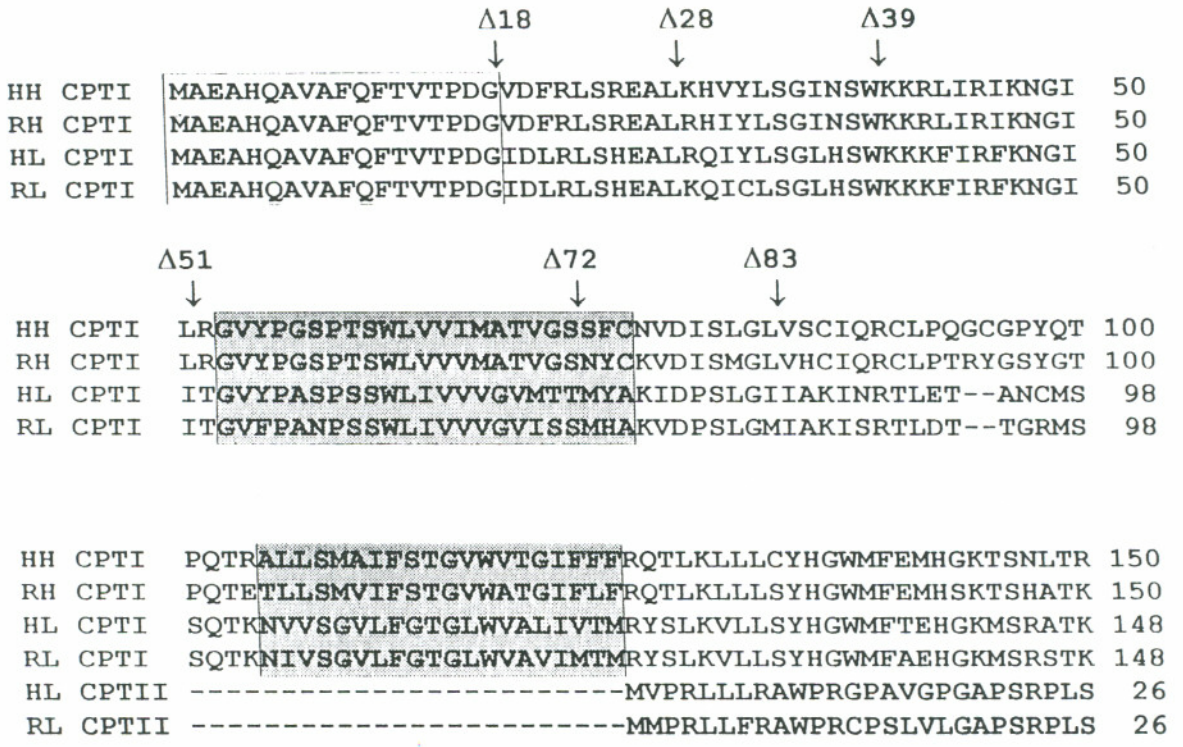

Figure 6.1 The amino acid sequence of the first 150 N-terminal residues of human and rat liver CPTs. The shaded areas represent the position of the two predicted membrane-spanning domains of all known CPTIs. The position of each of the deletion mutants is shown by an arrow. Sources of the sequences from the data bank were from refs. 3, 281, 282, and 294 as indicated in the text. HH, RH = human, rat heart; HL, RL = human, rat liver.

Figure 6.2 Immunoblot showing expression of wild-type and deletion mutant human heart M-CPTIs in the yeast *P. pastoris*. Mitochondria (50 μg 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 as described in Section 6.2. WT = wild-type M-CPTI; Δ = deletions 18, 28, 39, 51, 72 and 83, respectively.

Table 6.1

CPT Activity, Malonyl-CoA Sensitivity, and Binding in Yeast Strains Expressing Wild-type M-CPTI and N-Terminal Deletion Mutants

Strain	Activity (nmol/ mg • min)	IC_{50} (μm)	K_{D1} (nm)	K_{D2} (nm)	B_{max1} (pmol/ mg)	B_{max2} (pmol/ mg)
wild-type	2.5±0.4	0.07±0.01	5.7	35.0	13.0	34.8
Δ18	2.1±0.2	0.3±0.05	4.6	37.0	6.3	24.4
Δ28	6.7±1.3	7.5±0.3	—	684.9	—	8.6
Δ39	6.3±0.9	7.5±0.4	—	285.0	—	12.5
Δ51	1.4±0.2	3.5±0.2	—	115.7	—	5.3
Δ72	0.5±0.2	7.0±0.4	—	744.6	—	4.0
Δ83	no activity					

Mitochondria were isolated from the yeast strains separately expressing M-CPTI and the deletion mutants, and were assayed for CPT activity, malonyl-CoA sensitivity, and binding as described in Section 6.2. 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 ± 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.

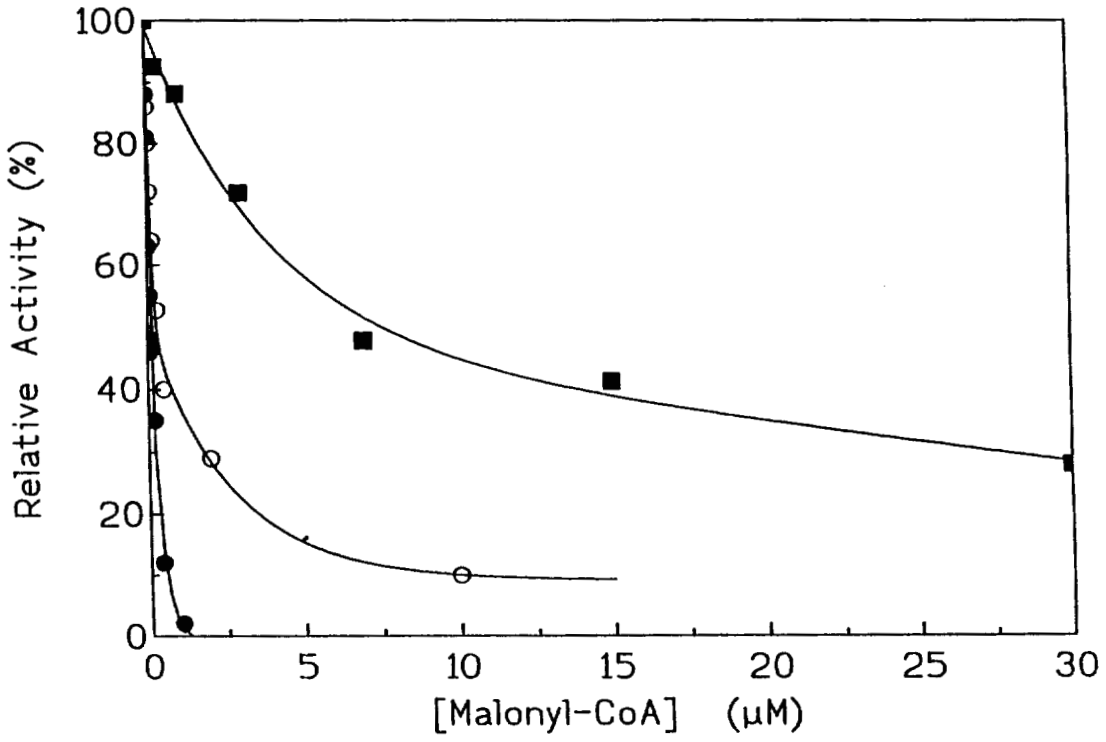

Figure 6.3 Effect of increasing concentrations of malonyl-CoA on the activities of yeast-expressed wild-type and deletion mutant M-CPTIs. Approximately 150 μg of mitochondrial protein were used for the assay. ● = wild type; ○ = Δ18; ■ = Δ28.

activity compared to the wild type at all levels of carnitine tested (Fig. 6.4A) under standard assay conditions. The calculated K_m for carnitine for Δ28 was 408 μM, which is similar to the 530 μM for the wild type, but the V_{max} (19.1 nmol/min.mg protein) for Δ28 was 2.5-fold higher than that of the wild-type strain (7.6 nmol/min.mg protein). With respect to the second substrate, palmitoyl-CoA, both the wild type and Δ28 showed non-Michaelis-Menten saturation kinetics at a fixed concentration of albumin (1% W/V) (Fig. 6.4B), characteristics similar to our previous report [294]. Δ28 exhibited significantly higher activity than the wild type at all levels of palmitoyl-CoA tested. However, both Δ28 and the wild type showed normal saturation kinetics when the molar ratio of palmitoyl-CoA to albumin was fixed at 6.1:1 (Fig. 6.4C). For Δ28, the calculated K_m for palmitoyl-CoA was 56.8 μM, and the V_{max} was 24.9 nmol/min.mg protein, which is similar to the wild-type values of 93.8 μM and 25.0 nmol/min.mg protein. Thus, deletion of the first 28 and 39 N-terminal amino acid residues abolishes malonyl-CoA sensitivity and increases catalytic activity of M-CPTI, while loss of malonyl-CoA inhibition in Δ51 and Δ72 was associated with decreased catalytic activity. Unlike Δ18 of L-CPTI [231], deletion of the first 18 N-terminal amino acids of M-CPTI had minimal effect on malonyl-CoA inhibition and catalytic activity.

6.3.5 ^{14}C-Malonyl-CoA Binding in Yeast-Expressed Wild-Type and Mutant M-CPTIs

Malonyl-CoA binding to the mitochondria from the yeast strain expressing Δ28 was significantly lower compared to that observed in the mitochondria from the wild-type strain and Δ18, 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 and Δ18 as shown by the Scatchard plots in Fig. 6.6, but only very-low-affinity binding was observed in the mitochondria from Δ28. Deletion of the first 28 N-terminal residues completely abolished high affinity malonyl-CoA binding (K_{D1}) and further decreased the low-affinity binding (K_{D2}) by 20-fold (Table 6.1). A complete loss in high affinity malonyl-CoA binding (K_{D1}) was also observed for Δ39, Δ51, and Δ72, which was associated with a decrease in the low affinity binding (K_{D2}).

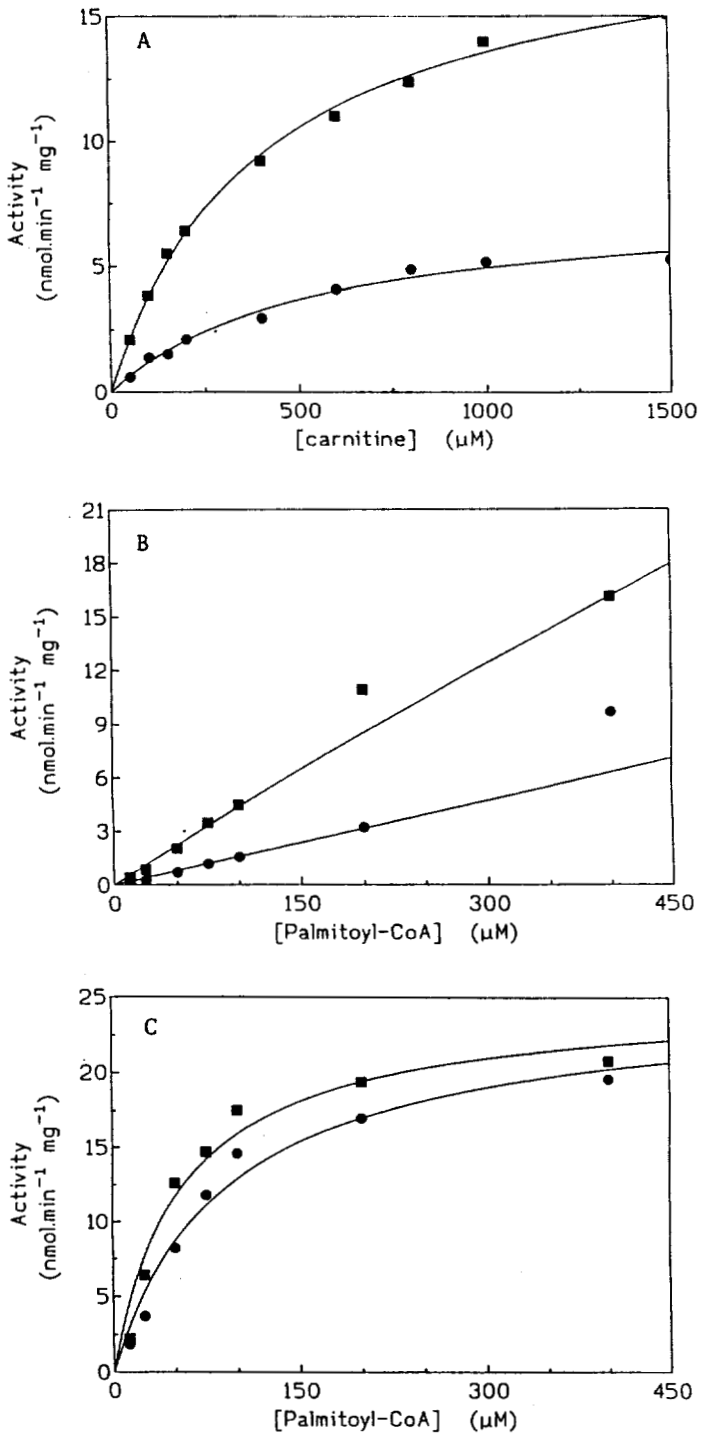

Figure 6.4 Kinetic analysis of wild-type and Δ28 mutant M-CPTI activities. Isolated mitochondria (150 μg protein) from the yeast strains expressing the wild-type (●) and Δ28 (■) mutant M-CPTI were assayed for CPTI activity in the presence of increasing concentrations of carnitine and palmitoyl CoA as described in Section 6.2. The figures show the resulting dose-response curves for M-CPTI, (**A**) carnitine; (**B**) palmitoyl CoA with fixed albumin concentration (1% W/V); (**C**) palmitoyl CoA with fixed molar ratio of palmitoyl CoA:albumin (6.1:1).

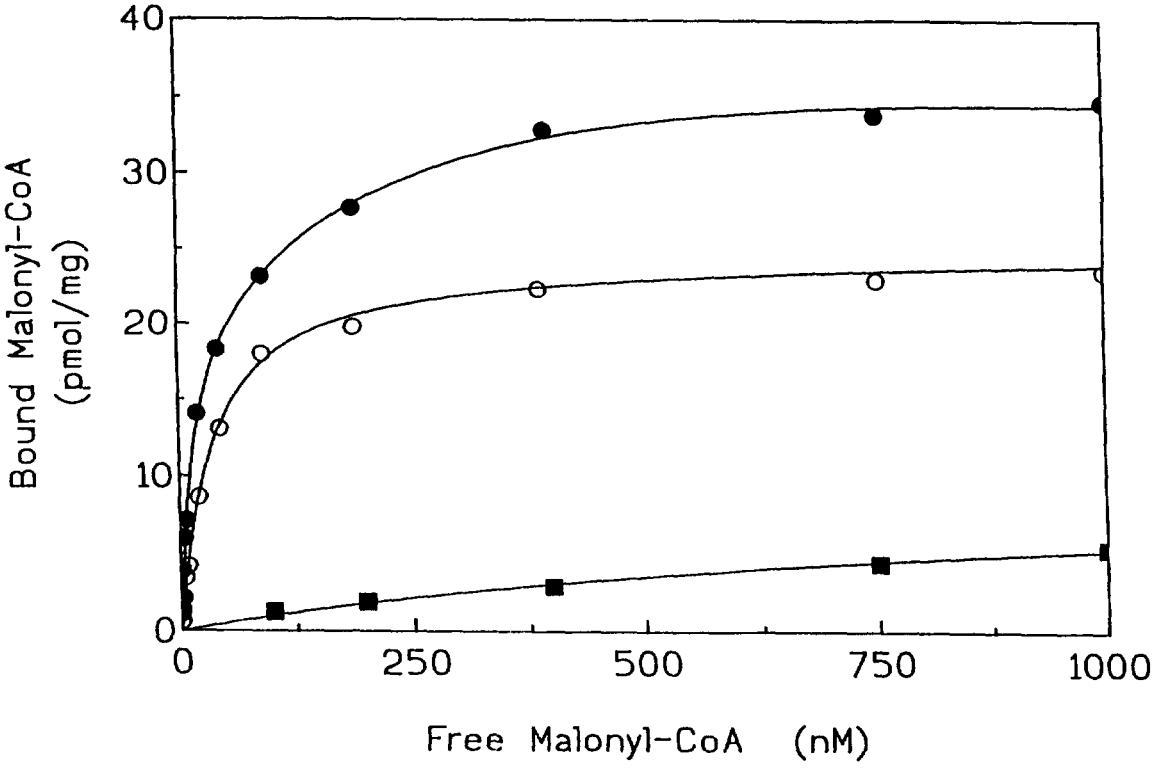

Figure 6.5 Binding of ^{14}C-malonyl CoA to mitochondria isolated from the yeast strain expressing the wild-type M-CPTI, Δ18, and Δ28. Approximately 200 μg of protein were used for the binding assay. Malonyl-CoA binding values for the wild-type and deletion mutants were corrected for malonyl-CoA binding to the mitochondria from the yeast strain with the vector but no insert. ● = wild type; ○ = Δ18; ■ = Δ28.

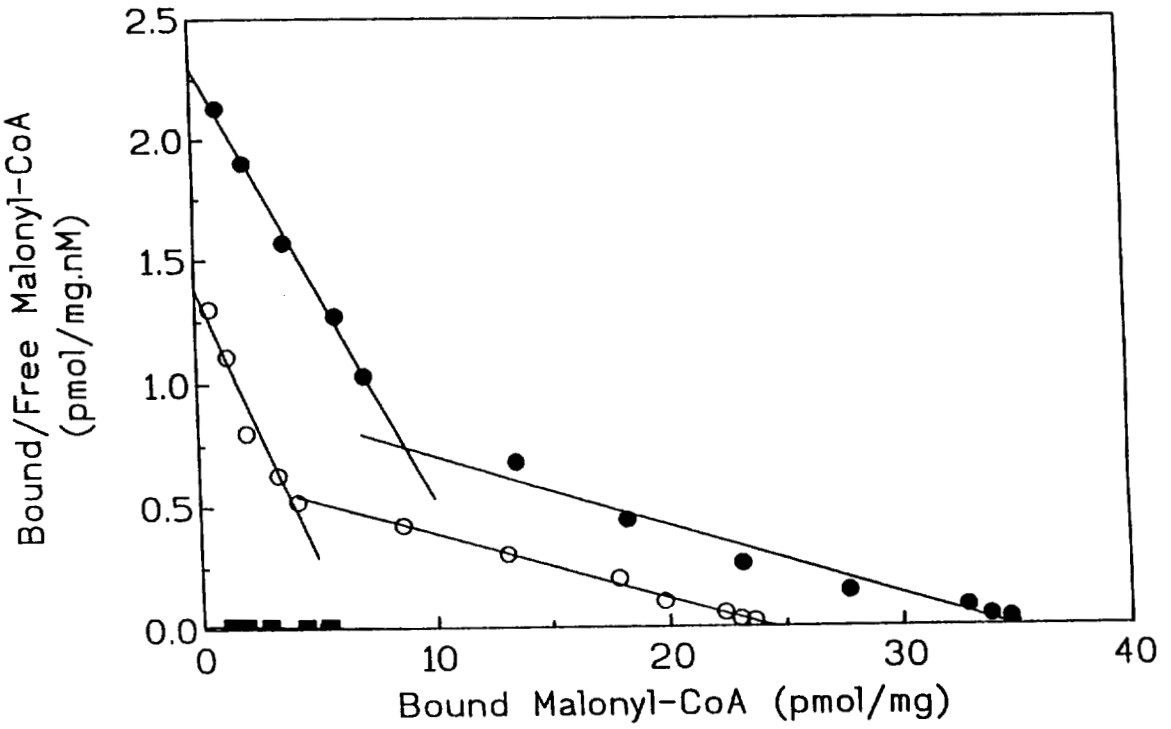

Figure 6.6 Scatchard plot for binding of ^{14}C-malonyl-CoA to mitochondria from yeast strains expressing wild-type and mutant M-CPTIs. ● = wild type; ○ = Δ18; ■ = Δ28.

The increase in K_{D2} for the low affinity binding site due to the deletions correlated with a decrease in the calculated B_{max2}, 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.

6.3.5 Activity and Malonyl-CoA Sensitivity of Chimeric CPTI Enzymes

Four chimeric L-CPTI enzymes, in which the first 41, 79, 130, and 197 N-terminal amino acid residues of human heart M-CPTI replaced the corresponding portion of rat L-CPTI were constructed and expressed in *P. pastoris*. Mitochondria from the yeast strains expressing the chimeric rat liver enzymes were monitored for changes in malonyl-CoA sensitivity as a result of transplanting the N-terminal residues of the human heart enzyme to the liver enzyme. Chimeras 41, 79, and 130 showed similar CPT activity as the wild-type L-CPTI which was 3-fold higher than that of the wild-type M-CPTI (Table 6.2). Chimera 197 had no CPT activity. The IC_{50} for malonyl-CoA inhibition of chimeras 41 and 130 were similar to the wild-type L-CPTI. Chimera 79 exhibited a 4.5-fold decrease in malonyl-CoA sensitivity compared to the wild-type L-CPTI, suggesting that interaction between the two membrane-spanning α-helices of the same isoform may be important for malonyl-CoA sensitivity. For the wild-type L-CPTI and chimeric enzymes, proteins of predicted sizes were synthesized and were expressed at similar steady-state levels as shown by western blot analysis using the L-CPTI-specific C-terminal antibodies (Fig. 6.7). Thus, replacement of the first 130 N-terminal amino acid residues of L-CPTI with the corresponding M-CPTI residues did not increase the malonyl-CoA sensitivity of the chimeric liver enzyme.

6.4 DISCUSSION

To determine the role of the N-terminal region of human heart M-CPTI on malonyl-CoA sensitivity and binding, a series of deletion mutations were constructed ranging in size from 18 to 83 N-terminal residues. All of the deletions except Δ83 had 20–268% of the wild-type M-CPTI activity. Δ28 and Δ39 were insensitive to

Table 6.2

CPTI Activity and Malonyl-CoA Sensitivity of Wild-type L-CPTI and Chimeric L-CPTIs

Strain	Activity (nmol/mg • min)	IC_{50} (μM)
wild-type L-CPTI	7.8 ± 0.5	2.0 ± 0.2
wild-type M-CPTI	2.5 ± 0.4	0.07 ± 0.01
ML-41	6.9 ± 0.6	1.5 ± 0.3
ML-79	6.2 ± 0.4	9.0 ± 1.0
ML-130	6.6 ± 0.4	2.0 ± 0.2
ML-197	no activity	

Mitochondria were isolated from the yeast strains separately expressing L-CPTI and the chimeric L-CPTIs,and were assayed for CPT activity and malonyl CoA sensitivity as described in Section 6.2. Results are mean ±SD of at least three independent experiments with different mitochondrial preparations. ML = muscle-liver chimera.

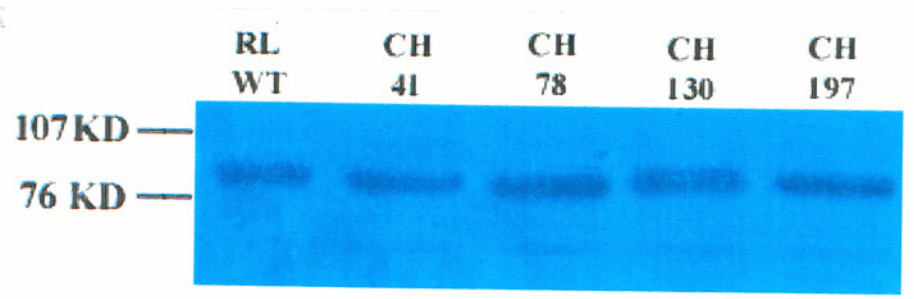

Figure 5.7 Immunoblot showing expression of wild-type L-CPTI and chimeric L-CPTs in the yeast *P. pastoris*. Mitochondria (30 μg of protein) from the wild-type L-CPTI yeast strain and the strains expressing each of the chimeric L-CPTIs were separated on a 7.5% SDS-PAGE and blotted onto a nitrocellulose membrane; the immunoblot was developed as described in Section 6.2 using L-CPTI-specific polyclonal antibodies. RL, WT = rat liver CPTI, wild-type; CH = human M-CPTI-rat L-CPTI chimera.

malonyl-CoA inhibition and had complete loss of high affinity malonyl-CoA binding. However, Δ18 showed only a 4-fold loss in malonyl-CoA sensitivity but had activity and high affinity malonyl-CoA binding similar to the wild type. This is in contrast to the L-CPTI Δ18 which showed complete loss in malonyl-CoA sensitivity and high affinity binding [231], suggesting that the same conserved first 18 residues play a different role in L-CPTI and M-CPTI. Mutant Δ72 had only 20% of the wild-type activity, and Δ83 had no activity. Our data show that loss of residues necessary for optimal catalysis started with the Δ51 mutant. The corresponding deletion mutants for L-CPTI had ~70% of the wild-type activity, but had lost malonyl-CoA sensitivity and high affinity binding [231], suggesting that for M-CPTI, unlike L-CPTI, the first transmembrane domain is essential for catalytic activity. This is the first report to demonstrate isoform differences in the role of the first transmembrane domain in M-CPTI and L-CPTI activity.

The V_{max} for carnitine suggests that Δ28 has lost residues that interfere with catalysis, which may comprise part of the high-affinity malonyl-CoA binding site. 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 binding site may merely sequester palmitoyl-CoA, reducing its availability—a form of substrate inhibition. This model is supported by the V_{max} data for palmitoyl-CoA. The V_{max} for carnitine, at fixed palmitoyl-CoA and albumin concentrations, suggests palmitoyl-CoA is limiting such that the effect of sequestration is observable. However, when the molar ratio of palmitoyl-CoA to albumin is fixed, the effect disappears. Alternatively, since the increase in CPT activity was only observed with Δ28 and Δ39, but not Δ18 and Δ51, deletion of the first 28 and 39 residues could induce a conformational change optimal for palmitoyl-CoA binding and catalysis.

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 [23,187,188]. It is now well established that M-CPTI is 30–100-fold more sensitive to malonyl-CoA inhibition than L-CPTI, but our binding data show no differences in the K_{D1} or K_{D2} between the heart

and liver yeast expressed wild-type CPTI isoforms, suggesting that the observed differences in malonyl-CoA sensitivity may not be due to differences in the K_Ds for the two isoforms.

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 amino acid side chains responsible for the differences in sensitivity to malonyl-CoA are not located in this region. More recently, replacement of the N-terminal domain of M-CPTI with that of L-CPTI resulted in a chimeric M-CPTI with malonyl-CoA sensitivity similar to wild-type M-CPTI [242]. The decrease in malonyl-CoA sensitivity observed with chimera 79 but not chimera 130 could be due to reduced interaction between the first transmembrane domain of M-CPTI and the second transmembrane domain of L-CPTI that may be necessary for malonyl-CoA sensitivity of CPTI, suggesting that the membrane-spanning α-helices must be changed as a pair. Our malonyl-CoA binding studies with the yeast-expressed wild-type and mutant CPTIs, and the chimera data, suggest that malonyl-CoA sensitivity of L-CPTI and M-CPTI is an intrinsic property of each enzyme and that the N-terminal domain of M-CPTI cannot confer malonyl-CoA sensitivity to L-CPTI or vice versa, or to CPTII [50,242]. We are currently constructing substitution mutations of N-terminal amino acid residues 19–28 to identify specific residue(s) involved in malonyl-CoA binding and inhibition of M-CPTI.

CHAPTER 7
SUMMARY

7.1 CPTI and CPTII are distinct, catalytically active enzymes encoded by different genes. CPT catalytic activity and malonyl-CoA sensitivity are contained within a single CPTI polypeptide in mammalian mitochondrial membranes. Detergent inactivates CPTI, but not CPTII. Detergent inactivation of CPTI is reversible. CPTI thus requires a membrane environment for activity.

7.2 While there is only one CPTII isoform, there are two isoforms of CPTI—the liver form (L-CPTI) and the muscle form (M-CPTI)—encoded by distinct genes, expressed in different tissues, and having different kinetic properties. The *Pichia pastoris*-expressed M-CPTI is about 30-fold more malonyl-CoA sensitive than L-CPTI (IC_{50} = 0.07 nM versus 2 μM) and has about 20-fold lower affinity for carnitine. Both L-CPTI and M-CPTI have two malonyl-CoA binding sites, a high-affinity binding site and a low-affinity binding site. *P. pastoris* is by far the best expression system to study the CPT system, because there is no endogenous CPT activity in this yeast strain. Yeast-expressed CPTs are localized in mitochondria.

7.3 The conserved first 6 N-terminal amino acid residues of rat L-CPTI are essential for malonyl-CoA inhibition and high-affinity binding as shown by deletion mutation analysis. Substitution of glutamic acid 3 with alanine abolished high-affinity malonyl-CoA binding and inhibition. Replacement of histidine 5 with alanine caused loss of high-affinity malonyl-CoA binding but only partial loss of malonyl-CoA inhibition. Glutamic acid 3 and histidine 5 in

L-CPTI are essential for malonyl-CoA inhibition and binding, but not for catalysis.

7.4 The conserved first 18 N-terminal amino acids in CPTI are important for malonyl-CoA inhibition and binding of L-CPTI, but are not critical for M-CPTI. Deletion of the conserved first 18 N-terminal amino acids in M-CPTI only caused a 4-fold increase in IC_{50} for malonyl-CoA inhibition and had little effect on malonyl-CoA binding. The residues important for malonyl-CoA inhibition and binding seem to reside between amino acid residues 18 and 28 of the N-terminal M-CPTI sequence. Mitochondria from the yeast strain expressing Δ28 of M-CPTI exhibited 2.5-fold higher activity compared with wild type, but were insensitive to malonyl-CoA inhibition and had complete loss of high-affinity malonyl-CoA binding.

7.5 Based on the chimera studies, it is concluded that malonyl-CoA sensitivity of L-CPTI and M-CPTI is an intrinsic property of each enzyme and that the N-terminal domain of M-CPTI cannot confer malonyl-CoA sensitivity to L-CPTI or vice versa, or to CPTII.

7.6 The amino acid residues responsible for malonyl-CoA sensitivity are separate from those responsible for catalysis. The malonyl-CoA binding site may be localized at the extreme N-terminal region of CPTI, but still needs to interact with the C-terminal region of CPTI. The difference in malonyl-CoA sensitivity between L-CPTI and M-CPTI may be due to the different amino acid residues responsible for malonyl-CoA inhibition in the different tissues.

7.7 We are currently constructing substitution mutations of N-terminal amino acid residues 19–28 to identify specific residues involved in malonyl-CoA binding and inhibition.

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BIOGRAPHICAL SKETCH

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