A Single Amino Acid Change (Substitution of the Conserved Glu-590 with Alanine) in the C-terminal Domain of Rat Liver Carnitine Palmitoyltransferase I Increases its Malonyl-CoA Sensitivity Close to That Observed with the Muscle Isoform of the Enzyme*

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Carnitine palmitoyltransferase I (CPTI) catalyzes the conversion of long-chain fatty acyl-CoAs to acylcarnitines in the presence of l-carnitine. To determine the role of the highly conserved C-terminal glutamate residue, Glu-590, on catalysis and malonyl-CoA sensitivity, we separately changed the residue to alanine, lysine, glutamine, and aspartate. Substitution of Glu-590 with aspartate, a negatively charged amino acid with only one methyl group less than the glutamate residue in the wild-type enzyme, resulted in complete loss in the activity of the liver isoform of CPTI (L-CPTI). A change of Glu-590 to alanine, glutamine, and lysine caused a significant 9- to 16-fold increase in malonyl-CoA sensitivity but only a partial decrease in catalytic activity. Substitution of Glu-590 with neutral uncharged residues (alanine and glutamine) and/or a basic positively charged residue (lysine) significantly increased L-CPTI malonyl-CoA sensitivity to the level observed with the muscle isoform of the enzyme, suggesting the importance of neutral and/or positive charges in the switch of the kinetic properties of L-CPTI to the muscle isoform of CPTI. Since a conservative substitution of Glu-590 to aspartate but not glutamine resulted in complete loss in activity, we suggest that the longer side chain of glutamate is essential for catalysis and malonyl-CoA sensitivity. This is the first demonstration whereby a single residue mutation in the C-terminal region of the liver isoform of CPTI resulted in a change of its kinetic properties close to that observed with the muscle isoform of the enzyme and provides the rationale for the high malonyl-CoA sensitivity of muscle CPTI compared with the liver isoform of the enzyme.

Carnitine palmitoyltransferase I (CPTI) catalyzes the conversion of long-chain fatty acyl-CoAs to acylcarnitines in the presence of l-carnitine, the first step in the transport of long-chain fatty acids from the cytoplasm to the mitochondria matrix, a rate-limiting step in β-oxidation (1, 2). Mammalian tissues express two isoforms of CPTI, a liver isoform (L-CPTI) and a muscle isoform (M-CPTI), that are 62% identical in amino acid sequence (3–8). As an enzyme that catalyzes the first rate-limiting step in β-oxidation, CPTI is regulated by its physiological inhibitor, malonyl-CoA (1, 2), the first intermediate in fatty acid synthesis, suggesting a coordinated control of fatty acid oxidation and synthesis. Previous studies of our laboratory and others have demonstrated that the muscle isoform of CPTI, M-CPTI, is significantly more sensitive to malonyl-CoA inhibition than the liver isoform, but the molecular/structural basis for the differences in malonyl-CoA sensitivity between M-CPTI and L-CPTI remain to be established (3–8). Because of its central role in fatty acid metabolism, understanding the molecular mechanism of the regulation of the CPT system is an important first step in the development of treatments for diseases, such as myocardial ischemia and diabetes, and in human-inherited CPTI deficiency diseases (9–11).

We developed a novel high level expression system for human heart M-CPTI, rat L-CPTI, and CPTII in the yeast Pichia pastoris, an organism devoid of endogenous CPT activity (6, 12–14). Furthermore, by using this system, we have shown that CPTI and CPTII are active distinct enzymes and that L-CPTI and M-CPTI are distinct malonyl-CoA-sensitive CPTs that are reversibly inactivated by detergents. Recent site-directed mutagenesis studies from our laboratory have demonstrated that glutamic acid 3 and histidine 5 in L-CPTI are necessary for malonyl-CoA inhibition and high affinity binding but not for catalysis (15, 16). For M-CPTI, our mutagenesis studies demonstrate that in addition to Glu-3 and His-5, Val-19, Leu-23, and Ser-24 are necessary for malonyl-CoA inhibition and high affinity binding, in agreement with the differences in malonyl-CoA sensitivity observed between M-CPTI and L-CPTI (17, 18). In addition, our site-directed mutagenesis studies of conserved residues in the C-terminal domain of L-CPTI demonstrated that conserved arginine and tryptophan residues are important for catalysis (19). In this report, our mutagenesis studies show for the first time that a change of the highly conserved C-terminal Glu-590 residue in L-CPTI to alanine, glutamine, or lysine significantly increased its sensitivity to malonyl-CoA inhibition.

**EXPERIMENTAL PROCEDURES**

L-CPTI mutants were constructed by the overlap extension PCR procedure using the primers shown in Table I with the wild-type plas-
mid DNA (pGAP-L-CPTI) as template (12, 20). For example, to construct the E590A mutant, the primers f-GWW3-r-E590A and r-MDR2-f-E590A were used to generate 1.0-kb and 500-bp PCR products, respectively, using the wild-type L-CPTI cDNA as a template. The two PCR products were purified, mixed, and used as a template for a second-round PCR with the primer f-GWW3-r-MDR2. The 1.5-kb PCR product was digested with AvaI-SacI, and the 1.0-kb DNA fragment containing the desired fragment was subcloned into AvaI-SacI-cut wild-type L-CPTI cDNA in the pGAP expression vector. Bacterial colonies obtained upon transformation of the mutagenesis reactions were

![FIG. 1](image)

**TABLE I**

| Mutant | Primer | Sequence |
|--------|--------|----------|
| E590A  | fE590A | 5'-GTCCTGCTCCAATATGCGGCTCCATAGCCCAGGC-3' |
|        | rE590A | 5'-GCCTGCTCCAATAGCCCATGAGCCGAGAC-3' |
| crE590A| fE590A | 5'-GTCCTGCTCCAATATGCGGCTCCATAGCCCAGGC-3' |
|        | rE590Q | 5'-GCCTGCTCCAATAGCCCATGAGCCGAGAC-3' |
| crE590Q| fE590Q | 5'-GTCCTGCTCCAATATGCGGCTCCATAGCCCAGGC-3' |
|        | rE590D | 5'-GCCTGCTCCAATAGCCCATGAGCCGAGAC-3' |
| crE90D | fE590K | 5'-GTCCTGCTCCAATATGCGGCTCCATAGCCCAGGC-3' |
|        | rE590K | 5'-GCCTGCTCCAATAGCCCATGAGCCGAGAC-3' |
| cfE590K| MDR2   | 5'-GCGCCGCTGACCTATACATTTAGATGATG-3' |
|        | Gw3    | 5'-ATCACCCCAACCCATATC-3' |

**FIG. 1.** Sequence alignment of portions of the C-terminal region of various acyltransferases. Asterisk, identical residues; colon, conserved residues.
Glut-590→Ala Mutant L-CPTI with Increased Malonyl-CoA Sensitivity

**Table II**

| Strain    | Activity (nmol/mg min) | IC₅₀ (µM) |
|-----------|------------------------|-----------|
| WT        | 7.8 ± 1.3              | 3.39 ± 0.42 |
| E590A     | 6.2 ± 1.7              | 0.21 ± 0.08 |
| E590Q     | 7.3 ± 0.7              | 0.39 ± 0.83 |
| E590D     | 3.7 ± 0.4              | 0.25      |

**Results**

Preincubation of isolated mitochondria from the yeast strain expressing rat L-CPTI at room temperature with dicyleohexylcarbodiimide, a glutamate-specific modifying reagent (22), resulted in an irreversible 50% loss in catalytic activity (data not shown). These preliminary chemical modification studies with dicyleohexylcarbodiimide provided evidence that a conserved glutamate residue(s) is important for maximal L-CPTI activity.

Alignment of the sequences of all of the carnitine and choline acetyltransferases from different species showed the presence of two conserved glutamate residues, Glu-601 and Glu-606, as well as two adjacent highly conserved arginine residues, Arg-601 and Arg-606, for important for L-CPTI activity and malonyl-CoA sensitivity (19, 23).

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

Construction of plasmids carrying substitution mutations E590A, E590Q, E590D, and E590K was performed as described under “Experimental Procedures.” P. pastoris was chosen as an expression system for L-CPTI and the mutants, because it does not have aendogenous CPT activity (6, 12–16). The P. pastoris expression plasmids expressed L-CPTI under control of the P. pastoris glyceraldehyde-3-phosphate dehydrogenase gene promoter (12, 24). Yeast transformants with the wild-type L-CPTI gene and the mutants were grown in liquid medium supplemented with glucose (12).

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 (12), is shown in Fig. 2, A and B. For the wild type and the mutants E590A, E590Q, E590D, and E590K, proteins of predicted sizes were synthesized with similar steady-state levels of expression.

**Effect of Mutations on L-CPTI Activity and Malonyl-CoA Sensitivity**—Substitution mutant E590D was inactive. A change of Glu-590 to Ala resulted in only a 26% loss in L-CPTI activity, but the mutant E590A exhibited a 16-fold increase in malonyl-CoA sensitivity as shown by the IC₅₀ values in Table II. For the wild type and the mutants E590A, E590Q, E590D, and E590K, proteins of predicted sizes were synthesized with similar steady-state levels of expression.

**Kinetic Characteristics of Mutant L-CPTIs**—Mutants E590A and E590Q exhibited normal saturation kinetics when the carnitine concentration was varied relative to a constant second substrate, palmitoyl-CoA (Fig. 4A), a property identical to that of the wild-type L-CPTI. For mutants E590A and E590Q, the calculated Kₘ values for carnitine were only 26–48% higher compared with the wild type as shown in Table III. However, the Vₘₕ values for carnitine for the E590A and E590Q mutants were only 14–34% lower compared with the wild-type L-CPTI enzyme (Table II). In short, our studies identify for the first time a conserved residue in the C-terminal region of L-CPTI, Glu-590, which when mutated to neutral and/or positively charged residues is important for increased sensitivity of the enzyme to malonyl-CoA inhibition.

**Generation of Mutations and Expression in P. pastoris**—Construction of plasmids carrying substitution mutations E590A, E590Q, E590D, and E590K was performed as described under “Experimental Procedures.” P. pastoris was chosen as an expression system for L-CPTI and the mutants, because it does not have endogenous CPT activity (6, 12–16). The P. pastoris expression plasmids expressed L-CPTI under control of the P. pastoris glyceraldehyde-3-phosphate dehydrogenase gene promoter (12, 24). Yeast transformants with the wild-type L-CPTI gene and the mutants were grown in liquid medium supplemented with glucose (12).

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wild type, respectively. Thus, the substitution of the conserved Glu-590 residue with alanine, glutamine, and/or lysine resulted in a reduction in catalytic activity and a significant increase in malonyl-CoA sensitivity. In contrast, a conservative substitution of Glu-590 with aspartate inactivated L-CPTI, suggesting that the longer side chain of glutamate is required for catalytic activity and malonyl-CoA sensitivity.

DISCUSSION

Our site-directed mutagenesis study of the highly conserved Glu-590 residue in the C-terminal region of L-CPTI demonstrated that substitution with alanine, glutamine, or lysine resulted in partial loss in activity and a significant increase in malonyl-CoA sensitivity. Glu-590 and Glu-603 in the C-terminal region of L-CPTI are the only two conserved glutamate residues in the family of acyltransferases (Fig. 1). Glu-603 is a conserved residue within the family of CPT enzymes, whereas other acyltransferases have aspartate at this position. We have recently demonstrated that a change of Glu-603 to alanine or histidine resulted in complete loss in L-CPTI activity (23), whereas a change to glutamine caused a significant loss in activity and malonyl-CoA sensitivity. In contrast with the complete loss of activity observed with the E590D mutant, substitution of Glu-603 with aspartate resulted in only partial loss in CPTI activity but a significant loss in malonyl-CoA sensitivity. A change of the highly conserved glutamate residue corresponding to Glu-590 in CPTII, Glu-487 to aspartate inactivated the enzyme (25), suggesting the importance of this highly conserved residue in CPTI and CPTII in maintaining the active site conformation of the two enzymes. Substitution of the highly conserved Glu-590 with alanine did not have a major effect on catalytic activity but caused a significant increase in L-CPTI malonyl-CoA sensitivity, indicating the opposing roles played by the conserved C-terminal glutamate residues in L-CPTI on activity and malonyl-CoA sensitivity. The site-directed mutagenesis study described here is aimed at elucidating the function of the highly conserved acidic residue, Glu-590, found in the proximity of the active site of L-CPTI.

To determine the role of the highly conserved Glu-590 on catalysis and malonyl-CoA sensitivity, we separately changed the Glu-590 residue to alanine, glutamine, aspartate, and lysine (E590A, E590Q, E603D, and E590K, respectively) and determined the effect of the mutations on L-CPTI activity and malonyl-CoA sensitivity. The presence of the extra methyl group in Glu-590 is probably crucial for maintaining the configuration of the L-CPTI active site. This finding suggests that a change of Glu-590 to aspartate may result in the carboxylate being outside the hydrogen bond distance of Asp-567, a highly

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**Fig. 3. Effect of increasing concentrations of malonyl-CoA on the activities of yeast-expressed wild-type and mutant L-CPTIs.** Approximately 150 µg of mitochondrial protein was used for the assay. A, wild type (○); B, E590A (▲), E590K (●), and E590Q (■). RL, rat liver.
conserved residue that is predicted to be at the CoA binding site of L-CPTI (26). Glu-590 may thus be required for L-CPTI stability and positioning of the imidazole ring of His-473 for efficient catalysis and inhibition, thus facilitating productive interaction with the substrates and the inhibitor (27). The mutant L-CPTIs with a replacement of Glu-590 with alanine (E590A), glutamine (E590Q), and lysine (E590K) showed a partial loss in activity but a significant increase in malonyl-CoA sensitivity. The partial loss in catalytic activity observed with the mutant L-CPTI (E590Q, E590Q, and E590K) was in each case associated with a significant increase in malonyl-CoA sensitivity, suggesting that these highly conserved glutamate residues may be important for substrate and inhibitor binding. In contrast, mutation of the corresponding conserved Glu-603 to alanine, histidine, and glutamine resulted in an inactive enzyme or an enzyme with significantly decreased activity and malonyl-CoA sensitivity, suggesting different roles played by these conserved residues in L-CPTI activity and malonyl-CoA sensitivity. The effect of the mutations on activity and malonyl-CoA sensitivity suggest that the highly conserved Glu-590 in the C-terminal region of L-CPTI may be important for substrate (palmitoyl-CoA) and inhibitor (malonyl-CoA) binding, probably through the common CoA moiety present in both compounds. Although there was a decrease in \( V_{max} \) for both substrates (carnitine and palmitoyl-CoA) with the E590A, E590Q, and E590K mutants, all of the mutants showed an increase in the affinity for palmitoyl-CoA but not carnitine, suggesting that these residues are involved in binding palmitoyl-CoA and malonyl-CoA but not carnitine. Thus, this region may be involved in binding the CoA moiety of palmitoyl-CoA and malonyl-CoA and/or may be the low affinity acyl-CoA binding site.

As a rate-limiting enzyme that transports long-chain fatty acids from the cytosol to the mitochondrial matrix, L-CPTI in the presence of carnitine catalyzes the conversion of long-chain acyl-CoA to acylcarnitines (1, 2). Similar to other acyltransferases, L-CPTI contains a general acid/base, His-473, a highly conserved amino acid residue that may form a hydrogen-bonding network or a salt bridge to a nearby conserved glutamate residue such as Glu-603 (27). We hypothesize that the substitution of Glu-590 with aspartate may disrupt a hydrogen-bonding network or a salt bridge, perhaps to the highly conserved Asp-567 residue that is predicted to be at the CoA binding pocket of L-CPTI. The hydrogen bonding between the negatively charged carboxyl groups of Glu-590 and Asp-567 may stabilize the positive charge or cation on the carnitine substrate in the wild-type enzyme, but disruption of the hydrogen-bonding network by substitution of Glu-590 with Asp, a negatively charged residue with only one methyl group less than Glu, destabilizes and inactivates L-CPTI. The complete loss in activity in the E590D mutant implicates the important role of Glu-590 in the maintenance of active site architecture, suggesting that substitution of Glu-590 with aspartate may disrupt a hydrogen-bonding network or a salt bridge to a residue like Asp-567 at the substrate binding site pocket of L-CPTI, which may stabilize the positive charge on the carnitine substrate. Disruption of the hydrogen-bonding network due to a change of Glu-590 to Asp may also result in the destabilization of the negative charge on the palmitoyl-CoA substrate due to charge repulsion, thereby inactivating L-CPTI. The substitution of Glu-590 with Gln (E590Q), a neutral residue with the same carbon chain length as Glu, with alanine, a neutral residue with a shorter carbon chain length than Glu, or Lys, a positively charged residue with a longer carbon chain length than Glu, significantly increased the malonyl-CoA sensitivity of L-CPTI with minor change in activity. We suggest that the positive charge in the mutant E590K stabilizes the negative charge on the malonyl-CoA, thus increasing the affinity and sensitivity of the enzyme to the inhibitor, but it may also destabilize the positive charge on the carnitine substrate causing a partial decrease in activity. Because a change of Glu-590 to Gln in-

**Table III**

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

| Strain | Carnitine | Palmitoyl-CoA |
|--------|------------|---------------|
|        | \( K_m \)  | \( V_{max} \) | \( K_m \)  | \( V_{max} \) |
| WT     | 59.3 ± 6.0 | 20.0 ± 0.5   | 153.5 ± 5.9 | 55.0 ± 0.7   |
| E590A  | 87.6 ± 11.4 | 17.2 ± 0.6  | 62.2 ± 7.4  | 23.2 ± 0.7  |
| E590Q  | 74.8 ± 3.1  | 13.5 ± 0.2  | 74.4 ± 8.1  | 21.8 ± 0.6  |

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Fig. 4. **Kinetic analysis of wild-type and mutant L-CPTI activities.** Isolated mitochondria (150 μg of protein) from the yeast strains expressing the wild type (○), E590A (△), and E590Q (●) mutants were assayed for CPT activity in the presence of increasing concentrations of carnitine (A) and palmitoyl-CoA (B).
creased the malonyl-CoA sensitivity of L-CPTI with no effect on activity, we suggest that the negatively charged acidic group of Glu-590 may be responsible for the decreased malonyl-CoA sensitivity of L-CPTI compared with M-CPTI. The role of the negatively charged –COOH group of Glu-590 in the reduction of malonyl-CoA sensitivity of L-CPTI compared with M-CPTI is further supported by our data demonstrating that a change of Glu-590 to Ala, a neutral residue of a much shorter chain length that Glu, significantly enhanced the malonyl-CoA sensitivity of L-CPTI with a partial decrease in activity.

Recent site-directed mutagenesis studies from our laboratory have demonstrated that glutamic acid 3 and histidine 5 in L-CPTI are necessary for malonyl-CoA inhibition and high affinity binding but not for catalysis (15, 16). For M-CPTI, our mutagenesis studies demonstrate that in addition to Glu-3 and His-5, Val-19, Leu-23, and Ser-24 are necessary for malonyl-CoA inhibition and high affinity binding, in agreement with the differences in malonyl-CoA sensitivity observed between M-CPTI and L-CPTI (17, 18). In addition, our site-directed mutagenesis studies of conserved residues in the C-terminal domain of L-CPTI demonstrated that conserved arginine and glutamate residues are important for catalytic activity and malonyl-CoA sensitivity (19, 23). Others have reported that the deletion of N-terminal residues 19–30 containing Ser-24 and Gln-30 or substitution of Ser-24 and Gln-30 with alanine in L-CPTI increased malonyl-CoA sensitivity but their effect was entirely dependent on the presence of Glu-3 because mutation of Glu-3 to alanine was found to override the effects of both the deletion and the combined effects of S24A and Q30A on malonyl-CoA sensitivity (28, 29), confirming our previous report that Glu-3 in the extreme N-terminal region of L-CPTI is the main determinant of malonyl-CoA sensitivity (16).

Mammalian tissues express two isoforms of CPT, a liver isoform (L-CPTI) and a muscle isoform (M-CPTI), that are 62% identical in amino acid sequence (3–8). M-CPTI is specifically expressed in heart, skeletal muscle, and testis (6, 8, 30). Previous work from our laboratory and others has shown that M-CPTI has a higher Kₘ for carnitine and an approximately 30-fold lower IC₅₀ for malonyl-CoA (3–8) inhibition than L-CPTI. In adult heart mitochondria, the total carnitine level is lower than in liver mitochondria but the carboxylate group of Glu-590 in the reduction of malonyl-CoA (3–8) inhibition than L-CPTI is the main determinant of malonyl-CoA sensitivity (16).

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In this report, we demonstrate that a single substitution of the conserved C-terminal Glu-590 residue to Ala, Gln, and Lys in L-CPTI results in a switch in the kinetic properties of the liver to the muscle isoform of the enzyme because the malonyl-CoA sensitivity of L-CPTI increased to a level close to that observed in M-CPTI. This is the first demonstration of a change in the kinetic properties of the liver isoform of CPTI close to that observed in the muscle isoform of the enzyme by a single site mutation of a conserved negatively charged C-terminal residue to neutral or positively charged residues and forms the structural basis for the high malonyl-CoA sensitivity of M-CPTI compared with L-CPTI.

In this report, we demonstrate that the highly conserved Glu-590 of L-CPTI is required for the structural stability of the enzyme and for the increased sensitivity of L-CPTI to malonyl-CoA inhibition, resulting in a switch in the kinetic property of the liver to the muscle isoform of the enzyme, M-CPTI. Despite its similar charge and potential for hydrogen-bonding formation, aspartate, which has a shorter side chain, cannot substitute for glutamate, suggesting that the extra methyl group of Glu-590 and/or its ability to serve as a strong hydrogen bond acceptor is needed for optimal catalysis, maintenance of active site integrity, and malonyl-CoA inhibition and binding. For the E590D mutant, our data suggest that the loss of a methyl group may result in the carboxylate being outside the hydrogen bond distance of the conserved Asp-567 residue that is at the substrate and/or active site pocket. This finding suggests that the longer side chain of glutamate is essential for substrate binding, catalytic activity, and malonyl-CoA sensitivity. Because only a maximum 16-fold increase in malonyl-CoA sensitivity was observed in these mutants compared with >30-fold higher sensitivity to the inhibitor reported with the wild-type M-CPTI, it is possible that this C-terminal region may constitute the low affinity malonyl-CoA binding site in L-CPTI (16).

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