Identification by Mutagenesis of Conserved Arginine and Tryptophan Residues in Rat Liver Carnitine Palmitoyltransferase I Important for Catalytic Activity*

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Carnitine palmitoyltransferase I catalyzes the conversion of long-chain acyl-CoA to acylcarnitines in the presence of l-carnitine. To determine the role of the conserved arginine and tryptophan residues on catalytic activity in the liver isoform of carnitine palmitoyltransferase I (L-CPTI), we separately mutated five conserved arginines and two tryptophans to alanine. Substitution of arginine residues 388, 451, and 606 with alanine resulted in loss of 88, 82, and 93% of L-CPTI activity, respectively. Mutants R601A and R655A showed less than 2% of the wild type L-CPTI activity. A change of tryptophan 391 and 452 to alanine resulted in 50 and 93% loss of activity, respectively. The mutations caused decreases in catalytic efficiency of 80–98%. The residual activity in the mutant L-CPTIs was sensitive to malonyl-CoA inhibition. Mutants R388A, R451A, R606A, W391A, and W452A had no effect on the $K_m$ values for carnitine or palmitoyl-CoA. However, these mutations decreased the $V_{max}$ values for both substrates by 10–40-fold, suggesting that the main effect of the mutations was to decrease the stability of the enzyme-substrate complex. We suggest that conserved arginine and tryptophan residues in L-CPTI contribute to the stabilization of the enzyme-substrate complex by charge neutralization and hydrophobic interactions. The predicted secondary structure of the 100-amino acid residue region of L-CPTI, containing arginines 388 and 451 and tryptophans 391 and 452, consists of four a-helices similar to the known three-dimensional structure of the acyl-CoA-binding protein. We predict that this 100-amino acid residue region constitutes the putative palmitoyl-CoA-binding site in L-CPTI.

Transport of long-chain fatty acids from the cytoplasm to the mitochondrial matrix involves the conversion of their acyl-CoA derivatives to acylcarnitines, translocation across the inner mitochondrial membrane, and reconversion to acyl-CoA (1, 2). Carnitine palmitoyltransferase I (CPTI) catalyzes the conversion of long-chain fatty acyl-CoAs to acylcarnitines in the presence of carnitine. Mammalian tissues express two isoforms of CPTI, a liver isoform (L-CPTI) and a heart/skeletal muscle isoform (M-CPTI), that are 62% identical in amino acid sequence (Refs. 3–7 and 9, GenBank™ accession number U62317). As an enzyme that catalyzes the first rate-limiting step in $\beta$-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 (1, 2). Because of its central role in fatty acid metabolism, a good understanding of the molecular mechanism of the regulation of the CPT system is an important first step in the development of treatments for diseases, such as myocardial ischemia and diabetes, and in human inherited CPT deficiency diseases (10–12).

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, 13–15). Furthermore, by using this system, we have shown that CPTI and CPTII are active distinct enzymes and that L-CPTI and M-CPTI are distinct malonyl-CoA-sensitive CPTIs that are reversibly inactivated by detergents. More recent 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 (16, 17). For M-CPTI, our deletion and substitution mutation analyses to date indicate that, in addition to Glu-3 and His-5, other specific residues within the 19–28 N-terminal amino acids are necessary for malonyl-CoA inhibition and high affinity binding, in agreement with the differences in malonyl-CoA sensitivity observed between M-CPTI and L-CPTI (18). In this work, site-directed mutagenesis studies of conserved residues in the predicted C-terminal catalytic domain of L-CPTI demonstrate for the first time that conserved arginine and tryptophan residues are important for catalysis.

**EXPERIMENTAL PROCEDURES**

**Construction of Rat Liver CPTI Mutants—** Mutants of L-CPTI were constructed using the “Quick Change” polymerase chain reaction-based mutagenesis procedure (Stratagene) with the pYGW11 (pGAP-L-CPTI) plasmid DNA as template. For example, to construct R388A, the forward primer R388AF, 5'-CCCTCACACTGTTCCACAGCCGCTGCCCCTG-3' and the reverse primer R388AR, 5'-TGGCCCAAGGGAGACCCGGGCTGTCAGAGGTCACATTG-3' were used for mutagenesis. Mutants R451A, R601A, R606A, W391A, and W452A were constructed as above but with the following pairs of primers: R451AF, 5'-GGAGATGCTTTTGGACCCGCGGTTTTCAGACCTC-3'; R451AR, 5'-GGAGCATCCGGGCTGTTTGGACGCGGACCCGCTGACAGACAGCCG-3'; R601AF, 5'-GGAGATGCTTTTGGACCCGCGGTTTTCAGACCTC-3'; R601AR, 5'-GGAGCATCCGGGCTGTTTGGACGCGGACCCGCTGACAGACAGCCG-3'; R606AF, 5'-GGAGATGCTTTTGGACCCGCGGTTTTCAGACCTC-3'; R606AR, 5'-GGAGCATCCGGGCTGTTTGGACGCGGACCCGCTGACAGACAGCCG-3'; W391AF, 5'-GGAGATGCTTTTGGACCCGCGGTTTTCAGACCTC-3'; W391AR, 5'-GGAGCATCCGGGCTGTTTGGACGCGGACCCGCTGACAGACAGCCG-3'; W452AF, 5'-GGAGATGCTTTTGGACCCGCGGTTTTCAGACCTC-3'; W452AR, 5'-GGAGCATCCGGGCTGTTTGGACGCGGACCCGCTGACAGACAGCCG-3'; R388AF, 5'-CCCTCACACTGTTCCACAGCCGCTGCCCCTG-3' and the reverse primer R388AR, 5'-TGGCCCAAGGGAGACCCGGGCTGTCAGAGGTCACATTG-3' were used for mutagenesis. Mutants R451A, R601A, R606A, W391A, and W452A were constructed as above but with the following pairs of primers: R451AF, 5'-GGAGATGCTTTTGGACCCGCGGTTTTCAGACCTC-3'; R451AR, 5'-GGAGCATCCGGGCTGTTTGGACGCGGACCCGCTGACAGACAGCCG-3'; R601AF, 5'-GGAGATGCTTTTGGACCCGCGGTTTTCAGACCTC-3'; R601AR, 5'-GGAGCATCCGGGCTGTTTGGACGCGGACCCGCTGACAGACAGCCG-3'; W391AF, 5'-GGAGATGCTTTTGGACCCGCGGTTTTCAGACCTC-3'; W391AR, 5'-GGAGCATCCGGGCTGTTTGGACGCGGACCCGCTGACAGACAGCCG-3'; W452AF, 5'-GGAGATGCTTTTGGACCCGCGGTTTTCAGACCTC-3'; W452AR, 5'-GGAGCATCCGGGCTGTTTGGACGCGGACCCGCTGACAGACAGCCG-3'.

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1 The abbreviations used are: ACBP, acyl-CoA binding protein; CPTI, carnitine palmitoyltransferase I; DEPC, diethylpyrocarbonate; L-CPTI, liver isoform of CPTI; M-CPTI, heart/skeletal muscle isoform of CPTI; NBS, N-bromosuccinimide; CPT, carnitine palmitoyltransferase; ACBP, acyl-CoA-binding protein.
N-Bromosuccinimide (NBS)—Mitochondria (200 μg) from the yeast strains expressing L-CPTI, CPTII, and M-CPTI were incubated with 10 mM phenylglyoxal in disruption buffer (pH 6.0) at 25 °C for 30 min as described by Shannmugasundaran et al. (20, 21). For the NBS treatment, 200 μg of mitochondria were incubated with 0.4 mM NBS on ice for 30 min.

**Western Blot—**Proteins were separated by SDS-polyacrylamide gel electrophoresis in a 7.5% gel and transferred onto nitrocellulose membranes. Immuno blots were developed by incubation with the L-CPTI-specific antibodies as described previously (17).

Sources of other materials and procedures were as described in our previous publication (17).

**RESULTS**

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

Alignment of the sequences of all carnitine and choline transferases from different species showed the presence of five conserved arginine and three conserved tryptophan residues (Fig. 1). For L-CPTI, these arginine residues are 388, 451, 601, 606, and 655 and tryptophan residues are 236, 391, and 452. To determine the role of these conserved arginine and tryptophan residues in L-CPTI on catalytic activity, they were each separately mutated to alanine (R388A, R451A, R601A, R606A, and R655A, W391A, and W452A).

**Generation of Mutations and Expression in P. pastoris—**Construction of plasmids carrying substitution mutations R388A, R451A, R601A, R606A, R655A, W391A, and W452A was performed as described under "Experimental Procedures." Mutations were confirmed by DNA sequencing. *P. pastoris* was chosen as an expression system for L-CPTI and the mutants, because it does not have endogenous CPT activity (6, 13–17). The *P. pastoris* expression plasmids expressed L-CPTI under the control of the *P. pastoris* glyceraldehyde-3-phosphate dehydrogenase gene promoter (13, 22). Yeast transformants with the wild type L-CPTI gene and the mutants were grown in liquid medium supplemented with glucose. As previously reported, no CPT activity was found in the control yeast strain with the vector but without the CPTI cDNA insert (13).

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 (13) is shown in Fig. 2, A and B. For the wild type and all the conserved arginine substitution mutations R388A, R451A, R601A, R606A, and R655A (Fig. 2A) and for the wild type and the tryptophan mutants W391A and W452A (Fig. 2B), 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 mutants R388A, R451A, and R606A had activity 7.0–18.0% of the wild type L-CPTI activity that was malonyl-CoA-sensitive;
the R606A mutant exhibited the lowest activity (Table I). Mutants R601A and R655A had less than 2% of the wild type L-CPTI activity and were less sensitive to malonyl-CoA inhibition than the wild type.

| Strain | Activity(nmol/mg·min) | Malonyl-CoA Inhibition (% | 2 µM | 100 µM |
|--------|------------------------|---------------------------|------|-------|
| Wild-type L-CPTI | 12.32 ± 0.361 (100) | 8.31 | 1.44 |
| R388A  | 1.46 ± 0.030 (11.8) | 0.36 | 0   |
| R451A  | 2.16 ± 0.100 (17.5) | 1.04 | 0   |
| R601A  | 0.23 ± 0.030 (1.9)  | 0.25 | 0.10 |
| R606A  | 0.25 ± 0.070 (6.9)  | 0.79 | 0.21 |
| R655A  | 0.18 ± 0.070 (5.5)  | 0.10 | 0.08 |
| W391A  | 6.040 (49)          | 1.53 | 0.21 |
| W452A  | 0.850 (6.9)         | 0.17 | 0   |

Replacement of Trp-391 and Trp-452 with alanine resulted in 50 and 93% loss of L-CPTI activity, respectively (Table I), but the residual activity was sensitive to malonyl-CoA inhibition.

Kinetic Characteristics of Mutant L-CPTIs—Mutants R388A, R451A, and R606A exhibited normal saturation kinetics when the carnitine concentration was varied relative to a constant second substrate, palmitoyl-CoA (Fig. 3A), a property identical to that of the wild type L-CPTI. For the R388A, R451A, and R606A mutants, the calculated $K_m$ values for carnitine were similar to the wild type value as shown in Table II, and the $V_{max}$ values were only 6–13% of the wild type value (Table II), indicating a major effect of the mutations on catalytic activity. The catalytic efficiency as estimated by $V_{max}/K_m$ for R388A, R451A, and R606A was decreased by 91, 89, and 95%, respectively. Due to the extremely low residual activity in mutants R601A and R655A, it was not possible to perform saturation kinetics and determine the $K_m$ or the $V_{max}$ values for carnitine or palmitoyl-CoA. With respect to the second substrate, palmitoyl-CoA, mutants R388A, R451A, and R606A exhibited normal saturation kinetics similar to the wild type (Fig. 3B) when the molar ratio of palmitoyl-CoA:albumin was fixed at 6.1:1. The calculated $K_m$ value for palmitoyl-CoA for mutants R388A and R451A was about 50% lower than the wild type, whereas for mutant R606A, it was 90% lower than the wild type (Table II). The $V_{max}$ values for mutants R388A, R451A, and R606A were 2–8% of the wild type values (Table II), and the catalytic efficiency was decreased by 90, 83, and 74%, respectively. Thus, substitution of the conserved arginine residues 388, 451, and 606 with alanine caused a substantial loss in catalytic activity but...
not in malonyl-CoA sensitivity. Substitution of arginines 601 and 655 with alanine resulted in nearly complete loss in CPT activity, which was accompanied by loss in malonyl-CoA sensitivity.

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

**TABLE II**

| Strain      | Carnitine $K_m$ (μM) | Carnitine $V_{\text{max}}$ (nmol/mg/min) | Palmitoyl-CoA $K_m$ (μM) | Palmitoyl-CoA $V_{\text{max}}$ (nmol/mg/min) |
|-------------|---------------------|------------------------------------------|---------------------------|-----------------------------------------------|
| Wild-type L-CPTI | 100.2              | 20.00                                    | 43.05                     | 49.23                                         |
| R388A       | 79.5                | 1.43                                     | 22.90                     | 2.52                                          |
| R451A       | 121.3               | 2.64                                     | 20.70                     | 3.99                                          |
| R606A       | 114.1               | 1.20                                     | 3.74                      | 1.13                                          |
| W391A       | 235.8               | 10.38                                    | 29.53                     | 14.99                                         |
| W452A       | 419.4               | 1.90                                     | 11.58                     | 1.23                                          |

**DISCUSSION**

The site-directed mutagenesis study described here is aimed at elucidating the function of several strictly conserved basic and aromatic amino acid residues found at the proximity of the active site of L-CPTI. Earlier chemical modification studies with CoA-metabolizing enzymes suggested that adjacent arginine and tryptophan residues located at the active site might be involved in CoA binding (20, 21). Studies with other enzyme systems using the arginine-specific reagent, phenylglyoxal, and the tryptophan-specific reagent, NBS, have shown that the negatively charged pyrophosphate group and the adenine moiety of CoA bind to adjacent positively charged arginine and hydrophobic (aromatic) tryptophan residues of the enzymes, respectively (20, 21, 23, 24). We found that chemical modification of isolated mitochondria from the yeast strain expressing L-CPTI by phenylglyoxal and NBS resulted in loss of catalytic activity. Five arginine and three tryptophan residues are fully conserved throughout the family of acyltransferases with known primary sequences. In this study, we separately changed each of the five conserved arginine and two of the conserved tryptophan residues to alanine and determined the CPTI activity of the mutant proteins. Arginine residues 388, 451, and 606, and tryptophan residues 391 and 452, when changed to alanine, resulted in mutant proteins that had considerably reduced L-CPTI activity that was malonyl-CoA-sensitive. Substitution mutation of arginine residues 601 and 655 with alanine resulted in mutant proteins that had little or no detectable L-CPTI activity. Despite the differences in enzyme activity observed between the mutants and the wild-type, the immunoblots with L-CPTI-specific antibodies revealed that all the mutants were expressed at similar steady-state levels as the wild type.

The reaction catalyzed by L-CPTI at the catalytic pocket, conversion of palmitoyl-CoA to palmitoylcarnitine in the presence of l-carnitine, has been hypothesized to involve deprotonation of the hydroxyl group of carnitine by a catalytic base (α-proton abstraction by His, Glu, or Gln) and attack by the resultant oxanion at the carbonyl of the thioester of palmitoyl-CoA to generate palmitoylcarnitine and free CoA (25). Since the CPT system has two substrates (palmitoyl-CoA and carnitine) with different physical properties, the active site pocket of the enzymes is predicted to contain separate or only partially overlapping binding pockets. Thus, a mutation that affects only the $K_m$ for one of the substrates might be predicted. No such mutation was found in this study. In fact, no change in $K_m$ of more than ~10-fold was observed for any of the mutant L-CPTIs in this study.

Mutants R388A, R451A, R606A, W391A, and W452A had little effect on the $K_m$ values for carnitine or palmitoyl-CoA but caused a considerable decrease in the $V_{\text{max}}$ for both substrates, suggesting that the main effect of the mutations was to decrease the stability of the enzyme-substrate complex. However, it is also possible that the mutations could lead to misfolding in 90% of the molecules, producing a lower $K_m$ and unchanged $V_{\text{max}}$ values. Since the mutations had minimal effect on the $K_m$, such a lack of $K_m$ alteration would suggest that separate substitution of the arginine and tryptophan residues is not sufficient to alter carnitine or palmitoyl-CoA binding. However, since these mutations decreased the $V_{\text{max}}$ by 10–40-fold, the substantial decrease in $V_{\text{max}}$ could be related to the alteration of intrinsic L-CPTI stability. In CPTII, a single nucleotide missense mutation of the conserved Arg-503 to cysteine, which corresponds to the conserved Arg-606 in CPTI, is the cause for CPTII deficiency disease in humans (26).

On the other hand, the R601A and R655A mutants were devoid of detectable activity. Thus, the presence of these conserved arginine residues is probably crucial for maintaining the configuration of the L-CPTI active site. Substitution mutation of the highly conserved Arg-505 to asparagine in bovine
Conserved Residues in L-CPTI Important for Catalysis

Liver carnitine octanoyltransferase, corresponding to the conserved Arg-655 in CPTI, was found to increase the Km for carnitine by more than 1650-fold (27). Based on the R505N mutation in carnitine octanoyltransferase, it was suggested that this conserved arginine residue in carnitine octanoyltransferase and other acyltransferases contributes to substrate binding by forming a salt bridge with the carboxylate moiety of carnitine (27). Mutant L-CPTI with a change of Arg-655 to Ala had insufficient activity to allow measurement of its Km value for carnitine. We suggest that conserved arginine and tryptophan residues in L-CPTI contribute to the stabilization of the transition state by charge neutralization and hydrophobic interactions, respectively.

Alignment of residues 381–481 of L-CPTI that contain Arg-388, Arg-451, Trp-391, and Trp-452 with a protein of known three-dimensional structure in the GenBank™ by the Swiss model software resulted in a known secondary structure of the acyl-CoA-binding protein (ACBP), a protein with 86 amino acid residues, being the best fit for the predicted secondary structure of the 100 C-terminal amino acids that constitute the putative palmitoyl-CoA binding region of L-CPTI (28, 29). ACBP and the 100-amino acid fragment of L-CPTI showed 32% similarity. The three-dimensional structure of the acyl-CoA-binding protein with bound palmitoyl-CoA consists of a skewed four a-helix bundle (30). The predicted secondary structure for the putative 100-amino acid residue palmitoyl-CoA binding region consists of four a-helices. Both ACBP and CPTI bind palmitoyl-CoA. ACBP binds long-chain fatty acyl-CoAs with high affinity, and the acyl-CoA-ACBP complex has been suggested to play a role in acyl-CoA-mediated cell signaling by interaction with, or donation of, long-chain acyl-CoA to CPTI and other proteins (31). Palmitoyl-CoA is a substrate for L-CPTI. We suggest that this 100-amino acid residue region constitutes the putative palmitoyl-CoA-binding site of L-CPTI.

The ACBP three-dimensional structure is a shallow bowl with a rim characterized by many polar and charged groups, whereas the inside and outside surfaces are predominantly hydrophobic with patches of uncharged hydroxyl groups (28, 30). It is predicted that the specificity of the ligand binding resides at the omega end of the acyl chain, together with strong electrostatic and hydrophobic interactions with the adenine 3’-phosphate of the CoA (28, 30). The phosphate and hydroxyl groups of the ribose are involved in an intense network of electrostatic and polar interactions with the polar parts of the side chains of hydrophobic and positively charged hydrophilic residues (28, 30). The large adenine ring stacks with the aromatic ring of a Tyr residue. In a separate study, it was demonstrated that the adenine ring of a CoA moiety interacts with the tryptophan residue of an enzyme that catalyzes the synthesis of acetyl-CoA, and furthermore, arginine residues electrostatically interact with the pyrophosphate moiety of CoA (20, 21).

In this study, we have investigated the functional importance of conserved arginine and tryptophan residues in L-CPTI on catalytic activity by site-directed mutagenesis. Mutations of conserved arginine and tryptophan residues affected catalytic activity, indicating the importance of electrostatic and hydrophobic contacts for the interaction of L-CPTI with the substrates. Electrostatic interactions are generally thought to play a role in the initial steps of ligand binding by guiding the productive collision of ligand and receptor. Chemical modification of mitochondria from yeast strains expressing CPTII by the histidine-specific reagent, diethylpyrocarbonate (DEPC), and site-directed mutagenesis have identified the conserved His-372 residue to be essential for catalytic activity (8). However, chemical modification of mitochondria from yeast strains expressing L-CPTI and M-CPTI by DEPC had no effect on catalytic activity, suggesting that the same conserved histidine residue in L-CPTI and M-CPTI is either not important for catalysis or may be important but inaccessible for DEPC modification.

Substitution mutagenization of arginine residues 601 and 665 with alanine resulted in mutant proteins that had little or no detectable L-CPTI activity. Thus, the presence of both of these conserved arginine residues is probably important for maintaining the configuration of the active site of L-CPTI. The region of CPTII between amino acid residues 550 and 619 has 11 additional highly conserved residues and may contain critical residues necessary for catalysis, such as Glu-571, Gln-575, and Glu-590, which may act as a catalytic base for the deprotonation of the hydroxyl group of carnitine.

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