Identification by Mutagenesis of a Conserved Glutamate (Glu487) Residue Important for Catalytic Activity in Rat Liver Carnitine Palmitoyltransferase II*

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Mammalian mitochondrial membranes express two active but distinct carnitine palmitoyltransferases: carnitine palmitoyltransferase I (CPTI), which is malonyl CoA-sensitive and detergent-labile; and carnitine palmitoyltransferase II (CPTII), which is malonyl CoA-insensitive and detergent-stable. To determine the role of the highly conserved C-terminal acidic residues glutamate 487 (Glu487) and glutamate 500 (Glu500) on catalytic activity in rat liver CPTII, we separately mutated these residues to alanine, aspartate, or lysine, and the effect of the mutations on CPTII activity was determined in the Escherichia coli-expressed mutants. Substitution of Glu487 with alanine, aspartate, or lysine resulted in almost complete loss in CPTII activity. Because a conservative substitution mutation of this residue, Glu487 with aspartate (E487D), resulted in a 97% loss in activity, we predicted that Glu487 would be at the active-site pocket of CPTII. The substantial loss in CPTII activity observed with the E487K mutant, along with the previously reported loss in activity observed in a child with a CPTII deficiency disease, establishes that Glu487 is crucial for maintaining the configuration of the liver isoform of the CPTII active site. Substitution of the conserved Glu500 in CPTII with alanine or aspartate reduced the $V_{\text{max}}$ for both substrates, suggesting that Glu500 may be important in stabilization of the enzyme-substrate complex. A conservative substitution of Glu500 to aspartate resulted in a significant decrease in the $V_{\text{max}}$ for the substrates. Thus, Glu500 may play a role in substrate binding and catalysis. Our site-directed mutagenesis studies demonstrate that Glu487 in the liver isoform of CPTII is essential for catalysis.

*Carnitine palmitoyltransferase (CPT) I and CPTII, in conjunction with carnitine translocase, transport long chain fatty acids from the cytoplasm to the mitochondrial matrix for β-oxidation (1, 2). Mammalian mitochondrial membranes express two active but distinct carnitine palmitoyltransferases (CPTI and CPTII), a malonyl CoA-sensitive, detergent-labile CPT, and a malonyl-CoA-insensitive, detergent-stable CPTII. CPTI is an integral membrane enzyme located on the outer mitochondrial membrane, and CPTII is a membrane-associated enzyme loosely bound to the matrix side of the inner mitochondrial membrane. A current model for the membrane topology of CPTI predicts exposure of the N- and C-terminal domains crucial for activity and malonyl-CoA sensitivity on the cytosolic side of the outer mitochondrial membrane (3). As an enzyme that catalyzes the rate-limiting step in fatty acid oxidation, CPTI is tightly regulated by its physiologic inhibitor, malonyl-CoA, the first intermediate in fatty acid synthesis (1, 2). This is an important regulatory mechanism in fatty acid metabolism and suggests coordinated control of fatty acid oxidation and synthesis. Mammalian tissues express two isoforms of CPTI, a liver isoform of CPTI and a heart/skeletal muscle isoform of CPTI, that are 62% identical in amino acid sequence (4–7, 9). Rat and human CPTII cDNAs have been cloned and sequenced (10, 11). The cDNA sequences predicted proteins of 658 amino acid residues (71 kDa) that had 82 and 85% identity at the amino acid and nucleotide levels, respectively. Because the size of the mRNA in different rat tissues was identical, CPTII appears to be the product of a single gene that is expressed uniformly in every tissue examined thus far (2). CPTII is a distinct, catalytically active, malonyl-CoA-insensitive enzyme, because a rat liver cDNA encoding CPTII synthesizes an active protein when expressed in Escherichia coli, in the yeasts Saccharomyces cerevisiae and Pichia pastoris, or in baculovirus or COS cells (11, 13–16). Detergent-solubilized yeast-expressed CPTII showed normal saturation kinetics with both substrates, carnitine and palmitoyl-CoA, and the calculated $K_m$ and $V_{\text{max}}$ were similar to that observed with the rat liver mitochondrial CPTII (15). With the S. cerevisiae-expressed CPTII, mutations of the conserved residues His372, Asp376, and Asp464 to alanine resulted in complete loss of CPTII activity, suggesting that these residues may be required for catalysis (14). We and others hypothesize that the reaction catalyzed by CPTI and CPTII in the direction of palmitoylcarnitine formation at the active site pocket involves deprotonation of the hydroxyl group of carnitine by a catalytic base (α-proton abstraction by His, Glu, or Gln) and attack by the resultant oxyanion at the carbonyl of the thioester of palmitoyl-CoA to generate palmitoylcarnitine and free CoA (14, 17, 18).

Carnitine palmitoyltransferase deficiencies are common disorders of mitochondrial fatty acid oxidation. Rare human genetic defects in fatty acid oxidation specifically ascribed to CPTI, CPTII, and the carnitine translocase have been reported (19, 20). CPTII deficiency, the most common inherited disorder

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The abbreviation used is: CPT, carnitine palmitoyltransferase.

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of lipid metabolism affecting skeletal muscle, is an autosomal recessive disorder with three distinct clinical phenotypes (19). The human CPTII gene is 20 kb in length, contains five exons, and is located at chromosome Ip32 (10). More than 25 different mutations and three polymorphisms have been identified in the CPTII gene (18, 22, 23). One missense mutation, S113L, accounts for ∼60% of the mutant alleles responsible for the adult myopathic form of the disease (24, 25). Recently, an E487K missense mutation in conjunction with S113L was reported in a child with CPTII deficiency characterized by recurrent episodes of myalgia and myoglobinuria triggered by fever (26).

**EXPERIMENTAL PROCEDURES**

**Construction of Rat Liver CPTII Mutants**—Mutants were constructed using the overlap extension PCR procedure (27) with the pPROEX-CPTII plasmid DNA as the template. Plasmid pPROEX-CPTII is a derivative of pPROEX-1, a protein expression vector in E. coli (Invitrogen) and the plasmid pYGW6 (15). To construct pPROEX-CPTII, the rat liver CPTII cDNA, was released and ligated to EcoI-cuts of the wild-type CPTII cDNA insert. The construction of the wild-type and mutant CPTIIs was performed as an expression system for the wild-type and mutant CPTII is catalytically active (13). We expressed the full-length rat liver CPTII cDNA in E. coli and demonstrated that, unlike CPTI, the expressed CPTII is catalytically active (13). We expressed the full-length rat liver CPTII cDNA in E. coli and demonstrated that, unlike CPTI, the expressed CPTII is catalytically active (13). 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**TABLE II**

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

| Strains  | Activity | % |
|----------|----------|---|
| Wild-type CPTII | 122.4 ± 0.48 | 100 |
| EC-E487A | 4.9 ± 0.09 | 4.0 |
| EC-E487K | 1.2 ± 0.05 | 1.0 |
| EC-E487D | 4.0 ± 0.07 | 3.3 |
| EC-E500A | 76.6 ± 0.33 | 57.6 |
| EC-E500D | 30.6 ± 0.05 | 25.0 |

FIG. 1. Sequence alignment of portions of the C-terminal region of various acyltransferases. *+, identical conserved residues; -, conserved residues.

Western blot analysis of the extract from the E. coli strain expressing the wild-type and mutant CPTIIs using an anti-His tag antibody showed the presence of a 71-kDa protein corresponding to CPTII (Fig. 2). For the wild-type and mutant CPTIIs, proteins of the predicted size were expressed at similar steady-state levels as determined by quantitative immunoblot using a pure His₆ protein as the standard. The quantitative Western blot and the densitometry analysis for the His₆ protein are shown in Fig. 2, B and C. Based on the quantitative immunoblot of the standard protein, the wild-type and mutant CPTII proteins were estimated to contain 7 pmol (0.49 μg) of CPTII in 20 μg of whole-cell extract protein.

Kinetic Properties of Wild-type and Mutant CPTIIs—Because of the extremely low activity in mutants E487A, E487D, and E487K, it was not possible to determine the Kₘ or Vₑₘ values for the carnitine or palmitoyl-CoA substrates. Mutants E500A and E500D exhibited normal saturation kinetics when the carnosine concentration was varied relative to a constant second substrate, palmitoyl-CoA (Fig. 3A), a property identical to the wild-type CPTII. For the E500A and E500D, the calculated Kₘ values for carnitine were 2-fold higher than the value for the wild-type CPTII (lane 4), a plot of the band intensity (densitometry units) versus the His₆ protein at different concentrations. C, a plot of the band intensity (densitometry units) versus the His₆ protein at different concentrations.

FIG. 2. A, immunoblots showing expression of wild type (lane 1), E. coli hbl(1/1) preyX-1 (lane 2), EC-E500A (lane 3), EC-E500D (lane 4), EC-E487A (lane 5), and EC-E487K (lane 6), EC-E487D (lane 7), and wild type (lane 8). Proteins (20 μg) of whole-cell extract from each strain expressing the wild-type and mutant CPTIIs were separated on a 10% SDS-PAGE and blotted onto a nitrocellulose membrane. The immunoblots were developed using anti-His tag antibodies as described under “Experimental Procedures.” B, immunoblots of the His₆ protein at different concentrations. C, a plot of the band intensity (densitometry units) versus the His₆ protein at different concentrations.

**DISCUSSION**

The site-directed mutagenesis study described here is aimed at elucidating the function of highly conserved acidic residues found at the proximity of the active site of CPTII. Two glutamate residues, Glu⁴⁸⁷ and Glu⁵⁰⁰ in rat liver CPTII, and the corresponding residues Glu⁵⁹⁰ and Glu⁶⁰³ in a liver isoform of CPTI, are conserved throughout the family of acyltransferases with known primary sequences. Specifically, Glu⁴⁸⁷ in CPTII and Glu⁵⁹⁰ in CPTI are highly conserved within the family of acyltransferases, but Glu⁵⁰⁰ in CPTII and Glu⁶⁰³ in CPTI are conserved residues within the family of CPT enzymes, whereas other acyltransferases have aspartate at this position (Fig. 1).

To determine the role of the conserved Glu⁴⁸⁷ and Glu⁵⁰⁰ residues in CPTII in catalysis, we separately changed the highly conserved glutamate residue (Glu⁴⁸⁷) to aspartate (E487A), aspartate (E487D), and lysine (E487K), and the second conserved glutamate residue (Glu⁵⁰⁰) in CPTII to alanine (E500A) and aspartate (E500D), and we determined the effect of the mutations on CPTII activity in the E. coli-expressed mutant enzyme. Substitution of Glu⁴⁸⁷ with alanine, lysine, or aspartate resulted in nearly complete loss in CPTII activity (Table II).

asppartate resulted in substantial loss in catalytic activity, but substitution of the highly conserved Glu⁴⁸⁷ with alanine, lysine, or aspartate resulted in nearly complete loss in CPTII activity (Table II).
maintaining the configuration of the liver isoform of CPTII active site. All of the Glu\(^{487}\) mutants had insufficient activity to allow measurement of \(K_m\) or \(V_{\text{max}}\) for either carnitine or palmitoyl-CoA. Because a conservative substitution of Glu\(^{487}\) with aspartate (E467D), a negatively charged amino acid that has only one less methyl group than the glutamate residue in the wild-type enzyme, resulted in 97% loss in activity, we suggest that Glu\(^{487}\) is at the active-site pocket of CPTII.

As the terminal enzyme that transports long chain fatty acids from the cytosol to the mitochondrial matrix, CPTII in the presence of CoA-SH reversibly catalyzes the conversion of long chain acylcarnitines to long chain acyl-CoAs. Similar to other acyltransferases, CPTII contains a general acid/base, His\(^{372}\), a highly conserved residue that may form a hydrogen-bonding network or a salt bridge to a nearby conserved glutamate residue such as Glu\(^{487}\) or Glu\(^{500}\). Substitution of Glu\(^{487}\) with alanine, lysine, or aspartate resulted in an inactive enzyme. Glu\(^{487}\) may thus be involved in facilitating catalysis by orienting the imidazole ring of His\(^{372}\) for optimum productive interaction with the substrate (12). We hypothesize that the Glu\(^{487}\) to alanine or lysine substitution may disrupt a hydrogen-bonding network or a salt bridge, perhaps to a residue like His\(^{372}\) at the active site of CPTII. However, a change of Glu\(^{487}\) to aspartate may result in the carboxylate being outside the hydrogen bond distance of His\(^{372}\), the predicted general acid/base at the active site (12).

The characterization of E487K, E487A, and E487D described here is consistent with previously reported studies of a child with a novel CPTII deficiency disease that exhibited clinical episodes of myalgia and myoglobinuria induced by intercurrent febrile illnesses (26). The patient was heterozygous for a G-to-A substitution at codon 487, changing the highly conserved Glu\(^{487}\) to lysine (E487K), whereas the other allele carried the common benign S113L missense mutation. However, for the rat liver CPTII, a single substitution mutation of Glu\(^{487}\) to lysine resulted in almost complete loss (99%) in activity, and even a single conservative change of Glu\(^{487}\) to aspartate (E487D) or alanine (E487A) caused >96% loss in catalytic activity, further supporting a pathogenic role for a mutation of this residue. Thus, substitution mutation of the highly conserved negatively charged Glu\(^{487}\) residue located in the C-terminal region inactivates CPTII, suggesting a critical role of this residue in catalysis. CPTII deficiency, inherited as an autosomal recessive trait, is the most common disorder of lipid metabolism affecting muscle and is the most frequent cause of hereditary myoglobinuria in adults that is triggered by prolonged exercise or fasting (or both) and by cold, stress, or fever, conditions associated with increased dependence of muscle on lipid metabolism (19, 24, 25). A hepatic form of CPTII deficiency associated with hypoketotic hypoglycemia, hepatopathy, cardiopathy, and sudden death has also been reported in infants (19).

To investigate the role of the second conserved glutamate residue in CPTII (Glu\(^{500}\)1 on catalytic function, Glu\(^{500}\) was changed to alanine and aspartate and the mutant enzyme was characterized. Substitution of the conserved Glu\(^{500}\) with alanine and aspartate resulted in partial loss of activity and a decrease in the \(V_{\text{max}}\) for both substrates. In addition, a change of the conserved Glu\(^{500}\) to aspartate caused a significant decrease in the \(V_{\text{max}}\) for both substrates with a 2-fold increase in the \(K_m\) for carnitine. For both substrates, a lower but similar decrease in the \(V_{\text{max}}\) and a 2-fold increase in the \(K_m\) for carnitine was observed with the E500A mutant, suggesting that the main effect of the mutations was to decrease the stability of the enzyme-substrate complex. However, the mutations could lead to misfolding in 90% of the molecules, producing a lower \(K_m\) and unchanged \(V_{\text{max}}\) values. Because the E500A and E500D mutations caused a substantial decrease in the \(V_{\text{max}}\), this could be related to the alteration of intrinsic CPTII stability. Furthermore, Glu\(^{500}\) appears to be critical for the structural stability of CPTII. A conservative substitution of Glu\(^{500}\) to aspartate resulted in >7- and 2.5-fold decreases in the \(V_{\text{max}}\) for the.

### Table III

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

| Strains         | Carnitine | Palmitoyl-CoA |
|-----------------|-----------|---------------|
|                 | \(K_m\)   | \(V_{\text{max}}\) | \(K_m\)   | \(V_{\text{max}}\) |
| Wild-type CPTII | 63.7      | 187.6         | 81.9      | 395.7         |
| EC-E500A        | 105.6     | 138.7         | 19.9      | 114.2         |
| EC-E500D        | 111.4     | 77.5          | 8.8       | 53.0          |

FIG. 3. A, kinetic analysis of wild-type and mutant CPTII activities. Proteins (150 μg) of whole-cell extract from wild type (squares), EC500A (circles), and EC500D (triangles) were assayed for CPT activity in the presence of increasing concentration of carnitine. B, same as A, except CPT activity was measured in the presence of an increasing concentration of palmitoyl-CoA.
substrates palmitoyl-CoA and carnitine, respectively, with a relatively minor change in carnitine binding but a significant increase in the affinity for palmitoyl-CoA. It is suggested that the Glu$^{497}$ mutation exists within a region containing a possible adenine binding loop, because the affinity of the enzyme for palmitoyl-CoA that has an adenine group is greatly increased, in comparison with the enzyme corresponding residue in CPTI, His$^{473}$ to alanine, inactivates both enzymes (14, 21). In this report, we have demonstrated that the conserved Glu$^{487}$ of rat liver CPTII is reactivated both enzymes (14, 21). In this report, we have demonstrated that the conserved Glu$^{487}$ of rat liver CPTII is reactivated both enzymes (14, 21). In this report, we have demonstrated that the conserved Glu$^{487}$ of rat liver CPTII is reactivated both enzymes (14, 21).

Sequence alignment of various acyltransferases shows the presence of highly conserved histidine and glutamate residues in CPTII. Mutation of the conserved His$^{372}$ residue in CPTII and the corresponding residue in CPTI, His$^{473}$ to alanine, inactivates both enzymes (14, 21). In this report, we have demonstrated that the conserved Glu$^{487}$ of rat liver CPTII is required for catalysis. Despite its similar negative charge and potential for hydrogen bonding, an aspartate residue cannot fulfill this requirement, suggesting that the extra methyl group in glutamate is needed for optimal catalysis and maintenance of active site integrity. A change of Glu$^{487}$ to lysine or alanine also resulted in an inactive enzyme. The loss in activity observed with the E487K is consistent with previously reported studies of a child with a novel CPTII deficiency disease (26). Substitution of the conserved Glu$^{500}$ in CPTII with alanine or aspartate reduced the catalytic efficiency, suggesting that this residue may be important in stabilization of the enzyme-substrate complex.

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