Structure and Function of Carnitine Acyltransferases

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ABSTRACT: Carnitine acyltransferases catalyze the exchange of acyl groups between carnitine and coenzyme A (CoA). These enzymes include carnitine acetyltransferase (CrAT), carnitine octanoyltransferase (CrOT), and carnitine palmitoyltransferases (CPTs). CPT-I and CPT-II are crucial for the β-oxidation of long-chain fatty acids in the mitochondria by enabling their transport across the mitochondrial membrane. The activity of CPT-I is inhibited by malonyl-CoA, a crucial regulatory mechanism for fatty acid oxidation. Mutation or dysregulation of the CPT enzymes has been linked to many serious, even fatal human diseases, and these enzymes are promising targets for the development of therapeutic agents against type 2 diabetes and obesity. We have determined the crystal structures of murine CrAT, alone and in complex with its substrate carnitine or CoA. The structure contains two domains. Surprisingly, these two domains share the same backbone fold, which is also similar to that of chloramphenicol acetyltransferase and dihydrolipoyl transacetylase. The active site is located at the interface between the two domains, in a tunnel that extends through the center of the enzyme. Carnitine and CoA are bound in this tunnel, on opposite sides of the catalytic His343 residue. The structural information provides a molecular basis for understanding the catalysis by carnitine acyltransferases and for designing their inhibitors. In addition, our structural information suggests that the substrate carnitine may assist the catalysis by stabilizing the oxyanion in the reaction intermediate.

KEYWORDS: carnitine; carnitine acetyltransferase; carnitine acyltransferase; carnitine palmitoyltransferase; chloramphenicol acetyltransferase; dihydrolipoyl transacetylase; fatty acid; function; structure

INTRODUCTION

Carnitine is a small, polar compound (FIG. 1A) that is found in all mammals. The most important function of this compound is to facilitate the transport of fatty acids across the inner mitochondrial membrane.[1,2] The β-oxidation of fatty acids in the mitochondrial matrix is a major source of cellular energy, especially in the heart and muscle.[3] Besides fatty acid oxidation, carnitine may also play a role in maintaining

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the cellular coenzyme A (CoA):acyl-CoA ratio, by acting as an acceptor for the acyl group.

The central role of carnitine in the energy metabolism of fatty acids is due to the fact that there are no transporters for the CoA esters of fatty acids in the inner mitochondrial membrane. Instead, the CoA esters must first be converted to carnitine esters (FIG. 1A).[4,5] This reaction is catalyzed by carnitine palmitoyltransferase-I

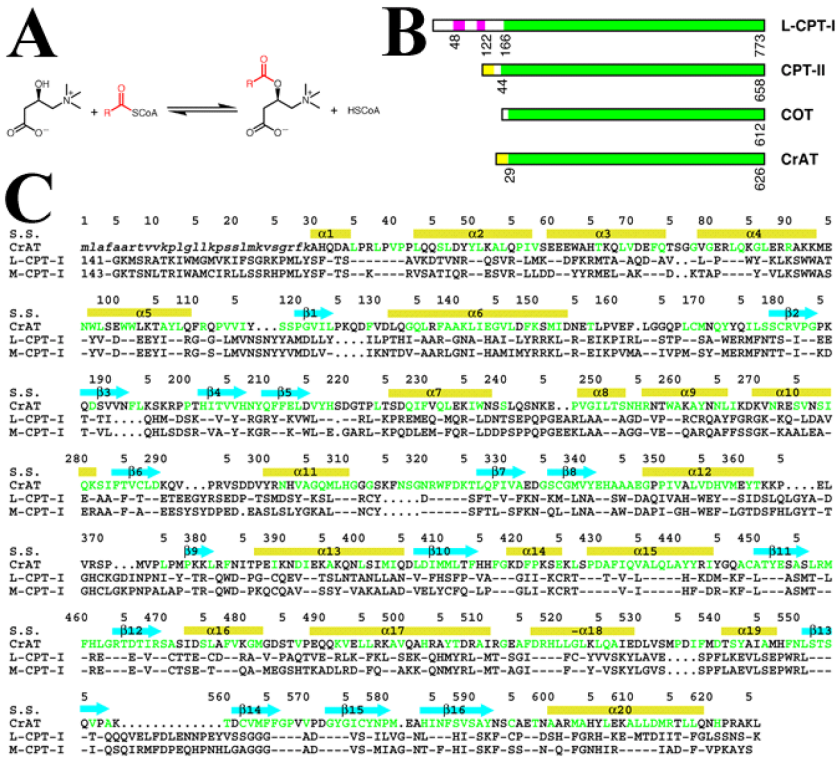

FIGURE 1. The carnitine acyltransferases. **(A)** The reaction catalyzed by the carnitine acyltransferases. **(B)** Domain organization of L-CPT-I, CPT-II, CrOT, and CrAT. The catalytic domains are shown in *green*, the two transmembrane segments of L-CPT-I in *magenta*, and the mitochondrial targeting sequences of CPT-II and CrAT in *yellow*. **(C)** Sequence alignment of mouse CrAT and human liver- and muscle-type carnitine palmitoyltransferase I (L-CPT-I and M-CPT-I). The secondary structure elements in the CrAT structure are labeled (S.S.). Residues shown in *green* are in the core of the protein, with less than 25% exposed surface area. The residue numbers are for CrAT. An additional 140 residues at the N-terminus of the L-CPT-I and M-CPT-I are not shown. A *dash* represents a residue that is identical to that in CrAT, while a *dot* represents a deletion. [NOTE: This figure appears in color on the NYAS Web site (www.nyas.org/annals).]

(CPT-I), which is localized in the outer membrane of the mitochondria (FIG. 1B). The carnitine esters are transported across the inner membrane and converted back to the CoA esters by CPT-II. The CoA esters then enter the β-oxidation pathway in the mitochondrial matrix.

Three isoforms of CPT-Is are present in humans. CPT-Ia is mostly found in the liver (also known as L-CPT-I); CPT-Ib is mostly found in the heart and skeletal muscle (M-CPT-I); and CPT-Ic is mostly found in the brain. The functional role of CPT-Ic in fatty acid metabolism remains to be established.[6] In comparison, there is only one CPT-II enzyme in humans.

The CPTs are an important control point for the regulation of fatty acid oxidation and are promising targets for the development of therapeutic agents against diabetes, obesity, and other human diseases. The most important regulatory mechanism is the potent inhibition of CPT-Is by malonyl-CoA.[4] In heart and skeletal muscle, malonyl-CoA is primarily produced by acetyl-CoA carboxylase 2 (ACC2), and ACC2 knockout mice have elevated fatty acid oxidation and reduced body fat content and body weight.[7,8] Studies with the compound C75, originally developed as a fatty acid synthase inhibitor, show that it can activate CPT-Is and reduce body weight.[9,10] Therefore, an agonist of CPT-Is may be efficacious for the treatment of obesity.

Mutation and dysregulation of CPTs are linked to serious human diseases. Recessive mutations of CPT-I and CPT-II can produce hypoketonemia and hypoglycemia in patients, while CPT-II deficiency is the most common cause of abnormal lipid metabolism in skeletal muscle.[4,5] Single-point mutations as well as insertions/deletions in the CPT genes can produce the clinical phenotype. The hypoglycemia observed in patients with reduced CPT-I activity suggests that antagonists of CPT-Is may be able to lower blood glucose levels. A covalent CPT-I inhibitor, etomoxir, can lower blood glucose levels in diabetic animals and humans, showing that such inhibitors may be efficacious for the treatment of type 2 diabetes.[5,11–13]

The CPTs belong to the family of enzymes known as the carnitine acyltransferases, which also includes carnitine octanoyltransferases (CrOTs) and carnitine acetyltransferases (CrATs, also known as CATs). These enzymes generally contain about 600 residues, with molecular weights of about 70 kDa (FIG. 1B). The CPT-Is contain an additional N-terminal domain of about 160 residues for membrane attachment as well as for malonyl-CoA sensitivity.[5] The catalytic domains of these enzymes are well conserved, with 35% or higher amino acid sequence identity between any pair of them (FIG. 1C). However, these enzymes do not share any recognizable amino acid sequence homology to other proteins in the sequence database.

The carnitine acyltransferases catalyze the exchange of acyl groups between carnitine and CoA (FIG. 1A).[4,5,14] This exchange reaction is fully reversible, with no need for energy input. As it is generally accepted that acyl-CoAs represent activated acyl groups, it follows that acylcarnitines must also represent activated acyl groups.[15] This may be linked to the solvation energies of the carnitine and acylcarnitine compounds.[5]

Our understanding of this important family of enzymes has been enhanced significantly by the recent reports on the crystal structure of mouse CrAT, alone and in complex with the substrates carnitine and CoA,[16] as well as the structure of human CrAT free enzyme.[17] The observations from these first structures, as well as their implications for the catalytic mechanism of these enzymes and their roles in fatty acid transport, are described below.[18]

THE OVERALL STRUCTURE

The structure of CrAT contains 16 β-strands (named β1–β16) and 20 α-helices (α1–α20) and can be divided into 2 domains, N and C domains (FIG. 2A). A surprising discovery from the structures is that the 2 domains share the same backbone fold, despite the lack of any recognizable amino acid sequence homology between them. The core of the 2 domains is a 6-stranded mixed β-sheet (β10–β13, β15, β16 in the C domain), with 3 α-helices covering one of its faces (α6, α7, α12 in the N domain) (FIG. 2A). The other face of the β-sheet in the N domain is covered by additional helices, whereas the other face of the β-sheet in the C domain is covered by the N domain. In fact, part of the β13–β15 crossover connection in the

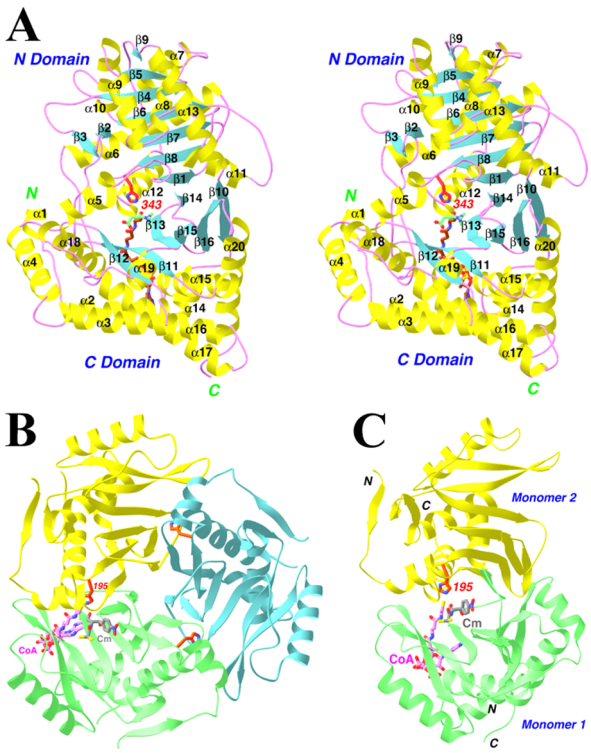

FIGURE 2. Structure of CrAT. **(A)** Stereo diagram showing a schematic representation of the structure of CrAT. The β-strands and α-helices are labeled, and the catalytic His343 residue is shown in *red*. The binding modes of carnitine (in *green*) and CoA (in *brown*) are also indicated. **(B)** Structure of the trimer of chloramphenicol acetyltransferase (CAT).[19] The catalytic His residue is shown in *red*. The substrates CoA and chloramphenicol (Cm) are shown and labeled in one of the active sites. **(C)** Structure of two monomers in the trimer of CAT, viewed in the same orientation as panel A. The active site is located at the interface between the two monomers. Produced with Ribbons.[32] [NOTE: This figure appears in color on the NYAS Web site (www.nyas.org/annals).]

C domain forms a β-strand (β14), and this strand is hydrogen-bonded to β1 of the N domain in an antiparallel fashion (FIG. 2A).

The structural observations with CrAT are confirmed with our recent structure of mouse CrOT (Jogl, Hsiao, and Tong, manuscript in preparation), which shares 35% amino acid sequence identity with CrAT. This suggests that the catalytic domains of all the carnitine acyltransferases may have the same overall structure as observed for CrAT and CrOT. The CPT-Is have an additional segment of about 50 residues at the extreme N terminus that is located in the cytoplasm (FIG. 1B). It is known that this segment has interactions with the catalytic domain,[5] but their conformation and docking site on the catalytic domain remain to be determined.

STRUCTURAL AND FUNCTIONAL CONSERVATION WITH CHLORAMPHENICOL ACETYLTRANSFERASE AND DIHYDROLIPOYL TRANSACETYLASE

The backbone fold of the N and C domains of CrAT is similar to that of chloramphenicol acetyltransferase (CAT) (FIG. 2B)[19] as well as the catalytic domain of dihydrolipoyl transacetylase (E2pCD),[20] another unexpected discovery from the structural studies on CrAT. The amino acid sequence identity between the structurally equivalent residues of these enzymes and CrAT is less than 10%. Because of the structural conservation between carnitine acetyltransferase and CAT, it is better to call the former CrAT to avoid confusion. This enzyme was generally referred to as CAT in the literature before the structural information became available.

Both CAT and E2pCD function as trimers (FIG. 2B) and catalyze the transfer of an acetyl group from acetyl-CoA to an organic substrate. The active site of these enzymes is located at the interface between neighboring monomers of the trimer (FIG. 2B). Most remarkably, the tertiary organization of the N and C domains of CrAT is similar to the quaternary organization of neighboring monomers in the trimers of CAT and E2pCD (FIG. 2C). Moreover, the catalytic histidine residue is located at the same position in all these enzymes. These observations highlight the structural and functional conservation between the carnitine acyltransferases, CAT, and E2pCD, despite the lack of any sequence conservation.

The carnitine acyltransferases may have evolved by gene duplication of a single-domain enzyme such as E2pCD or CAT. However, most enzymes that evolved through gene duplication maintain internal pseudo 2-fold symmetry. The carnitine acyltransferases may be unique in possessing a pseudo 3-fold symmetry between the duplicated domains. Moreover, the exposed face of the core β-sheet is covered by the neighboring monomer in the trimers of CAT and E2pCD (FIG. 2B). Such coverage is observed for the C domain, but is not possible for the N domain of CrAT (FIG. 2A). Instead, the carnitine acyltransferases contain additional α-helices in the N domain (α8, α10, α11) that cover the exposed face of the core β-sheet (FIG. 2A).

THE ACTIVE SITE TUNNEL

The active site of CrAT is located at the interface between the N and C domains (FIG. 2A). The catalytic base of carnitine acyltransferases has been identified by bio-

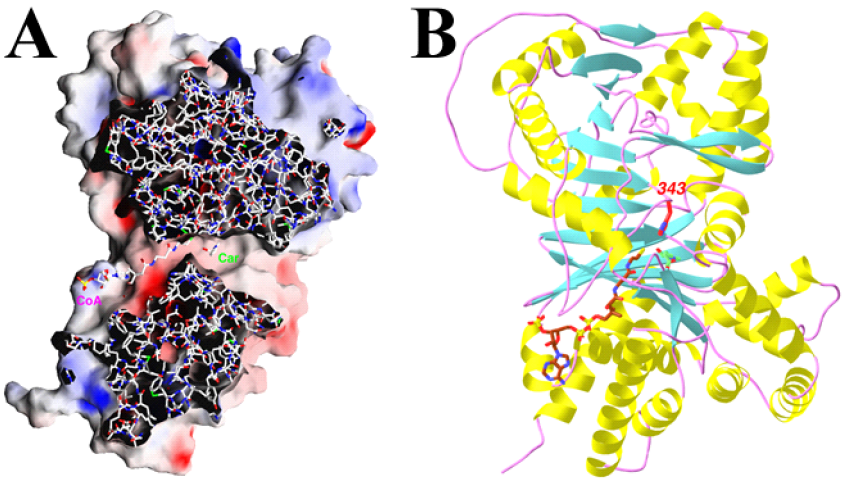

FIGURE 3. The active site tunnel. **(A)** Molecular surface of CrAT, showing the tunnel that extends through the center of the molecule. The top half of the structure was cut away in order to visualize the tunnel. Produced with Grasp.[33] **(B)** Schematic drawing of the structure of CrAT, in a similar view as panel A. Produced with Ribbons.[32] [NOTE: This figure appears in color on the NYAS Web site (www.nyas.org/annals).]

chemical and mutagenesis studies as a histidine residue, equivalent to His343 in mouse CrAT.[4,5] In the structure, His343 is located deep in the enzyme, in a tunnel that extends through the center of the protein (FIG. 3A). The catalytic residue can be reached from both openings of the tunnel on the surface of the structure. One of these openings is used for binding carnitine, while the other is used for binding CoA. Thus, the two substrates of the enzyme are bound at opposite sides of the catalytic His residue (FIG. 3B).

The His343 residue is in the connection between strand β8 and helix α12 in the N domain (FIG. 2A). The side chain of this residue is held in an unusual conformation such that the Nδ1 ring nitrogen is hydrogen-bonded to the main-chain carbonyl oxygen. Such a conformation is also observed for the catalytic His residues in CAT and E2pCD,[19,20] and may be important for the catalysis by these enzymes.

THE CARNITINE BINDING SITE

Residues from both the N and C domains participate in the formation of the carnitine binding site (FIG. 4A). Carnitine is bound in a partially folded conformation (FIG. 4B), which is one of the favored rotamers of this compound in solution.[21] There are only minor conformational changes in the enzyme upon the binding of carnitine.

The carboxylate group of carnitine is involved in an intricate network of hydrogen-bonding interactions, as well as electrostatic interactions with the side chain guanidinium group of Arg518 (from helix α18) (FIG. 4B). The side chain

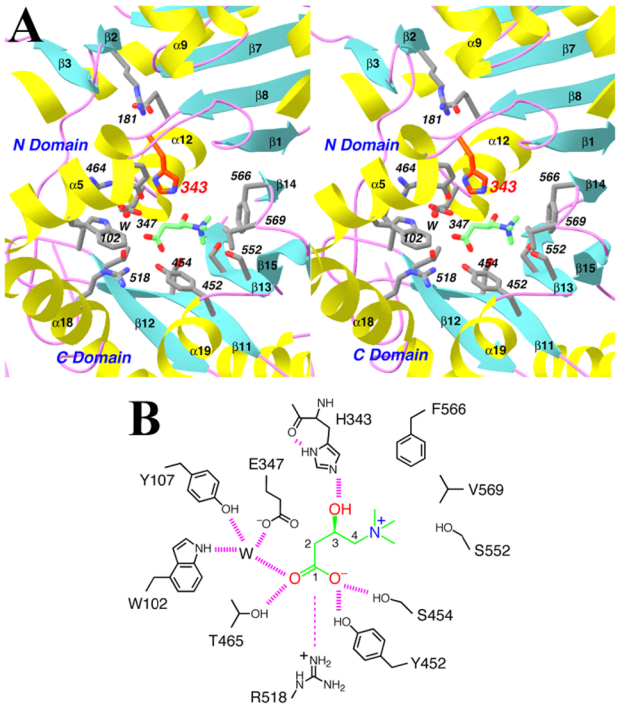

FIGURE 4. The carnitine binding site. **(A)** Stereo diagram showing the carnitine binding site of CrAT. The side chain of the catalytic His343 residue is shown in *red*, and carnitine is shown in *green*. A water molecule that mediates carnitine binding is shown as a *red sphere* and labeled "W". Produced with Ribbons.[32] **(B)** Schematic drawing of the interactions between carnitine and CrAT. Hydrogen-bonding interactions are shown as *thick dashed lines*, and the electrostatic interaction between the carboxylate group and Arg518 is shown as the *thin dashed line*. [NOTE: This figure appears in color on the NYAS Web site (www.nyas.org/annals).]

hydroxyls of Tyr452, Ser454, and Thr465 provide three hydrogen-bonding partners for the carboxylate group, whereas the fourth partner is a water molecule. This water molecule is held in place by hydrogen bonds to the side chains of Trp102, Tyr107, and Glu347, in a tetrahedral arrangement (FIG. 4A). Thr465 and Arg518 are strictly conserved among the carnitine acyltransferases (FIG. 1C). The Arg → Asn mutation in bovine CrOT produced a 1650-fold increase in the K_m for carnitine, but had little effect on the K_m for CoA or the k_{cat} of the enzyme.[22]

In comparison, the trimethylammonium group of carnitine does not show many specific interactions with the enzyme, and its positive charge is not recognized by a negatively charged residue from the enzyme. Instead, the group is located in a mostly hydrophobic environment, near the side chains of Ser552, Phe566, and Val569 (FIG. 4B). Ser552 is the first residue of the strictly conserved STS motif among the carnitine acyltransferases (FIG. 1C). Mutation of this residue to Ala in bovine CrOT

caused only a 17-fold increase in the K_m for carnitine.[23] Structural analysis suggests the positive charge on carnitine may be important for the catalytic activity of these enzymes (see below).

The 3-hydroxyl group of carnitine forms a hydrogen bond with the side chain Nε2 atom of the catalytic His343 residue (FIG. 4B). Hence, the carnitine substrate is positioned perfectly for the withdrawal of its hydroxyl proton by the catalytic base.

THE CoA BINDING SITE

The CoA molecule is bound in the active site tunnel, on the opposite side of the His343 side chain from the carnitine molecule (FIG. 3B). It is in the fully extended, linear conformation (FIG. 5A), with a 25-Å separation between the thiol group at one end and the adenine base at the other. This binding mode is different from that of CoA when bound to CAT and E2pCD,[19,20] where a folded conformation is observed (FIG. 5B). As a consequence, the adenine bases are separated by about 14 Å in the

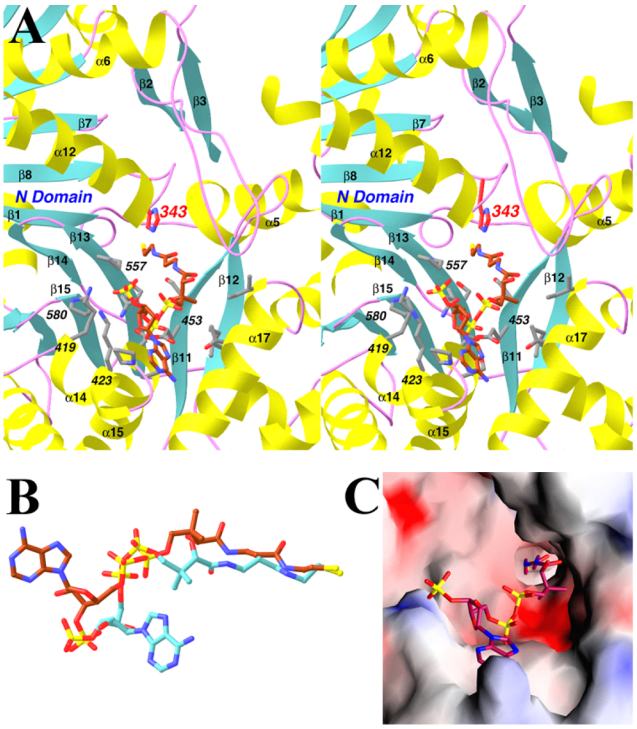

FIGURE 5. The CoA binding site. **(A)** Stereo diagram showing the CoA binding site of CrAT. The CoA molecule is shown in *brown*. Produced with Ribbons.[32] **(B)** Overlap of the binding modes of CoA to CrAT (in *brown*) and CAT (in *cyan*). **(C)** The active site tunnel for CoA binding. Panels B and C produced with Grasp.[33] [NOTE: This figure appears in color on the NYAS Web site (www.nyas.org/annals).]

two conformations of CoA. Amino acid differences between CrAT and CAT, especially the Pro580 residue near the end of strand β15, account for the different CoA binding modes.[16] Interestingly, Pro580 is conserved only in the CrATs, but not in the CrOTs or the CPTs (FIG. 1C). It will be interesting to determine the binding mode of CoA to these other carnitine acyltransferases.

The CoA binding tunnel is created in part by the splaying apart of the neighboring parallel strands β11 and β13 of the β-sheet in the C domain (FIG. 2A). This produces a small opening between the two strands, which accommodates the pantothenic arm of CoA (FIG. 5C). This region of the tunnel may not be wide enough for the adenine nucleotide portion of CoA to pass through, suggesting that the CoA molecule can reach the active site only from one of the openings of the active site tunnel.

Residues that line the CoA binding tunnel are generally conserved among the carnitine acyltransferases (FIG. 1C). Residues Lys419 and Lys423 recognize the 3′-phosphate group of CoA (FIG. 5A). Covalent modification of these Lys residues might be the molecular basis for the reduced substrate binding and catalytic activity of CrAT associated with aging.[24] Interestingly, two acidic residues, Asp430 and Glu453, are hydrogen-bonded to each other and help form the tunnel for the pantothenic arm of CoA (FIG. 5A). Mutation of either of these residues can reduce the activity of the enzyme.[5] Mutation of the equivalent of Glu453 in L-CPT-I to Asp produced an inactive enzyme, whereas mutation of this residue to Ala, Gln, and Lys had only minor impact on catalysis, but increased the malonyl-CoA sensitivity of the enzyme.[25]

THE FATTY ACID BINDING SITE

Based on the structure of CrAT in complex with carnitine, the binding mode of acetylcarnitine can be predicted (FIG. 6). The model suggests that the binding site

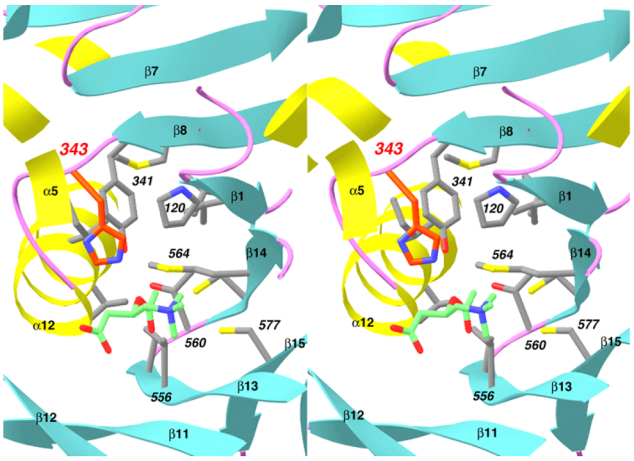

FIGURE 6. The fatty acid binding site. The possible binding site for acyl groups is revealed based on a model for the binding mode of acetylcarnitine. Produced with Ribbons.[32] [NOTE: This figure appears in color on the NYAS Web site (www.nyas.org/annals).]

for the fatty acids may be located in a hydrophobic pocket between helix α12 and strands β1, β8, β13, β14, and β15 (FIG. 6). Interestingly, the side chain of Met564, from strand β14, is positioned in the center of this pocket, which may be part of the molecular basis for the preference for short-chain acyl groups by CrAT. The equivalent residue in the CPT-Is is a glycine (FIG. 1C), which should create additional space for the binding of longer fatty acids. This is confirmed by our mutagenesis studies showing that the M564G mutant of CrAT has better activity with the hexanoyl-CoA substrate than the wild-type enzyme (Hsiao, Jogl, and Tong, manuscript in preparation). Overall, the sequence conservation among the carnitine acyltransferases near strand β14 is weak (FIG. 1C). This sequence diversity may have a direct impact on the size and shape of the fatty acid binding pocket and may help define the substrate preference of the different carnitine acyltransferases.

THE CATALYTIC MECHANISM: SUBSTRATE-ASSISTED CATALYSIS

For the catalysis by the carnitine acyltransferases, the His residue in the active site acts as a general base to extract the proton from the 3-hydroxyl group of carnitine or the thiol group of CoA, depending on the direction of the reaction.[4,5] This is consistent with observations in the crystal structures, and the substrates are optimally positioned in the complexes for catalysis (FIG. 7).

The structural analyses suggest that the oxyanion in the tetrahedral intermediate of the reaction may be stabilized by the positive charge on the trimethylammonium group of carnitine itself (FIG. 7). This is supported by the kinetic data showing that the positive charge of carnitine is not critical for binding, but is absolutely required for catalysis.[26] Thus, carnitine acyltransferases are an example of substrate-assisted catalysis.[27]

The oxyanion should also be able to hydrogen-bond to the side chain hydroxyl of Ser554 (FIG. 7), the second Ser residue in the strictly conserved STS motif (FIG. 1C). The equivalent Ser residue in CAT and E2pCD has been identified as the oxyanion hole of those enzymes.[28,29] However, Ser554 probably makes only a minor contri-

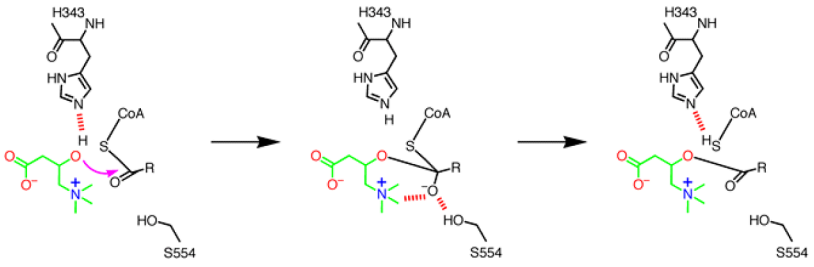

FIGURE 7. The catalytic mechanism of carnitine acyltransferases. The catalytic His343 residue can extract the proton from either carnitine or CoA. The oxyanion in the tetrahedral intermediate is stabilized by interactions with carnitine and the side chain hydroxyl of Ser554. [NOTE: This figure appears in color on the NYAS Web site (www.nyas.org/annals).]

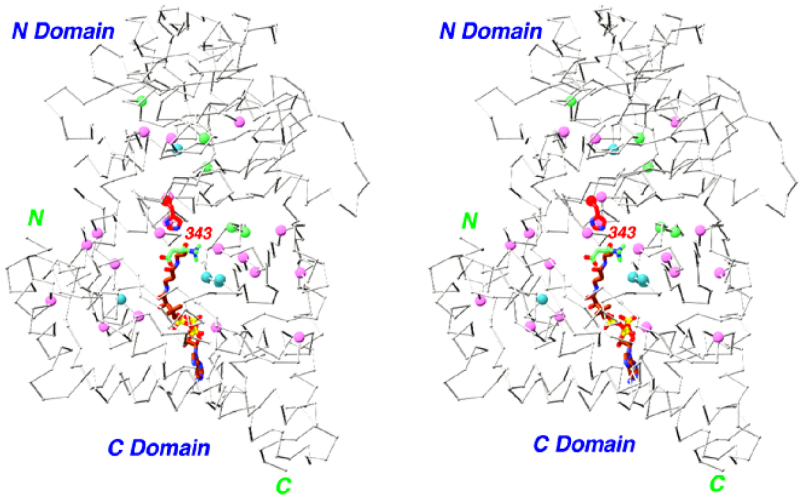

FIGURE 8. The disease-causing mutations in the structure of carnitine acyltransferases. Mutations in L-CPT-I (*green*), CPT-II (*magenta*), or CrOT (*cyan*) that affect the activity or stability of the enzyme are shown. The H343 side chain is shown in *red*. [NOTE: This figure appears in color on the NYAS Web site (www.nyas.org/annals).]

bution to the catalysis by the carnitine acyltransferases as mutation of this residue in CrOT produced only a 10-fold decrease in the k_{cat}, while having little impact on the K_m for carnitine.[23] This further supports the functional importance of the carnitine substrate itself in stabilizing the transition state of this reaction.

DISEASE-CAUSING MUTATIONS

Natural recessive mutations in L-CPT-I and CPT-II have been associated with several human diseases.[4,5,30,31] In addition, site-directed mutants have been created for CrOT to assess the functions of specific amino acid residues.[5] Based on the sequence homology between these enzymes and CrAT, the locations of the mutations can be mapped onto the structure of CrAT. Interestingly, the mutations are widely distributed in the three-dimensional structure of this enzyme (FIG. 8). A few of them are located near the active site and may have a direct impact on substrate binding or enzyme catalysis. For example, the G710E mutation of L-CPT-I replaces the glycine residue in the fatty acid binding pocket (equivalent of Met564 in CrAT; FIG. 1C) with a bulky, charged group, which can be expected to be detrimental for substrate binding. However, most of the mutations are located at least 10 Å away from the active site, suggesting that they must have an indirect mechanism of action.[30] Studies on these enzymes and their mutants, especially of CPT-II, are needed to fully understand the molecular basis for the deficiencies caused by these mutations.

Carnitine acyltransferases have crucial roles in fatty acid oxidation and are promising targets for the development of antidiabetes and antiobesity agents.[4,5,11–13] Structural studies on these enzymes have provided a clear understanding of their substrate binding and catalytic mechanism. It will be especially satisfying if the structural information can lead to the development of successful therapeutics in the clinic.

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