Structural and biochemical studies
of the substrate selectivity of carnitine acetyltransferase

Yu-Shan Hsiao, Gerwald Jogl, Liang Tong*

Department of Biological Sciences
Columbia University
New York, NY 10027

* Corresponding author.
Phone: (212) 854-5203; FAX: (212) 854-5207
e-mail: tong@como.bio.columbia.edu
Summary

Carnitine acyltransferases catalyze the exchange of acyl groups between coenzyme A (CoA) and carnitine. They have important roles in many cellular processes, especially the oxidation of long-chain fatty acids, and are attractive targets for drug discovery against diabetes and obesity. These enzymes are classified based on their substrate selectivity for short-chain, medium-chain, or long-chain fatty acids. Structural information on carnitine acetyltransferase (CrAT) suggests that residues Met564 and Phe565 may be important determinants of substrate selectivity, with the side chain of Met564 located in the putative binding pocket for acyl groups. Both residues are replaced by glycine in carnitine palmitoyltransferases (CPTs). To assess the functional relevance of this structural observation, we have replaced these two residues with small amino acids by mutagenesis, characterized the substrate preference of the mutants, and determined the crystal structures of two of these mutants. Kinetic studies confirm that the M564G or M564A mutation is sufficient to increase the activity of the enzyme towards medium-chain substrates, with hexanoyl-CoA being the preferred substrate for the M564G mutant. The crystal structures of the M564G mutant, both alone and in complex with carnitine, reveal a deep binding pocket that can accommodate the larger acyl group. We have determined the crystal structure of the F565A mutant in a ternary complex with both the carnitine and CoA substrates at 1.8 Å resolution. The F565A mutation has minor effects on the structure or the substrate preference of the enzyme.

Running title: The substrate selectivity of carnitine acetyltransferase
Introduction

Carnitine acetyltransferase (CrAT) belongs to the family of carnitine acyltransferases, which catalyze the exchange between acyl-CoA and acylcarnitine (1-4). The enzymes of this family are classified based on their substrate selectivity. CrAT prefers short-chain fatty acids, whereas carnitine octanoyltransferase (COT) prefers medium-chain fatty acids. The exchange reaction for the long-chain fatty acids is catalyzed by the carnitine palmitoyltransferases (CPTs). These enzymes generally contain about 600 residues, and have significant sequence homology. For example, the amino acid sequence identity between CrAT and the other carnitine acyltransferases is about 35%. However, these enzymes do not share any recognizable sequence homology with other proteins in the database.

The CPTs have crucial roles in the transport of long-chain fatty acids into the mitochondria for oxidation (2). In humans, three different CPT enzymes (CPT-Ia, CPT-Ib, and CPT-Ic) are associated with the outer membrane of the mitochondria, and a fourth CPT enzyme (CPT-II) is located in the mitochondrial matrix (3). Naturally-occurring mutations of these proteins are linked with hypoketonemia, hypoglycemia and other diseases (2,3). Inhibitors of these enzymes may be efficacious for the treatment of type 2 diabetes (5,6). At the same time, agonists of these enzymes can stimulate fatty acid oxidation, and may regulate body weight (7). Consequently, there is currently a considerable amount of interest in the carnitine acyltransferases.

We recently reported the crystal structure of mouse CrAT, alone and in complex with its substrate carnitine or CoA (8). The structure of the free enzyme of human CrAT has also been reported (9). The structures demonstrate that the carnitine acyltransferases are made up of two domains (N and C domains) of the same backbone fold, and the active site is located at the interface between the two domains (Fig. 1A) (10). The carnitine and CoA substrates are bound in a tunnel, on opposite sides of the catalytic His343 residue. The
carboxylate group of carnitine has four hydrogen-bonds with the enzyme, and its negative charge is balanced by the side chain of the strictly-conserved Arg518 residue. In contrast, the positively-charged trimethylammonium ion of carnitine interacts with several hydrophobic side chains. The structural information suggests that this cation may be important for stabilizing the oxyanion in the tetrahedral transition state of the reaction (8), an example of substrate-assisted catalysis (11).

The structures of the carnitine and CoA complexes identify a potential binding site for the acyl group of the substrate (Fig. 1B), and suggest that Met564 and Phe565 in this pocket may be strong determinants of the preference for short-chain fatty acids by CrAT (8). To assess the functional roles of these residues in the substrate selectivity of carnitine acyltransferases, we have created and characterized single-site mutants in this binding pocket of mouse CrAT based on the structural information.

Materials and Methods

**Mutagenesis, protein expression and purification.** Residues 30–626 of wild-type mouse CrAT was sub-cloned into the pET28a vector (Novagen) and over-expressed in *E. coli* (8). The expression construct contains an N-terminal hexa-histidine tag. The mutants were designed based on the structural information, created with the QuikChange kit (Stratagene), and verified by sequencing.

The wild-type and mutant proteins were purified following the same protocol, with nickel-agarose, anion exchange, and gel filtration chromatography. The protein was concentrated to 80 mg/ml in a solution containing 20 mM Tris (pH 8.5) and 200 mM NaCl, flash-frozen in liquid nitrogen in the presence of 5% (v/v) glycerol, and stored at –80°C. More than 20 mgs of highly-pure wild-type and mutant enzymes were obtained from the preparation. The protein samples migrated as a single peak from the gel filtration column.
**Protein Crystallization.** Crystals of the M564G mutant were obtained at 4°C by the sitting-drop vapor diffusion method. The reservoir solution contained 100 mM Tris (pH 8.5), 150 mM NaCl, 12% (w/v) PEG 3350, and the protein was at 25 mg/ml concentration. For cryo protection, the crystals were transferred to a solution containing 25% (v/v) ethylene glycol, and flash-frozen in liquid nitrogen for data collection at 100K. They belong to space group \( P4_3 \), with cell parameters of \( a=b=72.2 \) Å, and \( c=128.8 \) Å. There is one molecule of the mutant protein in the crystallographic asymmetric unit.

Crystals of the M564G mutant protein in complex with carnitine were obtained by soaking the free enzyme crystals with 2.5 mM carnitine for 16 hours.

Crystals of the F565A mutant were obtained at 4°C by the sitting-drop vapor diffusion method. The reservoir solution contained 100 mM Tris (pH 7.5), 100 mM NaCl, 15% (w/v) PEG 3350, and 2.3 mM carnitine, and the protein was at 38 mg/ml concentration. The crystals were then soaked in 5 mM acetyl-CoA for 2 hours before being flash-frozen for data collection. They belong to space group \( C2 \) with cell parameters of \( a=165.1 \) Å, \( b=89.6 \) Å, \( c=123.0 \) Å, and \( \beta=129.2^\circ \). There are two molecules of the mutant protein in the crystallographic asymmetric unit.

**Data collection, structure determination and refinement.** X-ray diffraction data were collected on an ADSC Quantum-4 CCD at the X4A beamline of the National Synchrotron Light Source (NSLS), as well as on a R-AxisIV imaging plate system mounted on a Rigaku RU-H3R rotating anode X-ray generator. The diffraction images were processed and scaled with the HKL package (12).

The initial structures of the mutants were determined by the molecular replacement method, with the program COMO (13,14). The structure refinement was carried out with the program CNS (15). Manual adjustment of the atomic model against the electron density was performed with the program O (16). The crystallographic information are summarized in Table 1.
Kinetic studies. The substrate preference of wild-type and mutant CrAT enzymes were determined by kinetic studies, following a protocol reported earlier (17). The reaction buffer contained 25 mM potassium phosphate (pH 7.4), 125 µM 4,4'-dithiobispyridine (dissolved in ethanol), 0.5 mM CoA, 2 mM carnitine, and 0.5 µg enzyme, in a volume of 0.2 ml. The reaction was monitored by the increase in absorbance at 324 nm. The assays were repeated 2-5 times for each enzyme preparation with each substrate in order to verify the observed reaction rates.

Atomic Coordinates. The atomic coordinates will be deposited in the Protein Data Bank, with the accession codes 1T7N, 1T7O, and 1T7Q.

Results and Discussion

Selection of residues for mutagenesis

Based on the crystal structure of mouse CrAT in complex with carnitine, we predicted the possible binding mode of acetylcarnitine (8). For the current studies, we modeled the compound octanoylcarnitine in the binding site, assuming a mostly extended conformation for the acyl group (Fig. 1B). The octanoyl group is chosen as a representative of medium-chain acyl groups. In the model, it is located in a pocket that is formed between helix α12 and strands β1, β8, β13, β14, and β15, supporting our earlier prediction that this may be the binding site for longer acyl groups. Interestingly, the residues that are in close proximity of the octanoyl group are poorly conserved among the carnitine acyltransferases (Fig. 1C). This also supports the identification of this pocket for binding acyl groups, as sequence variations are needed to modulate the size of this pocket to accommodate the different substrate preferences of these enzymes.

The sequence comparison shows that residues Met564 and Phe565 in strand β14 may play important roles in determining the substrate specificity. The side chain of Met564 is located in the center of the pocket, and actually clashes with the modeled conformation of
the octanoyl group (Fig. 1B). In all the other carnitine acyltransferases, this residue is replaced by a glycine (Fig. 1C), which should relieve the steric clash between the octanoyl group and the enzyme. The side chain of Phe565 is sandwiched between strand β1 and helix α11 (Fig. 1B). This residue is replaced with a glycine in the CPTs, and with a valine in COT (Fig. 1C). Reducing the bulk of this side chain could affect the positioning of the β1 and β14 strands, and thereby change the shape of the binding pocket. As a first attempt to understand the molecular basis for substrate selectivity by the carnitine acyltransferases, we have selected to mutate these two residues and characterize the effects of the mutation on the catalysis by the enzyme and the structure of the enzyme.

**Mutation at Met564 can change substrate preference**

Based on the structural and sequence information, we designed the M564G, M564A, and F565A single-site mutants. These mutant proteins were over expressed in *E. coli* and purified in large quantities, using the same protocol as that for the wild-type enzyme. The mutant proteins are monomeric in solution, and appear to be as stable as the wild-type enzyme.

The substrate preference of the wild-type and the mutant enzymes were determined by kinetic studies, using short-chain (acetyl-CoA and butyryl-CoA), medium-chain (hexanoyl-CoA and octanoyl-CoA), and long-chain (decanoyl-CoA and palmitoyl-CoA) substrates at saturating concentrations. The concentration of the carnitine substrate is also at the saturating level. The assays were repeated several times for each mutant and for each substrate, and consistent results were obtained from these experiments (Fig. 2).

The kinetic data show that the wild-type enzyme has higher activity with the short-chain substrates than the medium-chain substrates (Fig. 2). In comparison, the M564G and M564A mutants have significantly better catalytic activity with the medium-chain substrates. Especially, the preferred substrate for the M564G mutant appears to be hexanoyl-CoA, rather than acetyl-CoA (Fig. 2). This supports our hypothesis based on the
structural information that the Met564 residue is important for substrate selectivity, and removing the bulk of this side chain has altered the substrate preference profile of the enzyme. Interestingly, the M564A mutant has roughly equal activity with the short-chain and medium-chain substrates, suggesting that the extra methyl group has a strong influence on substrate selectivity.

On the other hand, the catalytic activity of the F565A mutant with the medium-chain substrates is still very weak (Fig. 2). In fact, the substrate preference of this mutant closely mimics that of the wild-type enzyme. Neither the wild-type enzyme nor any of the mutants have significant activity with the long-chain substrates (Fig. 2). Additional structural differences between CrAT and the CPTs may be necessary to accommodate these longer acyl groups.

An enlarged acyl group binding pocket in the structures of the M564G mutant

To assess the effects of the M564G mutation on the conformation of the enzyme, we have determined the crystal structures of this mutant, both alone and in complex with carnitine, at up to 1.9 Å resolution (Table 1). Several attempts at determining the binding modes of the acyl groups, especially the hexanoyl or octanoyl group, have so far not been successful. This is consistent with our earlier experiences with the wild-type enzyme, which showed that the acyl groups are hydrolyzed during the crystallization process (8).

The overall structure of the M564G mutant is similar to that of the wild-type enzyme, with rms distance of 0.52 Å for 591 equivalent Cα atoms between them. The M564G mutation introduced only minor changes in the conformation of this residue, with the Cα atom shifting by about 0.5 Å (Fig. 3A). Interestingly, larger conformational changes are observed in several other residues in the active site region. Most pronounced among these is the change in the position of the side chain of Val556, which partly fills the void left by the absence of the Met564 side chain (Fig. 3A). In the wild-type enzyme, Val556 interacts with the beginning of helix α12. In the mutant, these residues (345–352) show large
conformational changes, which may be coupled with the change in the Val556 side chain (Fig. 3A).

Carnitine is bound in the active site of the M564G mutant, at the same position as that observed in the wild-type enzyme (Fig. 3A). The carboxylate group of carnitine is involved in the same intricate network of hydrogen-bonding interactions with the enzyme. Although the main chain of Glu347 has undergone a conformational change due to the mutation, its side chain carboxylate is located at the same position as in the wild-type enzyme (Fig. 3A), maintaining its role in the binding of carnitine. There are only small structural changes in the enzyme upon carnitine binding, the rms distance between Cα atoms of the free enzyme and the carnitine complex is only 0.24 Å. The side chain of Ser454 assumes a different rotamer to hydrogen-bond to the carboxylate of carnitine, and this change was also observed in the wild-type enzyme (8).

The removal of the Met564 side chain in the M564G mutant produces a dramatic change in the surface of the protein, such that there is now a clearly defined, deep pocket in the mutant enzyme that can readily accommodate the hexanoyl group (Fig. 3B). In contrast, this pocket is much shallower in the wild-type enzyme, which may only have enough space for a butyryl group (Fig. 3C). This is consistent with kinetic observations on most CrATs (Fig. 2), which showed that the enzymes have good activity for acetyl, propionyl, and butyryl groups but poorer activity for longer acyl substrates (1).

**Minor structural changes in the F565A mutant**

We have determined the crystal structure of the F565A mutant at 1.8 Å resolution (Table 1). A comparison to the structure of the wild-type CrAT showed that the F565A mutation introduced only minor differences in the structure of the enzyme, including the positions of the β1 and β14 strands (Fig. 4). The side chain of Ile123 adopts a different rotamer in the F565A mutant, occupying a small portion of the void created by the mutation (Fig. 4). The Met564 side chain in the acyl group binding pocket has essentially
the same conformation as in the wild-type enzyme. The structural information therefore explains our kinetic observation that the F565A mutant still prefers short-chain acyl groups as substrates (Fig. 2).

It remains to be seen whether the F565A or F565G mutation can have a larger impact on substrate selectivity when it is coupled with the M564G mutation. In fact, the Val563 residue of CrAT is replaced by a glycine in the CPTs as well (Fig. 1C). We have created the triple mutant that replaced the 563-VMF-565 residues in CrAT with three Gly residues. Unfortunately, this mutant is unstable in solution and we were not able to characterize it kinetically or structurally.

**Structure of CrAT in a ternary complex with carnitine and CoA**

The F565A mutant crystal was grown in the presence of carnitine, and soaked for two hours in a solution containing 5 mM acetyl-CoA. The crystal was then transferred to a cryo-protectant solution containing an additional 25% ethylene glycol (EG) for 1 min before being flash-frozen. The shorter soaking time was chosen in an attempt to minimize the hydrolysis of the acetyl group by the enzyme. The crystallographic analysis, however, revealed electron density for a CoA molecule in the active site (Fig. 5A), without the acetyl group. Even more amazingly, we observed the binding of an EG molecule in the active site (Fig. 5B and see below). This suggests that the hydrolysis must have happened during the 1 min in the cryo-protectant solution, as EG cannot bind in the active site unless (acetyl-)CoA leaves the pocket.

Clear electron density for the carnitine substrate was also observed (Fig. 5C). Therefore, this is the structure of CrAT in a ternary complex with carnitine and CoA, which for the first time allows us to examine the simultaneous binding of both substrates to this enzyme.
The two molecules of the F565A mutant in the asymmetric unit of the crystal have the same conformation, with rms distance of 0.26 Å for their equivalent Cα atoms. The bound conformations of the CoA and carnitine substrates are also the same in the two active sites. The overall structure of the ternary complex of the F565A mutant (Fig. 5D) is highly similar to that of the wild-type enzyme, with rms distance of 0.35 Å to the structure of the wild-type free enzyme.

In the ternary complex, carnitine has the same interactions with the enzyme as those in the binary complex that we observed earlier (8) (Fig. 5E). The hydroxyl group of carnitine is hydrogen-bonded to the side chain of the catalytic His343 residue, with a distance of 2.7 Å. In comparison, the thiol group of CoA in the ternary complex is 1.2 Å away from its position in the binary complex (Fig. 5E), such that this group is not hydrogen-bonded to the His343 side chain (distance of 3.8 Å) in the ternary complex. Interestingly, the distance between the hydroxyl group of carnitine and the thiol group of CoA is only 2.8 Å in the ternary complex, suggesting a strong hydrogen-bonding interaction between the two substrates. The thiol group in the ternary complex may also have a weak interaction with the main chain amide group of Gly348, with a distance of 3.5 Å.

Two side chains in the active site, Glu347 and Ser454, assume different rotamers in the ternary complex as compared to the free enzyme structure (Fig. 4). The change in the side chain of Ser454 enables it to be hydrogen-bonded to the carboxylate of carnitine, and this change was also observed in the binary complex with carnitine (8). The side chain of Glu347 in the free enzyme conformation is in steric clash with the thiol group of CoA in the ternary complex. Interestingly, the conformation of this side chain in the ternary complex is the same as that in the binary complex with CoA (8), and it can maintain the hydrogen-bond to the water molecule that mediates the binding of the carboxylate group of carnitine (Fig. 5E).
The ethylene glycol molecule is placed in the acyl group binding site (Fig. 5E). One of its hydroxyl groups is hydrogen-bonded to the side chain of Ser554, mimicking the interaction from the carbonyl oxygen from the acyl group of the substrate, while the other hydroxyl group is hydrogen-bonded to the side chain of Tyr341. The ethylene group has van der Waals interactions with the side chains of Met564 and Phe566.

Our structural and biochemical studies show that the Met564 residue has an important role in determining the substrate preference of CrAT. Met564 is part of a cluster of three residues, 563-VMF-565, that are replaced by glycine in the CPTs. These residues are located in strand β14, which helps enclose the acyl group binding site. Besides these three amino acids, several other clusters of residues show significant sequence differences among the canitine acyltransferases, (Fig. 1C). It is likely that these other sites also play a role, and further studies will be needed to fully characterize the molecular basis for substrate selectivity of these enzymes.

Acknowledgements

We thank Randy Abramowitz and Xiaochun Yang for setting up the X4A beamline at NSLS; Yang Shen, Javed Khan, Corey Mandel, and Farhad Forouhar for help with the data collection at the synchrotron; Rona Ramsay for helpful discussions. This research is supported in part by a grant (DK67238 to LT) from the National Institutes of Health.
Table 1

Summary of crystallographic information

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>M564G in complex with carnitine</th>
<th>M564G in complex with carnitine and CoA</th>
<th>F565A in complex with carnitine and CoA</th>
</tr>
</thead>
<tbody>
<tr>
<td>X-ray source</td>
<td>X4A</td>
<td>Rigaku</td>
<td>X4A</td>
</tr>
<tr>
<td>Resolution range (Å)</td>
<td>30–1.9</td>
<td>30–2.3</td>
<td>30–1.8</td>
</tr>
<tr>
<td>Number of observations</td>
<td>222,754</td>
<td>61,432</td>
<td>500,787</td>
</tr>
<tr>
<td>$R_{merge}$ (%)$^a$</td>
<td>5.9 (20.1)</td>
<td>7.8 (21.4)</td>
<td>6.9 (38.5)</td>
</tr>
<tr>
<td>Number of reflections</td>
<td>50,988</td>
<td>24,609</td>
<td>119,268</td>
</tr>
<tr>
<td>Completeness (%)</td>
<td>98 (95)</td>
<td>87 (63)</td>
<td>93 (78)</td>
</tr>
<tr>
<td>$R$ factor (%)$^b$</td>
<td>18.5 (20.6)</td>
<td>20.3 (27.9)</td>
<td>19.1 (27.7)</td>
</tr>
<tr>
<td>free $R$ factor (%)</td>
<td>21.8 (25.0)</td>
<td>26.2 (34.9)</td>
<td>22.1 (28.4)</td>
</tr>
<tr>
<td>rmsd in bond lengths (Å)</td>
<td>0.006</td>
<td>0.007</td>
<td>0.006</td>
</tr>
<tr>
<td>rmsd in bond angles (°)</td>
<td>1.3</td>
<td>1.3</td>
<td>1.2</td>
</tr>
<tr>
<td>Number of waters</td>
<td>753</td>
<td>224</td>
<td>1148</td>
</tr>
</tbody>
</table>

$a. \quad R_{merge} = \sum \sum |I_{hi} - \langle I_h \rangle|/\sum \sum I_{hi}$. The numbers in parenthesis are for the highest resolution shell.

$b. \quad R = \sum |F^o_h - F^c_h|/\sum F^o_h$
Figure Legends

**Fig. 1.** Structure of carnitine acetyltransferase (CrAT). (A). Schematic drawing of the structure of mouse CrAT (8). The catalytic His343 residue is shown in red. The positions of the carnitine (in green) and CoA (magenta) substrates are also shown. (B). The putative binding site for the acyl group in CrAT. The structure of octanoylcarnitine is modeled into this binding site. (C). Alignment of the sequences of carnitine acyltransferases in the binding site for the acyl group. Residues that are in close proximity of octanoylcarnitine are highlighted in green. The two residues that were selected for mutagenesis studies are indicated with the green oval. Panels A and B produced with Ribbons (18).

**Fig. 2.** Substrate preference of wild-type and mutant CrAT enzymes. The reaction velocity of the wild-type enzyme with the acetyl-CoA substrate is scaled to 100. Error bars represent standard deviation in the measurements.

**Fig. 3.** Structure of the M564G mutant of CrAT. (A). Structural comparison of the wild-type free enzyme (in gray) and M564G mutant (in cyan) of CrAT. The position of carnitine in the M564G mutant is shown in green. The modeled position of octanoylcarnitine is shown in magenta. Produced with Ribbons (18). (B). Molecular surface of the M564G mutant near the acylcarnitine binding site. The modeled position of octanoylcarnitine is shown in green. (C). Molecular surface of the wild-type CrAT near the acylcarnitine binding site, in the same view as panel B. Residue Met564 is colored in magenta. Panels B and C produced with Grasp (19).

**Fig. 4.** Structural comparison of the wild-type free enzyme (in gray) and F565A mutant (in cyan) of CrAT. The modeled position of octanoylcarnitine is shown in magenta. Produced with Ribbons (18).

**Fig. 5.** Structure of the F565A mutant of CrAT in a ternary complex with carnitine and CoA. (A). Final 2Fₒ–Fₑ electron density at 1.8 Å resolution for CoA, contoured at 1σ. (B).
Electron density for ethylene glycol. (C). Electron density for carnitine. (D). Schematic drawing of the structure of the F565A mutant of CrAT in complex with carnitine, CoA, and ethylene glycol. The catalytic His343 residue is shown in red. The carnitine and CoA substrates are shown in green and brown, respectively. Ethylene glycol is shown in black. (E). The active site region of the F565A mutant of CrAT in complex with carnitine (green) and CoA (brown). The bound positions of carnitine and CoA in the binary complexes with the wild-type enzyme are shown in magenta (8). The water molecule that mediates the interactions between carnitine and the enzyme is labeled W. Panels A-C produced with Setor (20), and panels D and E with Ribbons (18).
References


Fig. 1
Fig. 2