Identification of Conserved Amino Acid Residues in Rat Liver Carnitine Palmitoyltransferase I Critical for Malonyl-CoA Inhibition

MUTATION OF METHIONINE 593 ABOLISHES MALONYL-CoA INHIBITION*

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Carnitine palmitoyltransferase (CPT) I, which catalyzes the conversion of palmitoyl-CoA to palmitoylcarnitine facilitating its transport through the mitochondrial membranes, is inhibited by malonyl-CoA. By using the SequenceSpace algorithm program to identify amino acids that participate in malonyl-CoA inhibition in all carnitine acyltransferases, we found 5 conserved amino acids (Thr314, Asn464, Ala478, Met593, and Cys608, rat liver CPT I coordinates) common to inhibitable malonyl-CoA acyltransferases (carnitine octanoyltransferase and CPT I), and absent in noninhibitable malonyl-CoA acyltransferases (carnitine acetyltransferase, carnitine octanoyltransferase and CPT II), and absent in noninhibitable malonyl-CoA acyltransferases (carnitine octanoyltransferase and CPT I), and absent in noninhibitable malonyl-CoA acyltransferases (carnitine acetyltransferase, carnitine octanoyltransferase and CPT II, carnitine acetyltransferase (CAT) and choline acetyltransferase (ChAT)). To determine the role of these amino acid residues in malonyl-CoA inhibition, we prepared the quintuple mutant CPT I T314S/N464D/A478G/M593S/C608A. In each case the CPT I amino acid selected was mutated to that present in the same homologous position in CPT II, CAT, and ChAT. Because mutant M593S nearly abolished the sensitivity to malonyl-CoA, two other Met593 mutants were prepared: M593A and M593E. The catalytic efficiency (Vmax/KM) of CPT I in mutants A478G and C608A and all Met585 mutants toward carnitine as substrate was clearly increased. In those CPT I proteins in which Met593 had been mutated, the malonyl-CoA sensitivity was nearly abolished. Mutations in Ala478, Cys608, and Thr314 to their homologous amino acid residues in CPT II, CAT, and ChAT caused various decreases in malonyl-CoA sensitivity of CPT I near the catalytic site and participates in the binding of malonyl-CoA in the low affinity site (Morillas, M., Gómez-Puertas, P., Rubí, B., Clotet, J., Ariño, J., Valencia, A., Hegardt, F. G., Serra, D., and Asins, G. (2002) J. Biol. Chem. 277, 11473–11480). Met593 may participate in the interaction of malonyl-CoA in the second affinity site, whose location has not been reported.

The enzyme carnitine palmitoyltransferase (CPT) catalyzes the conversion of long chain fatty acyl-CoAs to acylcarnitines, which is the first step in the transport of fatty acyl-CoA groups from the cytosol to mitochondria where they undergo β-oxidation. This reaction is inhibited by malonyl-CoA, and so this enzyme could be the most physiologically important regulatory step in mitochondrial fatty acid oxidation (1). This process allows the cell to signal the relative availability of lipid and carbohydrate fuels in liver, heart, skeletal muscle, and pancreatic β-cell (2). The mechanism of malonyl-CoA inhibition can be potentially mimicked by pharmacological malonyl-CoA-related agents for the treatment of metabolic disorders such as diabetes, insulin resistance, and coronary heart disease (3).

Mammals express two isoforms of CPT I, a liver isoform (L-CPT I) and a heart/skeletal muscle isoform (M-CPT I), which are the products of two different genes (4, 5). The identity in amino acids residues is high (62%) but they are differentially regulated by malonyl-CoA. The L-CPT I isoform is inhibited by malonyl-CoA to a much lesser extent than the M-CPT I isoform (the IC50 value for M-CPT I is about 2 orders of magnitude lower than for L-CPT I) (6). This property is probably involved in the finer regulation of fatty acid oxidation in heart and skeletal muscle in comparison to liver.

From studies on the pH dependence of the affinity of CPT I for its substrate and from the ability of palmitoyl-CoA to displace [14C]malonyl-CoA bound to skeletal muscle mitochondria it was hypothesized (7) that the palmitoyl-CoA and malonyl-CoA bind at different sites. A number of studies have shown that in rat liver CPT I there are two malonyl-CoA binding sites: one with greater capacity for binding and regulation of the inhibitor and not susceptible to competition from acyl-CoA, which behaves as an allosteric component (8–12); and a second acyl-CoA binding site, which is located near the catalytic site (13).

Various groups have attempted to establish the basis of the L-CPT I/malonyl-CoA interactions. The probable binding sites of malonyl-CoA in L-CPT I were deduced to be at the C terminus after preparation of several L-CPT I chimeras whose IC50 values for malonyl-CoA corresponded to the C-terminal region (14) of the chimera. However, the N terminus of L-CPT I was also shown to influence the enzyme/inhibitor interaction. Mutation of Glu3, His5, or His140 produced a loss of malonyl-CoA

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¶¶The abbreviations used are: CPT, carnitine palmitoyltransferase; L-CPT I, liver isoform of carnitine palmitoyltransferase I; M-CPT I, muscle isoform of carnitine palmitoyltransferase I; CAT, carnitine acetyltransferase; COT, carnitine octanoyltransferase; ChAT, choline acetyltransferase.
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palmityltransferase activity was determined by the radiometric method as described in Ref. 19 with minor modifications. The substrates were 1-methyl-3-H carnitine and palmityl-CoA. Enzyme activity was assayed for 4 min at 30 °C in a total volume of 200 μl.

For determination of the Km, for carnitine, palmityl-CoA was fixed at 135 μM (for L-CPT I). For determination of the Kc, for acyl-CoA, carnitine concentration was fixed at 400 μM. When malonyl-CoA inhibition was assayed, increasing concentrations of malonyl-CoA were included. The IC50, defined as the malonyl-CoA concentration that produces 50% inhibition of enzyme activity, was determined using 50 μM palmityl-CoA and 400 μM carnitine. Kc was estimated by analyzing the data from three experiments using the program Enzfitter (Biosoft), and IC50 was calculated by Excel software using linear regression analysis.

Values reported in the text are the means and standard deviations of three to five determinations. Curve fitting was carried out using Excel software. All protein concentrations were determined using the Bio-Rad protein assay with bovine albumin as standard.

Immunological Techniques—Western blot analysis was performed as described (19). The antibody for rat L-CPT I was kindly gift by Dr. V. A. Zammit (Hannah Research Institute, ayr, Scotland, United Kingdom) and was directed against peptide 428–441, in the cytosolic catalytic C-terminal domain.

RESULTS

Residues Conserved in Malonyl-CoA Inhibited Versus Noninhibited Carnitine-Choline Acyltransferases—An exhaustive analysis of the presence of residues shared by all the malonyl-CoA-regulated enzymes of the carnitine-choline acyltransferase family versus the malonyl-CoA nonregulated members of the same family was performed using the algorithm SequenceSpace (23, 24). This method uses a vectorial representation of each protein sequence as a point in a multidimensional space (SequenceSpace) and multivariate statistics, principal component analysis, to allow reduction of the number of dimensions. This representation allows us not only to define clusters of proteins according to specific properties by choosing the appropriate axes defined by the highest corresponding eigenvalues (also known as proper values), but also to project the individual residues on the same axes, and thus trace the positions conserved in the subfamilies defined. The main advantage of this method is the possibility of predicting which residues may be responsible for the specific characteristics of each protein subfamily or group of subfamilies as has been reported previously for short- and medium-long substrate specificity for the carnitine-choline acyltransferases protein family (19, 20) or effector recognition by some members of the Ras superfamily (25).

The two-dimensional projection of sequence vectors on the plane defined by the axes corresponding to eigenvalues 2 and 4 showed clustering of the enzyme subfamilies according to their malonyl-CoA inhibition properties (Fig. 1A). Proteins whose activity is not regulated by malonyl-CoA (CPT II, CAT, and ChAT subfamilies) were grouped, whereas the sequences of the proteins regulated by malonyl-CoA (COT, L-CPT I, and M-CPT I) occupy separate, and opposite, zones. The projection of the individual amino acid residues on the same plane, and thus trace the positions conserved in the subfamilies defined. The main advantage of this method is the possibility of predicting which residues may be responsible for the specific characteristics of each protein subfamily or group of subfamilies as has been reported previously for short- and medium-long substrate specificity for the carnitine-choline acyltransferases protein family (19, 20) or effector recognition by some members of the Ras superfamily (25).

Expression of Wild Type and Mutants in S. cerevisiae—We
prepared a quintuple mutant, T314S/N464D/A478G/M593S/C608A, and separately, the point mutants T314S, N464D, A478G, M593S, and C608A and all were expressed in *S. cerevisiae*. After we observed that mutant M593S nearly abolished the sensitivity to malonyl-CoA (see below), new point Met mutants were prepared: M593A and M593E. All transformed yeast cells expressed a protein with the same molecular mass (88 kDa) and the mutant enzymes were expressed in roughly the same proportion per milligram of protein as the wild type L-CPT I as deduced from immunoblot analysis (data not shown).

**Kinetic Properties of CPT I Wild Type and Mutants**—L-CPT I activities of the wild type, quintuple mutant variant T314S/N464D/A478G/M593S/C608A, and point mutants were similar (values ranged between 14 and 20 nmol min⁻¹ mg protein⁻¹) when the protein was overexpressed 20 h after galactose induction, showing that the various mutations assayed produce small changes in L-CPT I activity (Table I).

All mutants exhibited standard saturation kinetics when the carnitine concentration was varied relative to a constant concentration of the second substrate, palmitoyl-CoA, and when palmitoyl-CoA concentration was varied relative to a constant carnitine concentration, a property identical to that of the wild type L-CPT I (Fig. 3). The quintuple mutant produced small changes in the kinetic constants for carnitine and palmitoyl-CoA as substrates (Table I). Catalytic efficiency (*Vₘₐₓ/Kₘ*) was increased by a factor of 2.6 (carnitine) and 2.2 (palmitoyl-CoA). The catalytic efficiency for carnitine as substrate of those point
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### Table I

| L-CPT I | Activity ($\text{nmol min}^{-1} \text{mg protein}^{-1}$) | $K_m$ (µM) | $V_{\text{max}}$ (nmol min$^{-1}$ mg protein$^{-1}$) | Catalytic efficiency ($V_{\text{max}}/K_m$) |
|---------|---------------------------------|--------|-----------------|-----------------|
| Wild-type | 17.7 ± 0.9 | 12.3 | 4.9 ± 0.3 | 6.6 ± 0.8 | 0.58 (± 1.1) |
| T314S | 14.4 ± 2.1 | 15.0 | 1.7 ± 0.5 | 12.8 ± 0.1 | 0.15 (± 2.8) |
| N464D | 20.1 ± 3.1 | 8.7 | 4.1 ± 0.4 | 19.4 ± 1.4 | 0.28 (± 5.6) |
| A478G | 16.7 ± 0.7 | 39.5 | 15.1 ± 4.0 | 69.8 ± 9.3 | 0.21 (± 4.1) |
| C608A | 17.3 ± 1.7 | 27.5 | 24.3 ± 2.0 | 23.7 ± 5.0 | 0.46 (± 8.8) |
| M593S | 17.0 ± 0.8 | 319 | 7.4 ± 1.2 | 133 ± 18 | 1.07 (± 21) |
| M593A | 17.2 ± 0.9 | 155 | 6.1 ± 0.2 | 32.5 ± 4.6 | 0.58 (± 12) |
| M593E | 14.1 ± 1.8 | 220 | 6.3 ± 0.5 | 31.3 ± 2.6 | 0.21 (± 4.2) |
| QM | 13.6 ± 1.6 | 258 | 4.6 ± 1.5 | 13.1 ± 4.7 | 0.14 (± 2.6) |

Even at concentrations as high as 100 µM malonyl-CoA the CPT I quintuple mutant maintained 80% of the activity of the control without malonyl-CoA.

We then addressed the individual responsibility of the separate CPT I mutants for the malonyl-CoA sensitivity. Mutants T314S, N464D, M593S, and C608A expressed in *S. cerevisiae* were incubated with increasing amounts of malonyl-CoA, and CPT I activity was determined. Mutant A478G had been previously studied in Ref. 20 and showed decreased sensitivity to malonyl-CoA (IC$_{50}$ of 39.5 versus 12.3 µM of the wild type).

The kinetics of inhibition by malonyl-CoA depended on the mutant considered. Whereas mutant M593S (Fig. 4A) showed very low sensitivity at malonyl-CoA inhibition (IC$_{50}$ of 319 µM), the other mutations produced varied changes in malonyl-CoA sensitivity. L-CPT I C608A slightly modified the sensitivity to malonyl-CoA (IC$_{50}$ of 27.5 µM), the change in IC$_{50}$ of mutant T314S was small, whereas N464D showed similar sensitivity to malonyl-CoA to the wild type (Fig. 4B and Table I). Because the highest changes in sensitivity to malonyl-CoA and $K_m$ values for carnitine were observed in the methionine mutants (point and quintuple mutants), we additionally prepared two new mutants: M593A and M593E to examine whether Met$_{593}$ was essential to the malonyl-CoA interaction in L-CPT I. Results show that the sensitivity to malonyl-CoA was also nearly abolished in these mutants (IC$_{50}$ values of 155 and 220 µM, respectively) as in the M593S mutant, confirming the essential role of Met$_{593}$ in this interaction.

**Discussion**

We attempted to identify the amino acids in the C-terminal domain of L-CPT I that are responsible for the inhibition of the catalytic activity by malonyl-CoA. Over many years much work has been done to identify the domains in L-CPT I that may bind malonyl-CoA. Different groups have tested different empirical hypotheses and mutated amino acids, mostly in the amino-terminal region of L-CPT I. The results have shown that this domain plays a role in the regulation of CPT I by malonyl-CoA, because in some cases the sensitivity to the inhibitor is impaired.

A different approach was employed by our group very recently. This was based on the conservation of two histidine residues, which are present in the inhibitable malonyl-CoA carnitine acyltransferases (CPT I and COT) and absent in noninhibitable enzymes (CPT II and CAT). Mutation of both histidines resulted in the abolition of malonyl-CoA sensitivity in COT (26). Analogous results were observed in CPT I when its concentration at the mitochondrial membranes was not high. Mutation of other amino acids in the domain proximal to the catalytic site (Ala$^{78}$ and Pro$^{79}$) indicated that a malonyl-CoA-
inhibitable domain was probably the low-affinity malonyl-CoA binding site. Our previous studies showed that the location of malonyl-CoA in the structural model was compatible with competition of the inhibitor versus the substrate in the malonyl-CoA low affinity binding site (20).

The site-directed mutagenesis study used here to identify amino acids responsible for malonyl-CoA inhibition is based on the comparison of the sequences in a range of carnitine and choline acyltransferases, taking the positive or negative sensitivity to malonyl-CoA as a discriminatory criterion. The bio-computing study has shown that five amino acids are present in all CPT I (isoforms L- and M-) and in COT from various organisms and that they are absent not only in other nonmalonyl-CoA-inhibitable carnitine acyltransferases but also in ChAT. In rat L-CPT I these amino acids are Thr314, Asn464, Ala528, Met593, and Cys608. The corresponding positional amino acids in CPT II, CAT, and ChAT are Ser325, Asp363, Gly377, Ser490, and Ala505, respectively (coordinates of rat CPT II).

Therefore, we considered it highly probable that these amino acids were involved in the interaction of malonyl-CoA. Results confirmed in part this supposition. The quintuple mutant reduced malonyl-CoA sensitivity almost completely (80% activity at 100 μM malonyl-CoA (which is outside the physiological range)), supporting the initial hypothesis. The results obtained using separate single mutants indicate that not all of these amino acids have the same role in malonyl-CoA inhibition. Whereas M593S nearly abolished the sensitivity to malonyl-CoA like the quintuple mutant, A478G increased the IC50 from 12 to 39.5 μM (20). The other amino acids are less responsible for the inhibition.

The relevance of Met593 as a critical amino acid for malonyl-CoA sensitivity was confirmed by the results of mutation to other two amino acids, Ala and Glu. The mutants equally showed diminished sensitivity to malonyl-CoA like mutant M593S. Met593, when mutated to Ser as it appears in CPT II and CAT, decreased the sensitivity to malonyl-CoA in a stronger fashion than when it was mutated to other amino acids like Ala and Glu, which were unrelated to this position in other carnitine acyltransferases. Therefore, we conclude that the occurrence of Ser in this position has probably been evolutionarily conserved in nonmalonyl-CoA-sensitive carnitine acyltransferases because it prevents sensitivity to malonyl-CoA. In any case, it appears that Met593 is critical in the interaction of malonyl-CoA with L-CPT I.

It was of interest to measure the kinetic constants of all CPT I mutants. Several authors reported the competition between malonyl-CoA and carnitine (27, 28). The tissues in which the sensitivity of CPT I to malonyl-CoA is highest are those that require the highest concentration of carnitine to drive the reaction and the requirement for carnitine and sensitivity to malonyl-CoA appears to be inversely related. The authors concluded that the sites to which the two metabolites bind are closely associated (27, 7). Studies by Bird and Saggerson (28) showed on the one hand that malonyl-CoA reduced the effectiveness of carnitine as substrate, and on the other hand, that carnitine might diminish the regulatory effect of malonyl-CoA (29). Although a clear mechanism for this competition could not be established, the data strongly supported this idea. In the present study the various CPT I mutants have altered Km for malonyl-CoA in the same direction to Km for carnitine. Whereas the Km for C608A was half of the wild type, its Vmax was 3.6-fold higher. The mutant M593S had the same Km value for carnitine as the wild type but its Vmax increased 20-fold. The mutant A478G increased both the Km value and the Vmax with respect to the wild type values. The relationship between these values and catalysis is best revealed in the term catalytic efficiency. This term as calculated by the Vmax/Km ratio varies considerably among different mutants. Carnitine catalytic efficiencies for mutants M593S, M593A, M593E, C608A, and A478G increased 21-, 12-, 4.2-, 8.8-, and 4.1-fold with respect to the wild type. This means that mutations designed to decrease malonyl-CoA sensitivity strongly modified the catalytic efficiency of CPT I mutants measured in the absence of malonyl-CoA. Interestingly, the increase in catalytic efficiency appears to be roughly proportional to the extent of the alteration in malonyl-CoA sensitivity. The IC50 values for malonyl-CoA run in the same direction to the catalytic efficiency of the mutants. This indicates that those mutants that can locate carnitine better at the catalytic site might displace malonyl-CoA from its site, preventing the binding of the metabolite and thus the inhibition of CPT I.

Because L-CPT I has not been crystallized, we do not know the proximity of Met593 to the site of carnitine binding to perform the catalytic event. However, Met593 is very near the tripeptide TET602–604, which has been reported to play an

![Figure 4](image-url)

**Fig. 4. Effect of malonyl-CoA on the activity of yeast overexpressed L-CPT I (wild type) and several mutants.** A) L-CPT I wild type (open circles) and point methionine mutants M593S (black circles), M593A (black rhombus), M593E (black squares), and B) quintuple mutant (QM) (black circles) and point mutants T314S (open circles), N464D (open rhombus, broken line), A478G (open triangles, broken line), and C608A (open squares) overexpressed in yeast were incubated with increasing concentrations of malonyl-CoA and the enzyme activity was measured. Data are expressed relative to control values in the absence of inhibitor (100%) as the mean of three independent measurements.
important role in the accommodation of carnitine in catalysis. Cronin (30) showed that mutation of the homologous tripeptide VDN in choline acetyltransferases to TET greatly increased the catalytic efficiency of the reaction (137-fold) using carnitine as substrate. This proximity between Met693 and TET692–694 would explain the inverse correlation observed between the catalytic efficiency for carnitine and the IC50 for malonyl-CoA values of the mutants assayed. A new scenario appears in the mutual interaction between carnitine and malonyl-CoA in CPT I. The domain comprised, at least, between amino acid residues 593 and 604 is probably the site of interaction between carnitine and malonyl-CoA, which exclude each other. Higher catalytic efficiencies for carnitine in the mutants are followed by decreases in the inhibitory sensitivity to malonyl-CoA.

It is equally interesting to note that all mutants tested show higher catalytic efficiency for palmitoyl-CoA as substrate than the wild type. The increase in $V_{\text{max}}/K_m$ ranges from 2- to 3-fold. Previous work with a partially purified preparation of CPT I had indicated that the kinetics of the reaction with respect to carnitine concentration could be highly dependent on the concentration of the second substrate, palmitoyl-CoA (29). Experiments carried out by Bird and Saggerson (28) showed that in fasted animals, in which carnitine concentration was decreased, the IC50 values for malonyl-CoA increased up to 17-fold and the binding of [2-14C]malonyl-CoA was reduced by 35% at 50 $\mu$M palmitoyl-CoA and to even lower values at increasing palmitoyl-CoA concentrations.

Only two of these mutated amino acids are located in the three-dimensional CPT I structural model. Ala478 is one of the amino acids present in the low affinity site of malonyl-CoA interaction. This amino acid together with Pro477 and His483 conform a domain to which malonyl-CoA appears to bind (20). Mutation of this amino acid would explain a decrease in sensitivity to malonyl-CoA, and therefore it would also explain the increase in catalytic efficiency. On the other hand, Asn464 is also present in the catalytic core of the structural model of CPT I (20), but its location does not permit any conclusions about a participation in the malonyl-CoA inhibitory effect. In fact it is located on the opposite site to malonyl-CoA (data not shown). Therefore, it is not surprising that its mutation from Asn464 to Asp464 does not alter sensitivity to the inhibitor. As a corollary of this study, we conclude that the occurrence of the five other amino acids (Ser223, Asp363, Gly377, Ser490, and Ala505) at the positions, respective, identical to those amino acids seen in CPT I may be sufficient to prevent the sensitivity to malonyl-CoA not only to carnitine acyltransferases such as CPT II and CAT but also to ChAT.

The use of either the quintuple mutant or the methionine point mutants may allow studies on the influence of these negative dominant CPT Is, which are expected to be independent of malonyl-CoA concentration in a range of tissues such as liver, muscle, and the $\beta$-cell, in which the metabolism of fatty acids plays important roles in ketone body synthesis, resistance to insulin, and glucose-stimulated insulin secretion, respectively. Some of these topics are the subject of current investigations in our laboratory.

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