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The G553M Mutant of Peroxisomal Carnitine Octanoyltransferase Catalyses Acetyl Transfer and Acetyl-CoA Hydrolysis

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Summary. The structure of carnitine acetyltransferase revealed a putative binding site for longer acyl chains but access was blocked by methionine 564 (*G. Jogl* and *L. Tong* (2003) Cell 112, 113–122). The equivalent residue in all long chain carnitine acyltransferases is a conserved glycine. Mutation of glycine 553 to methionine in bovine COT resulted in loss of activity with all acyl-CoA substrates except acetyl-CoA, supporting the hypothesis that the methionine blocks access for longer acyl chains. The kinetic characteristics of acetyl transfer to carnitine were identical in the native and mutant enzyme. However, rapid acetyl-CoA hydrolysis in the mutant but not the wild-type indicates perturbation of the catalytic site.

Keywords. Carnitine; Substrate specificity; Acetyl-Coenzyme A; Site-directed mutagenesis; Altered catalysis.

Introduction

The carnitine system provides the means to exchange activated acyl groups between the limited, compartmentalised pools of CoA and to export excess acyl groups from the cell. The reversible removal or supply of activated acyl groups from or to the isolated CoA pools depends on the specific locations of the acyltransferases and on the acyl chain length specificity of the enzyme(s) in that location. For example, the mitochondrial CPT1 and CPT2 show long acyl-chain specificity to transfer fatty acids into the mitochondria for oxidation whereas COT in the peroxisomes has a broad specificity to facilitate the transfer of chain-shortened fatty acids to the mitochondria for further oxidation with better energy yield [1].

Carnitine acetyltransferase (CrAT) is the only member of the carnitine acyltransferase family for which the structure has been solved [2, 3]. In the structure of

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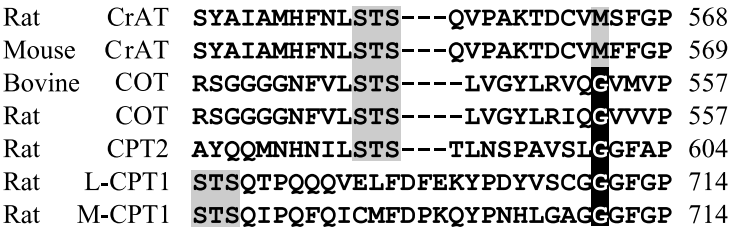

Fig. 1. Amino acid sequence alignment of mammalian carnitine acyltransferases; sequences from the SwissProt data bank were aligned using ClustalW; the M564 of CrAT and the conserved glycine in other members of this family are highlighted (Accession ID: Rat CAT-NP001004085, Mouse CAT-P47934, Bovine COT-O19094, Rat COT-P11466, Rat L-CPT1-NP113747, Rat M-CPT1-Q63704, Rat CPT2-NP037062)

CrAT, a putative site for the binding of long chain acyl groups was identified, but access to the pocket was blocked by M564 near the STS motif important for carnitine binding [2]. In all other members of the family, the equivalent residue is a glycine (Fig. 1), suggesting that the pocket could be accessible in COT and CPT. A disease-causing mutation in human CPT1 has glutamic acid at the equivalent position (E710), and no catalytic activity with palmitoyl-CoA [4]. To test the hypothesis that steric hindrance by the side chain of methionine prevents long chain acyl-CoA binding, the M564G mutant of CrAT was analysed and shown to be active with C2–C6 acyl-CoA [5, 6]. Conversely, an increase in the size of the residue at the equivalent position in COT should block binding of long acyl chains [6], so the mutant G553M of bovine COT was constructed, the protein purified, and its substrate specificity examined. The mutant lacked activity with any substrate except acetyl-CoA, providing support for the acyl binding area identified in the structure of CrAT.

Results and Discussion

Changed Acyl Chain-length Specificity

The optimised expression of the wild-type COT and its mutant yielded 63 units/g and 90 units/g of cells respectively, assayed with acetyl-CoA. The purity on SDS-PAGE was at least 99%, with no other bands at 50 μg loading.

Although wild-type bovine COT is active with substrates from C2–C18, the G553M mutant showed no activity whatsoever with decanoyl-CoA as the substrate.

Table 1. Substrate specificities of wt-COT and G553M-COT assayed with 50 μM acyl-CoA substrate and 2 mM *L*-carnitine

Acyl-CoA Substrate	Activity (μmol · min^{-1} · mg^{-1})	
	WT-COT	G553M-COT
C2-CoA	1.23	1.21
C4-CoA	10.8	0
C6-CoA	17.3	0
C8-CoA	22.7	0
C10-CoA	38.2	0

However, it gave the same activity as the wild-type with acetyl-CoA (Table 1). Other acyl chain lengths were also tested (Table 1) to show that the heterologous expression did not change the chain length specificity of the wild-type COT from that observed previously. The G553M mutant gave no activity with any acyl chain length substrate apart from acetyl-CoA (C2).

The change in the substrate specificity of COT induced by the G553M mutation from the native broad chain length range (C2–C18) to activity with only acetyl-CoA (C2) is a clear indication that acyl chain binding is prevented. Thus, the increase in the size of the residue at the position 553 has blocked binding of acyl chains greater than C2. This means that the proposed binding site indicated by *Jogl* and *Tong* [2] in the structure of CrAT is likely to be the acyl chain binding region in other members of the family. In the long chain carnitine acyltransferases, the conserved glycine ensures that there is no steric hindrance to acyl binding. This agrees with the recent work for rat and mouse CrAT and for rat COT [5, 6]. Together, the experiments for the two enzymes from three species provide evidence for a common active site structure in this family of enzymes.

Catalytic Parameters are Otherwise Unchanged

The kinetic constants for the heterologously expressed wt-COT were determined using decanoyl-CoA and found to be unchanged from those found with the bovine

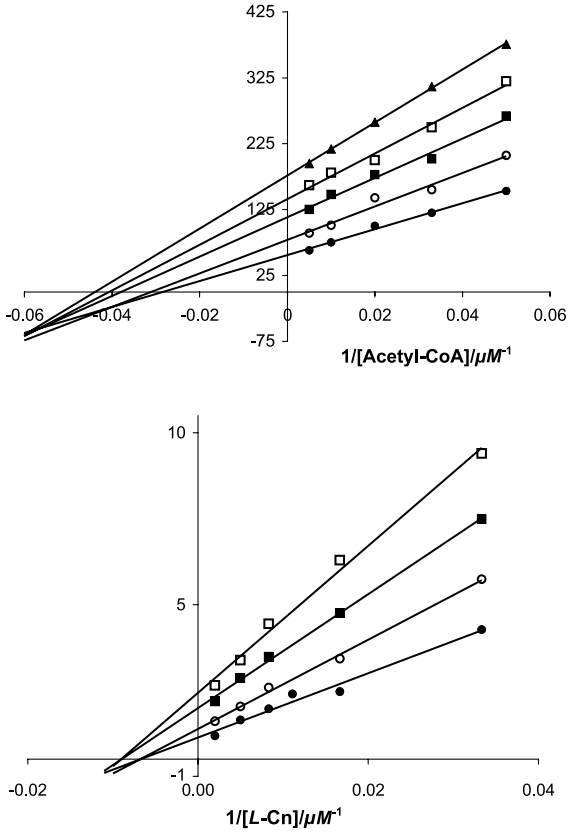

Fig. 2. *Lineweaver-Burk* plots for the acetyltransferase activity of G553M-COT (50 n*M*) assayed with series of increasing concentrations of *L*-carnitine (30–500 μM) and acetyl-CoA (20–200 μM)

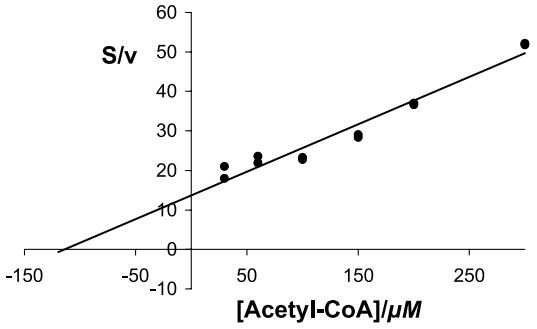

Fig. 3. *Hanes* plot for the acetyl-CoA hydrolase activity of G553M-COT (50 n*M*) assayed in the absence of *L*-carnitine with acetyl-CoA alone (20–200 μM)

liver enzyme [7]. The kinetic properties of wt-COT and G553M-COT were characterised for the first time using acetyl-CoA as the substrate. Figure 2 shows a *Lineweaver-Burk* plot of the data for G553M-COT to illustrate that the pattern is consistent with the rapid-equilibrium random mechanism determined for the native enzyme. From the data in Fig. 2 and three other determinations, the $v_{\max}$ for the acetyl-CoA to carnitine acetyltransferase activity (''forward reaction'') of G553M-COT was $4 \pm 1\ \mu\text{mol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$, the K_M for acetyl-CoA was $32 \pm 5\ \mu M$, and the K_M for *L*-carnitine was $116 \pm 20\ \mu M$ (mean $\pm$ sd of four determinations). For wt-COT, the $v_{\max}$ was $5\ \mu\text{mol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$, the K_M for acetyl-CoA was $30\ \mu M$, and the K_M for *L*-carnitine was $106\ \mu M$. Thus, the mutation alters neither the catalysis of acetyl transfer by the enzyme nor the binding of either substrate.

Reverse Reaction

In the reverse direction (acetyl-*L*-carnitine to CoA, using $200\ \mu M$ CoA), the $v_{\max}$ was $5\ \mu\text{mol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$ and the apparent K_M for acetyl-*L*-carnitine was $150\ \mu M$.

Inhibition by Long-chain Acyl-CoA Esters

When $100\ \mu M$ decanoyl-CoA was added to an assay of G553M-COT with $100\ \mu M$ acetyl-CoA and $2\ mM$ *L*-carnitine, the activity was inhibited by almost 70%. This indicated that decanoyl-CoA could bind to the enzyme and prevent the acetyl-CoA binding. The apparent K_i for decanoyl-CoA inhibition of the acetyltransferase activity at $100\ \mu M$ acetyl-CoA was $20\ \mu M$ and $22\ \mu M$ in two separate experiments (data not shown). The inhibition by decanoyl-CoA suggests that the longer chain substrates can still bind but not in a catalytically competent mode. The low affinity for decanoyl-CoA (indicated by the apparent K_i value of $21\ \mu M$) suggests that the binding is due to the CoA moiety only. Similar inhibition of CrAT by palmitoyl-CoA has been reported [10, 12].

A Novel Hydrolase Activity is Present in the G553M Mutant

In the absence of carnitine, a rapid hydrolysis of acetyl-CoA was observed in the G553M mutant whereas there was no hydrolysis by the wild-type enzyme. Previous

kinetic studies revealed no hydrolase activity in COT isolated from bovine liver [8], so this indicates a change in the catalytic site. The hydrolysis is saturable (Fig. 2) and analysis of the data gives a v_{max} of 8.3 μmol $\cdot$ min^{-1} $\cdot$ mg^{-1} and a K_M for acetyl-CoA of 113 $\pm$ 15 μM (mean $\pm$ sd of four determinations). The hydrolase activity was also inhibited by decanoyl-CoA, with an apparent K_i value of 30 μM (against 100 μM acetyl-CoA). The appearance of the hydrolase activity means that an active site group in G553M COT is able to activate water directly or catalyse the hydrolysis *via* an active site group such as serine. Chemical modification experiments have provided evidence for a reactive group, possible serine, that prevented labelling of the catalytic histidine [9]. At low protein concentrations, carnitine inhibits the rate of release of CoA, rather than stimulating it. This indicates that carnitine either competes with a putative water for activation or its binding prevents activation of the water or active site group responsible for the hydrolysis. In the structure of COT just published, there is a water molecule hydrogen bonded to the main chain amide of Ala332 that may be responsible for the hydrolysis of the acyl esters [13].

Further evidence for steric alteration of the active site is seen in the decrease stability of the mutant protein. The G553M protein, although stable during purification, becomes labile after freezing, its activity decreasing over the course of a few hours. The appearance of acetyl-CoA hydrolase activity in the absence of carnitine is consistent with observations of hydrolysis in CrAT during crystallisation. Only carnitine or CoA were found in the active site of the crystal whether the crystal was grown in acetyl-*L*-carnitine or soaked with acetyl-CoA respectively [2].

Conclusions

The increased size of the methionine side group at 553 instead of the H of the conserved glycine normally found at this site blocked catalytic activity with any acyl chain except acetyl, as predicted from the structure of CrAT. Any other mutation here is likely to have similar consequences for the acyl chain length specificity that is critical to the normal function of the cell. Further, the perturbation of the active site indicated by the appearance of hydrolase activity in COT as a result of the mutation also may play a role in the deleterious consequences of naturally occurring mutations in the long chain acyltransferases, such as G710E in CPT1 [4].

Experimental

All acyl-CoA derivatives and *L*-carnitine were purchased from Sigma. Blue-Sepharose 6 (fast flow) was purchased from Pharmacia. The primers were obtained from MWG-Biotech, Germany.

Site-directed Mutagenesis and Expression of Bovine COT and its G553 Mutant

The bovine COT in a pET11-vector was expressed in *E. coli* strain BL21(DE3)pLysS. The G553M mutation was introduced by PCR using the primers 5′-TGGCAAAAAGCTAAGGAGAA-3′ (forward) and 5′-TACCATGGGAACCATCACCATCTGGACTCTTAAATAA-3′ (reverse, with the *Nco*1 site and the methionine anticodon underlined) with pET-bCOT^{wt} as template. The product was digested with *Bsr*G1 and *Nco*1 and used to replace the corresponding fragment in pET-bCOT^{wt}. The sequence was

confirmed by DNA sequence analysis. Expression in *E. coli* strain Rosetta(DE3) was performed following the manufacturer's instructions (Novagen, Nottingham, UK).

Purification of Heterologously Expressed COT and G553M COT

The wild type and mutant COT inocula were grown at 37°C in 50 cm^3 Terrific Broth medium containing 50 $\mu g/cm^3$ chloramphenicol and 34 $\mu g/cm^3$ carbenicillin. Protein expression was induced with isopropyl-β-D-thiogalactopyranoside (0.5 *mM*) at 18°C overnight. Cells were harvested and washed in 20 m*M Tris* · HCl-2 m*M EDTA*-1 m*M PMSF*, *pH* 7.5, at 5 g wet mass/dm^3. After a freeze-thaw cycle, the cell suspension was broken by sonication, centrifuged at 22000 *g* for 20 min, and the supernatant ultracentrifuged at 120000 g for 1 hour.

Purification was based on the method of *Ramsay et al*. [7]. The supernatant was loaded onto a column of Blue Sepharose 6 fast flow (3 cm × 15 cm) equilibrated with 20 m*M Tris* · HCl-2 m*M EDTA*-1 m*M PMSF*, *pH* 7.5, at flow rate of 1 cm^3/min, washed, then COT activity eluted with a linear gradient of 0.05–0.5 *M* KCl in buffer. The dialysed sample was applied to small column (18 cm × 1.5 cm) of Blue Sepharose 6 fast flow equilibrated with 20 m*M Tris* · HCl-2 m*M EDTA*, *pH*-7.5, washed with the same buffer containing 50 m*M* KCl, and affinity eluted with octanoyl-CoA (10 mg/100 cm^3 of elution buffer). The enzyme was dialysed, concentrated, and stored at −20°C in buffer containing 20% glycerol and 0.5 m*M* dithiothreitol. The protein concentration was determined by BCA™ protein assay kit. The purity of the enzyme was assessed by 10% SDS-PAGE.

Assays and Kinetic Analysis

The carnitine acyltransferase activity was measured by the production of thiopyridone detected at 324 nm in a Shimadzu 2101PC spectrophotometer after adding enzyme to 20 m*M* potassium phosphate, *pH* 7.4, containing 125 μM dithiobispyridine, 50 μM acyl-CoA, and 2 m*M L*-carnitine. Kinetic constants were determined at 30°C using six concentrations of *L*-carnitine (30–500 μM) at 5 concentrations of acetyl-CoA (20–200 μM). The apparent kinetic constants were calculated by fitting the data to the *Michaelis-Menton* equation using the Shimadzu kinetic software. Linear secondary plots yielded the true kinetic constants. The rate of hydrolysis was measured in the absence of *L*-carnitine.

The activity in the reverse direction was measured as the increase in the thioester at 232 nm (extinction coefficient, 4500 $M^{-1} \cdot cm^{-1}$) from acetyl-*L*-carnitine (30–200 μM) and CoA (200 μM).

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