Inhibition by etomoxir of rat liver carnitine octanoyltransferase is produced through the co-ordinate interaction with two histidine residues

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Rat peroxisomal carnitine octanoyltransferase (COT), which facilitates the transport of medium-chain fatty acids through the peroxisomal membrane, is irreversibly inhibited by the hypoglycaemia-inducing drug etomoxir. To identify the molecular basis of this inhibition, cDNAs encoding full-length wild-type COT, two different variant point mutants and one variant double mutant from rat peroxisomal COT were expressed in *Saccharomyces cerevisiae*, an organism devoid of endogenous COT activity. The recombinant mutated enzymes showed activity towards both carnitine and decanoyl-CoA in the same range as the wild type. Whereas the wild-type version expressed in yeast was inhibited by etomoxir in an identical manner to COT from rat liver peroxisomes, the activity of the enzyme containing the double mutation H131A/H340A was completely insensitive to etomoxir. Individual point mutations H131A and H340A also drastically reduced sensitivity to etomoxir. Taken together, these results indicate that the two histidine residues, H131 and H340, are the sites responsible for inhibition by etomoxir and that the full inhibitory properties of the drug will be shown only if both histidines are intact at the same time. Our data demonstrate that both etomoxir and malonyl-CoA inhibit COT by interacting with the same sites.

Key words: carnitine acyltransferases, malonyl-CoA, site-directed mutagenesis.

INTRODUCTION

Carnitine acyltransferases facilitate the transport of fatty acyl-CoAs through various organelles. The enzyme involved depends on the length of the fatty acyl moiety to be transported. Carnitine acetyltransferase acts with acetyl-CoA as substrate [1], while carnitine octanoyltransferase (COT) facilitates the transport of medium-chain fatty acids from peroxisomes to mitochondria through the conversion of shortened fatty acyl-CoA from peroxisomal β-oxidation into fatty acyl-carnitine [2]. Carnitine palmitoyltransferases (CPTs) I and II [3] facilitate the transport of long-chain acyl groups into the mitochondrial matrix, where they undergo β-oxidation. One characteristic that differentiates the various carnitine acyltransferases activities is their sensitivity to inhibition by malonyl-CoA. In mitochondria the two isoforms CPT I and II are differentially regulated by malonyl-CoA. Liver-isoform (L-) and muscle-isoform (M-) CPT I [4,5] activities are down-regulated by this metabolite, but CPT II is not [6]. COT is also inhibited by malonyl-CoA, but to a lesser extent than CPT I. Physiological inhibition of CPT I and COT by malonyl-CoA plays a pivotal role in the regulation of the β-oxidation pathways in both mitochondria [3] and peroxisomes [7].

The interaction between malonyl-CoA and CPT I involves two sites: one that affects the acyl-CoA-binding domain [8] and the other lying towards the N-terminus. The latter site has a greater capacity for regulation by malonyl-CoA and behaves as an allosteric component [9–11]. The involvement of His residues in modulation by malonyl-CoA is suggested by the finding that a decrease in pH (which is associated with the protonation of the imidazol group of histidine) increases the affinity of CPT I for malonyl-CoA [12,13]. Similar data on variations of enzyme activity by malonyl-CoA at different pHs have been reported for COT [14].

The various carnitine acyl-transferases also show differential sensitivity to inhibition by etomoxir, which is an aryl-substituted 2-oxirane carboxylic derivative [15–17] (Figure 1). The pharmacological effects of etomoxir and its pharmacological analogue 2-tetradecylglycidic acid (TDGA) are due to the fact that they are specific inhibitors of CPT I, which is the rate-limiting enzyme

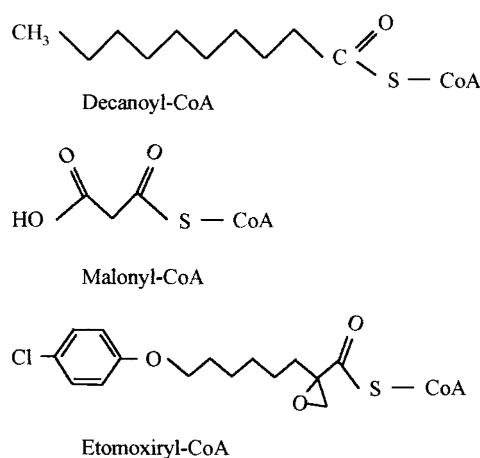

Figure 1 Structures of decanoyl-CoA, malonyl-CoA and etomoxiryl-CoA

Abbreviations used: COT, carnitine octanoyltransferase; CPT, carnitine palmitoyltransferase; L-CPT I, liver isoform of CPT I; M-CPT I, muscle isoform of CPT I; TDGA, 2-tetradecylglycidic acid.

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of fatty acid oxidation in mitochondria. In contrast, etomoxir does not inhibit either CPT II or other β-oxidation enzymes such as acyl-CoA dehydrogenase. Binding studies suggest that etomoxir interacts with CPT I at two sites: (i) within the substrate-binding site; and (ii) at a second site, which is like an allosteric component. The binding depends on the chemical substituent at carbon 2 of the compound [18,19]. These inhibitory properties of etomoxir resemble those of malonyl-CoA, and competitive binding studies using rat liver mitochondria revealed that etomoxir and malonyl-CoA probably interact at the same locus [18].

Etomoxir and TDGA are orally effective inhibitors of fatty acid oxidation in liver, muscle, adipose tissue [20] and heart [21] and they induce anti-ketogenic and hypoglycaemic activity in animal models of Type II diabetes. Etomoxir has hypoglycaemic and a hypotriglyceridaemic effects in healthy human subjects, attributable to the suppression of hepatic fatty acid oxidation and food intake [22]. In clinical trials, etomoxir and TDGA showed promise as anti-ketotic, hypoglycaemic agents, and also improved insulin sensitivity in Type II diabetic patients [23] since they reduced hepatic glucose production and plasma lipids (free fatty acids and triacylglycerides) in these patients. The decrease in plasma lipids observed in diabetic patients [24] after etomoxir administration may involve a variation not only in mitochondrial β-oxidation but also in peroxisomal β-oxidation. The understanding of the regulation of peroxisomal COT by malonyl-CoA and etomoxir is expected to contribute to the design of novel drugs to control mitochondrial and peroxisomal fatty acid oxidation.

To this end we decided to test whether COT is also inhibited by etomoxir, to analyse the inhibitory kinetics, and to compare these data with those of malonyl-CoA. This study takes advantage of our previous identification of the residues of COT responsible for malonyl-CoA inhibition. This approach led us to identify H131 and H340 as the residues involved in the response to malonyl-CoA, since their mutation abolishes malonyl-CoA inhibition [25].

In this report we show that: (i) rat liver peroxisomal COT and yeast-expressed COT are inhibited by etomoxir with similar kinetics; (ii) etomoxir does not interact with the catalytic histidine (H327) in COT, but it does interact with other two histidines H131 and H340, since the double mutation abolishes etomoxir inhibition, maintaining full COT activity; and (iii) inhibition by etomoxir depends on the integrity of H131 and H340: mutation of either abolishes the inhibition caused by etomoxir, irrespective of the integrity of H327.

MATERIALS AND METHODS

Isolation of rat liver COT cDNA

The fragment encompassing the entire coding sequence for rat COT, with GenBank® accession number U26033 [26], nt 33–2670, was obtained and subcloned in the *Eco*RI site of pBluescript SK$^+$ vector (Stratagene), producing pBSCOT as described previously [25]. The plasmid pBSCOT was used to produce the plasmid pBSCOT*Xba*I, which included the fragment *Xba*I–*Xba*I (nt 510–2009).

Construction of plasmids for COT expression in *Saccharomyces cerevisiae*

For expression experiments, the fragment encompassing nt 52–2009, including the coding region of COT, was subcloned into the *S. cerevisiae* expression plasmid pYES2 (Invitrogen). A *Hin*dIII site was introduced by PCR immediately 5′ of the ATG start codon of COT to enable cloning into the unique *Hin*dIII site of plasmid pYES2. The primers used were: COT*Hin*dIII.for, 5′-AGCTTATAAAATGGAAAATCAATTGGCTAAG-3′; and COT*Xba*I.rev, 5′-GGTTCATGTCTAGAGCAG-3′, which includes a *Xba*I site present in the sequence of COT [25]. Primer COT*Hin*dIII.for also contains a consensus sequence, optimized for efficient translation in yeast. Amplifications were performed, using a thermal cycler with *Pfu* DNA polymerase (Stratagene) in 35 cycles of 94 °C, 52 °C and 72 °C, each for 1 min. PCR products were purified (Qiagen) and digested with *Hin*dIII and *Xba*I. pYES2 was similarly digested and ligated to the PCR product. The plasmid was digested with *Xba*I and ligated with the COT fragment *Xba*I–*Xba*I (nt 510–2009) obtained from pBSCOT, producing pYESCOTwt.

Construction of site-directed mutants

Plasmid pYESCOTwt was used for site-directed mutagenesis of histidines 131 and 340 to alanine by the asymmetric PCR method [27]. The following primers were used for the pYESCOT^{H131A} construction: the mutated megaprimer fragment, obtained with H131A.for, 5′-GCATACTACTGTGGGCCAACTTGAACTA-CTGGC-3′ (the mutated nucleotides are underlined), and COT520.rev, 5′-TCATGTCTAGAGTATTTCCAGATTTAT-G-3′, and the COT*Hin*dIII.for primer. The fragment obtained was digested with *Hin*dIII and *Xba*I and subcloned into the plasmid pYESCOTwt.

For construction of pYESCOT^{H340A} the plasmid pBSCOT*Xba*I was used. The mutated megaprimer fragment was obtained with H340A.rev, 5′-GGAGCTTCTCATCAACGTACGCAGCAA-TGTTCACCATAA-3′ (the mutated nucleotides are underlined), and COT734.for, 5′-GCTGGAAATGAACCTGTTGGG-3′, which was used with the primer COT1221.rev, 5′-GCTGCTT-TGAGGRGRRGGGC-3′ to obtain a fragment from nt 734 to 1221 of COT. After digestion with *Apa*I and *Acc*I, the fragment was subcloned into the pBSCOT*Xba*I. The mutated fragment *Xba*I–*Xba*I was then subcloned into the pYESCOTwt digested with *Xba*I, yielding pYESCOT^{H340A}.

The plasmid pYESCOT$^{H131A/H340A}$ was generated by excising the sequence *Hin*dIII–*Xba*I from pYESCOT^{H131A} and the *Xba*I–*Xba*I sequence from pYESCOT^{H340A}, and subcloned into pYES2. The appropiate substitutions and the absence of unwanted mutations were confirmed by sequencing the inserts in both directions with an Applied Biosystems 373 automated DNA sequencer.

Expression of COT variants in *S. cerevisiae*

The expression plasmids pYESCOTwt, pYESCOT^{H131A}, pYESCOT^{H340A} and pYESCOT$^{H131A/H340A}$, and the empty plasmid pYES2, were transformed in the *S. cerevisiae* strain YPH499 (*MAT a ura3-52 leu2-D1 ade2-101 lys2-801 his3-D200 trp1-d63*) using a modification of methods described in [28]. Positive *S. cerevisiae* colonies grown under complete minimal medium lacking uracil, CM(−ura), with 2 % glucose as a carbon source [29], were grown in 10 ml of yeast liquid CM(−ura) supplemented with 2 % glucose. The culture was transferred to 200–400 ml of the same medium, and grown to a D_{660} of 3. Cells were recovered by centrifugation, washed in CM(−ura) with 2 % galactose, centrifuged again and finally resuspended in 100 ml of the same medium. Cultures were harvested during the exponential growth phase by centrifugation at 2000 ***g*** for 5 min at 4 °C, washed in 20 ml of ice-cold buffer A (150 mM KCl/5 mM Tris/HCl, pH 7.2), centrifuged again, and resuspended in 1 ml of buffer B (which is buffer A supplemented with 1 mM PMSF/

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0.5 mM benzamidine/10 ng/ml leupeptine/100 ng/ml pepstatin). Crude cell extracts were prepared by disrupting the resuspended cells with 0.5 ml of cold, acid-washed glass beads (Sigma, catalogue reference G-9268). Cell debris was removed by centrifugation at 500 ***g*** for 3 min at 4 °C. Homogenates were centrifuged further at 15000 ***g*** for 10 min at 4 °C and pellets were resuspended in buffer B and frozen immediately and stored at −70 °C.

Activation of etomoxir to etomoxiryl-CoA

Extracts from yeast cells transformed with the empty plasmid were resuspended in 50 μl of buffer B and combined with an equal volume of 'activation mixture': 200 mM Tris/HCl, pH 7.2, 100 mM KCl, 13 mM $MgCl_2$, 12 mM ATP, 0.7 mM GSH, 100 μM CoA and 1.2 % (w/v) fatty acid-free BSA. Etomoxir was added to a final concentration of 200 μM, and the mixture was incubated for 30 min at 22 °C to allow conversion of etomoxir into etomoxiryl-CoA. For calculations we assumed that the conversion was complete. In inhibition assays, increasing concentrations of the inhibitor were included (1–30 μM).

Measurement of COT activity

COT activity was assayed at 30 °C by the forward exchange method using L-[methyl-^{3}H]carnitine and decanoyl-CoA as described previously [25] with minor modifications. Rat liver peroxisomes (4 μg) or yeast extracts in 80 μl were pre-incubated at 30 °C for 1 min in the presence or absence of malonyl-CoA or etomoxiryl-CoA. The reaction was started by addition of 120 μl of the standard enzyme-assay mixture containing 400 μM L-[methyl-^{3}H]carnitine (0.3 μCi), 50 μM decanoyl-CoA, 105 mM Tris/HCl (pH 7.2), 2 mM KCN, 15 mM KCl, 4 mM $MgCl_2$, 4 mM ATP, 250 mM GSH and 0.1 % (w/v) fatty acid-free BSA. Enzyme activity was assayed at 30 °C for 8 min for liver peroxisomes and 4 min for yeast extracts (reaction was linear under the conditions described). The reaction was arrested by addition of 200 μl of 1.2 M HCl, followed by centrifugation at 10000 ***g*** for 2 min. The butanol phase was rinsed with 200 μl of water and an aliquot was transferred to a vial for liquid scintillation counting (Ecolite, ICN).

Kinetic parameters

Substrate-saturation data were fitted to rectangular hyperbolae by non-linear regression using the computer program Enzfitter (Biosoft). K_m values were estimated by analysing the data from three experiments performed in duplicate using the same program. Malonyl-CoA and etomoxiryl-CoA concentrations, ranging from 2 to 200 μM and from 1 to 30 μM respectively, were used for estimation of the IC_{50} value. IC_{50} is defined as the concentration that produces 50 % inhibition of enzyme activity. The COT activity and IC_{50} values are given as a means ± S.D. for at least three independent assays with different preparations.

Isolation of rat peroxisomes

Peroxisomes were prepared from livers of fed male Wistar rats weighing 160–200 g. Peroxisomes were isolated in 0.3 M sucrose/5 mM Tris/HCl/1 mM EDTA, pH 7.4, using differential centrifugation and then purified further in a Nycodenz cushion as described in [30]. The purified peroxisomes were dispersed in 250 mM sucrose/10 mM Tris/HCl/1 mM EDTA, pH 7.4, at a protein concentration of 2 mg/ml and stored at −70 °C.

Generation of anti-COT antibody

Rabbit polyclonal antibodies against amino acids 69–83 of rat COT were raised as follows: a peptide KLLERAKGKRNWLEEC was synthesized using the solid-phase method [31] with additional inclusion of the C-terminal cysteine residue. The peptide was cross-linked to *N*-maleimidobenzoyl-*N*-hydroxysuccinimide-activated keyhole-limpet haemocyanin and used to immunize rabbits. The peptide showed low identity to other carnitine acyltransferases. Anti-COT antibodies were generated according to conventional procedures [32]. γ-Globulins derived from peptide 69–83 were designated A69.

Western-blot analysis

Proteins were separated by SDS/PAGE in a 10 % gel [33] and electrophoretically transferred on to nitrocellulose membranes for 2 h using a Mini Trans-Blot Electrophoretic Cell according to the manufacturer's instructions (Bio-Rad). The membrane was blocked at room temperature for 1 h with PBS containing 0.1 % Tween and 5 % non-fat dried milk. The membrane was washed three times in PBS/Tween at room temperature. Immunoblots were developed by incubation with the COT-specific polyclonal antibody A69 (1:100 dilution), or pre-immune sera, followed by an anti-rabbit IgG conjugated to horseradish peroxidase (1:10000 dilution). The antigen–antibody complex was detected using an ECL® Western-blotting system (Amersham Pharmacia Biotech)

Statistics

All experiments were run at least three times. Values reported in the text are the means ± S.D. from 3–5 determinations. Student's unpaired *t* test was used for the statistical evaluations. Correlation coefficients were calculated by the method of least squares. The calculations were performed with the program GraphPad Instat (version 2.03, GraphPad Software).

Drugs and chemicals

(+)Etomoxir (2-[6-(4-chlorophenoxy)hexyl]oxirane-2-carboxylate) sodium salt was obtained from Dr H. P. O. Wolf (GMBH, Allensbach, Germany). It was prepared as a stock solution of 1 mM in DMSO. L-[methyl-^{3}H]Carnitine was from Amersham Pharmacia Biotech; malonyl-CoA and decanoyl-CoA were from Sigma. Yeast culture media products were from Difco.

Computer-aided analysis of sequence data

Alignments were made by combining two algorithms, PILEUP and PRETTY programs from Wisconsin Package version 9.1 (Genetics Computer Group, Madison, WI, U.S.A.).

Miscellaneous

Escherichia coli strain DH5α was used to propagate various plasmids and their derivatives. The plasmid pBluescript (Stratagene) was used for cloning DNA fragments. Protein was determined by the Bradford assay [34] with BSA as a standard. All restriction enzymes and T4 DNA ligase were purchased from Fermentas.

RESULTS

Effect of etomoxir and malonyl-CoA on peroxisomal COT activity

The inhibitory potencies of malonyl-CoA and etomoxiryl-CoA towards rat liver peroxisomal COT are shown in Figure 2. The concentration of etomoxir needed to suppress enzyme activity by

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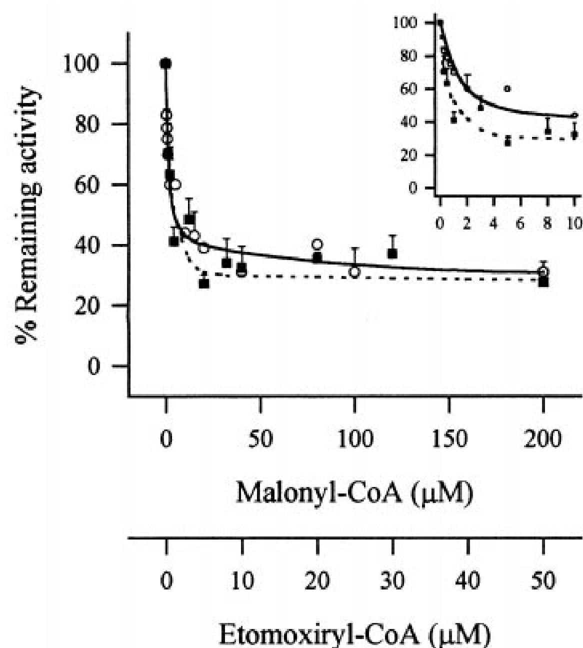

Figure 2 Effect of etomoxiryl-CoA and malonyl-CoA on the activity of peroxisomal COT

Rat liver peroxisomes were incubated with increasing concentrations of etomoxiryl-CoA (■, broken lines) and malonyl-CoA (○, solid lines) and the enzyme activity was measured. Data are expressed relative to control values in the absence of the inhibitors (100%), as the mean from three independent measurements. The IC_{50} values for the inhibition of COT were 1.2 μM for etomoxiryl-CoA and 4.2 μM for malonyl-CoA. Activities remaining at either 30 μM etomoxiryl-CoA or malonyl-CoA were 31 and 39%. Insert: expanded dose–response curve for the two inhibitors.

50% (IC_{50}) in peroxisomes was approx. 1.2 μM, whereas the analogous IC_{50} for malonyl-CoA was 4.2 μM. In contrast to malonyl-CoA, which interacts with CPT I and COT in reversible fashion, etomoxir acted irreversibly, as judged by the failure of multiple washings of peroxisomes to reduce the inhibition.

Analysis of histidines potentially involved in the inhibition of peroxisomal COT by etomoxiryl-CoA

To determine which of the His residues of COT were potentially responsible for the inhibitory action of etomoxir, we examined all the His residues common to a number of carnitine acyltransferases. Figure 3 summarizes all the His residues in seven carnitine acyltransferases. Histidines a, b and c, as shown in Figure 3 (H5, H133/135 and H140/142 of the L- and M-isoforms of mitochondrial CPT I) cannot be responsible for the binding to etomoxir because they are absent in peroxisomal COT (the first amino acid of COT corresponds to position 152 of the L- and M- isoforms of mitochondrial CPT I), provided that both carnitine transferases have conserved the same etomoxiryl-CoA-inhibitable sites. In addition, histidines e, g, h, l and m, which are present in CPT I and not conserved in peroxisomal COT, are unlikely to be candidates for etomoxir inhibition. Moreover, those His residues present at the same position in all carnitine transferases (histidines f, i and k) cannot be responsible because CPT II is insensitive to the inhibitor. According to this reasoning, only two residues, present in the etomoxir-inhibitable enzymes and absent in non-inhibitable carnitine transferases, were candidates for etomoxir inhibition: His d, corresponding to peroxisomal COT H131 and His j, corresponding to peroxisomal COT H340 from the rat. We then decided to mutate these two His residues. Our approach was to express the mutated enzymes in heterologous systems, to evaluate the activity and inhibitory effect of etomoxir.

Expression and kinetics of wild-type and mutated COT in *S. cerevisiae*

The pYES2 plasmids containing the wild-type and the various mutant versions of COT were expressed in *S. cerevisiae* as described in the Materials and methods section. In cells transformed with the empty plasmid, COT activity was undetectable

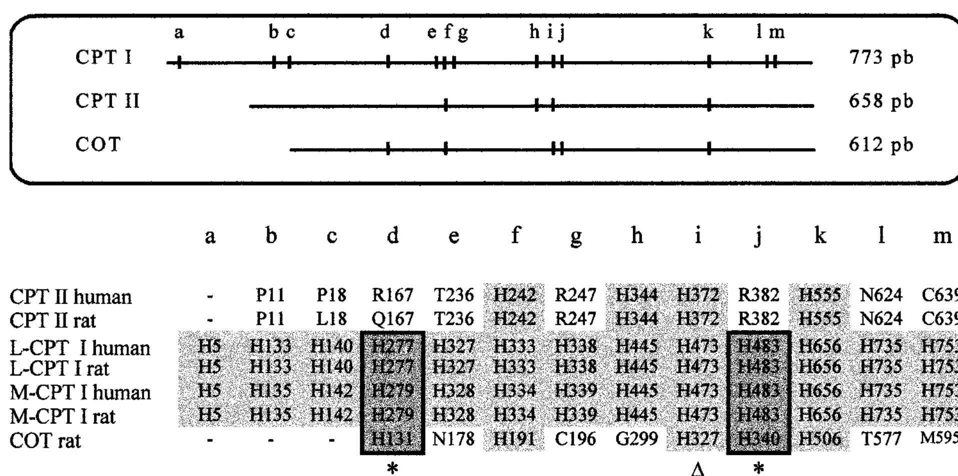

Figure 3 Alignment of conserved histidines in seven carnitine acyltransferases

Sequences were obtained from the GenBank nucleotide sequence database: human CPT II (accession number P23786) [41], rat CPT II (J05470) [6], human L-CPT I (L39211) [42], rat L-CPT I (L07736) [4], human M-CPT I (D64178) [43], rat M-CPT I (D43623) [44] and rat COT (U26033) [26]. Alignment of sequences was obtained with PILEUP and PRETTY programs from Wisconsin Package version 9.1 (Genetics Computer Group). At the top, the distribution of the thirteen conserved histidines is shown. At the bottom, * indicates the conserved histidines in etomoxir-inhibitable enzymes (CPT I and COT), △ indicates the conserved histidine in all carnitine transferases and presumably involved in the acyl-CoA binding [25,45].

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Table 1 Characteristics of COT activity in yeast strains expressing COT and in rat liver peroxisomes

Extracts were isolated from yeast cells transformed with empty expression vector (pYES2), from yeast expressing the wild-type and mutant enzymes of COT, and from rat liver peroxisomes, as described in the Materials and methods section. K_m values for carnitine were determined at 50 μM decanoyl-CoA and K_m values for decanoyl-CoA were determined at 400 μM carnitine. Results are the means ± S.D. from 3–6 separate experiments.

	Carnitine		Decanoyl-CoA	
Strain	K_m (μM)	V_{max} (nmol/min per mg)	K_m (μM)	V_{max} (nmol/min per mg)
PYES2	–	0	–	0
pYESCOTwt	172 ± 46	205 ± 24	2.0 ± 0.2	67 ± 4
pYESCOT^{H131A}	160 ± 32	98 ± 15	2.6 ± 0.2	159 ± 7
pYESCOT^{H340A}	117 ± 21	210 ± 25	6.0 ± 0.9	270 ± 15
pYESCOT$^{H340A/H131}$	106 ± 19	302 ± 36	13.0 ± 2.7	332 ± 25
Rat liver peroxisomes	218 ± 23	23 ± 7	0.2 ± 0.06	19 ± 1

(Table 1). When the wild-type COT was expressed, we obtained a preparation of COT protein with a specific activity of 205 nmol/min per mg of protein. Under the same assay conditions, the specific activity of rat liver peroxisomes was 23 nmol/min per mg of protein.

Interestingly, the growth rate of yeast cells transformed with the expression plasmids pYESCOTwt, pYESCOT^{H131A}, pYESCOT^{H340A} and pYESCOT$^{H131A/H340A}$ was dramatically reduced when the cultures were switched to galactose to induce expression of the different COT variants. In contrast, expression of pYESCOT^{H327A}, encoding a version of the enzyme in which the putatively catalytic histidine 327 had been replaced by Ala [25], did not have such a strong effect on cell growth (results not shown). We considered that expression of catalytically active forms of COT might interfere with the yeast metabolism, which would impede cell growth. We thus decided to grow the cells in glucose, to allow vigorous growth, and to switch the carbon source to galactose only when expression of COT was sought.

Mutations on either H131 or H340 produced small changes in the K_m and V_{max} for carnitine and decanoyl-CoA as substrates (Table 1). K_m values for carnitine ranged between 172 μM (wild type) and 106 μM (double mutant). K_m values for decanoyl-CoA ranged between 2 μM (wild type) and 13 μM (double mutant). V_{max} values of the different enzymes varied less for carnitine than for decanoyl-CoA (Figure 4). For the purposes of this study, these small changes in kinetic parameters of the mutated enzymes can be considered of secondary importance in comparison with the dramatic changes observed in the inhibition by etomoxir.

To rule out the possibility that the various transformed yeast cultures expressed variable amounts of peroxisomal COT we performed an immunoblot analysis. As shown in Figure 5, all transformed yeast cells expressed a protein with the same molecular mass and in roughly the same proportion per mg of total protein.

Inhibitory action of etomoxiryl-CoA on the wild type and mutants of peroxisomal COT expressed in *S. cerevisiae*

Figure 6(A) shows the etomoxiryl-CoA-dependent inhibition of yeast-expressed wild-type COT. These data showed a decrease in the activity similar to that observed in rat liver peroxisomes, indicating that yeast is a suitable organism in which to study inhibition of COT by etomoxiryl-CoA. Figure 6(B) shows the pattern of inhibition by etomoxiryl-CoA on the *S. cerevisiae*-

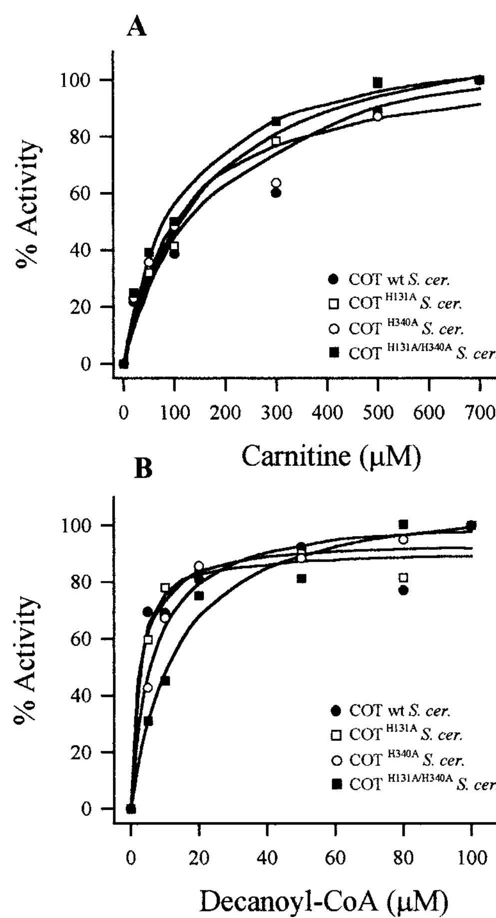

Figure 4 Dependence on carnitine and decanoyl-CoA concentration of the wild-type and mutant COT variants

Yeast extracts of wild-type and mutant COT variants were incubated at increasing concentrations of carnitine (**A**) or decanoyl-CoA (**B**). When decanoyl-CoA concentration was modified, L-carnitine concentration was 400 μM, and when carnitine was modified the decanoyl-CoA concentration was fixed at 50 μM. Data are the means from three independent experiments.

expressed mutant H131A, which was only slightly inhibited at the etomoxir concentrations assayed. Mutant H340A responded to etomoxir in a similar way (Figure 6C) to mutant H131A. The activity of peroxisomal COT was hardly inhibited at concentrations of etomoxiryl-CoA up to 30 μM. Therefore, the inhibition is nearly lost as a result of each of the two mutated histidines. Figure 6(D) shows the inhibitory pattern of

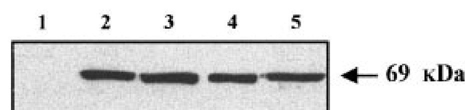

Figure 5 Immunoblot analysis of recombinant COT variants in *S. cerevisiae*

Samples were prepared and analysed as described in the Materials and methods section. *S. cerevisiae* extracts (10 μg) was separated by SDS/PAGE and subjected to immunoblotting by using specific A69 antibodies for COT. This is a representative experiment, which gave analogous immunoblots when repeated three times. Lane 1, extracts from *S. cerevisiae* transformed with empty plasmid; lanes 2–5, wild-type COT and COT mutants H131A, H340A and H131A/H340A, respectively. The arrow indicates the migration position and the molecular mass of COT from rat liver peroxisomes.

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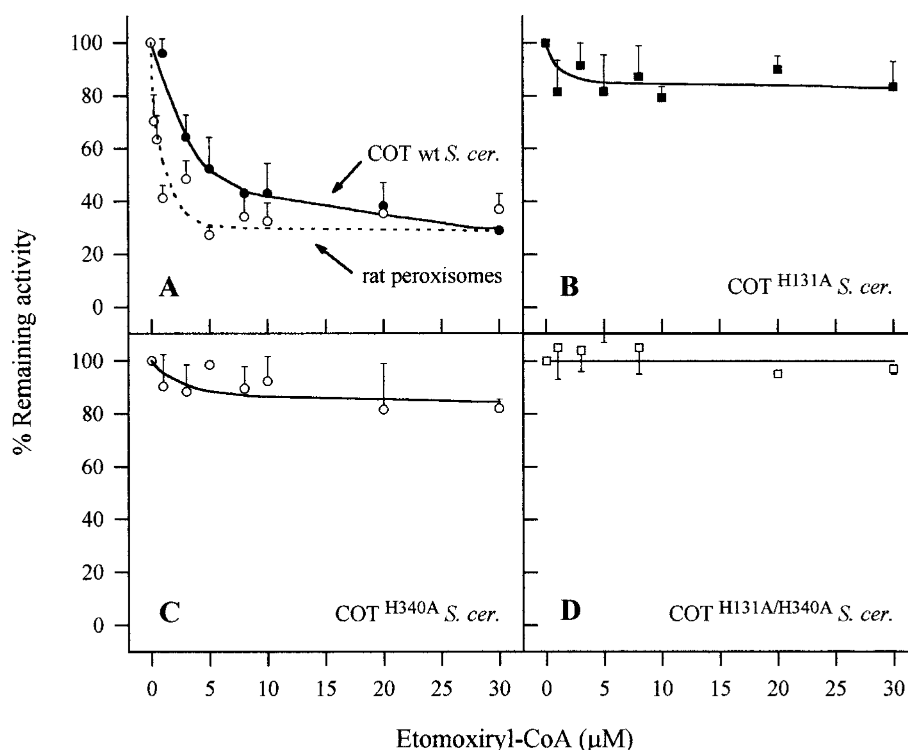

Figure 6 Effect of etomoxiryl-CoA on the activity of the wild-type and point mutants of COT

S. cerevisiae cells were transformed with the cDNAs for the wild-type (**A**), the point mutants H131A (**B**) and H340A (**C**), and the H131A/H340A double mutant (**D**). Assays were carried out as described in the Materials and methods section. Extracts were incubated with increasing concentrations of etomoxiryl-CoA. Data are expressed relative to control values in the absence of etomoxiryl-CoA (100%), as the mean of three independent measurements. (**A**) also shows the curve corresponding to peroxisomal COT (as a control), taken from Figure 2.

etomoxiryl-CoA on the *S. cerevisiae*-expressed double mutant H131A/H340A of peroxisomal COT. This mutant variant of the enzyme was not inhibited by etomoxir at any concentration in the range 1–30 μM.

DISCUSSION

A few pharmacological analogues of the physiological inhibitor malonyl-CoA have been studied for their ability to regulate CPT I, including the glycidic acids such as TDGA, clomoxir and etomoxir. They act as irreversible inhibitors of CPT I [8,35]. They are functionally active only after metabolic conversion into their CoA esters and presumably inhibit CPT I via covalent binding to the protein through the reaction of their epoxide moiety.

It had long been assumed that regulation of carnitine acyltransferases by etomoxir and related compounds affects only mitochondrial CPT I. However, the work of Skorin et al. [36] and Lilly et al. [37] showed that TDGA inhibits COT and CPT I with similar intensity in rat liver mitochondria and peroxisomes. Lilly et al. [37] also showed that COT was inhibited by etomoxir, which is related to TDGA. Our studies here show that COT is inhibited by etomoxir in a concentration-dependent manner, with similar kinetics to malonyl-CoA.

The effects of the pharmacological compounds derived from oxirane and of the physiological inhibitor malonyl-CoA are similar, and it has been suggested that they may share the same locus of interaction with CPT I [18]. This was deduced from the following: (i) malonyl-CoA blocks the binding of the labelled inhibitor, (ii) the inhibitor abolishes the binding of labelled malonyl-CoA and (iii) the number of binding sites for [^{3}H]TDGA-CoA on muscle mitochondria was the same as that for [^{14}C]malonyl-CoA. It was clear that the inhibitors bind to CPT I at a common locus in such a way that the percentage occupancy is related directly to the extent of inhibition of CPT I. We favour the notion that malonyl-CoA and etomoxir also share the same locus of interaction with COT.

Studies by A'Bhaird and Ramsay [14] show that malonyl-CoA binds to rat peroxisomal COT at two separate sites. At one of these sites, which is within the catalytic domain, malonyl-CoA competes with the substrate, either palmitoyl-CoA or decanoyl-CoA, in a similar way to the inhibition produced by several CoA esters, like glutaryl-CoA, 3-hydroxy-3-methylglutaryl-CoA and methylmalonyl-CoA. In addition, there is another, more specific site, to which malonyl-CoA binds as an allosteric effector, but to which the other CoA-related products cannot bind. Several reports have suggested that the interaction of malonyl-CoA with carnitine acyltransferases is exerted through histidine residues [12,13], and since then different authors have attempted to identify the histidines responsible for the inhibition.

In the search for the histidines from COT that are responsible for the interaction with etomoxir, we extended the alignment of the carnitine transferases to all known sequences, some of them regulated by etomoxir and some others not regulated. From this comparison it was found that the three histidine residues (H5, H133 and H140) suggested as candidates for the inhibition of CPT I [3] by malonyl-CoA and, by extension, by etomoxir, were unlikely to be etomoxir-binding sites. This was because they are absent in COT, based on the assumption that the same amino acid residues are responsible for the inhibition in all malonyl-

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CoA-regulatable carnitine transferases. In a later study the same authors [38] showed that although H5 and H140 may play a role in the malonyl-CoA response, the larger C-terminal region dictates the degree of sensitivity to malonyl-CoA, and also for etomoxir binding. These conclusions were established after the expression of L- and M-CPT I chimaeras in COS cells. Very recently, Jackson et al. [39], using six chimaeric proteins formed by different domains of L- and M-CPT I, have shown that the same I_{50} values for malonyl-CoA were seen in the chimaeras containing the same C-terminus, whether from L- or M-CPT I, after expression in the yeast *Pichia pastoris*.

The other candidate histidines, save two, were also ruled out, some because they are also conserved in CPT II and others because they are not present in COT. Therefore, the two histidines present in the carnitine acyltransferases regulated by etomoxir and candidates for interaction and binding to etomoxir were H131 and H340.

S. cerevisiae does not contain COT activity (Table 1), so it is a suitable organism for the study of expression of this protein. Mutations H131A and H340A in peroxisomal COT did not have an important effect on the kinetic behaviour of the mutants with carnitine, as the differences in K_m values observed for carnitine are small in either purified or peroxisomal COTs [14]. This was expected, as previous results showed that a different residue (Arg-505) of the bovine COT had a role in binding to carnitine [40]. The small but detectable change in K_m for decanoyl-CoA in the H340A and H131A/H340A mutants suggests that the loss of a positive charge within the catalytic domain may alter the binding of the substrate.

The double mutant H131A/H340A showed no inhibition at low or high concentrations of etomoxir, despite the fact that the enzyme was fully active. This is the best demonstration that these two histidine residues are responsible for the inhibition by etomoxir. The individual contributions of the mutations to the inhibition by etomoxir were also tested. In both cases (H131A and H340A) there was a slight inhibition at concentrations of etomoxir of up to 20–30 μM.

Taken together, the results of the present study, demonstrate that: (i) H131 and H340 of COT are responsible for the action of etomoxir on the protein, since in the double mutant, although the enzyme activity is not changed, the inhibition by etomoxir is lost, and (ii) etomoxir produces its inhibitory effects by binding simultaneously to both histidine residues, since when either is mutated there is almost no inhibition. The bridge that probably connects both histidines to etomoxir is lost when one is mutated, and therefore the inhibition produced in the wild-type COT is lost. The coincidence of similar results with those obtained with malonyl-CoA is additional evidence that malonyl-CoA and etomoxir-CoA bind to COT at the same site, as was proposed for CPT I.

In conclusion, these results can serve as a model to study the effect of specific mutations in amino acids that bind malonyl-CoA and etomoxir in other carnitine acyltransferases. The exact understanding of how etomoxir-CoA interacts with those enzymes to which it binds may facilitate the development of other drugs that interfere with the metabolism of fatty acids in several tissues like liver, heart and muscle and in pancreatic β-cells. Studies on these topics are in progress in our laboratory.

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