Novel methylenecyclopropyl-based acyl-CoA dehydrogenase inhibitor

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Received 28 June 1998; received in revised form 26 August 1998

Abstract A novel hexyl-substituted methylenecyclopropyl acetyl-CoA was tested as an enzyme-specific acyl-CoA dehydrogenase inhibitor. Its CoA ester generated in situ from the carboxylic acid and CoASH, displayed marked differences in inhibition specificity as compared to methylenecyclopropyl acetyl-CoA, consistent with the substrate specificities of the target enzymes. Thus methylenecyclopropyl acetyl-CoA inactivated short-chain-specific acyl-CoA dehydrogenase rapidly, medium-chain-specific acyl-CoA dehydrogenase much more slowly and had no effect on long-chain- or very long-chain-specific acyl-CoA dehydrogenases. The hexyl-substituent on the methylenecyclopropyl ring gave an inhibitor which rapidly inactivated MCAD and LCAD whilst VLCAD was inhibited more slowly and SCAD was essentially unaffected. In some cases (e.g. SCAD and MCPA-CoA) inhibition was accompanied by flavin bleaching. In other cases (e.g. LCAD and C_6MCPA) less pronounced bleaching suggests a different chemistry of inhibition.

Key words: Acyl-CoA dehydrogenase; Covalent inhibitor; Alkyl-substituted methylenecyclopropane acetyl-CoA; Flavin alkylation; Active-site-directed inhibitor

1. Introduction

Acyl-CoA dehydrogenases are mitochondrial flavoproteins which catalyse the oxidation of saturated acyl-CoA esters to give the corresponding 2,3-*trans*-unsaturated compounds [1]. Six such enzymes have been described in mammals [2,3]; short-chain-specific (SCAD), medium-chain-specific (MCAD), long-chain-specific (LCAD) and very long-chain-specific acyl-CoA dehydrogenase (VLCAD) have overlapping substrate specificity profiles and catalyse the first of the four steps serially repeated in the β-oxidation spiral of fatty acid oxidation [1,4]. Isovaleryl-CoA dehydrogenase (IVD) and 2-methyl-branched-chain acyl-CoA dehydrogenase (2MeBCD) catalyse the oxidation of carbon skeletons from branched chain amino acid degradation [5].

Methylenecyclopropyl acetyl-CoA (MCPA-CoA), a metabolite of the unusual amino acid hypoglycin, is the causative agent of Jamaican vomiting sickness [6]. Both in vitro and in vivo, MCPA-CoA inhibits SCAD, MCAD, IVD and 2MeBCD, whilst LCAD is unaffected [7–11]. There is also one report of MCAD being unaffected by MCPA-CoA [12]. MCPA-CoA, a 'suicide' or mechanism-based inhibitor [7], causes irreversible inhibition through covalent modification of the flavin prosthetic group [7,13]. The lower homologue, methylenecyclopropyl formyl-CoA (MCPF-CoA), exhibits a rather different target specificity, giving potent inhibition of 2MeBCD and IVD whilst SCAD is rather weakly inhibited and MCAD and LCAD are unaffected [14,15]. Inhibition by this compound is irreversible but the mechanism of action is unknown [14,15]. Recently it was reported that spiropentane acetyl-CoA (SPA-CoA) preferentially inhibited octanoyl-CoA dehydrogenase activity in liver homogenates [16]. The branched-chain acyl-CoA dehydrogenase activities appeared to be unaffected by SPA-CoA [16].

The different effects of MCPA-CoA, MCPF-CoA and SPA-CoA indicate the potential for targeting inhibitors towards specific acyl-CoA dehydrogenases. We have used a hexyl-substituted derivative of MCPA to investigate this possibility further and now report on the effects of this novel inhibitor on the catalytic activity and absorption spectra of SCAD, MCAD, LCAD and VLCAD.

2. Materials and methods

2.1. Chemicals

Coenzyme A (trilithium salt), Sephadex G-200 and monoQ columns were from Pharmacia. ATP was from Boehringer Mannheim. Acyl-CoA esters were from Sigma. Hydroxyapatite was from Bio-Rad. Cellulose powder (CC31 microgranular) was from Whatman Lab Sales. All other reagents were of the highest grade commercially available.

The methylenecyclopropane carboxylic acids used in this study, MCPA and C_6MCPA, were a generous gift from Professor Charles Stirling, Department of Chemistry, University of Sheffield. Methylenecyclopropane synthesized by Koster's procedure [17] was lithiated by treating with butyl lithium in tetrahydrofuran [18] and subsequently alkylated [19]. The alkyl methylenecyclopropane was again lithiated and then reacted with ethylene oxide. The resulting alcohol was oxidized with chromium trioxide [20] to yield the desired carboxylic acid. Proton NMR showed that the acids were not 100% pure, but, in view of the subsequent procedures to be used and the instability of the compounds, they were used without further purification.

2.2. Enzymes

Medium-chain-length-specific acyl-CoA synthetase was purified from pig liver mitochondria [21]. SCAD, MCAD and LCAD were purified from bovine liver. SCAD, purified as described previously [22] was prepared in yellow (CoA persulphide-free) and green (CoA persulphide-liganded) forms [23,24]. The side fraction described in [22], containing MCAD and LCAD, was used for their further purification. MCAD and LCAD were separated on a hydroxyapatite-cellulose column [25]. MCAD was further purified by gel-filtration on a Sephadex G-200 column (100×1.5 cm) equilibrated with KH_2PO_4/K_2HPO_4 buffer (50 mM, pH 7.6) containing EDTA (0.3 mM) and

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Abbreviations: SCAD, short-chain-specific acyl-CoA dehydrogenase; MCAD, medium-chain-specific acyl-CoA dehydrogenase; LCAD, long-chain-specific acyl-CoA dehydrogenase; VLCAD, very long-chain-specific acyl-CoA dehydrogenase; IVD, isovaleryl-CoA dehydrogenase; 2MeBCD, 2-methyl-branched-chain acyl-CoA dehydrogenase; MCPA, methylenecyclopropyl acetic acid; MCPF, methylenecyclopropyl formic acid; SPA, spiropentane acetic acid; C_6MCPA, MCPA with a hexyl-substituted cyclopropane ring

PII: S0014-5793(98)01155-7

eluted at approximately 20 ml/h with the same buffer. LCAD was further purified by fplc anion-exchange on monoQ (5/5 column) equilibrated with HEPES buffer (20 mM, pH 7.5). LCAD was eluted with a KCl gradient (two column volumes at 0 mM KCl ,step to five column volumes at 200 mM KCl followed by a linear gradient of 200 mM to 500 mM KCl over 30 column volumes; flow rate = 1 ml/min). During purification acyl-CoA dehydrogenase activity was monitored by assaying with butyryl-CoA, lauroyl-CoA and 3-phenylpropionyl-CoA, the latter being a specific substrate for MCAD. SCAD preparations displayed no activity with lauroyl-CoA or 3-phenylpropionyl-CoA indicating them to be free of MCAD and LCAD. LCAD preparations displayed no activity with 3-phenylpropionyl-CoA indicating them to be free of MCAD. VLCAD was a generous gift from Professor T. Hashimoto [3].

2.3. Enzyme assays

Acyl-CoA synthetase was assayed by following the decrease in free CoASH on incorporation into acyl-CoA thioesters [26]. Reaction mixtures (1 ml) contained: ATP (4 mM), $MgCl_2$ (4 mM), CoASH (trilithium salt, 0.4 mM), fatty acid substrate (0.1–1 mM) and acyl-CoA synthetase in KH_2PO_4/K_2HPO_4 buffer (100 mM, pH 7.1). Acyl-CoA dehydrogenases were assayed at 25°C using a dye-linked system [22]. Assays (1 ml) contained: KH_2PO_4/K_2HPO_4 buffer (100 mM, pH 7.1), dichlorophenolindophenol (0.0001% (w/v)), *N*-ethylmaleimide (200 μM), acyl-CoA substrate (50 μM), enzyme and phenazine ethosulphate (0.6 mM for SCAD assays, 6 mM for MCAD assays, 3 mM for LCAD and VLCAD assays). Assays were initiated by phenazine ethosulphate addition exactly 2 min after addition of the enzyme.

2.4. Inhibition incubations

The CoA thioesters of MCPA and C_6MCPA were generated in situ by using pig liver mitochondrial acyl-CoA synthetase. The complete system consisted of an acyl-CoA synthetase reaction mixture to which the acyl-CoA dehydrogenase of interest was added (usually 1 μM by flavin absorbance). The carboxylic acid substrate was the MCPA compound to be studied. Samples were periodically removed and assayed for acyl-CoA dehydrogenase activity (10 μl samples) and for CoASH consumption (= thioester synthesis) (150 μl samples) exactly as in acyl-CoA synthetase assays [26]. The amount of acyl-CoA synthetase used was adjusted according to which MCPA compound was being used so that the rate of thioester synthesis in the incubation mixture was approximately 80 nmol/h/ml.

2.5. Absorption spectra

For all measurements of spectra the test cuvette contained the standard acyl-CoA synthetase reaction mixture plus acyl-CoA dehydrogenase. The reference cuvette contained only the acyl-CoA synthetase reaction mixture. Inhibition was initiated by addition of the appropriate MCPA compound. Spectra were recorded at 10 nm/s in a Cary 219 UV/vis spectrophotometer at room temperature. In some experiments during incubation with the inhibitors, the 30 s scan time inevitably entails a distortion of the true instantaneous spectrum. This effect should be slight, however, since incubation times were 1–2 h. Aliquots were removed for assay of acyl-CoA dehydrogenase activity as described above.

3. Results and discussion

3.1. Effects of methylenecyclopropyl compounds on activity

The structures of the compounds used in this study are shown in Fig. 1. The hexyl-substituent on C_6MCPA can be considered to give a compound with a C_{10} 'backbone' whilst by the same argument MCPA can be considered to have a C_4 'backbone'. The progressive inhibitory effects on purified preparations of SCAD, MCAD, LCAD and VLCAD of MCPA-CoA and C_6MCPA-CoA (both generated in situ using acyl-CoA synthetase) are shown in Fig. 2. In no case was inhibition observed in the absence of ATP and CoASH or when MCPA or C_6MCPA were omitted from an otherwise complete reaction mixture. This indicates that the CoA thioesters of these compounds are the active species. Inhibition was always irreversible, no activity being regained on extensive dialysis against potassium phosphate buffer (100 mM, pH 7.1). The two inhibitors display very different target specificity with respect to the enzymes they inhibit; these differences are consistent with the substrate specificities of the target enzymes. The main target of MCPA-CoA was SCAD (using yellow enzyme free of CoA persulphide). By comparison, MCAD was much more slowly inhibited whilst LCAD and VLCAD were unaffected. These data are in broad agreement with earlier reports that MCPA-CoA inhibits SCAD and MCAD but not LCAD [10,11]. However, whilst Tanaka's laboratory, working with purified rat liver enzymes, reported that MCAD was inhibited slightly faster than SCAD [10] it was shown by Tserng et al. [16] that MCPA-CoA inhibits SCAD more rapidly than MCAD in bovine liver homogenates. The relatively slower inhibition of MCAD observed with the bovine enzymes presumably reflects subtle differences in the substrate specificity of the bovine and rat enzymes. Bovine SCAD is active with C_4 to C_8 (optimum C_4) acyl-CoA esters [2,25,27] and consequently MCPA-CoA is of the correct 'size' to be acted on by this enzyme. Bovine MCAD is active with C_4 to C_{18} (optimum C_6 to C_8) acyl-CoA esters [2,25,27] and so can act slowly on MCPA-CoA. Bovine LCAD is active with C_6 to C_{20} (optimum C_{10} and C_{12}) acyl-CoA esters [2,21,23] and rat VLCAD is active with C_{10} to C_{24} (optimum C_{16} and C_{18}) acyl-CoA esters [3] and consequently MCPA-CoA is of too short a 'chain-length' to be acted on by these latter two enzymes.

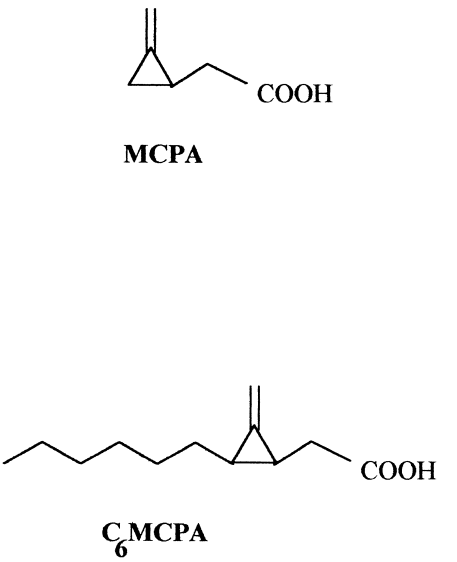

Fig. 1. Structure of the free acid forms of MCPA and C_6MCPA.

Green SCAD (i.e. enzyme liganded with CoA persulphide [24]) exhibits a very different time-course of inhibition from yellow (CoA persulphide-free) SCAD (Fig. 2A). The significant lag period before any inhibition is observed with green SCAD presumably reflects the need for MCPA-CoA to displace CoA persulphide from the enzyme active site before inhibition can occur; similarly, normal catalytic assays of green SCAD display a significant lag (during which time butyryl-CoA displaces CoA persulphide) before maximum activity is observed [23]. In the present work samples were incubated with 50 μM butyryl-CoA and 200 μM *N*-ethylmaleimide for exactly 2 min before initiating activity assays by addition of PES. Reaction traces did not display any lag, indicating

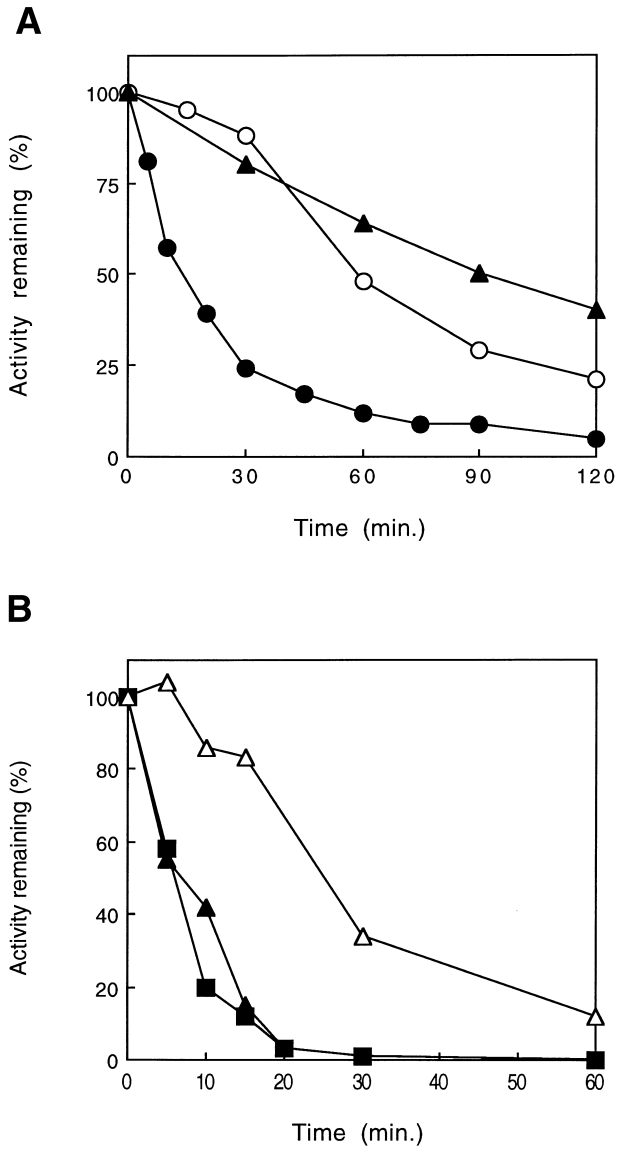

Fig. 2. Effect of MCPA-CoA and C_6MCPA-CoA (generated in situ) on the activity of purified SCAD, MCAD, LCAD and VLCAD. Incubations contained 1 μM acyl-CoA dehydrogenase, 0.4 mM CoASH, 4 mM ATP, 4 mM $MgCl_2$, MCPA (200 μM) or C_6MCPA (100 μM) and porcine acyl-CoA synthetase (100 μg) in potassium phosphate buffer (100 mM, pH 7.1). A: ●, SCAD (yellow)/MCPA; ○, SCAD (green)/MCPA; ▲, MCAD/MCPA. B: ■, MCAD/C_6MCPA; ▲, LCAD/C_6MCPA; △, VLCAD/C_6MCPA. In each case data are from a single experiment which is representative of three independent experiments.

that the CoA persulphide had been completely displaced from the enzyme active site (by butyryl-CoA and/or by reaction with *N*-ethylmaleimide). Protection against inhibition by CoA persulphide is a further line of evidence that MCPA-CoA is acting at the active site of SCAD.

C_6MCPA-CoA rapidly inhibited both MCAD and LCAD whilst VLCAD was inhibited more slowly and only after a distinct lag period (Fig. 2B). By contrast, SCAD was essentially unaffected. The C_{10} 'backbone' C_6MCPA-CoA is too long to be acted on by SCAD and is only just long enough to be acted on by VLCAD. The C_{10} 'backbone' of C_6MCPA-CoA is, however, of a suitable 'chain-length' for it to be acted on rapidly by both MCAD and LCAD. The lag period before VLCAD is inhibited by C_6MCPA-CoA is consistent with a low affinity of VLCAD for this inhibitor as compared to MCAD or LCAD. Since C_6MCPA-CoA was generated in situ from C_6MCPA the concentration of the inhibitor increased linearly with time throughout the inhibition incubation. Thus the lag in the inhibition of VLCAD results from the need for a sufficiently high concentration of C_6MCPA-CoA to be generated before inhibition is observed. In contrast, inhibition of MCAD and LCAD was observed within 5 min, indicating a much lower concentration of C_6MCPA-CoA is required to inhibit these enzymes. When the amount of acyl-CoA synthetase (and hence rate of thioester synthesis) was reduced in inhibition incubations of C_6MCPA with MCAD and LCAD, a lag similar to that seen with VLCAD and consistent with the above argument was observed (data not shown).

3.2. *Effect on absorption spectra*

The effects of MCPA-CoA and C_6MCPA-CoA on the absorption spectra of the acyl-CoA dehydrogenases were examined in order to gain insight into the nature of the inhibition, i.e. whether inhibition was due to modification of the flavin prosthetic group and/or the enzyme protein itself. The enzyme-inhibitor combinations examined were SCAD/MCPA-CoA, MCAD/C_6MCPA-CoA, and LCAD/C_6MCPA-CoA, i.e. each enzyme was incubated with its 'optimum' inhibitor. Inhibition of SCAD by MCPA-CoA resulted in bleaching of the characteristic flavin absorption spectrum (Fig. 3A) as has been described for *Megasphaera elsdenii* butyryl-CoA dehydrogenase [28] and porcine kidney MCAD [7], for which it has been shown that inhibition is due to modification of the FAD prosthetic group [7]. Inhibition of MCAD by C_6MCPA-CoA also resulted in bleaching of the flavin (Fig. 3B) suggesting that in this case inhibition is also due to modification of the prosthetic group.

By contrast, inhibition of LCAD by C_6MCPA-CoA was accompanied by only slight bleaching of the flavin (Fig. 3C). In the representative experiment shown (Fig. 3C) after 60 min incubation activity had been inhibited 96% whereas the absorbance at 448 nm had decreased only by 32%. The flavin remained capable of reduction by $Na_2S_2O_4$. Lack of flavin bleaching is characteristic of inhibitors that act via modification of the enzyme protein [29–31], suggesting that in this case C_6MCPA-CoA causes inhibition mainly by modification of the LCAD protein itself.

The observation that C_6MCPA-CoA inhibits both MCAD and LCAD but apparently in different ways is noteworthy. In MCAD in which inhibition by C_6MCPA-CoA appears to be due to flavin modification the α-proton-abstracting base is Glu-376 [31,32]. In SCAD, for which it is well documented that inhibition by MCPA-CoA is due to modification of the flavin [7,13,33], this residue is conserved and is thought also to act as the proton-abstracting base [33]. However, in LCAD this residue is replaced by glycine and another residue, Glu-261 (which corresponds to Glu-255 in the MCAD sequence) acts as the α-proton-abstracting base [34]. Thus, differences of the detailed topology of the active sites of MCAD and LCAD may explain the apparently different mechanisms of inhibition of these two enzymes by the same inhibitor. In this connection it is noteworthy that recent work in Ghisla's laboratory [35] shows that the different position of the catalytic base in

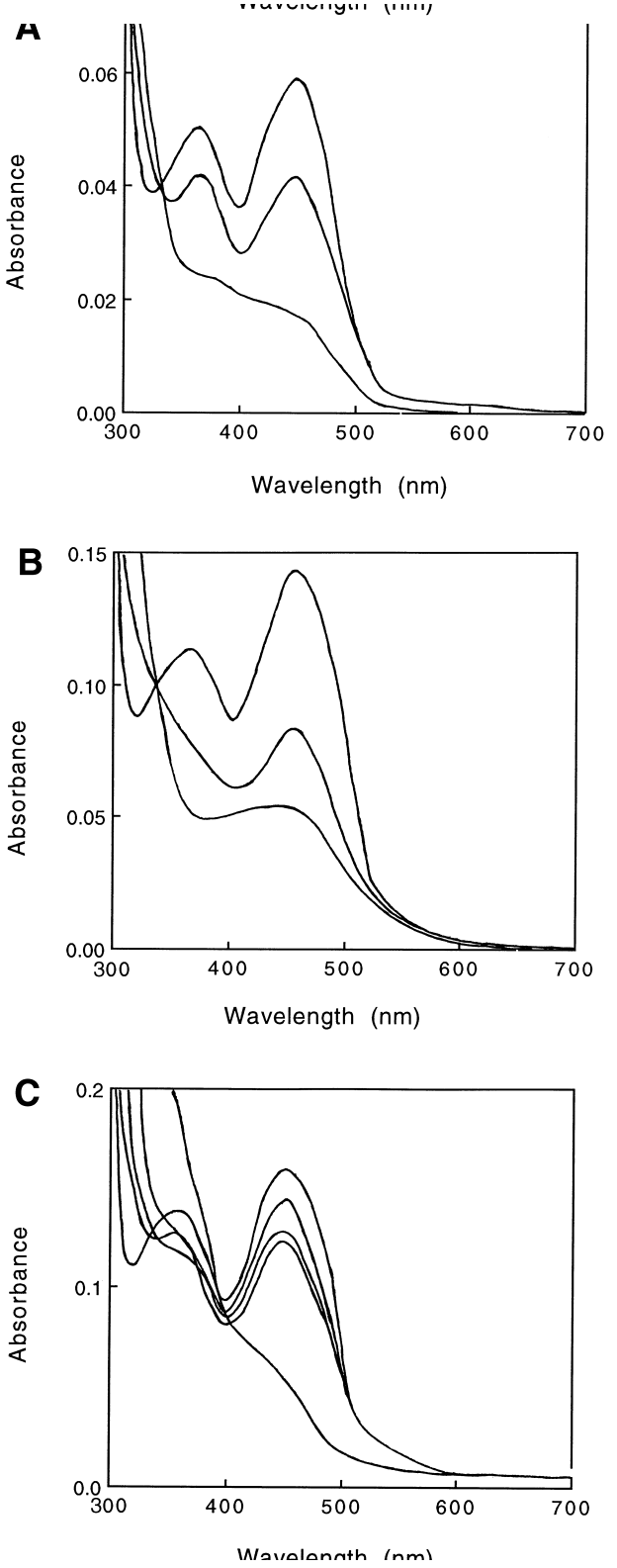

Fig. 3. Effect of methylenecyclopropyl compounds on the absorption spectra of purified acyl-CoA dehydrogenases. Spectra were obtained as described in Section 2. A: SCAD/MCPA, incubation contained 200 μM MCPA and 200 μg porcine acyl-CoA synthetase. B: MCAD/C_6MCPA, incubation contained 200 μM C_6MCPA and 100 μg porcine acyl-CoA synthetase. C: LCAD/C_6MCPA, incubation contained 200 μM C_6MCPA and 300 μg porcine acyl-CoA synthetase. In each case the spectrum is from a single experiment which is representative of three similar independent experiments.

MCAD and LCAD is important in determining the acyl-chain-length specificity of these enzymes, i.e. the position of the catalytic base profoundly affects the topology of the acyl-CoA binding site.

The present studies clearly illustrate the potential for designing enzyme-specific acyl-CoA dehydrogenase inhibitors. In view of the overlapping acyl-chain-length specificities of the acyl-CoA dehydrogenases it is not surprising that C_6MCPA-CoA did not specifically inhibit just one enzyme. The compound 3-phenylpropionyl-CoA is a specific substrate for MCAD. Thus it is possible that substitution of the methylenecyclopropyl ring with an a benzyl group may produce an MCAD-specific inhibitor.

Acknowledgements: We thank Professor T. Hashimoto for his generous gift of rat liver VLCAD and Professor Charles Stirling and his colleagues for their extensive efforts to optimize the synthesis of the MCPA compounds used in this work.

This work was funded by a Project Grant from the Wellcome Trust whose support is gratefully acknowledged.

The Krebs Institute was a designated Centre for Molecular Recognition Studies under the Molecular Recognition Initiative of SERC during the period of this work.

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