Studies on the Inactivation of Bovine Liver Enoyl-CoA Hydratase by (Methylenecyclopropyl)formyl-CoA: Elucidation of the Inactivation Mechanism and Identification of Cysteine-114 as the Entrapped Nucleophile

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Abstract: The inhibitory properties of (methylenecyclopropyl)formyl-CoA (MCPF-CoA), a metabolite derived from a natural amino acid, (methylenecyclopropyl)glycine, against bovine liver enoyl-CoA hydratase (ECH) were characterized. We have previously demonstrated that MCPF-CoA specifically targets ECHs, which catalyze the reversible hydration of α,*β*-unsaturated enoyl-CoA substrates to the corresponding *β*-hydroxyacyl-CoA products. Here, we synthesized (*R*)- and (*S*)-diastereomers of MCPF-CoA to examine the stereoselectivity of this inactivation. Both compounds were shown to be competent inhibitors for bovine liver ECH with nearly identical second-order inactivation rate constants (k_{inact}/K_I) and partition ratios (k_{cat}/k_{inact}), indicating that the inactivation is nonstereospecific with respect to ring cleavage. The inhibitor, upon incubation with bovine liver ECH, labels a tryptic peptide, ALGGGXEL, near the active site of the protein, where X is the amino acid that is covalently modified. Cloning and sequence analysis of bovine liver ECH gene revealed the identity of the amino acid residue entrapped by MCPF-CoA as Cys-114 (mature sequence numbering). On the basis of gHMQC (gradient heteronuclear multiple quantum coherence) analysis with [3-^{13}C]-labeled MCPF-CoA, the ring cleavage is most likely induced by the nucleophilic attack at the terminal carbon of the exomethylene group (C_2'). We propose a plausible inactivation mechanism that involves relief of ring strain and is consistent with examples found in the literature. In addition, these studies provide important clues for future design of more efficient and selective inhibitors to control and/or regulate fatty acid metabolism.

Enoyl-CoA hydratase (ECH) catalyzes the reversible hydration of α,*β*-unsaturated enoyl-CoA substrate **1** to the corresponding *β*-hydroxyacyl-CoA product, **2**. This physiologically important reaction is the second step in the *β*-oxidation pathway of fatty acid metabolism and is also required for the catabolism of branched-chain amino acids.[1] ECHs have been isolated from many sources and have been shown to differ in their preference toward substrates of varied acyl chain lengths, as well as with regard to the presence or absence of substituents at α- or *β*-C.[2−4] For example, three types of ECH have been identified in higher animals. The short chain ECH and a trifunctional protein, which acts not only as an ECH but also as an (*L*)-3-hydroxyacyl-CoA dehydrogenase and a 3-ketoacyl-CoA thiolase, are both found in mitochondria.[5,6] A bifunctional protein which possesses ECH and 3-hydroxyacyl-CoA dehydrogenase activities is present in peroxisomes.[7] Among them, the mitochondrial short chain ECH, also known as crotonase (EC 4.2.1.17), is the best studied.

Mitochondrial short chain ECH has been purified from bovine liver,[4] rat liver,[8] pig heart,[9] pig kidney,[10] and a few other sources. It is an extremely efficient catalyst that processes its optimum substrate, crotonyl-CoA (C_4), at a rate ($k_{cat}/K_m = 5 \times 10^7$ M^{-1} s^{-1})[2] approaching the diffusion-controlled limit. The rate of the bovine liver ECH-catalyzed reaction decreases linearly with the chain length of the enoyl-CoA substrate up to C_{16}.[2] The overall stereochemistry of ECH-catalyzed bond formation/cleavage at the α and *β* positions of the enoyl-CoA substrate has been established to be syn.[11] The reaction occurs at the si face of the conjugated thioester substrate leading to the formation of an (*S*)-3-hydroxy-acyl-CoA as the hydration product in almost all ECHs examined. However, a (*R*)-specific ECH involved in the biosynthesis of polyhydroxy alkanoates (PHA) has recently been cloned from *Aeromonas caviae*.[12]

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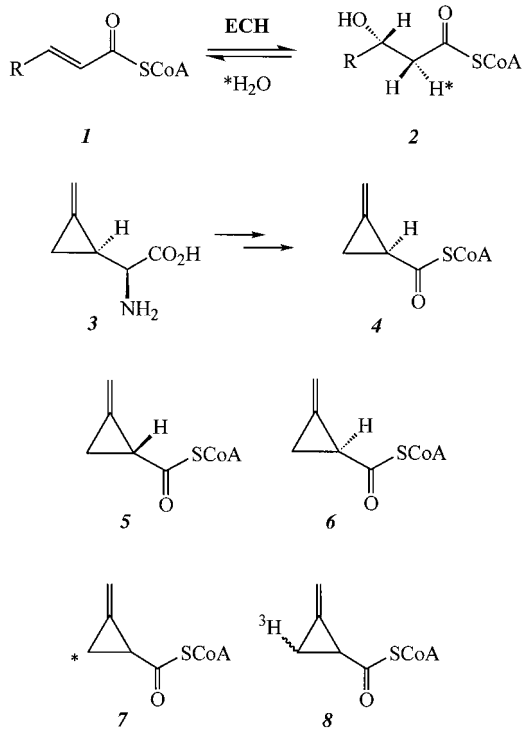

Interestingly, studies of the kinetic isotope effects of ECH-catalyzed reactions have indicated that a single transition state leads to the formation of both C_α−H and C_β−OH bonds, suggesting that the overall reaction occurs in a concerted fashion.[13,14] Extensive experiments have also established that, prior to chemical transformation, the substrate and/or its analogues undergo a reorganization of their conjugated π-electrons in the active site of the enzyme.[15−18] This active site induced polarization of the π-bond effectively enhances the electrophilicity at C_β of the enoyl-CoA substrate and thus facilitates the addition of a water molecule across the α,β-unsaturated thioester. More recently, rat liver ECH has been shown to add a water molecule across crotonyl-oxyCoA, albeit with reduced catalytic efficiency.[19]

The X-ray structures of rat liver ECH complexed with the tight-binding inhibitors acetoacetyl-CoA and octanoyl-CoA have been solved to 2.5 and 2.4 Å resolution, respectively.[20,21] From the mutagenesis studies and the crystal structure of this enzyme,[15,22] Glu164 has been firmly assigned as the general acid that donates a proton to C_α of the substrate (the key residues of rat ECH, such as Gly141, Cys143, Glu144, and Glu164, are numbered according to the encoded sequence including the N-terminal signal sequence, whereas the amino acid residue numbering for bovine liver ECH refers to the mature sequence of the protein, minus the signal sequence). The residue Glu144 was inferred by the structural analysis to function as the catalytic base responsible for the activation of the conserved water molecule in the active site. The role of Glu144 has since been confirmed by mutagenesis studies.[23] In addition, the crystal structure also reveals the presence of a strong hydrogen bond (d = 2.7 Å) between the C═O of the substrate and the N−H backbone of Gly141, corroborating the polarization of the conjugated π-system of substrate in the active site as deduced by spectroscopic studies.[15−18] Because both Glu144 and Glu164 are located on the same side of the *trans*-enoyl double bond, such disposition is in agreement with the syn hydration/dehydration stereochemistry.[11,24]

While the physiological significance of ECHs is apparent and its catalytic mechanism is well-documented, very few inhibitors are known for this class of enzymes.[2,25] Examples include several cinnamoyl-CoA derivatives and acetoacetyl-CoA,[2] all of which are competitive inhibitors against ECHs with K_i values in the micromolar range. However, these compounds lack specificity and are toxic against most coenzyme A binding enzymes involved in fatty-acid metabolism.[26−28] Recently, 5,6-dichloro-7,7,7-trifluoro-4-thia-5-heptenoyl-CoA has been reported to be an active site thiol-directed inactivator for rat liver ECH.[29] Interestingly, (methylenecyclopropyl)formyl-CoA (**4**, MCPF-CoA), a metabolite of (methylenecyclopropyl)glycine (**3**) isolated from kernels of litchi fruits, has been shown to have hypoglycemic activity due to its ability to inhibit ECH and thus interrupt the β-oxidation pathway of fatty acid metabolism.[30−33] Our recent study confirmed that MCPF-CoA (**4**) is an irreversible inhibitor for bovine liver ECH.[34,35] Although the inhibition was established to be time-dependent, active site-directed, and irreversible, the details of the inactivation mechanism remained unclear. To determine whether the two stereoisomers of **4** would elicit different inhibition behaviors, the (*R*)- and (*S*)-epimers of MCPF-CoA (**5** and **6**, respectively) were prepared and tested for their competence as inhibitors against bovine liver ECH. The [3-^{13}C]-labeled analogue of MCPF-CoA (**7**) was also synthesized as a probe to help elucidate the chemical nature of the enzyme−inactivator adduct. The radiolabeled analogue [3-^{3}H]MCPF-CoA (**8**) was used to label the protein which, after tryptic digestion and sequence analysis, allowed the location of the active site nucleophile being trapped during inactivation to be determined. Because the protein sequence of bovine liver ECH is unknown, the corresponding gene was cloned and

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sequenced to facilitate the identification of the modified residue. Reported in this paper are the results of these experiments and our comments on the mechanistic insights deduced from these observations.

Experimental Section

General. The ^{1}H and ^{13}C NMR chemical shifts are reported on the δ scale relative to an internal standard or appropriate solvent peak with coupling constants given in hertz. Flash chromatography was performed in columns of various diameters with J. T. Baker (230−400 mesh) silica gel by elution with the solvents reported. Analytical thin-layer chromatography (TLC) was carried out on Merck silica gel 60 G-254 plates (25 mm) and developed with the solvents mentioned. TLC spots were visualized either with UV light or by heating plates previously stained with solutions of $KMnO_4$ (1%), vanillin/ethanol/H_2SO_4 (1:98:1), or phosphomolybdic acid (7% EtOH solution). Drying agent used in the routine workup was anhydrous magnesium sulfate. Solvents, unless otherwise specified, were analytical reagent grade and distilled once prior to use. For anhydrous reactions, the solvents were pretreated prior to distillation as follows: tetrahydrofuran (THF) was dried over sodium and benzophenone; methylene chloride, dimethyl sulfoxide (DMSO), and dimethyl formamide (DMF) were dried over calcium hydride; pyridine and triethylamine were dried over KOH.

The concentration of protein was determined by Bradford's method[36] using bovine serum albumin as the standard. However, for routine purposes, the enzyme concentration was estimated using the extinction coefficient of 16 000 $M^{-1}\cdot cm^{-1}$ at 280 nm.[4] The level of expression, the solubility of the gene product, and the purity of the purified protein were assessed by sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) as described by Laemmli.[37] The amino acid sequencing was carried out by the Microchemical Facility at the Institute of Human Genetics of the University of Minnesota. The DNA sequencing was performed by the Bioanalytical Center of the University of Minnesota, St. Paul. DNA sequence analyses were performed with Wisconsin Sequence Analysis Package of Genetics Computer Group (Madison, WI) and GeneWorks version 2.5 of IntelliGenetics, Inc. (Mountain View, CA). Methods and protocols for recombinant DNA manipulations were according to the manufacturers' manuals or from general references.[38]

Materials. Premade bovine liver cDNA library, Uni-ZAP XR Library, phagemid vector pBluescript SK(±), T3 and T7 sequencing primers, f1 helper phage (VCSM13 (f1), ExAssist (M13)), *pico*Blue Immunoscreening kit, *E. coli* phage lysate, immunoaffinity-purified goat anti-rabbit antibodies, nitroblue tertrazolium (NBT), 5-bromo-4-chloro-3-indoyl phosphate (BCIP), Tween 20, and cloned *pfu* DNA polymerase were products of Stratagene (La Jolla, CA). Nitrocellulose membranes, *Hind*III digested bacteriophage lambda DNA marker, and agarose were purchased from GIBCO BRL (Gaithersburg, MD). Geneclean II was from BIO101, Inc. (La Jolla, CA) and Bradford reagent was from BioRad (Richmond, CA). Ecoscint A of National Diagnostics (Manville, NJ) was used as scintillation cocktail. Nucleotide triphosphates (dATP, dCTP, dGTP, and dTTP (10 mM solution)), Magic Mini Preps, Wizard Miniprep and Wizard Megaprep DNA purification systems, and buffers used in PCR (polymerase chain reaction) were obtained from Promega (Madison, WI). The expression vectors pTrc99A, *E. coli* JM105, DEAE-Sepharose (DEAE = (diethylamino)ethyl), and phenyl-Sepharose CL-6 were purchased from Pharmacia (Uppsala, Sweden). Custom-designed oligonucleotide primers were made by Integrated DNA Technologies (Coralville, IA). Rabbit antiserum against the bovine liver ECH was custom prepared by HTI Biochemicals (Ramona, CA). The molecular weight standards, biochemicals, and chemicals were purchased from Sigma or Aldrich (Milwaukee, WI) and were of the highest purity available.

Isolation of Bovine Liver ECH cDNA Clones. A bovine liver Lambda cDNA library in a Uni-ZAP XR vector was purchased from Stratagene (La Jolla, CA). This Lambda cDNA library was screened with rabbit antibodies generated against bovine liver ECH using the *pico*Blue Immunoscreening kit (Stratagene). Two positive clones were detected following plaque screening ($\sim 1 \times 10^6$ clones). Subsequent excision and recircularization of the pBluescript SK(−) phagemid containing the cloned DNA insert followed protocols recommended by the manufacturer (Stratagene). The resulting phagemids, designated as pBluescript SK(±)-SD_{a1} and pBluescript SK(±)-SD_{b1}, were characterized by gene walking, and their cDNA was sequenced by an automated DNA sequencer.

PCR Amplification and Cloning of Bovine Liver ECH Gene. The gene was amplified by standard recombinant DNA techniques and was subcloned into *Eco*RI and *Bam*HI sites of the expression vector pTrc99A. The start primer, 5′-GCG**GAATTC**CATATGAGCGCAG-CTTTCGAATAC-3′, contained a *Eco*RI restriction site (in bold), a TA-rich region, a start codon, and the codons for the first six amino acid residues of the desired enzyme. The halt primer, 5′-GCG**GGATCC**T-CACTGGTCTTTGAAGTT-3′, introduced a *Hin*dIII restriction site (in bold) immediately downstream from the stop codon of the ECH gene. These primers were used to amplify the desired gene from pBluescript SK(±)-SD_{a1} by polymerase chain reaction (PCR). The PCR reactions were run in a total volume of 100 μL and contained 10 μL DMSO, 1 μL glycerol, 10 μL 10× cloned *pfu* buffer, 4 μL dNTP mix (10 mM each of dATP, dCTP, dGTP, and dTTP), 0.5 μL each of start and halt primer (100 pmol/μL), 1 μL pBluescript SK(±)-SD_{a1} DNA (20 ng), and 1.0 μL cloned *pfu* DNA polymerase (2.5 units). After mixing, the reaction mixture was overlaid with a drop of mineral oil and was subjected to the following thermal cycles: five cycles of one type (94 °C, 5 min; 50 °C, 5 min; 72 °C, 3 min) and thirty cycles of a second type (94 °C, 1 min; 55 °C, 1 min; 72 °C, 1.5 min), followed by incubation at 72 °C for an additional 10 min. The PCR product was purified using GeneClean II, digested with the appropriate restriction enzymes, and ligated into the *Eco*RI/*Hin*dIII sites of the transcription vector pTrc99A. This recombinant plasmid was isolated and was used to transform *E. coli* JM101. The plasmid DNA from positive clones was used to transform *E. coli* JM105.

Purification of Recombinant Bovine Liver ECH. An overnight culture of *E. coli* JM105/pSD_6 (30 mL), grown in Luria−Bertani (LB) medium supplemented with ampicillin (100 μg/mL) at 37 °C, was evenly divided and added to three 1 L portions of the same LB/ampicillin medium in Pyrex Erlenmeyer flasks. The flasks were shaken at 250 rpm at 25 °C until the OD_{600} reached 0.66. Thereafter, isopropyl β-D-thiogalactoside (IPTG) was added to the culture to a final concentration of 1.0 mM, and the incubation was continued for an additional 6 h at 25 °C. The cells were harvested by centrifugation at 4000*g* for 15 min at 4 °C. Subsequent operation to purify the enzyme was carried out at 4 °C and is described below.

Step 1: Crude Extract. The cell pellet was resuspended in 100 mL of 50 mM potassium phosphate buffer containing 3 mM EDTA and 1 mM DTT (dithiothreitol) (pH 7.4). This suspension was sonicated with five 1 min bursts with a 1 min cooling period between each burst to disrupt the cells. Cell debris was removed by centrifugation (17 000*g*, 20 min). The supernatant was collected and designated the crude extract.

Step 2: Ammonium Sulfate Fractionation. The crude extract (75 mL) from step 1 was treated with solid ammonium sulfate (18.22 g), added portionwise over 1 h up to 40% saturation. This suspension was centrifuged at 17 000*g* for 25 min to remove the precipitates, and to the supernatant was added more solid ammonium sulfate (24.5 g) to a final concentration of 85% saturation. This cloudy solution was stirred for an additional hour, and the precipitated proteins were collected by centrifugation (17 000*g*, 25 min). The protein pellet was resuspended in a minimal amount of 50 mM potassium phosphate buffer containing 3 mM EDTA, pH 7.4, and dialyzed against the same buffer overnight with three changes of buffer.

Step 3: DEAE-Sepharose Chromatography. The dialysate from step 2 was clarified by centrifugation (17 000*g*, 20 min) and applied to a DEAE-Sepharose column (1 cm × 17 cm) preequilibrated with 50 mM potassium phosphate buffer containing 3 mM EDTA, pH 7.4. The column was eluted with the same buffer (500 mL total volume) with a flow rate of 1 mL/min. The active fractions that were not retained were pooled and concentrated by ultrafiltration on an Amicon concen-

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trator using a YM-10 membrane. The concentrated solution was then dialyzed against 2 L of 50 mM potassium phosphate buffer containing 0.5 M NaCl and 3 mM EDTA, pH 7.4, overnight, with four changes of buffer.

Step 4: Phenyl-Sepharose Chromatography. The dialyzed solution from step 3 was applied to a phenyl-Sepharose column (1 cm × 17 cm) that had been equilibrated with 50 mM potassium phosphate buffer containing 0.5 M NaCl and 3 mM EDTA, pH 7.4. After loading, the column was washed with 20 mL of the same buffer, followed by elution with a linear gradient of NaCl from 0.5 to 0 M in 50 mM potassium phosphate buffer containing 3 mM EDTA, pH 7.4 (1 L total volume). The flow rate was 1 mL/min, and 10 mL fractions were collected. The active fractions were combined, concentrated by ultrafiltration (YM-10), and stored at −80 °C for later analysis. The level of protein expression and the purity was assessed by SDS−PAGE analysis.

Enzyme Assays. The activity of ECH was assayed as described by Steinman and Hill,[4] except for the omission of ovalbumin from the reaction mixture and the use of 10 mM potassium phosphate buffer (pH 7.0). Crotonyl-CoA (80 μM) was used as the substrate, and changes in absorbance at 263 nm (ϵ 6700 $M^{-1}\cdot cm^{-1}$), which corresponds to the α,β-unsaturated moiety of the substrate, were monitored. A unit of activity is defined as the amount of ECH required to catalyze the hydration of 1 μmol of substrate per minute.

Kinetic Analysis of Inactivation. In a typical inactivation experiment, an appropriate amount of MCPF-CoA was added to the bovine liver ECH (9 μM) in 250 μL of 50 mM potassium phosphate buffer, pH 7.6, at 25 °C. At various time intervals, aliquots (10 μL) were removed and diluted into 1 mL of the same buffer containing crotonyl-CoA (10 μM), and the residual enzyme activity was determined as described above.

Determination of Partition Ratio. A series of sample solutions each containing 9 μM bovine liver ECH and an appropriate amount of (*R*)- or (*S*)-MCPF-CoA were prepared in 400 μL of 50 mM potassium phosphate buffer, pH 7.6, to give $[I]_o/[E]_o$ ratios ranging from 1.0 to 50. These samples were incubated at 25 °C for 2 h. Subsequently, an aliquot of 10 μL was measured from each sample, and the residual enzyme activity was determined as described above. The control experiments were carried out in the same manner without inactivator. The partition ratio was deduced by plotting the residual enzyme activity versus $[I]_o/[E]_o$.

Isolation and Sequence Analysis of Labeled Peptides. Bovine liver ECH (1.5 mg, 56 nmol) was incubated with 20 equiv of [^{3}H]MCPF-CoA (**8**; 0.56 mCi/mmol) in 50 mM potassium phosphate buffer (pH 7.6) at room temperature overnight. The inactivated enzyme was concentrated by Microcon (Amicon) and repeatedly washed with the same potassium phosphate buffer to remove the excess MCPF-CoA until the radioactivity of the filtrates matched the background readings. The radioactivity of the labeled protein was measured and its concentration determined. The labeled protein was then digested using trypsin (60 μg, porcine pancreas type IX) under nitrogen overnight in the dark. The tryptic fragments, after treatment with 1 mM DTT and filtered through Microcon, were separated by HPLC on a Vydac C_{18} (4.6 mm × 25 cm) column using a 0−60% (B/A) linear gradient formed from 0.1% (v/v) trifluoroacetic acid (TFA) in water (solvent A) and 0.1% TFA in acetonitrile (solvent B) over a period of 2 h. The elution was monitored at 220 nm, and the flow rate was 1 mL/min. The two radioactive peaks (peaks A and B) were separately pooled and further purified under the same HPLC conditions. The two radiolabeled tryptic fragments were sequenced by automated Edman degradation. The effluents obtained after each cycle were also counted for radioactivity.

***N*-[(*R*)-1′-Phenyl-2′-hydroxyethyl]-(1*R*)-methylenecyclopropylcarboxamide (12) and *N*-[(*R*)-1′-Phenyl-2′-hydroxyethyl]-(1*S*)-methylenecyclopropylcarboxamide (13).** The synthesis of (methylenecyclopropyl)formic acid (**11**) has been described previously.[35] To a solution of acid **11** (1.84 g, 18.7 mmol) and triethylamine (2.6 mL, 18.7 mmol) in THF (70 mL) was added isobutyl chloroformate (2.43 mL, 18.7 mmol) at 0 °C. The solution was stirred at room temperature for 1 h, which was followed by the addition of (*R*)-phenylglycinol (2.6 g, 18.7 mmol) in 10 mL THF.[39,40] The resulting mixture was stirred for an additional 3 h at room temperature and then filtered. The filtrate was concentrated under reduced pressure to obtain the crude amides **12** and **13**. The crude products were purified repeatedly by flash chromatography (30% ethyl acetate in hexanes) to separate the diastereomers. The combined yield of purified compounds **12** (1.2 g) and **13** (1.1 g) was 48%. The enantiomeric purity of each preparation was determined by HPLC using a Spheris ORB silica gel (1 cm × 25 cm) that was eluted with 10% 2-propanol in methylene chloride (flow rate 4 mL/min, monitoring wavelength 254 nm). Under these conditions, the retention times of isomers **12** and **13** were found to be 5.9 and 6.8 min, respectively. The enantiomeric purities of both isomers were found to be greater than 99%. **12**: ^{1}H NMR ($CDCl_3$) δ 7.37−7.28 (5H, m, Ar H), 6.43 (1H, br s, NH), 5.54 (2H, br s, =CH_2), 5.01 (1H, dt, J = 12.1 and 5.0, H-1′), 3.82 (2H, t, J = 5.0, H-2′), 2.92 (1H, br s, OH), 2.16 (1H, m, H-1), 1.73 and 1.58 (1H each, m, H-3); ^{13}C NMR ($CDCl_3$) δ 171.7 (C=O), 139.0 (Ar C), 130.6 (C-2), 128.9, 127.9, 126.7 (Ar), 105.3 (=CH_2), 66.5 (C-2′), 56.1 (C-1′), 20.0 (C-1), 11.2 (C-3). **13**: ^{1}H NMR ($CDCl_3$) δ 7.38−7.27 (5H, m, Ar H), 6.33 (1H, br s, NH), 5.55 (2H, br s, =CH_2), 5.04 (1H, dd, J = 11.5 and 5.0, H-1′), 3.85 (2H, m, H-2′), 2.77 (1H, br s, OH), 2.17 (1H, m, H-1), 1.72 and 1.62 (1H each, m, H-3). ^{13}C NMR ($CDCl_3$) δ 171.7 (C=O), 139.1 (Ar C), 130.7 (C-2), 128.9, 127.9, 126.7 (Ar C), 105.2 (=CH_2), 66.4 (C-2′), 56.0 (C-1′), 20.0 (C-1), 11.2 (C-3). High resolution FAB-MS: calcd for $C_{13}H_{16}NO_2$ $(M + 1)^+$, 218.1181; found, 218.1165.

(*R*)-(Methylenecyclopropyl)formic Acid (14) and (*S*)-(Methylenecyclopropyl)formic Acid (15). An equal volume of 4 N sulfuric acid (25 mL) was added to a solution of **12** (1.2 g, 5.3 mmol) in THF (25 mL). After refluxing for 4 h, the solution was cooled to room temperature and extracted with ether. The combined organic extracts were dried over anhydrous magnesium sulfate and filtered. The filtrate was concentrated in vacuo, the residue was purified by flash chromatography (10% ethyl acetate in hexanes), and **14** was obtained in a final yield of 89%. ^{1}H NMR ($CDCl_3$) δ 5.52 (2H, d, J = 1.8, =CH_2), 2.22 (1H, m, H-1), 1.85 and 1.68 (1H each, m, H-3); ^{13}C NMR ($CDCl_3$) δ 179.0 (C=O), 129.9 (C-2), 105.0 (=CH_2), 17.9 (C-1), 12.2 (C-3). High resolution CI-MS (CH_4): calcd for $C_5H_7O_2$ $(M + 1)^+$, 99.0446; found, 99.0445. Compound **15** was prepared by a similar procedure in 87% yield. The NMR spectra of **15** are identical to those obtained for **14**.

(*R*)-(Methylenecyclopropyl)formyl-CoA (5) and (*S*)-(Methylenecyclopropyl)formyl-CoA (6). To a solution of **14** in methylene chloride was added an equimolar amount of triethylamine under an argon atmosphere. After the mixture was stirred for 10 min, an equimolar amount of isobutyl chloroformate was added dropwise at 0 °C. The reaction was agitated vigorously, and fuming was noted during the process. The reaction was stirred at room temperature for 1 h. Thereafter, the solvent was removed under reduced pressure, and the residue containing the mixed anhydride was redissolved in THF (5 mL) to give a cloudy solution. Meanwhile, a solution of coenzyme A was prepared by dissolving its sodium salt (50 mg, 50 μmol) in distilled water (5 mL) that had been deoxygenated by repeated freeze−thaw cycles under vacuum. The pH of this solution was brought up to 8.0 by the addition of 1 N NaOH. To this solution was slowly added the mixed anhydride solution via a cannula using positive argon pressure. The pH of the resulting mixture was adjusted to 8.0, and the reaction mixture was stirred for 10 min. The pH of the reaction was then changed to 5.0−5.5 by the addition of dilute perchloric acid. The organic solvent (THF) was removed under reduced pressure, and the remaining aqueous solution was extracted twice with ether to remove any residual organic soluble materials. The aqueous solution was then lyophilized. The crude product was purified by HPLC using a C_{18} column (10 mm × 250 mm, 5 μm) and eluted with 30% methanol in 50 mM potassium phosphate buffer (pH 5.3, flow rate 3 mL/min, monitoring wavelength 260 nm). The fractions containing the product were pooled, concentrated under reduced pressure to remove methanol, and lyophilized. The resulting residue was desalted on the same column, which was washed with water (3 mL/min) for 20 min followed by methanol. The eluant containing the product was evaporated under reduced pressure and then lyophilized, providing (*R*)-MCPF-CoA (**5**) as a white powder. The

(39) Helmchen, G.; Nill, G.; Flockerzi, D.; Schuhle, W.; Youssef, M. S. K. *Angew. Chem., Int. Ed. Engl.* **1979**, *18*, 63−64.

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overall yield was 87%. Similarly, compound **6** was prepared in a yield of 84%. ^{1}H NMR (^2H_2O) of **5** (the chemical shifts of the MCPF signals are shown italicized): δ 8.58, 8.32 (1H each, s, adenine H), 6.20 (1H, d, J = 6.0, ribose anomeric H), *5.62* and *5.56* (1H each, d, J = 2.2, =CH_2), 4.93−4.78 (1H, buried under ^{2}HOH peak), 4.71 and 4.62 (1H each, s, ribose H), 4.27 (2H, s, ribose CH_2O), 4.04 (2H, s), 3.83 (1H, m), 3.60 (1H, m), 3.47 (2H, t, J = 10.8), 3.38 (2H, t, J = 10.8), 3.02 (2H, m), *2.79* (1H, t, J = 6.5, H-1), 2.44 (2H, t, J = 10.0), *1.89* (2H, m, H-3), 0.91, 0.78 (3H each, s, Me); the measured sample was repeatedly dissolved in ^2H_2O and lyophilized prior to ^{1}H NMR analysis. High resolution FAB-MS calcd for $C_{26}H_{40}N_7O_{17}P_3S$ $(M + 1)^+$, 848.1492; found, 848.1431. The spectra of **5** and **6** are superimposable.

***(Z)*-3-Iodo-2-butenol (17).** To a solution of 2-butyn-1-ol (**16**, 1.0 g, 14.3 mmol) in 15 mL of anhydrous ether was added dropwise a solution of Red-Al (7.1 mL, 22.8 mmol) at −78 °C under argon. The reaction was allowed to warm to room temperature, and stirring was continued overnight. Subsequently, the mixture was cooled to −78 °C and quenched with I_2 (11.2 g, 44.3 mmol). The resulting solution was gradually warmed to room temperature with continuous stirring within 30 min. It was concentrated under reduced pressure and chromatographed directly on a silica gel column (1:1 ether/pentane) to afford **17**, as a colorless oil, in 73% yield (3.3 g). ^{1}H NMR ($CDCl_3$) δ 5.77 (1H, tq, J = 6.0 and 1.2, H-2), 4.15 (2H, dq, J = 6.0 and 1.2, H-1), 2.54 (3H, q, J = 1.2, Me).

[3-$^{13}C_1$](2-Iodo-2-methylcyclopropyl)methanol (18). To a solution of **17** (0.25 g, 1.27 mmol) and [^{13}C]CH_2I_2 (0.34 g, 1.27 mmol) in 10 mL of methylene chloride was added dropwise a 2 M solution of trimethylaluminum in hexanes (1.34 mL, 2.68 mmol) over 15 min at 0 °C under nitrogen. The resultant mixture was stirred at room temperature for 24 h. The reaction was then chilled to 0 °C and quenched with 5 mL of 1N NaOH. After being stirred for an additional hour, the organic layer was separated, and the aqueous layer was washed with 10 mL of methylene chloride twice. The organic extracts were combined, washed with water, dried, filtered, and concentrated under reduced pressure. The crude product was purified by flash column chromatography (1:4 ether/pentane) to give **18** in 74% yield (0.2 g). Because this product is a mixture of a few diastereomers, multiple sets of signals made the spectrum too complicated to be analyzed. ^{1}H NMR ($CDCl_3$) δ 3.97 (dd, J = 12.2, 4.7), 3.73 (t, J = 4.7), 3.48 (m, CH_2OH, diastereomeric mixture), 1.96 (s), 1.94 (s, Me, diastereomeric mixture), 1.67 (1H, br s, CH_2OH), 1.02 (1H, dt, J_{Ha-C} = 59.3, J_{Ha-C-H} = $J_{Ha-CC-H}$ = 6.9, CH_2), 0.95 (1H, dm, J_{Hb-C} = 161.5, CH_2), 0.55 (1H, m, H-1); ^{13}C NMR ($CDCl_3$) δ 22.4 (C-3).

[3-$^{13}C_1$](Methylenecyclopropyl)methanol (19). To a solution of potassium *t*-butoxide (0.2 g, 1.69 mmol) in 5 mL of anhydrous dimethyl sulfoxide (5 mL) was added neat **18** over a period of 30 min at 70 °C. The resultant mixture was vigorously stirred for 7 h, and subsequently, it was cooled to room temperature and poured over ice. The crude product was extracted in ethyl ether (3 × 25 mL), and the combined organic layers were washed with water, dried, filtered, and carefully concentrated in vacuo. The crude product was purified by flash column chromatography (3:1 pentane/ethyl ether) to give the desired product, **19**, in 72% yield (56 mg). ^{1}H NMR ($CDCl_3$) δ 5.48 (1H, ddd, J = 9.6, 2.2, and 0.9, CH_2=C), 5.44 (1H, q, J = 2.1, CH_2=C), 3.65, 3.60 (1H each, t, J = 5.4, 5.7, CH_2OH), 3.51 (2H, m, CH_2OH, diastereomeric mixture), 1.81 (1H, m, CHCH_2OH), 1.41, 1.21 (1H each, dm, J_{H-C} = 102, CH_2); ^{13}C NMR ($CDCl_3$) δ 7.2 (C-3). High resolution FAB-MS calcd for $^{13}C_1C_4H_9O$ $(M + 1)^+$, 86.0653; found, 86.0668.

[3-$^{13}C_1$](Methylenecyclopropyl)formic Acid (20). Compound **19** (56 mg, 0.66 mmol) was dissolved in acetone (10 mL) and treated with Jones reagent that was prepared by mixing chromium oxide (26.72 g) with concentrated sulfuric acid (23 mL), followed by water dilution to a final volume of 100 mL. Addition of Jones reagent was continued until the red color persisted for at least 1 min. The resulting mixture was stirred at room temperature for 30 min to ensure the completion of oxidation. The excess oxidizing reagent was quenched with 2-propanol. The reaction solution was then diluted with water, followed by repeated extraction with ether. The combined organic extracts were dried, filtered, and concentrated in vacuo. The crude product was purified by flash chromatography (10% ethyl acetate/hexanes) to give desired acid **20** in 67% yield (44 mg). ^{1}H NMR ($CDCl_3$) δ 5.56 (2H, m, CH_2=C), 2.26 (1H, m, 1-H), 1.88 (1H, ddddd, J = 166.2, 9, 4.5, 2.4, and 2.4, H-3), 1.71 (1H, dtt, J = 166.2, 9, and 2.4, H-3); ^{13}C NMR ($CDCl_3$) δ 12.27 (C-3).

[3-$^{13}C_1$](Methylenecyclopropyl)formyl-CoA (7). Compound **7** was prepared from **20** (6.0 mg, 0.06 mmol) in 62% yield (32.0 mg) by coupling with coenzyme A (50 mg, 65.1 mmol) according to the same procedure used to synthesize **5** as described above. ^{1}H NMR (D_2O) δ 8.38, 8.09 (1H each, s, adenine H), 6.00 (1H, d, J = 6.5, ribose anomeric H), *5.37* (2H, m, CH_2=C), 4.44 (1H, s, HOCHCMe_2), 4.09 (2H, br s, C(Me)$_2$CH_2O), 3.87 (1H, s, HOCHCMe_2), 3.67 (1H, dd, J = 10 and 5, ribose), 3.40 (1H, dd, J = 10 and 5, ribose), 3.28 (2H, t, J = 6.5), 3.16 (2H, m), 2.85 (3H, m, overlap CHCO_2H, CoA), *2.44* (1H, dm, J_{H-C} = 167.5, H-3), 2.25 (2H, m), *1.71* (1H, dm, J_{H-C} = 167.5, H-3), 0.73, 0.60 (3H each, s, Me); ^{13}C NMR (D_2O) δ 14.14 ($^{13}CH_2$). FAB-MS calcd for $^{13}C_1C_{25}H_{40}N_7O_{16}P_3S$ $(M + H)^+$, 849.2; found, 849.2.

Two-Dimensional ^{1}H{^{13}C} gHMQC Analysis. Bovine liver ECH (11.2 mg, 0.4 μmol) was incubated with 4 molar equiv of ^{13}C-labeled MCPF-CoA (**7**) in 50 mM potassium phosphate buffer (pH 7.6) at room temperature. The reaction was incubated for 6 h to ensure complete inactivation and then ultrafiltered through an Amicon YM-10 filter to remove unreacted MCPF-CoA and any possible turnover products. The resulting mixture was concentrated to 500 μL, to which 50 μL of ^2H_2O was added, transferred to an NMR tube, and subjected to gHMQC (gradient heteronuclear multiple quantum coherence) analysis on a Varian U-500 spectrometer (number of transients = 256; temperature = 23 °C; time ∼ 12 h).

Results

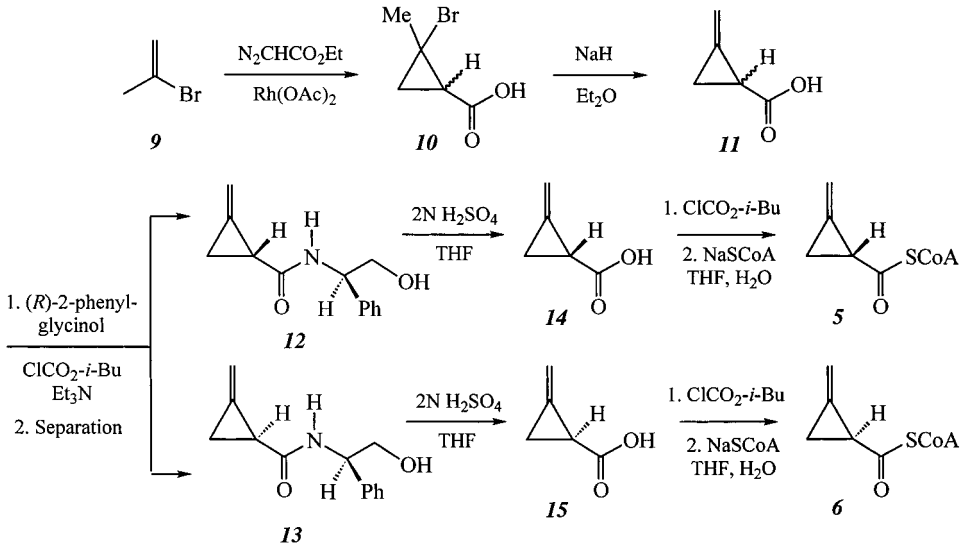
Preparation of (*R*)- and (*S*)- MCPF-CoA and Inactivation of Bovine Liver ECH. As mentioned above, MCPF-CoA (**4**) is a specific inactivator against mitochondrial short chain ECHs, such as those from bovine liver and pig kidney.[34,35] Recent experiments have shown that the toxicity of MCPF-CoA toward these enzymes results from covalent trapping of an enzyme active site nucleophile by this methylenecyclopropyl derivative. Furthermore, the inhibitory effect of MCPF-CoA is more pronounced against the bovine liver ECH as compared to the pig kidney enzyme.[35] Surprisingly, studies with rat liver ECH revealed that MCPF-CoA is a competitive inhibitor of this enzyme with a K_i of 30 μM and that the loss of activity is reversible in nature.[35] Evidently, despite the fact that a high degree of sequence homology (≥80%)[41,42] has been noted for all ECHs regardless of their origins,[8] subtle structural differences must exist between bovine liver and rat liver ECH as reflected by the disparity of the modes of inhibition caused by MCPF-CoA. This result not only highlighted the importance of effective binding for the action of inhibition but also prompted us to consider that the two stereoisomers of MCPF-CoA (**5** and **6**) may experience deviate chiral discrimination imposed by the bovine liver enzyme and may thus exhibit distinct inhibitory activities. Considering the significant role of ECHs in fatty acid metabolism and amino acid catabolism, selective inhibitors for different ECHs may emerge as a useful means to control and/or regulate these physiological processes for therapeutic intervention. With this hope in mind, we decided to examine the inhibitory effects of (*R*)- and (*S*)-MCPF-CoA on the activity of bovine liver ECH.

Preparation of pure diastereoisomers of MCPF-CoA (**5** and **6**) was accomplished according to a procedure developed earlier to synthesize (*R*)- and (*S*)-(methylenecyclopropyl)acetyl-CoA.[43] As shown in Scheme 1, the synthesis is initiated by a rhodium

(41) Minami-Ishii, N.; Taketani, S.; Osumi, T.; Hashimoto, T. *Eur. J. Biochem.* **1989**, *185*, 73−78.

(42) Kanazawa, M.; Ohtake, A.; Abe, H.; Yamamoto, S.; Satoh, Y.; Takayanagi, M.; Niimi, H.; Mori, M.; Hashimoto, T. *Enzyme Protein* **1993**, *47*, 9−13.

Scheme 1



acetate catalyzed cyclopropanation of 2-bromopropene (**9**) with ethyl diazoacetate. Derivatization of **11** with (*R*)-2-phenylglycinol led to a diastereomeric mixture (**12** and **13**), which could be readily separated by flash chromatography.[39,40] The resolved amide was converted to (methylenecyclopropyl)formic acid (**14** and **15**) by acid hydrolysis. Subsequent coupling to coenzyme A[43] afforded the desired (1*R*)- and (1*S*)-MCPF-CoA (**5** and **6**, respectively).

The effect of the MCPF-CoA isomers on the catalytic activity of ECH was analyzed by successive titration of bovine liver ECH with aliquots of each isomer. A plot of the observed residual activity versus the total equivalents of inhibitor added gave the same partition ratio of 10 for both (1*R*)- and (1*S*)-MCPF-CoA (Figure 1). The time-dependent loss of enzyme activity follows Michaelis−Menten saturation kinetics. As illustrated in Figure 2, a double reciprocal plot of the observed first-order rate (k_{obs}) versus inhibitor concentration led to the inactivation rate constant k_{inact} of 3.36×10^{-3} min^{-1} and the apparent dissociation constant K_I of 49.2 μM for the (1*R*)-isomer (**5**). The values of k_{inact} and K_I for the (1*S*)-isomer (**6**) were similarly determined to be 2.65×10^{-3} min^{-1} and 57.1 μM, respectively. The nearly identical kinetic parameters and partition ratios strongly suggest that (1*R*)- and (1*S*)-MCPF-CoA are equally competent inactivators against bovine liver ECH. Therefore, the inactivation of bovine liver ECH by MCPF-CoA is nonstereospecific.

Identification of Modified Residue in Bovine Liver ECH. Previous study using ^{3}H-labeled MCPF-CoA had fully substantiated the covalent nature of the inactivation of bovine liver ECH.[34] To further characterize the mechanism of this process, we proceeded to determine the identity of the amino acid residue entrapped by the methylenecyclopropyl moiety. As described in the Experimental Section, incubation of bovine liver ECH with [^{3}H]MCPF-CoA (**8**) yielded an inactive protein containing nearly 1 equiv of [^{3}H]MCPF-CoA per monomer, which is consistent with the labeling of one amino acid residue in the active site of the enzyme. Tryptic digestion of the labeled protein gave a mixture of peptides that was fractionated by reversed phase HPLC (Figure 3). In principle, only one tryptic fragment should be labeled; however, radioactivity was found in two separate fractions. The two radioactive peptides were isolated

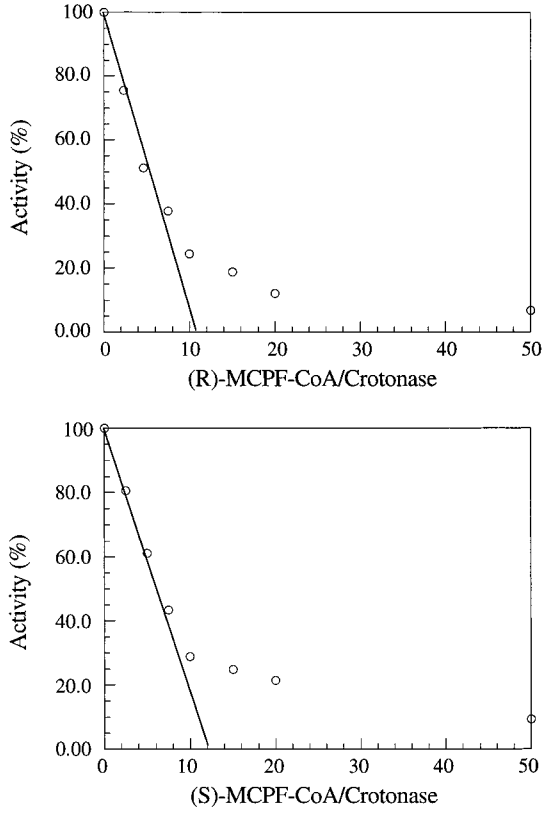


Figure 1. Effect of (*R*)- and (*S*)-MCPF-CoA on the catalytic activity of bovine liver ECH. These figures show the percentage of residual activity versus the ratio of MCPF-CoA to ECH.

and sequenced by standard Edman degradation. Interestingly, the first eight amino acid residues were identical for both peptides and had the sequence ALGGG**X**EL, indicating that they were formed from incomplete proteolysis catalyzed by a contaminating protease, possibly chymotrypsin. It should be noted that a majority of the radioactivity was released during one cycle of the Edman degradation of each fragment and was found to be associated with the sixth amino acid (Figure 4), which was designated **X**. However, the lack of nucleotide and/or peptide sequence information on bovine liver ECH precluded the determination of the identity of **X**. Nevertheless, the deduced peptide sequence aligned perfectly with a well conserved region

(43) Lai, M.-t.; Liu, L.-d.; Liu, H.-w. *J. Am. Chem. Soc.* **1991**, *113*, 7388−7397.

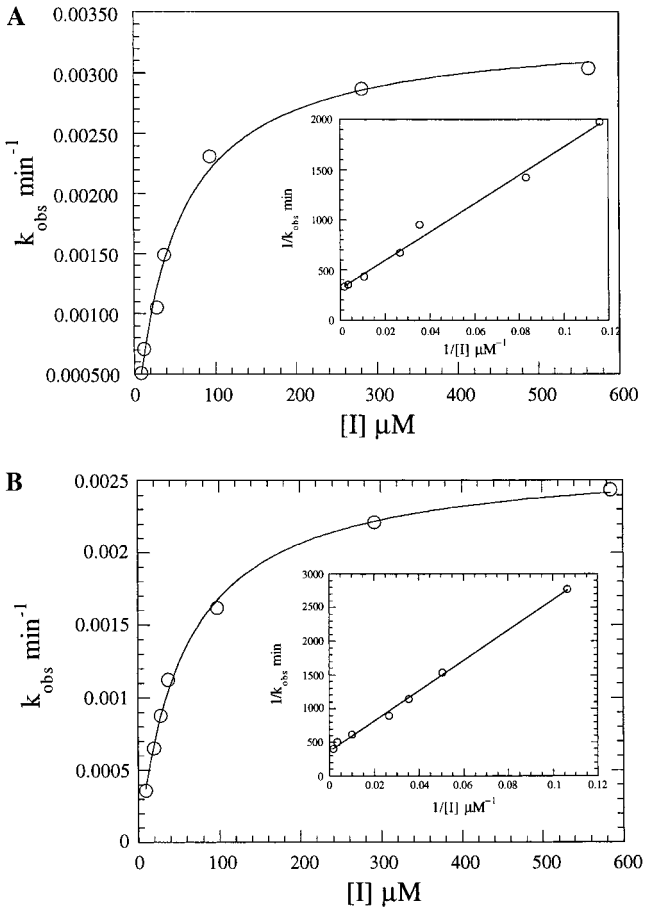


Figure 2. Effect of (*R*)- and (*S*)-MCPF-CoA on the catalytic activity of bovine liver ECH. Panels A and B are plots of k_{obs} as a function of (*R*)- and (*S*)-MCPF-CoA concentration, respectively. The inset shows the double reciprocal plot of k_{obs} versus MCPF-CoA concentration.

in the catalytic core of the mitochondrial short-chain ECHs from rat liver,[41] human liver,[42] and many others from different species (Figure 5). For example, this fragment corresponds to residues 138−145 in the rat liver enzyme, which includes Gly141 as well as Glu144 (rat liver ECH numbering), both of which have been shown to be important for catalysis. Interestingly, the alignment reveals that the counterpart of **X** in these sequences could be either cysteine, leucine, phenylalanine, or methionine. Because **X** should be nucleophilic, cysteine is a prime candidate for the entrapped active site residue.

Isolation and Nucleotide Sequence Analysis of cDNA Clones of Bovine Liver ECH. To gain more information about the identity of **X**, we proceeded to isolate the cDNA of bovine liver ECH. A bovine liver Lambda cDNA library (Stratagene) was screened by polyclonal antibodies raised against this enzyme using the dot blot test as described in the Experimental Section. Two positive clones were identified after screening $\sim 1 \times 10^6$ clones from the bovine liver library. These positive clones were subjected to in vivo excision in the host *E. coli* SOLR strain, and the resulting constructs were labeled as pBluescript SK-(±)-SD_{a1} and pBluescript SK(±)-SD_{b1}. Both clones carried a cDNA fragment of the same size (~1.6 kb), and sequencing both ends of the cloned DNA revealed that these two clones are identical. Both strands of the coding region were sequenced by the gene walking technique with custom designed primers. The resulting DNA sequence along with the translated amino acid sequence for bovine liver ECH is shown in Figure 6 (GenBank accession number AY049961).

The translated sequence of this enzyme is highly homologous to the mammalian mitochondrial ECHs. The overall identity of bovine liver ECH to rat liver and human liver ECHs is 87 and 85%, respectively, at the amino acid sequence level. As shown in Figure 6, it is clear that the isolated bovine liver ECH gene is truncated at the 5′-end, missing the signal sequence and the start codon.[44] Fortunately, the entire coding sequence for the mature protein remains intact in the cloned constructs (pBluescript SK(±)-SD_{a1} and SK(±)-SD_{b1}), as indicated by the presence of the N-terminal amino acid sequence which was deduced by N-terminal sequencing a commercial sample of bovine liver ECH. Most importantly, sequence alignment of the tryptic digested labeled peptide fragment with the wild-type bovine liver ECH has allowed the unambiguous identification of residue **X** as Cys114 (mature sequence numbering).

Purification of Bovine Liver ECH from *Escherichia coli* JM105/pSD_6. To ensure that the cloned sequence mentioned above indeed corresponds to bovine liver ECH, the cDNA encoding the mature protein (261 amino acids) in pBluescript SK(±)-SD_{a1} was amplified by PCR and subcloned into a pTrc99A expression vector. A start codon was engineered flanking the 5′-end of the mature sequence of the protein. Expression in *Escherichia coli* JM105 of the resulting construct, pSD_6, was induced by IPTG, and a significant portion of the

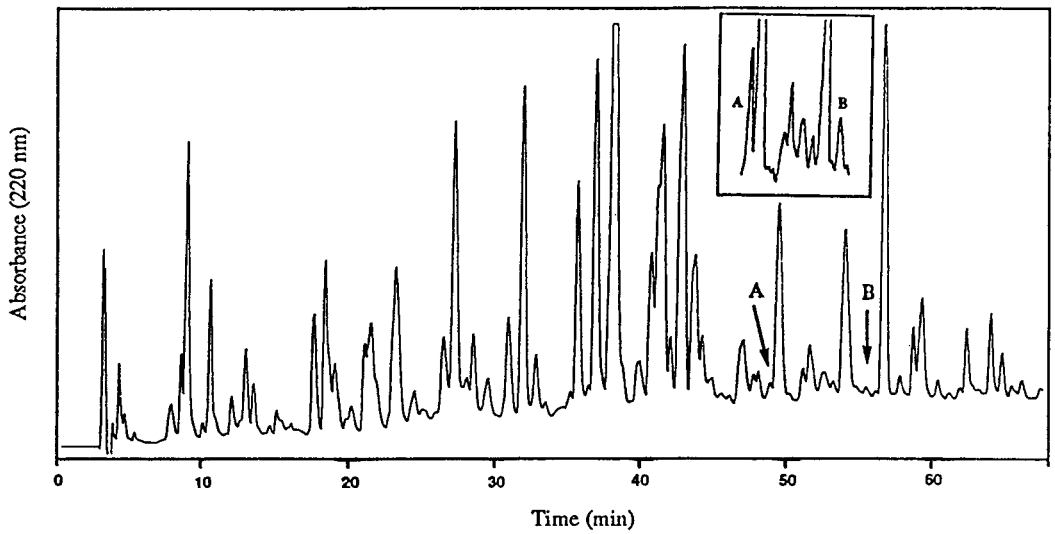


Figure 3. Fractionation of the tryptic digest of [^{3}H]MCPF-CoA (**8**) labeled bovine liver ECH by HPLC. See Experimental Section for details.

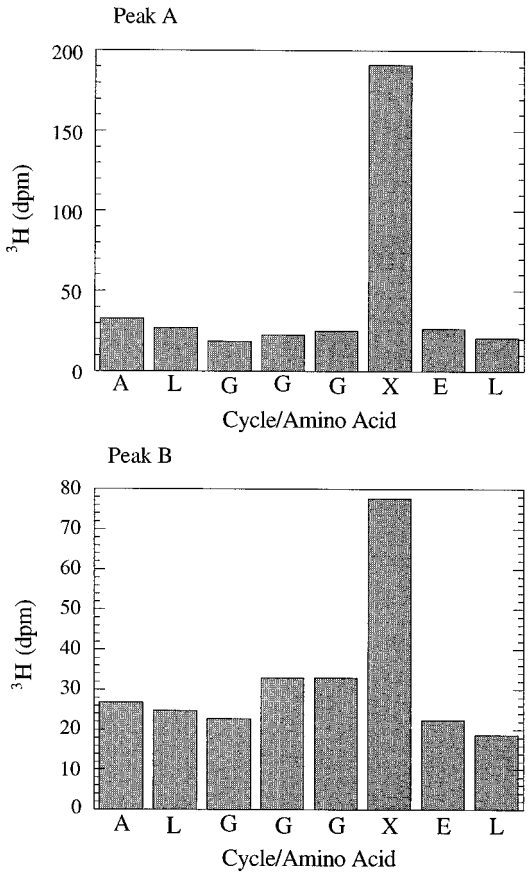


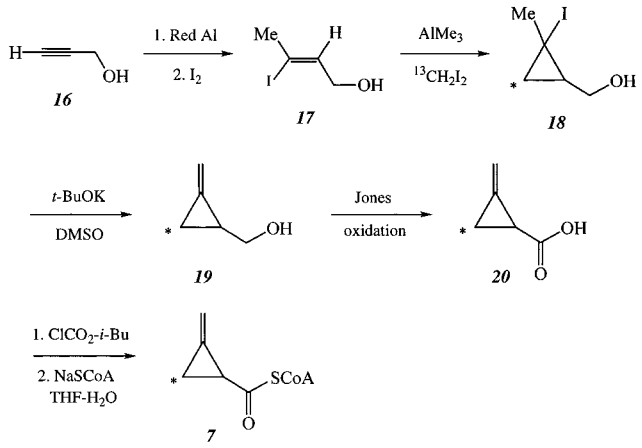
Figure 4. Determination of amino acid sequence for peptides A and B by automatated Edman degradation. After each cycle, the effluents were collected, and an aliquot of each was counted for radioactivity. Profiles A and B show the data obtained for peptides A and B, respectively. X represents the modified amino acid residue.

Rattus norvegicus	**AA**VNG**G**YALG**GG**C**E**LAMMCD
Homo sapiens	**AA**VNG**G**YTLG**GG**C**E**LAMACD
Archaeoglobus fulgidus	**AA**LNG**I**TAG**GG**L**E**LAMACD
Bacillus halodurans	**AA**VNG**G**YALG**A**G**C**E**L**ALLCD
Deinococcus radiodurans	**AA**IG**G**YALG**GG**L**E**LALCCD
Escherichia coli	**AA**VS**G**FALG**GG**F**E**LALHCD
Leishmania major	**AA**IE**G**KALG**GG**M**E**LALSLD
Mesorhizobium loti	**AA**VH**G**YALG**GG**F**E**LALACD
Rhodococcus fascians	**AA**VNG**I**AFG**GG**C**E**ITEAVP
Sulfolobus solfataricus	**AA**VNG**Y**PFG**GG**C**E**LAMMCD
Thermoplasma volcanium	**AA**VE**G**WALA**GG**C**E**IALSAD
Bovine liver	------ALG**GG**X**E**L-----

Figure 5. Amino acid sequence alignment of representative ECHs from different sources with the labeled peptide derived from bovine liver ECH.

protein was produced in soluble form. The expressed protein was purified to near homogeneity after DEAE-Sepharose and Phenyl-Sepharaose column chromatography. The recombinant protein has a molecular mass of 28 kDa per monomer and resembles that of the wild-type protein isolated from bovine liver.[4] Its catalytic activity is also similar to the wild-type enzyme, with a k_{cat} of 2.06×10^3 s^{-1} and a K_m of 15.6 ± 1.3 μM. In addition, the N-terminal sequence of the recombinant protein was determined to be identical to that of the wild-type enzyme. Thus, the above results confirmed that the cDNA borne by pBluescript SK($\pm$)-SD$_{a1}$ is indeed the desired bovine liver ECH gene.

Scheme 2



Characterization of the Covalent Adduct Between Bovine Liver ECH and MCPF-CoA. To perform structural characterization of the inhibitor−enzyme adduct to glean more insights into the inactivation mechanism, we decided to chemically synthesize [3-^{13}C$_1$]-labeled MCPF-CoA (**7**). As delineated in Scheme 2, reduction of 2-butynol (**16**) with Red-Al followed by quenching with I$_2$ gave 3-iodo-2-butenol (**17**). Cyclopropanation of the resulting crotyl alcohol (Al(Me)$_2$/^{13}CH$_2$I$_2$) introduced the stable isotope at C-3 of the ring carbon to give a racemic alcohol **18** in 54% overall yield.[45] Alcohol **18** was subjected to dehydroiodination, followed by Jones oxidation of the resulting (methylenecyclopropyl)methyl alcohol (**19**), to give (methylenecyclopropyl)formic acid (**20**). The corresponding CoA thioester was prepared by the mixed anhydride method and purified by HPLC to afford final product **7** in 62% yield. The labeled carbon (C-3) in **7** has a chemical shift of 14.4 ppm.

Four equivalents of **7** was incubated with bovine liver ECH (0.4 μmol) in 50 mM potassium phosphate buffer (pH 7.6) at 25 °C, and the progress of the inactivation was monitored using the standard assay protocol. After 6 h, at which time further inactivation was insignificant, the incubation mixture was concentrated to 500 μL by ultrafiltration and then subjected to the gHMQC (gradient heteronuclear multiple quantum coherence) experiment.[46] As illustrated in Figure 7, the gHMQC spectrum showed one pertinent signal at 105 ppm in the ^{13}C dimension and the corresponding cross-peaks at δ 4.8 and 5.0 in the ^{1}H dimension. The carbon and proton chemical shifts are characteristic for a terminal olefinic methylene group. Thus, the gHMQC results are most consistent with a nucleophilic attack on C-2′, followed by ring fragmentation to yield a thioether adduct (**21**) (Scheme 4, route A).

Discussion

Cyclopropyl compounds have proven to be valuable mechanistic probes in the study of enzyme-catalyzed reactions, especially where free radical chemistry is involved. Our interest in this class of compounds as inhibitors for fatty-acid degradation enzymes evolved from a study of the toxicity of hypoglycin A (**22**), which is the causative agent of Jamaican Vomitting Sickness.[47−49] This compound is metabolized in vivo to (methylenecyclopropyl)acetyl-CoA (MCPA-CoA, **23**), which in-

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Rat          1 MAALRALLPRACNSLLSPVRCPEFRRFASGANPQYIITEKKGKNSSVGLI
Bovine       1 ---------------------------FASSAAFEYIITAKKGKNSNVGLI
consensus    1                           FAS A F YIIT KKGkNS VGLI

Rat         51 QLNRPKALNALCNGLIEELNQALETFEEDPAVGAIVLTGGEKAFAAGADI
Bovine      25 QLNRPKALNALCNGLIVELNQALQAFEEDPAVGAIVLTGGEKVFAAGADI
consensus   51 QLNRPKALNALCNGLI ELNQAL  FEEDPAVGAIVLTGGEK FAAGADI

Rat        101 KEMQNRTFQDCYSGKFLSHWDHITRIKKPVIAAVNGYALGGGCELAMMCD
Bovine      75 KEMQSLTFQNCYAGGFLSHWDQLTRVKKPVIAAVNGYALGGGCELAMMCD
consensus  101 KEMQ  TFQ CY G FLSHWD iTRiKKPVIAAVNGYALGGGCELAMMCD

Rat        151 IIYAGEKAQFGQPEILIGTIPGAGGTQRLTRAVGKSLAMEMVLTGDRISA
Bovine     125 IIYAGEKAQFGQPEILIGTIPGAGGTQRLTRAVGKSLAMEMVLTGDRISA
consensus  151 IIYAGEKAQFGQPEILlGTIPGAGGTQRLTRAVGKSLAMEMVLTGDRISA

Rat        201 QDAKQAGLVSKIFPVETLVEEAIQCAEKIANNSKIVAMAKESVNAAFEM
Bovine     175 QDAKQAGLVSKIFPVETVVEEAIQCAEKIASNSKIVTAMAKESVNAAFEM
consensus  201 QDAKQAGLVSKIFPVETlVEEAIQCAEKIA NSKIi AMAKESVNAAFEM

Rat        251 TLTEGNKLEKKLFYSTFATDDRREGMSAFVEKRKANFKDH
Bovine     225 TLAEGVKLEKKLFYSTFAPKTGRKAWPAFVEKRKANFKDQ
consensus  251 TL EG KLEKKLFYSTFA    R g  AFVEKRKANFKD
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Figure 6. Nucleotide and the translated amino acid sequence of bovine liver and rat liver ECHs (GenBank accession number AY049961).

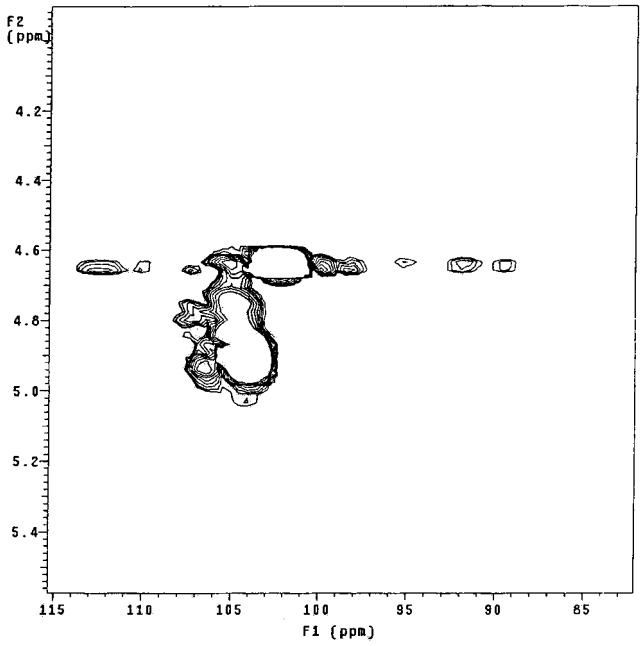
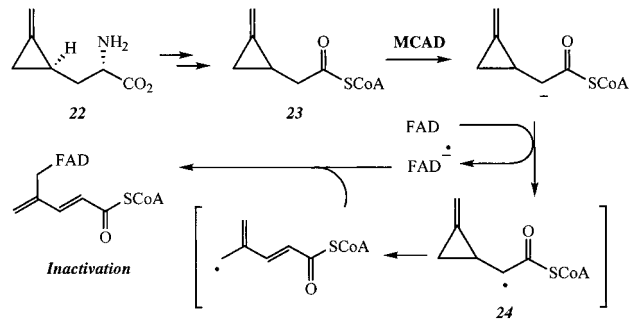


Figure 7. $^{13}C-^{1}H$-detected gHMQC spectrum (500 MHz, $^{2}H_2O$) showing the pertinent resonances of the covalent adduct between bovine liver ECH and [3-$^{13}C_1$](methylenecyclo-propyl)formyl-CoA (**7**).

hibits both short-chain and medium-chain acyl-CoA dehydrogenases.[50−52] The inactivation is initiated by the cleavage of the cyclopropyl ring followed by covalent modification of the active site bound FAD coenzyme (Scheme 3). The ring-opening step is likely induced by a cyclopropylcarbinyl radical (**24**), and the rapid ring opening renders this process practically spontaneous.[53−57] Thus, it is not surprising that both C-1 epimers of MCPA-CoA are effective inhibitors for acyl-CoA dehydrogenases, because the bond cleavage at β-C of MCPA-CoA is not enzyme-catalyzed. Interestingly, MCPF-CoA (**4**), a lower homologue of MCPA-CoA, does not exhibit inhibitory activity against acyl-CoA dehydrogenases, but specifically inhibits bovine liver ECH in a time-dependent, irreversible fashion.[34,35] The inactivation of bovine liver ECH occurs at the active site of the enzyme and involves covalent modification by 1 equiv of MCPF-CoA.[34]

Scheme 3



The crystal structures of rat liver ECH and several other proteins that bind coenzyme A thioesters have been solved.[58] The predominant theme in these enzymes is that the coenzyme A moiety serves as the primary binding determinant. Therefore, with the coenzyme A scaffold locked into the active site of bovine liver ECH, it is reasonable to assume that the cyclopropyl ring, where the inactivation occurs, would acquire a unique spatial orientation within the active site dictated by the stereochemistry at C-1. Therefore, one isomer of MCPF-CoA is

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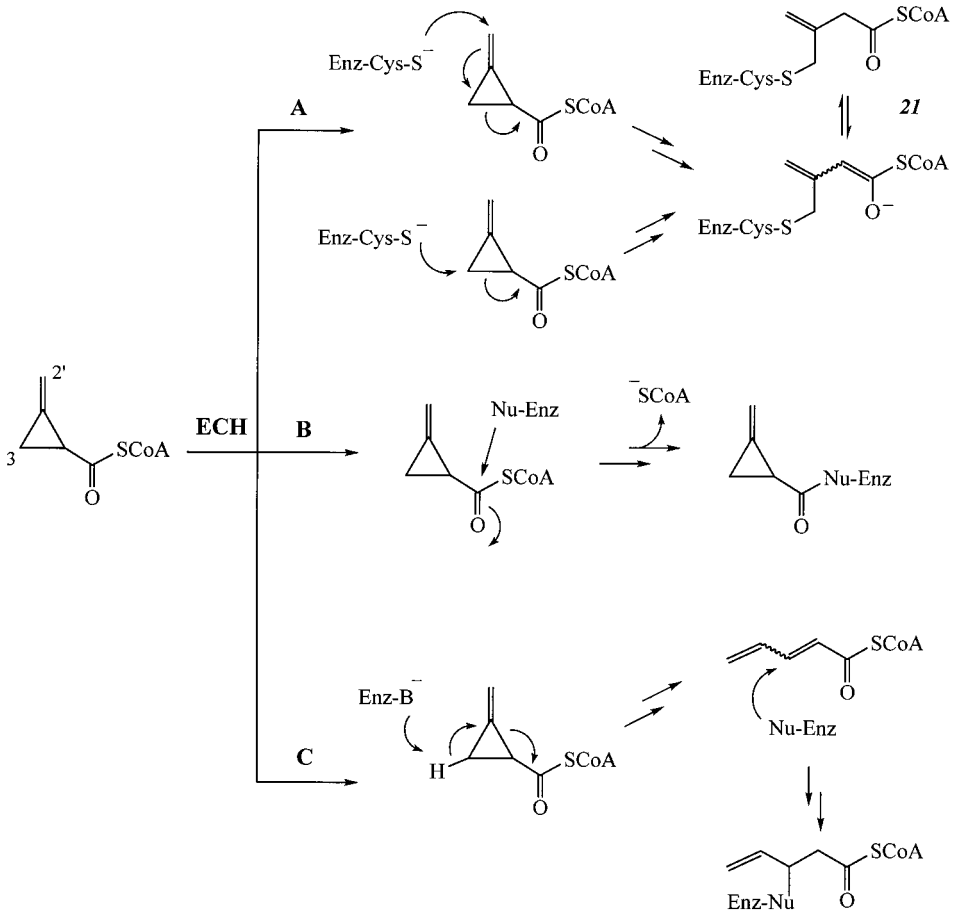
Scheme 4



expected to exert a greater inhibitory potency toward the enzyme than the other and may in fact be the sole contributor to the inactivation of the enzyme. To investigate the stereochemical preference of this inactivation, diastereomerically pure (*R*)- and (*S*)-MCPF-CoA were prepared. To our surprise, both (*R*)- and (*S*)-MCPF-CoA display similar kinetic behavior and therefore are equally competent in causing the inactivation of bovine liver ECH. As mentioned earlier, the lack of stereospecificity observed in the MCPA-CoA-mediated inactivation of acyl-CoA dehydrogenases could be readily explained by invoking the presence of a radical intermediate in the reaction mechanism. However, ECHs catalyze a stereospecific hydration reaction without the assistance of any cofactors. Therefore, the basis for the lack of stereospecificity in the inactivation of bovine liver ECH by MCPF-CoA is not immediately obvious, although it is possible that a rate-determining step independent of the bound inactivator, such as the ionization of an active site residue, may be involved in the inactivation process.

Intrigued by the apparent lack of discrimination displayed by bovine liver ECH toward the stereoisomers of MCPF-CoA, experiments were carried out to determine which residue(s) at the enzyme active site is (are) modified during the inactivation. Accordingly, bovine liver ECH was incubated with excess tritiated racemic MCPF-CoA (**8**), and the resulting inactivated enzyme was subjected to proteolytic digestion. Upon separation of the peptide fragments by reverse-phase HPLC, only two radiolabeled peptides were detected. Although the two peptides were not classical tryptic peptides, both had an identical N-terminal sequence, ALGGGXEL, and a significant amount of radiolabel was associated with single residue "X". These results indicate that covalent modification of bovine liver ECH by both diastereomers of MCPF-CoA occurs at a single site and are consistent with the observation that this inactivation is nonstereospecific. Although the cDNA sequence of bovine liver ECH was not available at the time, sequence alignment of crotonases isolated from different sources showed a high degree of homology, and the deduced short peptide sequence aligned perfectly with a well conserved segment which corresponds to residues 138−145 of the rat liver ECH (Figure 5). Therefore, our initial speculation was that a cysteine residue may be involved in the capture of MCPF-CoA by bovine liver ECH. Interestingly, the X-ray structure of the rat liver ECH indicates the importance of this cysteine residue in the formation of a putative H-bond with Gln161 (rat liver ECH numbering), an essential element for the proper positioning of the catalytic residues.[20,21]

It should be noted that bovine liver ECH contains five cysteines per subunit and none are implicated in disulfide bond formation.[4,59] Earlier reports had shown that thiol-directing reagents such as *p*-chloromercuribenzoate and iodoacetate inactivate the bovine liver ECH, and it was suggested on this basis that a thiol group may be catalytically essential for this enzyme. However, later studies indicated that none of the five cysteinyl residues are crucial for catalytic activity.[3] Interestingly, an affinity reagent, *p*-bromoacetamido-*trans*-cinnamoyl-CoA (BCC), inactivates the bovine liver ECH by steric blockage of the substrate-binding site and by alkylation of a proximal cysteine residue.[3] It is conceivable that MCPF-CoA, like BCC, exhibits an analogous mode of binding and that the same cysteinyl residue is the target of covalent modification. Although these results are intriguing, further characterization of the identity of the putative cysteine residue toward the inactivation was prudent in order to reach definitive conclusions. Thus,

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cloning and sequencing of the gene encoding bovine liver ECH were performed.

On the basis of the sequence of the cDNA clone for bovine liver ECH, we were able to establish that the amino acid corresponding to "X" labeled with MCPF-CoA is indeed a cysteine residue that is located at position 114 in the mature protein. It is clear that bovine liver ECH and its rat liver counterpart share a very high degree of sequence identity (Figure 6). In fact, the major differences in the amino acid sequence are primarily limited to the N- and C-terminal domains. The core catalytic domains of the two enzymes are virtually identical, with only a few amino acid substitutions, all of which are conservative in nature. It is reasonable to assume that bovine liver ECH and rat liver ECH have similar structures, catalytic properties, and kinetic parameters. However, MCPF-CoA acts as a time-dependent irreversible inhibitor for the bovine liver enzyme and as a competitive, reversible inhibitor for the rat liver enzyme. The high level of sequence identity of the two enzymes makes these differences in inhibition patterns all the more intriguing. However, in the absence of structural information about the bovine liver enzyme, we can only speculate that there may be subtle differences in the active site architecture of the two enzymes that govern the interaction of each with MCPF-CoA.

As far as the chemical basis of inactivation is concerned, three possible mechanisms of inactivation of bovine liver ECH by MCPF-CoA had been proposed (Scheme 4). However, two of them were considered unlikely on the basis of our early experiments.[34] For instance, a transesterification mechanism involving the formation of an acyl−enzyme adduct and the liberation of coenzyme A (Scheme 4, route B) was ruled out on the basis of the resistance of the enzyme−inhibitor adduct toward alkaline hydrolysis and on the association of the adenine chromophore with the inactivated enzyme despite prolonged dialysis. Similarly, a mechanism involving C-3 deprotonation followed by anion-induced ring opening (Scheme 4, route C) was rejected on the basis of the lack of tritium washout from [3-^{3}H]MCPF-CoA (**8**) when it was incubated with the enzyme. Therefore, a nucleophile-induced ring-opening mechanism appears to be the only viable alternative to explain the irreversible inactivation of the enzyme by MCPF-CoA.

Interestingly, such a mechanism could conceivably operate via two different pathways. As shown in Scheme 4, the nucleophilic attack could occur at C-3, leading to the cleavage of the C_1-C_3 bond to form the enzyme−inhibitor covalent adduct. Alternatively, the nucleophilic attack could occur at the olefinic C-2′ carbon, resulting in the formation of a C_2-C_3 double bond and the cleavage of the C_1-C_3 bond. Both inactivation routes result in an identical structure of the ultimate covalent adduct, irrespective of whether the initial nucleophilic attack occurred at C-3 or C-2′. However, it should be noted that in case of C-3 attack, the existing olefinic bond is retained between C-2 and C-2′, while C-3 and C-1 are transformed into allylic carbons. On the contrary, for the mechanism involving a C-2′ attack, an olefinic bond is formed between C-2 and C-3, while C-2′ and C-1 are transformed into allylic carbons. To distinguish between these two pathways, [3-^{13}C]MCPF-CoA (**7**) was synthesized as a probe to determine the fate of C-3 of MCPA-CoA after the inactivation had occurred. As described previously, the NMR data of the inactivated sample are most consistent with the assignment of the ^{13}C label as an alkene carbon in the adduct. Therefore, the molecular basis of the inactivation likely involves the nucleophilic attack of Cys-114 at the C-2′ olefinic carbon of MCPF-CoA, leading to ring scission and the permanent modification of the enzyme.

Several cyclopropane-containing mechanism-based inactivators in which the target enzyme activates the cyclopropane for nucleophilic addition by oxidation or protonation of the appended groups rendering them more electron withdrawing have been reported.[60,61] However, no such catalytic activation of MCPF-CoA appears to be necessary as the initial step for inactivation. Although the regioselectivity and mechanism of the methylenecyclopropyl ring fragmentation is poorly defined, the main driving force must arise from the relief of the great ring strain (40.8 kcal/mol).[62] Substrate activation via electron polarization of the π-bond to enhance the electrophilicity of β-C is a prerequisite of catalysis for ECH.[15−18] Given the preponderance of the ECH active site toward π-bond electron reorganization, it is conceivable that MCPF-CoA is activated by a similar push−pull mechanism, thus facilitating the entrapment of the active site nucleophile. Hence, the inactivation chemistry appears to be dictated by a normal turnover mechanism with the help of proper contacts and binding modes within the enzyme active sites. Considering the role of ECH in the metabolism of fatty acids and the catabolism of branched-chain amino acids, its inhibitors may emerge as a useful means to control and/or regulate these physiological processes for therapeutic intervention. Thus, the results described herein may provide important clues for future design of more efficient and selective inhibitors to control and/or regulate fatty acid metabolism.

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JA011226K

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