The Toxicity of Methylenecyclopropylglycine: Studies of the Inhibitory Effects of (Methylenecyclopropyl)formyl-CoA on Enzymes Involved in Fatty Acid Metabolism and the Molecular Basis of Its Inactivation of Enoyl-CoA Hydratases

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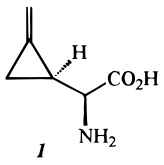
Abstract: (Methylenecyclopropyl)formyl-CoA (MCPF-CoA), a toxic metabolite of methylenecyclopropylglycine (MCPG), is known to have hypoglycemic activity due to its ability to interrupt the β-oxidation pathway of fatty acid metabolism. Earlier experiments have shown that acetoacetyl-CoA thiolase, 3-ketoacyl-CoA thiolase, acyl-CoA dehydrogenases, and enoyl-CoA hydratase (ECH) are cellular targets that can be inhibited by MCPF-CoA and/or MCPG. To gain more insights with respect to the target specificity and the mode of action, we have carried out a detailed investigation of the effects of MCPF-CoA on a variety of enzymes involved in fatty acid metabolism. Our studies confirmed that MCPF-CoA is a potent inactivator for ECHs but shows little effect on other β-oxidation enzymes tested in this study. Our results also revealed that MCPF-CoA manifests distinct modes of inhibition among ECHs isolated from different sources, being a competitive inhibitor for rat liver ECH and an irreversible inactivator for the bovine liver as well as pig kidney ECH. Given the high sequence homology of the mammalian ECH genes studied so far, the structures of these proteins are expected to be similar. Thus, the effects of MCPF-CoA toward different ECHs must be governed in part by the interaction of MCPF-CoA with the active site of each ECH whose architecture may be subtly different. More importantly, the incubation results with bovine liver ECH established that MCPF-CoA inactivates this enzyme via a mechanism involving the covalent trapping of an active site nucleophile by the methylenecyclopropane ring. Since MCPF-CoA is a rare irreversible inhibitor for ECHs, it could serve as a new lead for designing more effective agents for modulating ECH activity so as to control and/or regulate fatty acid metabolism.

Methylenecyclopropylglycine (**1**, MCPG) was first isolated



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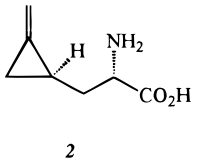

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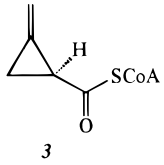

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in 1962 from the kernels of litchi fruits by Gray and Fowden and has been shown to cause hypoglycemia in mice[1] and fasting rats.[2,3] MCPG is a homologue of hypoglycin A (**2**), the better known and more potent hypoglycemic amino acid, and the causative agent of Jamaican vomiting sickness.[4−6] It is generally accepted that MCPG follows a metabolic route analogous to that found for hypoglycin[7] in which it is first converted in vivo into a toxic metabolite, (methylenecyclopropyl)formyl-CoA (**3**, MCPF-CoA),[2,3] which interrupts the β-oxidation pathway of fatty acid metabolism. Although both MCPG and hypoglycin A induce hypoglycemic activity, the major sites at which their toxicity is exhibited are distinctly different. Earlier experiments had established that the targets of hypoglycin A (**2**) are medium-chain (MCAD) and short-chain acyl-CoA dehydrogenases (SCAD).[8−10] MCPG (**1**), however, was found to be most inhibitory toward 3-ketoacyl-CoA and acetoacetyl-CoA thiolases and showed little effect on acyl-CoA dehydrogenases in fasting

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rats.[3] Interestingly, while enoyl-CoA hydratase (ECH) appears to be immune to MCPG in fasting rats,[3] when the metabolite MCPF-CoA (**3**) was incubated directly with purified enzymes, ECH could be effectively inactivated.[2] It has also been demonstrated that this inhibition is more potent against ECH from pig kidney but notably less inhibitory against the bovine liver enzyme.[2] The contrary target specificity exhibited by MCPG (**1**) and MCPF-CoA (**3**) toward enzymes participating in fatty acid metabolism and the distinct effects of MCPG and MCPF-CoA on the activity of ECHs are intriguing. Since the inhibitory properties of MCPG and MCPF-CoA are potentially useful for drug design aimed at controlling fatty acid metabolism, further investigations of their target specificity and modes of action promise to be fruitful. In a preliminary communication, we have described our initial studies of the inactivation of bovine liver ECH by MCPF-CoA (**3**).[11] Described herein are the results of our studies of the effects of MCPF-CoA on a variety of enzymes involved in fatty acid metabolism and a full account of the inhibition of a few different ECHs by MCPF-CoA.

Experimental Section

General. The ^{1}H and ^{13}C NMR chemical shifts are reported in 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 indicated solvents. TLC spots were visualized either with UV light or by heating plates previously stained with solutions of vanillin/ethanol/H_2SO_4 (1:98:1) or phosphomolybdic acid (7% EtOH solution). The 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. It should be noted that the tritium-labeled compounds prepared in this work were not submitted for spectral analysis due to the possibility of radioactive contamination. However, satisfactory analytical results were obtained with the corresponding unlabeled analogues. For enzyme purification, all operations were performed at 4 °C. The concentration of protein was determined by Bradford's method[12] using bovine serum albumin as the standard. The relative molecular weights and purity of enzyme samples were determined using sodium dodecyl sulfate−polyacrylamide gel electrophoresis as described by Laemmli.[13] The N-terminal amino acid sequences were determined by the Microchemical Facility at the Institute of Human Genetics of the University of Minnesota.

Materials. DEAE-cellulose (DE−52) was purchased from Whatman (Clifton, NJ), and phosphocellulose and CM-cellulose were from Sigma (St. Louis, MO). Hydroxyapatite and the Bradford reagent were products of BioRad (Richmond, CA). Ecoscint A (National Diagnostics, Manville, NJ) was used as a scintillation cocktail. All other biochemicals and chemicals were purchased from Sigma and Aldrich (Milwaukee, WI) and were of the highest purity available.

Enzymes. Carnitine acetyltransferase from pigeon breast muscle, 3-hydroxyacyl-CoA dehydrogenase from pig heart, and enoyl-CoA hydratase (ECH) from bovine liver were purchased from Sigma. The rat liver enoyl-CoA hydratase was purified from a recombinant strain (*Escherichia coli* BL21(DE3)pLyS/pET20ech1) by a reported procedure.[14] Similarly, the short-chain acyl-CoA dehydrogenase (SCAD) from *Megasphaera elsdenii* was also purified from a recombinant strain (*Escherichia coli* BL21(DE3)pLyS/pWTSCADT7), which contains the cloned SCAD gene.[15] Medium-chain acyl-CoA dehydrogenase (MCAD) from pig kidney was purified according to the procedure of Thorpe.[16] The 3-ketoacyl-CoA thiolase was purified from pig heart muscle by the procedure of Schulz and Staack.[17] The acetoacetyl-CoA thiolase from rat liver was purified by a procedure similar to that used for the purification of other thiolases.[18−20] The purity of each of these enzymes was estimated to be ≥90% by SDS−PAGE analysis, and their identities were confirmed by N-terminal sequencing analysis. The chromatographic, electrophoretic, and spectral properties of the purified proteins were identical to those cited in the literature. The pig kidney enoyl-CoA hydratase was purified by a protocol developed in house. The purification of this enzyme is summarized below.

Purification of Enoyl-CoA Hydratase from Pig Kidney. (A) Enzyme Assay. The activity of ECH was assayed as described by Steinman and Hill,[21] except for the omission of ovalbumin from the reaction mixture and the use of 10 mM potassium phosphate buffer (pH 7.0). Crotonoyl-CoA (80 μM) was used as the substrate and changes in absorbance at 263 nm (ϵ 6700 M^{-1} cm^{-1}), which corresponds to the α,β-unsaturated enoyl moiety of 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.

(B) Enzyme Purification. Step 1. Preparation of Acetone Powder Extract. Approximately 700 g of frozen pig kidneys were partially thawed, and the cortex layer was chopped into 1−2 cm cubes. These pieces were combined with 1.5 L of cold acetone (−20 °C) in a Waring blender. After the addition of 0.35 g of α-toluenesulfonyl fluoride (PMSF) in 2-propanol (3 mL), the mixture was blended for 3.5 min at top speed, and the resulting acetone suspension was filtered with a Büchner funnel lined with Whatman No. 4 filter paper. The retentate was allowed to run dry and was extracted by stirring for 12 h with 1.5 L of 20 mM buffer A (potassium phosphate, 5% (v/v) glycerol, and 5 mM 2-mercaptoethanol, pH 6.3) to which 0.18 g of PMSF in 2-propanol (4 mL) had been added. The insoluble material was then removed by centrifugation for 45 min at 14000*g*.

Step 2. Phosphocellulose Chromatography (Batch). The clear supernatant from step 1 was collected and adjusted to pH 6.3 with 1 N acetic acid. The resulting solution was stirred gently for 90 min with 360 mL of phosphocellulose that had been pre-equilibrated with 50 mM buffer A. This mixture was vacuum filtered on a Büchner funnel, and the resin was washed with a total of 3 L of 50 mM buffer A. The phosphocellulose was allowed to dry, suspended in 300 mL of the same buffer, and poured into a column (2.7 × 40 cm). The column was washed with 300 mL of 50 mM buffer A followed by elution of the protein with 1 L of 200 mM buffer A. Fractions of 20 mL were collected. The pooled active fractions (21−29) were then subjected to ammonium sulfate fractionation. The desired protein was precipitated between 35% and 70% saturation.

Step 3. Phosphocellulose Column Chromatography. The precipitated proteins from step 2 were collected, redissolved in 50 mL of 20 mM buffer A, and dialyzed overnight against 4 L of the same buffer to which 18 mg of PMSF in 2-propanol (1 mL) had been added. The dialyzed protein solution was applied to a phosphocellulose column (2.7 × 40 cm) pre-equilibrated with the same buffer. The column was washed with three column volumes of the same buffer, and the protein was then eluted with a linear gradient of 20−200 mM potassium phosphate buffer (pH 6.3), containing 10% glycerol (v/v) and 5 mM mercaptoethanol (2 L total). Fractions of 18 mL were collected throughout the elution. Fractions (77−95) with high specific activity were pooled and concentrated by an Amicon concentrator (PM-10 membrane).

Step 4. Hydroxyapatite Column Chromatography. The concentrate from step 3 was directly applied to a hydroxyapatite column (2.5 × 15 cm) pre-equilibrated with 200 mM potassium phosphate buffer

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(pH 6.3) containing 10% glycerol (v/v) and 5 mM 2-mercaptoethanol. The column was washed with four column volumes of the same buffer followed by a gradient of 200−700 mM potassium phosphate buffer (pH 6.3) containing 10% (v/v) glycerol and 5 mM 2-mercaptoethanol (1.2 L total). Fractions of 15 mL were collected, and fractions (58−75) with high specific activity were combined and concentrated by ultrafiltration with a PM-10 membrane. After the addition of glycerol to a final concentration of 20% (v/v) and dithiothreitol to 5 mM, the purified enzyme was stored at −80 °C.

Enzyme Assays. Other enzymes used in this study were assayed as follows: the activities of acyl-CoA dehydrogenases were determined by using phenazine methosulfate (PMS) or phenazine ethosulfate (PES) as the external electron carrier to mediate the transfer of reducing equivalents from the substrate (butyryl-CoA for SCAD[15] and octanoyl-CoA for MCAD[16]) to 2,6-dichlorophenolindophenol (DCPIP);[22] the activity of carnitine acetyl-CoA transferase was measured by monitoring the appearance and disappearance of acyl-CoA derivatives at 324 nm;[23] the activity of 3-hydroxylacyl-CoA dehydrogenase was determined by following the bleaching of NADH at 340 nm;[24] and the activity of acetoacetyl-CoA thiolase was assayed by following the disappearance of the Mg^{2+}-enolate complex of acetoacetyl-CoA spectrophotometrically at 303 nm.[17]

Synthesis. (A) Ethyl 2-Bromo-2-methylcyclopropanecarboxylate (5). Ethyl diazoacetate (10.85 g, 95 mmol) was added slowly via an automatic Sage syringe over a period of 2 days to a solution of rhodium-(II) acetate (45 mg, 0.1 mmol) in 2-bromopropene (**4**; 25 g, 0.21 mol) at room temperature under an argon atmosphere with constant stirring. The resulting mixture was filtered to remove the precipitated rhodium salts. The filtrate was concentrated and then distilled under reduced pressure (bp 70−80 °C at 35 Torr) to give **5** as a colorless oil in 86% yield. Since the product was a mixture of trans and cis isomers, the NMR spectra of **5** showed two sets of overlapping signals: ^{1}H NMR ($CDCl_3$) δ 4.13, 4.09 (2 H each; q, $J = 7.1$; OCH_2's), 2.23 (1 H; dd, $J = 9.3$, 6.6; 1-H of trans isomer), 1.80, 1.77 (3 H each; s; 2-Me's), 1.74−1.65 (2 H; m; 1-H, 3-H of cis isomer), 1.52 (1 H; dd, $J = 9.3$, 6.6; 3-H of trans isomer), 1.36 (1 H; t, $J = 6.6$; 3-H of trans isomer), 1.23, 1.22 (3 H each; t, $J = 7.1$; Me's), 1.15 (1 H; dd, $J = 7.6$, 5.2; 3-H of cis isomer); ^{13}C NMR ($CDCl_3$) δ 170.1, 169.2 (C=O's), 61.0, 60.9 (OCH_2's), 33.2, 32.9 (C-2's), 30.7, 29.6 (C-1's), 28.7, 24.2 (C-3's), 23.8, 22.8 (2-Me's), 14.3, 14.2 (Me's).

(B) Ethyl Methylenecyclopropanecarboxylate (6). To a rapidly stirring, refluxing slurry of sodium hydride (3.0 g, 125 mmol) and **5** (16.6 g, 80 mmol) in ether (125 mL) under an argon atmosphere was added 1 mL of ethanol. After being refluxed with stirring overnight, the reaction mixture was filtered and concentrated under reduced pressure. The residue was then distilled under reduced pressure (bp 55−65 °C at 35 Torr) to give **6** as a colorless oil in 80% yield: ^{1}H NMR ($CDCl_3$) δ 5.44 (2 H; d, $J = 2.0$; $=CH_2$), 4.07 (2 H; q, $J = 7.2$; OCH_2), 2.18 (1 H; m; 1-H), 1.73, 1.55 (1 H each; m; 3-H's), 1.19 (3 H; t, $J = 7.2$; Me); ^{13}C NMR ($CDCl_3$) δ 171.9 (C=O), 130.3 (C-2), 104.4 ($=CH_2$), 60.6 (OCH_2), 18.0 (C-1), 14.1 (C-3), 11.3 (Me).

(C) (Methylenecyclopropyl)formic Acid (7). To a solution of **6** (2.5 g, 20 mmol) in a mixture of 100 mL of methanol and water (v/v 4:1) was added 10 g of anhydrous potassium carbonate. After being stirred at room temperature for 2 days, the solution was acidified with 2 N HCl. The mixture was then extracted with methylene chloride, and the combined organic extracts were dried and concentrated in vacuo to afford **7** in 92% yield: ^{1}H NMR ($CDCl_3$) δ 5.52 (2 H; d, $J = 1.8$; $=CH_2$), 2.22 (1 H; m; 1-H), 1.85, 1.68 (1 H each; m; 3-H's); ^{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 $C_5H_7O_2$ $(M + H)^+$ 99.0446, found 99.0445.

(D) (Methylenecyclopropyl)formyl-CoA (3). To a solution of **7** (30 mg, 0.27 mmol) in methylene chloride (5 mL) was added triethylamine (38 μL, 0.27 mmol) under an argon atmosphere. After the mixture was stirred for 10 min, isobutyl chloroformate (34.7 μL, 0.27 mmol) was added dropwise at 0 °C. The reaction was agitated vigorously; fuming was noted during mixing. Stirring was continued at room temperature for 1 h. The solvent was removed under reduced pressure, and the remaining mixed anhydride was redissolved in THF (5 mL) to give a cloudy solution. Meanwhile, a solution of coenzyme A was prepared by dissolving the sodium salt of coenzyme A (50 mg, 50 μmol) in distilled water (5 mL) that had been deoxygenated by repeated freeze/thaw cycles under high vacuum. The solution was adjusted to pH 8.0 by adding 1 N NaOH. To this CoA solution was added, via a cannula, the mixed anhydride solution under positive argon pressure. The pH of the resulting mixture was readjusted to 8.0, and stirring was continued for an additional 10 min. The pH value of the solution was then changed to 5.0−5.5 by adding diluted perchloric acid. The organic solvent (THF) was evaporated 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 MCPF-CoA was chromatographed on a HPLC C_{18} column (10 × 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).[25] The MCPF-CoA-containing fractions were pooled, concentrated under reduced pressure to remove methanol, and then lyophilized. The resulting acyl-CoA product was desalted on the same C_{18} column, which was washed (3 mL/min) first with water for 20 min followed by methanol. The MCPF-CoA-containing methanol eluent was evaporated under reduced pressure and then lyophilized to give pure MCPF-CoA (**3**) as a white powder. The overall yield was 52%. ^{1}H NMR (^2H_2O) δ 8.58, 8.32 (1 H each; s; adenine H's), 6.20 (1 H; d, $J = 6.0$; ribose anomeric H), *5.62*, *5.56* (1 H each; d, $J = 2.2$; $=CH_2$), 4.93−4.78 (1 H; buried under ^{2}HOH peak), 4.71, 4.62 (1 H each; s; ribose H's), 4.27 (2 H; s; ribose CH_2O), 4.04 (2 H; s), 3.83 (1 H; m), 3.60 (1 H; m), 3.47 (2 H; t, $J = 10.8$), 3.38 (2 H; t, $J = 10.8$), 3.02 (2 H; m), *2.79* (1 H; t, $J = 6.5$; 1-H), 2.44 (2 H; t, $J = 10.0$), *1.89* (2 H; m; 3-Hs), 0.91, 0.78 (3 H each; s; Me's). The NMR sample was repeatedly dissolved in ^2H_2O and lyophilized prior to ^{1}H NMR analysis, and the chemical shifts of the MCPF signals are shown in italic. High-resolution FAB-MS: calcd for $C_{26}H_{40}N_7O_{17}P_3S$ $(M + 1)^+$ 848.1492, found 848.1431.

(E) 2-(2-Propynyloxy)tetrahydropyran (9). To a solution of 3,4-dihydro-*2H*-pyran (23.5 g, 279 mmol) in 150 mL of anhydrous ether was added *p*-toluenesulfonic acid (0.1 g) and propargyl alcohol (**8**, 3.9 g, 69 mmol) at 0 °C. The resulting mixture was stirred at room temperature for 5 h, followed by the addition of concentrated ammonium hydroxide (1 mL) and methanol (10 mL). The solvent was evaporated, and ether was added. This mixture was filtered to remove precipitated ammonium *p*-toluenesulfate. The filtrate was concentrated, and the crude product was purified by flash column chromatography (5% ethyl acetate in hexanes) to give pure **9** in 98% yield: ^{1}H NMR ($CDCl_3$) δ 4.80 (1H; m; ketalic-H), 4.28, 4.21 (1 H each; dd, $J = 15$, 2.4; side-chain OCH_2), 3.83, 3.50 (1 H each; m; ring-OCH_2), 2.39 (1 H; m; H−C≡C), 1.87−1.51 (6 H; m; ring-Hs); ^{13}C NMR ($CDCl_3$) δ 96.7 (ketalic-C), 79.6 (C-*C*≡C), 73.9 (C−C≡*C*H), 62.0 (=C-*C*-O), 54.0 (ring-OCH_2), 30.2, 25.3, 19.0 (ring-Cs).

(F) [3-^{3}H]-2-(2-Propynyloxy)tetrahydropyran (10). Compound **9** (4.0 g, 28.6 mmol) was dissolved in 100 mL of THF and cooled to −78 °C. To this solution was added dropwise *n*-BuLi (12.6 mL of a 2.5 M solution in hexanes, 31.4 mmol). After stirring for 2 h at 0 °C, the reaction was cooled to −78 °C and partially quenched with [^{3}H]-H_2O. The stirring was continued for an additional 1 h at 0 °C, and the excess lithium reagent was then thoroughly quenched with ice-cold saturated ammonium chloride solution. The resulting mixture was stirred for 10 min and extracted with ether. The combined organic extracts were dried, filtered, and concentrated. Purification by flash chromatography (5% ethyl acetate in hexanes) afforded the desired product in 90% yield. It should be noted that the experimental procedures were optimized using ^2H_2O prior to the use of radioactive material. ^{1}H NMR of the deuterated **10** is identical to that of **9** except for the lack of acetylenic proton at δ 2.39.

(G) 2-([3-^{3}H]-2-Propenyloxy)tetrahydropyran (11). Compound **10** (3.0 g, 21.3 mmol) was mixed with Lindlar's catalyst (300 mg) and

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quinoline (120 mL) in 100 mL of methanol. The reaction mixture was vacuumed and purged with H_2 several times and was then allowed to proceed under atmospheric hydrogen. After ~1 h, when most of the reactant was consumed, the reaction was filtered and concentrated. The residue was redissolved in methylene chloride and washed with 1 N HCl solution to remove quinoline. The organic layer was washed with brine, dried, and concentrated. The crude product was purified by flash chromatography (5% ethyl acetate in hexanes) to give the desired product in 80% yield: ^{1}H NMR ($CDCl_3$) of the unlabeled **11** δ 5.91 (1H; m; C*H*═CH_2), 5.27 (1 H; d, J = 17.1; cis-*R*HC═C*H*H), 5.14 (1 H; d, J = 10.4; trans-*R*CH═C*H*H), 4.63 (1 H; t, J = 3.3; ketalic-H), 4.22, 3.97 (1 H each; dd, J = 13.0, 6.0; ═C−CH_2O), 3.86, 3.50 (1 H each; m; ring-OCH_2), 1.86−1.49 (6 H; m; ring-Hs); ^{13}C NMR ($CDCl_3$) δ 134.6 (C═*C*-C), 116.5 (*C*═C−C), 98.0 (ketalic-C), 68.0 (═C-*C*-O), 62.2 (ring-O*C*H_2), 30.6, 25.5, 19.4 (ring-Cs).

(H) 2-[(2-Chloro-2-methyl-[3-^{3}H]-cyclopropyl)methoxy]tetrahydropyran (12). A solution of compound **11** (1.5 g, 10.5 mmol) and 1,1-dichloroethane (9.36 g, 94.5 mmol) in anhydrous ether (100 mL) was cooled to −40 °C. A solution of *n*-butyllithium in hexanes (35 mL of a 2.5 M solution in hexanes, 87.5 mmol) was added to the reaction mixture over a period of 3 h, during which time the reaction temperature was maintained between −40 and −35 °C. After being stirred at −30 °C for an additional 14 h, the reaction was warmed to 0 °C and quenched with water. The aqueous layer was extracted with ether, and the combined ether extracts were dried, filtered, and concentrated. The crude product was purified by flash chromatography (5% ethyl acetate in hexanes) to give **12** in 63% yield: ^{1}H NMR ($CDCl_3$) of the unlabeled **12** δ 4.66 (1 H; m; ketalic-H), 3.89 (1 H; m; ring-OCH), 3.85 (1 H; dd, J = 10.9, 7.5; side-chain OCH), 3.59 (1 H; dd, J = 10.9, 6.7; side-chain OCH), 3.49 (1 H; m; ring-OCH), 1.83 (1 H; m), 1.72 (1 H; m), 1.62 (3 H; s; CH_3), 1.58−1.50 (4 H; m), 1.21 (1 H; m; 2-H), 0.93 (1 H; dd, J = 9.6, 3.6; 3-H); ^{13}C NMR ($CDCl_3$) δ 98.9/98.6 (ketalic-C), 68.4 (side-chain-OCH_2), 62.5/62.1 (ring-OCH_2), 44.5/44.3 (C-2), 30.8/30.7, 28.5, 25.5, 25.1/24.7, 20.3 (C-3), 19.8/19.4.

(I) 2-{[3-^{3}H]-(Methylenecyclopropyl)methoxy}tetrahydropyran (13). A solution of compound **12** (650 mg, 3.16 mmol) in anhydrous dimethyl sulfoxide (30 mL) was heated to 70 °C. To this mixture was added potassium *t*-butoxide (675 mg, 6.03 mmol), and the reaction was stirred at 70 °C for 1 h. The solution was then cooled to room temperature, diluted with 100 mL of ether, and washed with brine. The organic layer was collected, dried, and concentrated. The crude product was purified by flash chromatography (5% ethyl acetate in hexanes) to give **13** in 85% yield: ^{1}H NMR ($CDCl_3$) of the unlabeled **13** δ 5.43, 5.38 (1H each; d, J = 1.8; CH_2═), 4.64 (1 H; m; ketalic-H), 3.82, 3.69 (1 H each; m; side-chain and ring OCHs), 3.50−3.46 (2 H; m; side-chain and ring OCHs), 1.76−1.18 (6 H; m), 0.90 (1 H; m).

(J) ([3-^{3}H]-Methylenecyclopropyl)formic Acid (14). A methanol solution (20 mL) of compound **13** (450 mg, 2.7 mmol) was treated with *p*-toluenesulfonic acid monohydrate (150 mg, 0.8 mmol) and stirred at room temperature for 24 h. At the end of the reaction, potassium carbonate (140 mg) was added, and stirring was continued for an additional 1 h. Methanol was removed by distillation, and the residue was resuspended in water. The aqueous phase was extracted with ether, and the combined ether layers were dried and concentrated in vacuo to give a colorless oil in 67% yield. This crude product was subjected to Jones oxidation without further purification.

The above compound, ([3-^{3}H]-methylenecyclopropyl)methanol (150 mg, 1.8 mmol), was dissolved in 30 mL of acetone and treated with Jones reagent that was prepared by mixing chromium oxide (26.7 g) with concentrated sulfuric acid (23 mL), followed by dilution with water to a final volume of 100 mL. Addition of Jones reagent was continued at 0 °C until the reddish color persisted for at least 1 min. The resulting solution was stirred for 1 h at room temperature, and the excess oxidizing reagent was quenched with 2-propanol. The resulting solution was then diluted with water followed by repeated extractions with ether. The combined ether layers were extracted with 10% NaOH (3 × 50 mL), and the aqueous extracts were acidified to pH <2 with 6 N HCl. The resulting solution was re-extracted with three 50 mL portions of ether. The organic phases were combined, dried, and concentrated in vacuo to give a colorless oil in 70% yield: ^{1}H NMR of the unlabeled **14** is identical to that of **7**.

(K) ([3-^{3}H]-Methylenecyclopropyl)formyl-CoA (15). With the same procedure used for the conversion of **7** to **3**, compound **15** was synthesized from **14** in 75% yield. The specific activity of **15** was 0.56 mCi/mmol.

Incubation of MCPF-CoA with Enzymes Involved in Fatty Acid Oxidation. The effects of MCPF-CoA (**3**) on the activities of nine enzymes involved in fatty acid metabolism were examined. In each case, excess MCPF-CoA was incubated with the target enzyme at room temperature, and aliquots of the incubation mixture were removed at different time intervals to determine the residual enzyme activity using the specific assays for each enzyme described above. Controls were also prepared in which the same volume of buffer was added instead of MCPF-CoA solution. In the studies of carnitine acetyl-CoA transferase, MCAD, and SCAD, 200 equiv of MCPF-CoA was used in each incubation. With 3-ketoacyl-CoA thiolase, acetoacetyl-CoA thiolase, and (L)-3-hydroxyacyl-CoA dehydrogenase, 100 equiv of MCPF-CoA was used in each incubation. To test the inactivation of ECHs from bovine liver, pig kidney, and rat liver, we employed roughly 20 equiv of MCPF-CoA.

Kinetic Analysis of Inactivation of Bovine Liver ECH by MCPF-CoA. In a typical inactivation experiment, an appropriate amount of MCPF-CoA was incubated with the enzyme solution (9 μM in 50 mM potassium phosphate buffer, pH 7.6) at 25 °C. At various time intervals, aliquots of the incubation mixture were diluted, and 2 μL of the resulting solution was used in the standard assay cocktail (1 mL). The remaining enzyme activity was then determined as described earlier. For the "protection" experiment, the enzyme was incubated with 200 μM each of acetoacetyl-CoA (AcAc-CoA) and MCPF-CoA. The residual enzyme activity was monitored over time as described above.

Test for the Reversibility of the Inactivation. A sample containing MCPF-CoA (20 molar equiv) and ECH isolated from bovine liver (5 nmol) and/or rat liver (2 nmol) was incubated for 12 h at room temperature to ensure complete inactivation of the enzyme. The inactivated enzyme was dialyzed against 50 mM potassium phosphate buffer (pH 7.6) over 2 days at 4 °C with seven buffer changes. The residual enzyme activity was determined before and after dialysis. A control experiment was performed under identical conditions without the inactivator.

Tritium Wash-out Study. One molar equivalent of the tritium-labeled **15** was incubated with bovine liver ECH in 500 μL of 50 mM potassium phosphate buffer (pH 7.6) at 25 °C. An aliquot (100 μL) of the reaction mixture was removed at time zero and counted for radioactivity. After being incubated for 12 h, the reaction was quenched with activated charcoal (10% solution).[26] The resulting suspension was mixed vigorously for 1 min, and the charcoal was precipitated by centrifugation. The supernatant was removed, and the radioactivity was analyzed by a scintillation counter. These data were calibrated against controls, prepared in parallel with boiled enzyme (100 °C, 10 min).

Determination of Stoichiometry of Tritium Incorporation. The bovine liver ECH (40 nmol) was incubated with 10 equiv of [^{3}H]-MCPF-CoA (**15**) in 50 mM potassium phosphate buffer (pH 7.6) for 12 h to ensure complete inactivation of the enzyme. The inactivated enzyme was dialyzed against the same buffer at 4 °C for 2 days with seven buffer changes. An aliquot (50 μL) of the incubation mixture was removed before and after dialysis to count for radioactivity. The readings were calibrated against a control prepared with boiled enzyme. These data yielded the ratio of inactivator per enzyme subunit.

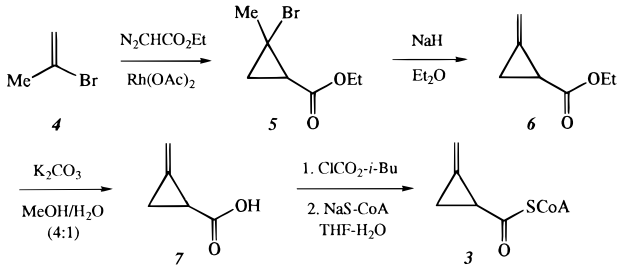
Testing the Stability of Enzyme−Inhibitor Adduct. Bovine liver ECH (7.5 nmol) was inactivated for 12 h with 20 equivalents of **15** in 50 mM potassium phosphate buffer (pH 7.6). The modified ECH was denatured by boiling, the suspension was centrifuged, and the precipitate was washed (3×) with the same phosphate buffer. The precipitate was redissolved in a minimal amount of 0.1 M Tris·HCl buffer (pH 10), which contained 6 M guanidine·HCl. After overnight incubation, the resulting mixture was ultrafiltered using a Microcon-10 (Amicon), and the denatured protein was washed extensively with fresh incubation buffer. The filtrates were pooled, and the released radioactivity was analyzed by scintillation counting.

(26) Lai, M.-t.; Li, D.; Oh, E.; Liu, H.-w. *J. Am. Chem. Soc.* **1993**, *115*, 1619−1628.

Table 1. Summary for Purification of Enoyl-CoA Hydratase from Pig Kidney

step	total protein (g)	total activity (units)	specific activity (units/mg)	purification fold	yield (%)
homogenate	42.5	23657	0.557	1	100
phosphocellulose (batch)	4.31	20953	4.86	8.7	88.6
$(NH_4)_2SO_4$ precipitate	1.41	10933	7.73	13.9	46.2
phosphocellulose (gradient)	0.192	6823	35.5	63.7	28.8
hydroxyapatite	0.0113	3315	293.3	526.6	14.0

Scheme 1



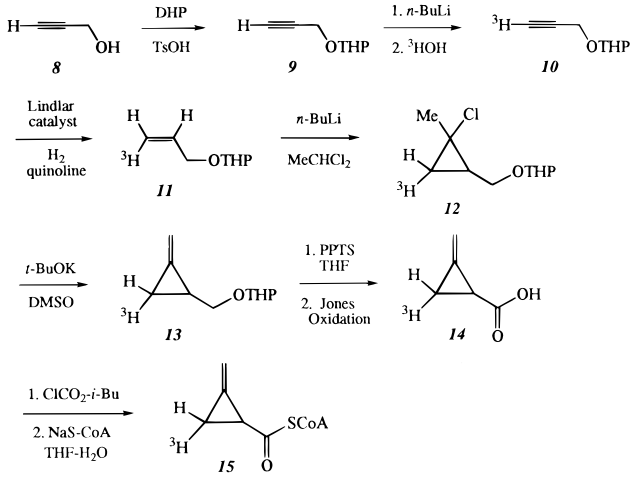
Results

Purification of Pig Kidney Enoyl-CoA Hydratase. Table 1 summarizes the purification of pig kidney ECH by a three-step chromatographic procedure that is similar to those used for the purification of other ECHs. Greater than 500-fold purification of the pig kidney ECH was achieved. While this enzyme has been used in an earlier study,[2,27] we have independently developed the purification protocol outlined in the Experimental Section. The physical properties of the purified pig kidney ECH are similar to those of the bovine liver enzyme.[21]

Synthesis of (Methylenecyclopropyl)formyl-CoA. As shown in Scheme 1, synthesis of MCPF-CoA (**3**) followed a similar sequence used in our preparation of (methylenecyclopropyl)-acetyl-CoA (MCPA-CoA).[28] Briefly, the synthesis was initiated by a rhodium acetate-catalyzed cyclopropanation of 2-bromopropene (**4**) with ethyl diazoacetate to yield compound **5**. Sodium hydride-induced elimination followed by base hydrolysis of the ester gave compound **7**. Condensation of (methylenecyclopropyl)formic acid (**7**) with isobutyl chloroformate and coupling with coenzyme A afforded the desired MCPF-CoA (**3**). Preparation of the tritium-labeled MCPF-CoA (**15**) was achieved by the reactions depicted in Scheme 2, which is analogous to a reported sequence for making a methylenecyclopropyl derivative.[29]

Incubation of Carnitine Acetyltransferase with MCPF-CoA. At least three carnitine acyltransferases that are associated with mitochondria are known; one is specific for short-chain fatty acids (carnitine acetyltransferase) and two are specific for the longer-chain fatty acids (carnitine palmitoyltransferase I and II).[30,31] Although the substrate specificities are distinct, their catalytic characteristics are expected to be similar. Since MCPF resembles short-chain fatty acids, its effect on this class of

Scheme 2



enzymes was tested with carnitine acetyltransferase. Overnight incubation of carnitine acetyltransferase isolated from pigeon breast muscle with excess MCPF-CoA (**3**) showed that MCPF-CoA has little effect on the catalytic activity of this enzyme.

Incubation of Acyl-CoA Dehydrogenase with MCPF-CoA. Currently, seven acyl-CoA dehydrogenases have been isolated from mammalian mitochondria.[32−34] Four are straight (short-, medium-, long-, and very long-) chain acyl-CoA dehydrogenases with distinct but overlapping acyl chain length specificities, and the other three enzymes are involved in amino acid metabolism (isovaleryl-, 2-methyl-branched-, and glutaryl-CoA dehydrogenase). Clearly, these enzymes exhibit diverse substrate specificities; however, their catalytic mechanisms are believed to be the same. While MCPF-CoA (**3**) has been shown to be a potent inhibitor for 2-methyl-(branched-chain)-acyl-CoA dehydrogenase,[2] overnight incubation of excess MCPF-CoA with medium-chain acyl-CoA dehydrogenase (MCAD) isolated from pig kidney led to the loss of only 20% of its activity. A similar result was also obtained with short-chain acyl-CoA dehydrogenase (SCAD) isolated from *Magasphaera elsdenii*. These findings are consistent with both the in vivo as well as in vitro data reported previously by Melde et al.[2,3] Considering the large excess of inhibitor used in these experiments and the long duration of incubation, the decrease of activity may not be physiologically significant.

Incubation of (L)-3-Hydroxyacyl-CoA Dehydrogenase with MCPF-CoA. The effect of MCPF-CoA (**3**) on the catalytic activity of (L)-3-hydroxyacyl-CoA dehydrogenase was examined by the incubation of the pig heart enzyme[24] with excess MCPF-CoA at room temperature. Since the enzyme activity remained practically unchanged even after overnight incubation, MCPF-CoA is not an inhibitor or inactivator for (L)-3-hydroxyacyl-CoA dehydrogenase.

Incubation of Thiolase with MCPF-CoA. Two types of thiolases differing in their chain length specificities are present in mitochondria:[18] the 3-ketoacyl-CoA thiolase (thiolase I), which is active toward substrates ranging from acetoacetyl-CoA to long-chain 3-ketoacyl-CoAs at low substrate concentrations ($K_m < 20\ \mu M$), and acetoacetyl-CoA thiolase (thiolase II), which only acts on acetoacetyl-CoA. In this study, 3-ketoacyl-CoA

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(28) Lai, M.-t.; Liu, L.-d.; Liu, H.-w. *J. Am. Chem. Soc.* **1991**, *113*, 7388−7397.
(29) Baldwin, J. E.; Parker, D. W. *J. Org. Chem.* **1987**, *52*, 1475−1477.
(30) Miyazawa, S.; Ozasa, H.; Osumi, T.; Hashimato, T. *J. Biochem.* **1983**, *94*, 529−542.
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(32) Schulz, H. In *Fatty Acid Oxidation: Clinical, Biochemical, and Molecular Aspects*; Tanaka, K., Coates, P. M., Eds.; Alan R. Liss: New York, 1990; pp 23−36.
(33) Engel, P. C. In *Chemistry and Biochemistry of Flavoenzymes*; Müller, F., Ed.; CRC Press: New York, 1992; Vol. III, pp 597−655.
(34) Izai, K.; Uchida, Y.; Orii, T.; Yamamoto, S.; Hashimoto, T. *J. Biol. Chem.* **1992**, *267*, 1027−1033.

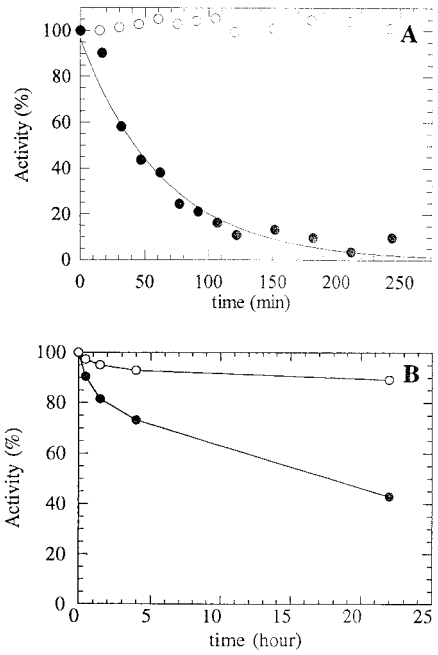

Figure 1. (A) Time-dependent inactivation of bovine liver ECH by MCPF-CoA (○, control; ●, with MCPF-CoA). Bovine liver ECH (9.2 μM) was incubated with MCPF-CoA (187 μM) in a reaction volume of 500 μL. At the indicated times, aliquots of the reaction were taken out, diluted 10-fold, and assayed as described in the Experimental Section. The control reaction had an equivalent volume of buffer instead of MCPF-CoA. (B) Time-dependent inactivation of pig kidney ECH by MCPF-CoA (○, control; ●, with MCPF-CoA).

thiolase isolated from pig heart[19] and acetoacetyl-CoA thiolase purified from rat liver were chosen as representatives of these two types of thiolases. Interestingly, while the activity of 3-ketoacyl-CoA thiolase was essentially unchanged after overnight incubation, the residual activity of acetoacetyl-CoA thiolase decreased to 80% of the control. Thus, MCPF-CoA (**3**) has some, albeit small, effects on thiolases. These observations are in sharp contrast to an earlier report in which the activities of acetoacetyl-CoA thiolase and of 3-ketoacyl-CoA thiolase were decreased to 25% and <10% of the controls in MCPG (**1**) treated fasting rats.[3]

Incubation of Pig Kidney ECH and Bovine Liver ECH with MCPF-CoA. As illustrated in Figure 1, the activity of the pig kidney ECH decreased by nearly 50% after overnight incubation with MCPF-CoA (**3**). The bovine enzyme was inactivated at a much faster rate ($k_{obs} = 18.2 \times 10^{-3}$ min^{-1}, $t_{1/2}$ = 38 min), losing almost 90% of its activity in 4 h. Since the enzyme activity was not recovered after extensive dialysis in both cases, MCPF-CoA is clearly an effective irreversible inhibitor for these isoforms of ECH. The rate of inactivation of the bovine enzyme was found to be significantly decreased when an equimolar amount of acetoacetyl-CoA (AcAc-CoA), which is a potent competitive inhibitor for crotonases,[35] was included in the reaction mixture. As shown in Figure 2, the enzyme sample incubated with MCPF-CoA retained only 10% of its activity after 4 h, whereas the enzyme sample incubated with equimolar amounts of AcAc-CoA and MCPF-CoA retained 80% of its activity over the same duration. Since the binding of AcAc-CoA is reversible in nature, it stands to reason that AcAc-CoA cannot protect the enzyme indefinitely. Thus, the enzyme sample containing AcAc-CoA was found to be inactivated to the same extent as the unprotected sample after overnight incubation (data not shown). However, AcAc-CoA can clearly protect the enzyme against MCPF-CoA during the initial stages of inactivation. Therefore, the inactivation of ECHs by MCPF-CoA is active-site-directed.

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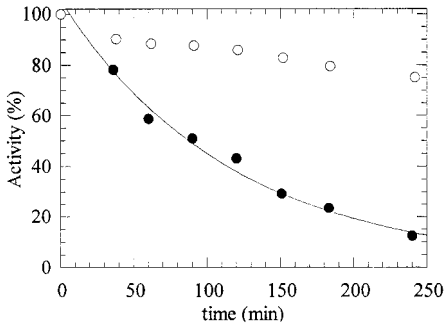

Figure 2. AcAc-CoA-mediated protection of bovine ECH against inactivation by MCPF-CoA (○, with AcAc-CoA and MCPF-CoA; ●, with MCPF-CoA alone). Bovine liver ECH (9.2 μM) was incubated with MCPF-CoA (200 μM) and AcAc-CoA (200 μM) in a reaction volume of 100 μL. At the indicated times, aliquots of the reaction were diluted 10-fold and assayed as described. The sample with MCPF-CoA alone had an equivalent volume of buffer instead of AcAc-CoA.

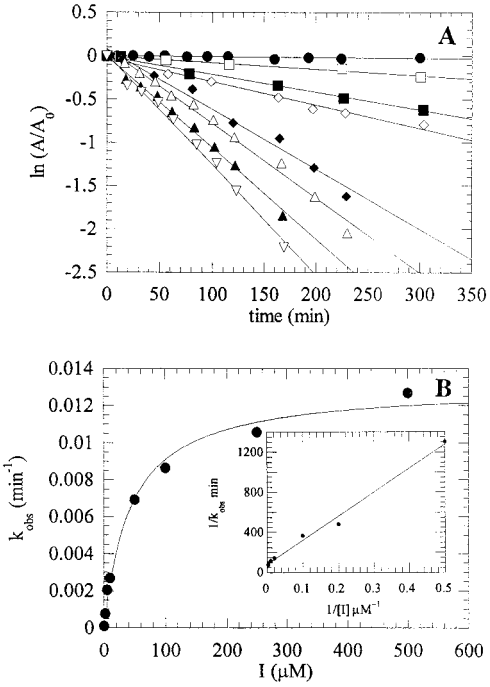

Figure 3. (A) Time- and concentration-dependent inactivation of bovine liver ECH by MCPF-CoA (●, 0 μM MCPF-CoA; □, 2 μM; ■, 5 μM; ◇, 10 μM; ◆, 50 μM; △, 100 μM; ▲, 250 μM; ▽, 500 μM). Bovine liver ECH (916 nM) was incubated with the indicated amounts of MCPF-CoA in a reaction volume of 100 μL. At the indicated times, aliquots of the reaction were directly used to assay the activity of the enzyme. The values of k_{obs} were determined from the slopes of the linear fit for the inactivation data obtained with the respective concentrations of MCPF-CoA. (B) Plot of k_{obs} as a function of MCPF-CoA concentration ([I]). The inset shows the double-reciprocal plot of k_{obs} versus MCPF-CoA concentration. The data from plot (B) was used to calculate k_{inact} and K_I, which are reported in the text.

Kinetic Study of Inactivation of Bovine Liver ECH by MCPF-CoA. The kinetic parameters of the inhibition of MCPF-CoA (**3**) on the activity of bovine liver ECH were determined by analyzing the plot of k_{obs} versus inhibitor concentration as shown in Figure 3. Figure 3A shows a plot of the natural log of the fraction of remaining enzyme activity versus time at various concentrations of MCPF-CoA. The values of k_{obs} as

determined from the slopes of the individual lines were plotted against the concentration of MCPF-CoA, as shown in Figure 3B. The k_{inact} and K_I of the inactivation were determined from Figure 3B and have values of 13.1×10^{-3} min^{-1} and 43.7 μM, respectively. Although the rate of inactivation is slow, the K_I is close to the K_m values (~10−30 μM) found for most substrates of this enzyme. Thus, MCPF-CoA appears to fit well in the active site of bovine liver ECH.

Further Characterization of the Inactivation of Bovine Liver ECH by MCPF-CoA. A 1:1 stoichiometric ratio between MCPF-CoA (**3**) and bovine liver ECH was determined by incubating 10 equiv of [^{3}H]MCPF-CoA (**15**) with the enzyme overnight, followed by thorough dialysis. This result provided strong evidence confirming that the inactivation is indeed irreversible and involves covalent bond formation. In a similar experiment, the enzyme was incubated with one equivalent of **15** for 12 h and quenched with a solution of activated charcoal (10%). After centrifugation to precipitate the charcoal, less than 1% of the radioactivity was detected in the supernatant. Evidently, no loss of ^{3}H-label occurs during inactivation. It was thus concluded that the abstraction of the ring-H of MCPF-CoA (**3**) is not part of the inactivation mechanism.

To determine whether liberation of coenzyme A occurs during the inactivation, we performed an analogous experiment using unlabeled **3**. Briefly, after quenching the reaction mixture with activated charcoal and centrifugation, the supernatant was treated with 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), and the change in absorption at A_{412} (ϵ 14,150 M^{-1}·cm^{-1}) was monitored.[36] The fact that the absorbance at A_{412} remained invariant as compared to the control strongly indicated that the release of CoA as a byproduct in this process is negligible. In addition, the UV−vis spectrum of the inactivated enzyme showed that approximately 1 equiv of CoA, as calculated from the absorption at 260 nm,[37] remained associated with the inactivated enzyme, even after prolonged dialysis (data not shown). Thus, the inactivation of ECH by MCPF-CoA (**3**) via a direct nucleophilic attack at the thioester group to form an acyl-enzyme adduct and free CoA is unlikely.

To assess the stability of the enzyme−inhibitor adduct, bovine liver ECH was inactivated with 20 equiv of **15**. The modified ECH was denatured by boiling, and the precipitate was incubated overnight in Tris·HCl buffer (pH 10) containing 6 M guanidine hydrochloride. Since the radioactivity released after incubation was found to be only 6.8%, this result suggested that the enzyme−inhibitor adduct is relatively stable under alkaline denaturing conditions.

Inhibition of Rat Liver ECH by MCPF-CoA. Upon incubation of MCPF-CoA (**3**) with the recombinant rat liver ECH, time-dependent loss of enzyme activity was observed (Figure 4). It is noteworthy that the rate of inactivation of the rat liver enzyme was almost 7-fold slower than that of its bovine counterpart ($k_{obs} = 2.7 \times 10^{-3}$ min^{-1}, $t_{1/2} = 4.28$ h). However, extensive dialysis of the inactivated enzyme or filtration of the inactivated enzyme through a size-exclusion column (G-10) resulted in full recovery of enzyme activity. Clearly, the inhibition of the rat liver ECH is reversible. Since no turnover products could be detected by HPLC, and no released coenzyme-A was discernible by DTNB titration, the reversible inhibition as a result of the formation of an incipient covalent acyl-enzyme intermediate that is highly labile can be ruled out.

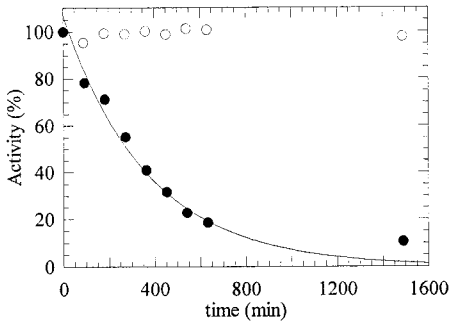

Figure 4. Time-dependent inactivation of rat liver ECH by MCPF-CoA (○, control; ●, with MCPF-CoA). Rat liver ECH (10.3 μM) was incubated with MCPF-CoA (187 μM) in a reaction volume of 50 μL. At the indicated times, aliquots of the reaction were taken out, diluted 50-fold, and assayed as described in the Experimental Section. The control reaction had an equivalent volume of buffer instead of MCPF-CoA.

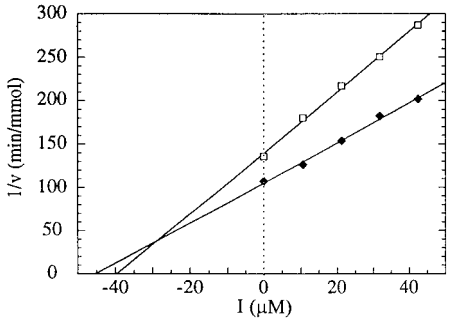

Figure 5. Plot of the reciprocal of the reaction velocity observed with rat liver ECH (v) as a function of MCPF-CoA concentration ([I]) in the presence of 10 μM (□) and 20 μM (◆) crotonoyl-CoA.

Thus, the competitive binding of MCPF-CoA (**3**) to the active site of rat liver ECH is the most likely mode of inhibition.

The slow but reversible loss of activity of the rat liver enzyme is puzzling. It is possible that the initial EI complex undergoes a slow conformational change to a more tightly bound EI* complex, causing a gradual loss of activity. Assuming that the initial encounter between the inhibitor and the enzyme is fast and reversible, we determined the "steady-state" inhibition parameters as shown in Figure 5. The Dixon plot of the reciprocal enzyme activity as a function of inhibitor concentration at different substrate concentrations led to the dissociation constant, or K_I, of 30 μM. This dissociation constant is close to the K_m value of 20 μM determined for crotonyl-CoA, the optimal substrate of this enzyme.

Discussion

Methylenecyclopropylglycine (**1**, MCPG) is a hypoglycemic agent that interrupts the β-oxidation of fatty acids in mice and fasting rats. The β-oxidation of fatty acids in mitochondria proceeds through a spiral sequence, where each cycle consists of four sequential enzymatic steps catalyzed by acyl-CoA dehydrogenase, enoyl-CoA hydratase, 3-hydroxyacyl-CoA dehydrogenase, and β-ketothiolase.[38,39] In addition, carnitine, a transporter molecule, and carnitine acyltransferases are required to mediate the unidirectional transport of fatty acids across the mitochondrial membrane.[40] Although the reactions catalyzed by these enzymes are quite diverse, the substrates of these enzymes

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(37) The stoichiometry of the bound CoA cannot be precisely determined due to the differences in the predicted molar absorptivity values of the possible enzyme−inhibitor adducts shown in Scheme 3.

(38) Schulz, H. *Biochim. Biophys. Acta* **1991**, *1081*, 109−120.

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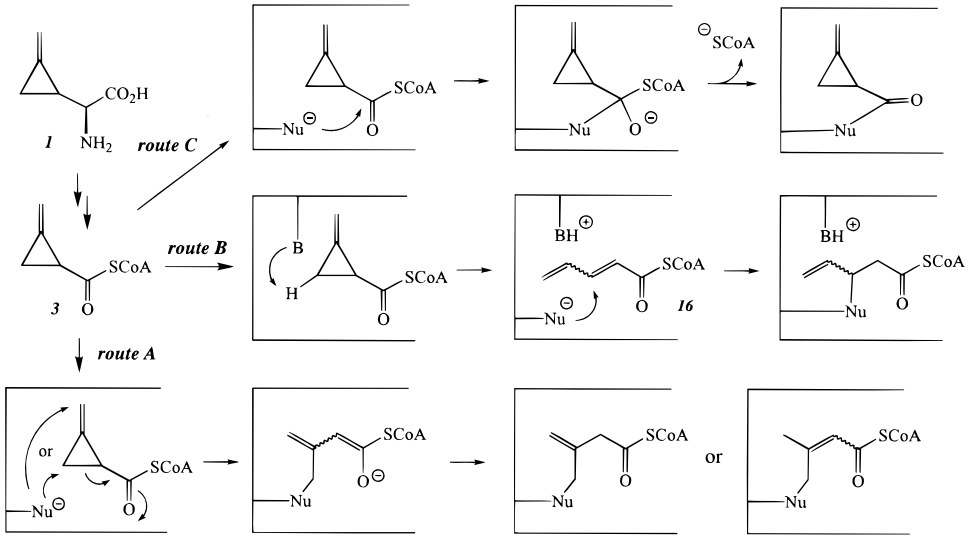
(40) Bieber, L. L. *Annu. Rev. Biochem*. **1988**, *57*, 261−283.

Scheme 3



are all coenzyme A derivatives. Given the compactness as well as the hydrophobic nature of the methylenecyclopropyl group, it was envisioned that MCPF-CoA (**3**) may potentially fit in the active site of each of these enzymes and express a broad spectrum of toxicity. However, as mentioned earlier, MCPG (**1**) was found to be most inhibitory toward thiolases in fasting rats,[3] and MCPF-CoA (**3**) appeared to be most potent against ECHs in vitro.[2] To fully define the specificity of this hypoglycemic agent and to determine its modes of action against its targets, we investigated the effects of MCPF-CoA on the catalytic activities of a variety of enzymes involved in mitochondrial fatty acid β-oxidation.

Our results showed that MCPF-CoA (**3**) exhibits no apparent effect on most enzymes involved in the catabolism of saturated fatty acids, except for acetoacetyl-CoA thiolase, MCAD, and SCAD, where limited inhibition was noted. Our studies also confirmed that MCPF-CoA is indeed a potent inhibitor for ECHs,[3,11] including those isolated from bovine liver, rat liver, and pig kidney. However, in contrast to an earlier report,[3] the toxicity of MCPF-CoA is more pronounced toward bovine liver ECH as compared to the pig kidney enzyme. More significantly, distinct modes of inhibition were found against the rat liver ECH and the bovine liver counterpart. Our data indicate that the inhibition of the rat liver enzyme by this inhibitor is competitive and reversible in nature, whereas the inactivation of the bovine liver enzyme is irreversible. The disparity of the inhibitory effects of MCPF-CoA toward these two closely related enzymes is intriguing and warrants a closer look. It is possible that subtle differences in their sequence are responsible for the observed difference in the inhibition characteristics. To determine the basis for this puzzling difference in behavior, we are trying to obtain sequence information for the bovine liver ECH to enable a direct comparison to the rat liver enzyme[41] whose X-ray structure has been recently determined.[42,43]

As delineated in Scheme 3, a few possible mechanisms of the inactivation of bovine liver ECH by MCPF-CoA (**3**) can be envisioned.[11] The cyclopropane ring of MCPF-CoA, by virtue of its ring strain and the electron-withdrawing substituent, may be readily susceptible to nucleophilic attack by an active site residue causing enzyme inactivation (route A). A base-catalyzed ring cleavage of MCPF-CoA to generate a reactive intermediate (**16**) capable of trapping an enzyme nucleophile is an alternative mechanism for the inactivation (route B). A direct attack at the thioester carbonyl of MCPF-CoA by an active site nucleophile to form an acyl-enzyme adduct may also account for the toxicity of MCPF-CoA (route C). Clearly, routes B and C are less likely, given that the ring hydrogen of MCPF-CoA is nonacidic and that CoA is not a unique structural entity for **3**. Their eventual exclusion as viable mechanisms was based on the following findings: the enzyme−inhibitor adduct was stable under denaturing and alkaline conditions, no released CoA could be detected in the incubation mixture, the UV−vis spectrum of the inactivated enzyme showed the presence of bound CoA, and little tritium (<1%) was released when **15** was used in the incubation. Thus, our results are most consistent with route A, in which MCPF-CoA (**3**) inactivates bovine liver ECH via trapping an active site nucleophile by the intact methylenecyclopropane ring.

It is worth mentioning that a similar mechanism has been proposed for 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.[44,45] For example, as illustrated in Scheme 4, a spirocyclopropyl pteridine (**17**) is an inactivator for dihydrofolate reductase.[46] In this case, protonation to activate the cyclopropane ring followed by the trapping of an enzyme nucleophile to form a covalent adduct has been suggested as the mechanism for the inactivation. Similarly, benzyl-1-(aminomethyl)-cyclopropane-1-carboxylate (**18**) has been shown to be a mechanism-based inhibitor for monoamine oxidase.[47] In this case, the tethered aminomethyl group in **18** is oxidized to an iminium ion in **19** to activate the cyclopropane ring, which then reacts with the reduced flavin causing enzyme inactivation. Likewise, the inactivation of alcohol dehydrogenase and lactate dehydrogenase

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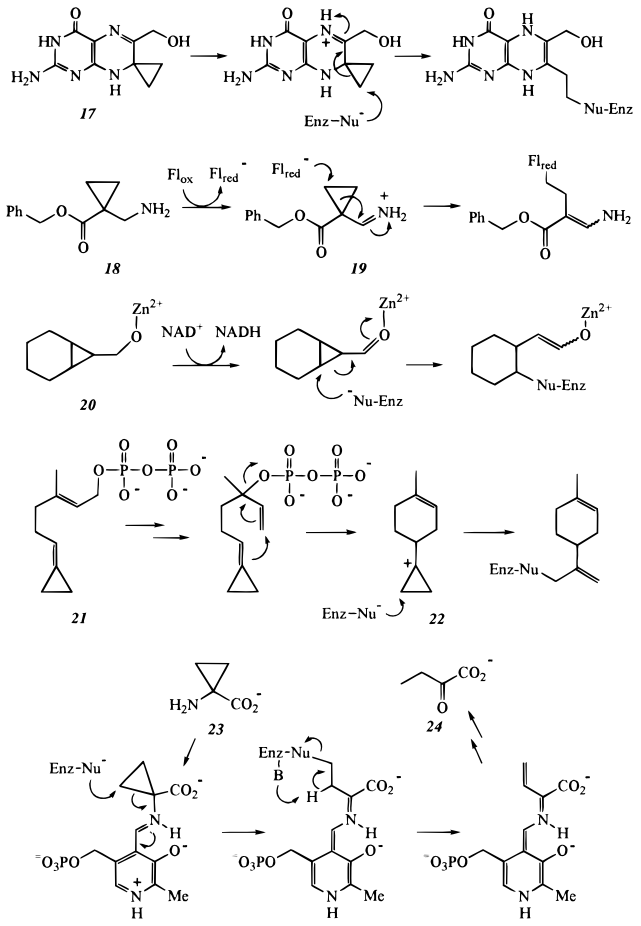
Scheme 4



by cyclopropylmethanol analogues, such as **20**, has been proposed as a result of the addition of an enzyme nucleophile to the fused three-membered ring, which is activated by the oxidation of its hydroxymethyl substituent to the corresponding aldehyde.[48] Another interesting example is an inhibitor for monoterpene cyclases, 6-cyclopropylidene-3*E*-methyl-hex-2-en-1-yl pyrophosphate (**21**).[49] This compound is believed to be processed by the cyclase to a reactive intermediate **22**, which is responsible for trapping an active site nucleophile. An analogous mechanism has also been proposed for the catalysis of 1-aminocyclopropane-1-carboxylate (ACC) deaminase, a pyridoxal 5′-phosphate (PLP)-dependent enzyme, which converts ACC (**23**) to α-ketobutyrate (**24**).[50] The ring fragmentation in this case is likely triggered by the attack of an enzyme nucleophile, followed by β,γ-elimination to afford the observed product. The initial Schiff base formation is essential to activate the cyclopropane ring, rendering it more susceptible to nucleophilic attack.

However, no such ring activation as a result of catalytic conversion seems to be necessary for the inactivation of bovine liver ECH by MCPF-CoA (**3**). It has been shown that the enoyl-CoA substrate is polarized in the active site of rat liver ECH by the two hydrogen bonds formed between the thioester carbonyl oxygen and the backbone NH groups of Gly-141 and Ala-98, and the presence of Glu-164 and Glu-144 proximal to the C_α-C_β bond of the substrate serves as the general acid/base in catalysis.[42,43] Such an electron polarization induced by a local electric field provides the driving force for the activation of enoyl-CoA in the active site.[51,52] A similar mode of substrate activation has also been proposed for the catalysis of keto steroid isomerase.[53] It is conceivable that MCPF-CoA is activated via the same push−pull mechanism, which enhances the electrophilicity at β-C and, thus, facilitates the trapping of an active site nucleophile. The large ring strain associated with the methylenecyclopropane system may also contribute to the reactivity of MCPF-CoA.

In summary, we have established that MCPF-CoA (**3**), a toxic metabolite of MCPG (**1**), is a potent inhibitor for ECHs. Since MCPF-CoA shows little effect on other β-oxidation enzymes tested in this study, it appears to be a more selective inhibitor against ECHs. Our results also showed that MCPF-CoA manifests distinct modes of inhibition among ECHs. Given the high homology of all mammalian ECH genes studied so far, the sequence and the structure of bovine liver ECH is expected to closely resemble that of the rat liver enzyme. However, the different inhibition mechanisms of these two ECHs caused by MCPF-CoA clearly indicate the existence of subtle differences between these two proteins, perhaps due to sequence variations at or near the active site. Since MCPF-CoA is a unique inhibitor for ECHs, it holds the potential to serve as a lead for the rational design of more effective agents aimed at modulating ECH activity, with a view to control and/or regulate fatty acid metabolism.

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