Novel Inactivation of Enoyl-CoA Hydratase via β-Elimination of 5,6-Dichloro-7,7,7-trifluoro-4-thia-5-heptenoyl-CoA†

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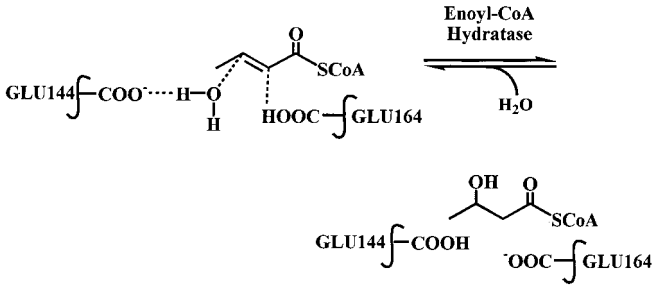
ABSTRACT: 5,6-Dichloro-7,7,7-trifluoro-4-thia-5-heptenoyl-CoA (DCTFTH-CoA) is an analogue of a class of cytotoxic 4-thiaacyl-CoA thioesters that can undergo a β-elimination reaction to form highly unstable thiolate fragments, which yield electrophilic thioketene or thionoacyl halide species. Previous work demonstrated that the medium-chain acyl-CoA dehydrogenase both bioactivates and is inhibited by these CoA thioesters through enzyme-catalyzed β-elimination of the reactive thiolate moiety [Baker-Malcolm, J. F., Haeffner-Gormley, L., Wang, L., Anders, M. W., and Thorpe, C. (1998) *Biochemistry 37*, 1383−1393]. This paper shows that DCTFTH-CoA can be directly bioactivated by the enoyl-CoA hydratase (ECH) with the release of 1,2-dichloro-3,3,3-trifluoro-1-propenethiolate and acryloyl-CoA. In the absence of competing exogenous trapping agents, DCTFTH-CoA effects rapid and irreversible loss of hydratase activity. The inactivator is particularly effective at pH 9.0, with a stoichiometry approaching 1 mol of DCTFTH-CoA per enzyme subunit. Modification is associated with a new protein-bound chromophore at 360 nm and an increase in mass of 89 ± 5 per subunit. Surprisingly, ECH exhibiting less than 2% residual hydratase activity retains essentially 100% β-eliminase activity and continues to generate reactive thiolate species from DCTFTH-CoA. This leads to progressive derivatization of the enzyme with additional UV absorbance, covalent cross-linking of subunits, and an eventual complete loss of β-eliminase activity. A range of exogenous trapping agents, including small thiol nucleophiles, various proteins, and even phospholipid bilayers, exert strong protection against modification of ECH. Peptide mapping, thiol titrations, UV−vis spectrophotometry, and mass spectrometry show that inactivation involves the covalent modification of Cys62 and/or Cys111 of the recombinant rat liver ECH. These data suggest that enoyl-CoA hydratase is an important enzyme in the bioactivation of DCTFTH-CoA, in a pathway which does not require involvement of the medium-chain acyl-CoA dehydrogenase.

Enoyl-CoA hydratase (ECH,[1] EC 4.2.1.17, crotonase) catalyzes the rapid, concerted (*1*, *2*), syn addition (*3*) of water across the double bond of a range of *trans*-2-enoyl-CoA derivatives (*4*, *5*), yielding the corresponding L-3-hydroxyacyl-CoA derivatives (Scheme 1). Recent crystallographic and mutagenesis studies suggest that Glu144 activates a water molecule for attack on the β-carbon of *trans*-2-enoyl-CoA substrates and Glu164 serves as a general acid to protonate the developing enolate at the α-position (*6*−*12*). In addition to normal catalysis, enoyl-CoA hydratase promotes α-proton exchange for a number of saturated acyl-CoA analogues (e.g., butyryl- and octanoyl-CoA; *6*). Strong polarization of the thioester carbonyl group, observed with thermodynamically unfavorable hydratase substrates, such as 4-(*N*,*N*-dimethylamino)cinnamoyl-CoA (DAC-CoA; *13*), presumably contributes to lowering the acidity of the α-proton of the bound acyl-CoA thioesters.

Scheme 1



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[1] Abbreviations: ECH, enoyl-CoA hydratase; CoA(SH), coenzyme A; DAC-CoA, 4-(*N*,*N*-dimethylamino)cinnamoyl-CoA; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); MCPF-CoA, methylenecyclopropylformyl-CoA; DCTH-CoA, 5,6-dichloro-4-thia-5-hexenoyl-CoA; BTTB-CoA, 4-(2-benzothiazole)-4-thiabutanoyl-CoA; DCTFTH-CoA, 5,6-dichloro-7,7,7-trifluoro-4-thia-5-heptenoyl-CoA; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane HCl; MALDI-TOF-MS, matrix-assisted laser desorption ionization time-of-flight mass spectrometry; TFA, trifluoroacetic acid; CTFTH-CoA, 6-chloro-5,5,6-trifluoro-4-thiahexanoyl-CoA; NPTB-CoA, 4-(4-nitrophenyl)-4-thiabutanoyl-CoA; DNPTB-CoA, 4-(2,4-dinitrophenyl)-4-thiabutanoyl-CoA; DTT, dithiothreitol; GSH, glutathione; NAC, *N*-acetylcysteamine; NEM, *N*-ethylmaleimide; BSA, bovine serum albumin; RNase A, ribonuclease A; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine.

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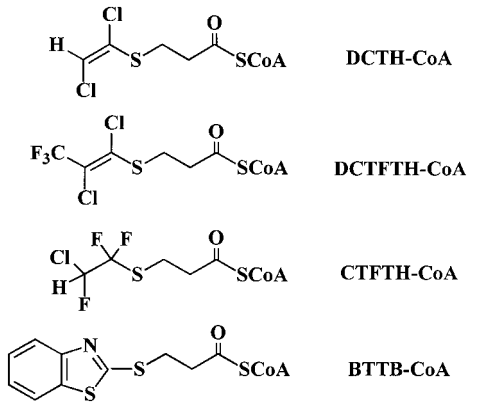
Chart 1: 4-Thiaacyl-CoA Analogues Used in This Work[a]



[a] DCTH-CoA, 5,6-dichloro-4-thia-5-hexenoyl-CoA; DCTFTH-CoA, 5,6-dichloro-7,7,7-trifluoro-4-thia-5-heptenoyl-CoA; CTFTH-CoA, 6-chloro-5,5,6-trifluoro-4-thiahexanoyl-CoA; BTTB-CoA, 4-(2-benzothiazole)-4-thiabutanoyl-CoA.

The enoyl-CoA hydratases appear rather refractory to inactivation by most traditional group specific reagents. For example, various sulfhydryl reagents partially inhibit ECH at millimolar concentrations (*5*, *14*, *15*). DTNB treatment leads to partial inactivation of the hydratase after the first thiol group per subunit is modified (*16*, *17*). Only *p*-chloromercuribenzoate, at a concentration of 1 mM, inhibits the enzyme completely (*5*, *14*, *15*).

This sluggish reactivity extends to potential affinity labels of ECH. Thus, treatment with *p*-bromoacetamido-*trans*-cinnamoyl-CoA shows a limiting k_{inact} of 0.03 min^{-1} at saturating inhibitor concentrations (*18*). Halogenated substrate analogues, such as 3-chloro-, 4-chloro-, and 2-bromocrotonyl-CoA, do not inhibit the enoyl-CoA hydratase and, in fact, prove to be fairly good substrates for the hydration reaction, despite the proximity of a reactive group to the catalytic residues (*18*). Furthermore, while 2-alkynoyl-CoA analogues are potent mechanism-based inhibitors of the acyl-CoA dehydrogenases (*19*−*21*), they are facilely hydrated by ECH to the corresponding 3-keto analogues (*22*). This reaction might be of physiological relevance in the detoxification of potentially injurious 2-alkynoic acids (*22*).

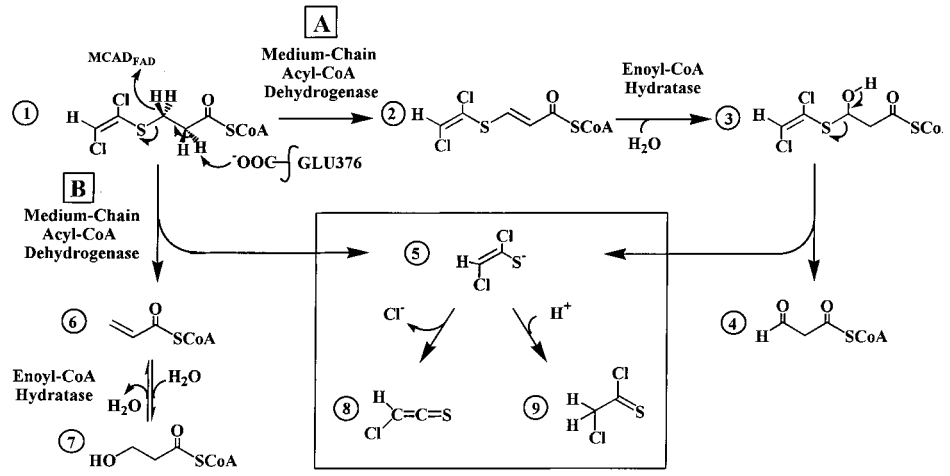
Enoyl-CoA hydratase is, however, inactivated by methylenecyclopropylformyl-CoA (MCPF-CoA) produced during the degradation of methylenecyclopropylglycine found in litchi seeds (*23*). Ghisla and colleagues have shown that a high molar excess of MCPF-CoA irreversibly inhibits pig kidney ECH (*24*). Liu and associates extended these studies (*25*, *26*) and report that MCPF-CoA slowly inhibits bovine, pig, and recombinant rat ECH (with inactivation half-complete in about 0.7, 20, and 5 h, respectively, using 187 μM thioester and 10 μM ECH).

The work presented here demonstrates an unexpectedly facile mode of irreversible inactivation of enoyl-CoA hydratase revealed during ongoing studies of the mechanism of the bioactivation of cytotoxic 4-thia-fatty acids by β-oxidation (*27*−*29*). Some of the 4-thiaacyl-CoA analogues used in this work are shown in Chart 1. Anders and colleagues have shown that one of the most intensively studied 4-thia-fatty acids, 5,6-dichloro-4-thia-5-hexenoic acid (DCTH), is a potent hepatotoxin and nephrotoxin (*30*, *31*). Two interrelated β-oxidation pathways for the activation of DCTH-CoA have been identified so far (Scheme 2; *27*, *28*).

In the first pathway, the medium-chain acyl-CoA dehydrogenase oxidizes DCTH-CoA (**1**) to the corresponding *trans*-2-enoyl-CoA analogue (**2**), which serves as the substrate for enoyl-CoA hydratase (Scheme 2, pathway A). The resulting thiohemiacetal (**3**) decomposes nonenzymatically to malonyl-semialdehyde-CoA (**4**) and 1,2-dichloroethenethiolate (**5**). Direct β-elimination catalyzed by the medium-chain acyl-CoA dehydrogenase provides the second route to 1,2-dichloroethenthiolate (**5**, Scheme 2, pathway B). Elimination is accompanied by the eventual covalent inactivation of the dehydrogenase by an electrophilic fragment or fragments, generated from rearrangement of the 1,2-dichloroethanethiolate (**5**). Two candidates are presented in the boxed area in Scheme 2: the chlorothioketene (**8**) and the chlorothionoacyl chloride (**9**). In vivo, one or more of these reactive species are thought to acylate critical cellular targets, leading to the cytotoxicity observed for DCTH (*32*−*40*).

Note that both pathways A and B in Scheme 2 require participation of the acyl-CoA dehydrogenase. However, the observation that enoyl-CoA hydratase catalyzes α-proton exchange with a variety of acyl-CoA thioesters (*6*) and the finding that it could activate 4-(2-benzothiazole)-4-thiabutanoyl-CoA (BTTB-CoA; *29*; see below) suggested that it, too, might activate cytotoxic 4-thiaacyl-CoA analogues directly. This is the case. One of these compounds, DCTFTH-CoA (Chart 1), proves to be a particularly potent irreversible

Scheme 2



inactivator of ECH. In comparison, DCTFTH-CoA is approximately 300-fold faster than MCPF-CoA (*26*), when the recombinant rat liver ECH is incubated under comparable conditions (10 μM ECH, 19-fold excess of inhibitor, and pH 7.6; see below). Further, rat liver ECH, in contrast to the bovine enzyme, is reversibly inhibited by MCPF-CoA (*26*). Not only does the work presented here with DCTFTH-CoA provide a number of unusual mechanistic features, but it also gives valuable insight into the bioactivation of these cytotoxic fatty acid analogues.

MATERIALS AND METHODS

Materials. CoASH (lithium salt), crotonyl-CoA, *N*-ethylmaleimide, glutathione (reduced form), dithiothreitol, histidine, lysine, tyrosine, adenosine, urea, 5,5′-dithiobis(2-nitrobenzoic acid), rabbit muscle glyceraldehyde 3-phosphate dehydrogenase, rabbit muscle aldolase, bovine pancreas ribonuclease A, bovine serum albumin, and native calf thymus DNA were purchased from Sigma. *N*-Acetylcysteamine, 4-(*N*,*N*-dimethylamino)cinnamic acid, 2,5-dihydroxybenzoic acid, 3,5-dimethoxy-4-hydroxycinnamic acid, and trifluoroacetic acid were purchased from Aldrich. Ammonium bicarbonate, EDTA, and HPLC grade acetonitrile, methanol, monobasic potassium phosphate, thymidine, and cytosine were purchased from Fisher. Protein assay mix was from Bio-Rad. Guanidine HCl and Tris-HCl were from ICN. Sequencing grade modified trypsin was from Promega. Guanosine was from Acros. Pyrene maleimide was from Molecular Probes. 1,2-Dimyristoyl-*sn*-glycero-3-phosphocholine from Avanti Polar-Lipids was a gift from M. K. Jain (University of Delaware).

Recombinant enoyl-CoA hydratase from rat liver mitochondria was overexpressed from pET20ech1 and purified as described previously (*9*) with slight modifications. Cells from a 1 L fermentation were centrifuged, resuspended in lysis buffer [50 mM potassium phosphate buffer (pH 7.4), 3 mM EDTA, 0.5 mM DTT, and 100 μg/mL lysozyme] for 30 min, and processed through a French press. Cellular debris was removed by centrifugation and the supernatant brought to 1% (w/v) streptomycin sulfate. The resulting precipitate was removed by centrifugation and the supernatant brought to 60% saturation in ammonium sulfate and applied to a 2 cm $\times$ 15 cm octyl-Sepharose column. ECH eluted at approximately 20% NH_4SO_4 in 20 mM phosphate buffer (pH 7.4) using either a decreasing step or a linear gradient. The collected fractions were combined and dialyzed prior to the CoA-Sepharose affinity step, as previously described (*9*). For some experiments, ECH was additionally purified by two crystallizations from ethanol (*9*), followed by FPLC gel filtration using a Superdex 200 column in 200 mM phosphate buffer (pH 7.4) and 0.3 mM EDTA. The concentration of recombinant rat liver ECH was determined using an extinction coefficient of 14.6 mM^{-1} cm^{-1} at 280 nm for the protein subunit as determined by comparison to a value of 12.33 mM^{-1} cm^{-1} at 280 nm in 6 M guanidine hydrochloride and 50 mM potassium phosphate (pH 6.5) (*41*). The concentration of the DCTFTH-CoA-modified enzyme was determined using the Bio-Rad protein assay in which a standard curve of native enoyl-CoA hydratase was utilized. The concentrations of aldolase, ribonuclease A, bovine serum albumin, and glyceraldehyde 3-phosphate dehydrogenase were determined at 280 nm using the following extinction coefficients: 37.5, 7.8, 39.0, and 36.7 mM^{-1} cm^{-1}, respectively, as stated in the 1999 Worthington Biochemical Corp. Enzyme Manual.

Instrumental Analyses. Static absorbance measurements were recorded at 25 °C on a Hewlett-Packard 8452A diode-array spectrophotometer. Where noted, turbidity corrections were made using the scatter correct routine included in the 8452A software program. Enzyme assays were carried out using a Perkin-Elmer 552A spectrophotometer at 25 °C. Data were analyzed using Graphpad InPlot (version 4.03). HPLC separations were performed on a Hewlett-Packard 1100 Series liquid chromatograph equipped with a diode-array detector and analyzed using the Hewlett-Packard LC ChemStation software. All solvents and buffers were filtered using 0.2 μm nylon filters (Whatman), and samples were filtered using either microfilterfuge tubes (Rainin; 0.2 micron) or Centricon-10 microconcentrators (Amicon). Peptide sequencing was performed using an Applied Biosystems gas phase protein sequencer (model 470A/120A/900A) according to the manufacturer's instructions. MALDI-TOF mass spectrometric analysis was performed using a Perseptive Biosystems Voyager-DE instrument in the linear mode. 3,5-Dimethoxy-4-hydroxycinnamic and 2,5-dihydroxybenzoic acids were used as matrixes. Typically, 1.0 μL of a 10 mg/mL solution of the matrix (in 30% acetonitrile/0.1% TFA) was applied to the sample plate, immediately followed by 1.0 μL of the sample. The combined spot was dried in vacuo. Spectra were collected in the positive-ion mode. The manufacturer's recommended data collection methods were utilized and modified slightly depending on the sample of interest. Electrospray mass spectrometric analysis was performed using a Micromass Quattro II triple-quadrupole mass spectrometer. The electrospray mass spectra were deconvoluted using the manufacturer's software. ^{19}F NMR spectra were recorded on a Bruker-400 instrument equipped with a 5 mm ^{19}F probe (QNP) operating at 376.5 MHz for fluorine. Spectra were recorded at room temperature, and chemical shifts are expressed in parts per million downfield with respect to external trichlorofluoromethane.

Preparation of 4-Thiaacyl-CoA Compounds. The following acids were synthesized as described previously: 5,6-dichloro-4-thia-5-hexenoic acid (DCTH; *42*), 6-chloro-5,5,6-trifluoro-4-thiahexanoic acid (CTFTH; *30*), 4-(2-benzothiazole)-4-thiabutanoic acid (BTTB; *30*), 4-(4-nitrophenyl)-4-thiabutanoic acid (NPTB; *28*), and 4-(2,4-dinitrophenyl)-4-thiabutanoic acid (DNPTB; *28*). 5,6-Dichloro-7,7,7-trifluoro-4-thia-5-heptenoic acid (DCTFTH) was synthesized as described previously (*28*) with the following modifications. 3-Mercaptopropionic acid (18.8 mmol) was added dropwise to a suspension of lithium hydride (37.8 mmol) in 15 mL of dry dimethylformamide with stirring at 0 °C. After 15 min, 1,1,2-trichloro-3,3,3-trifluoro-1-propene (32.3 mmol; PCR Inc., Gainesville, FL) was added, and the reaction mixture was stirred for 7 h at room temperature. The reaction was quenched with 10 mL of saturated aqueous ammonium chloride, and the mixture was acidified with 4 mL of concentrated HCl and extracted with ethyl acetate. The combined organic layers were dried over anhydrous sodium sulfate. To safely remove excess 1,1,2-trichloro-3,3,3-trifluoro-1-propene, 10 mL of toluene was added and the mixture was distilled until the temperature reached 110 °C. The remaining solution was diluted with 50 mL of ethyl acetate and washed with five 50 mL portions of 1 N HCl.

The organic layer was dried over anhydrous sodium sulfate and evaporated under reduced pressure. The crude product was filtered through silica gel and evaporated under reduced pressure. A white solid impurity was removed by crystallization from 20% ethyl acetate in hexane, and the residual oil was distilled under reduced pressure to yield a mixture of cis and trans isomers: ^{1}H NMR ($CDCl_3$) δ 2.7 (t, 0.6H), 2.8 (t, 1.4H), 3.2 (t, 0.6H), 3.3 (t, 1.4H); ^{19}F NMR (CD_2Cl_2) δ −58.7 (s, 0.9F), −60.5 (s, 2.1F).

All of the 4-thia-fatty acids, as well as 4-(*N*,*N*-dimethylamino)cinnamic acid, were converted to their corresponding CoA thioesters by the mixed anhydride method (*43*), utilizing isobutylchloroformate as a substitute for ethylchloroformate. Purification and desalting procedures utilized a semipreparative octadecylsilica column (Zorbax) with a methanol gradient as described previously (*44*). All CoA thioesters were detected at 260 nm and were further characterized by NMR or MALDI-TOF-MS analysis. Extinction coefficient values for the thioesters of DCTH-, CTFTH-, BTTB-, NPTB-, and DNPTB-CoA were used as reported previously (*28*). The extinction coefficients of the acid and coenzyme A thioester form of DCTFTH were reevaluated and determined to be 5.0 mM^{-1} cm^{-1} at 268 nm and 20.0 mM^{-1} cm^{-1} at 260 nm, respectively. Concentrations of the thiolate elimination products of BTTB-CoA, NPTB-CoA, and DNPTB-CoA were determined from their reported extinction values (*28*). The extinction coefficients used to quantitate crotonyl-CoA and DAC-CoA were 20.0 and 16.5 mM^{-1} cm^{-1} (*13*), respectively, at 260 nm.

Hydratase and β-Elimination Assays. Hydratase assay mixtures contained 150 μM crotonyl-CoA and 0.1−10 nM ECH in 50 mM potassium phosphate buffer (pH 7.6) containing 0.3 mM EDTA. Activity was measured at 25 °C by following the decrease in absorbance at 280 nm ($\Delta\epsilon_{280}$ = 3.6 mM^{-1} cm^{-1}) for the conversion of crotonyl-CoA to 3-hydroxybutyryl-CoA (*45*).

Enoyl-CoA hydratase elimination studies were performed as previously described for the medium-chain acyl-CoA dehydrogenase HPLC partition studies (*28*). ECH (0.4 or 0.5 μM) was incubated at 25 °C with 25 μM DCTFTH-, DCTH-, BTTB-, and 4-thiaoctanoyl-CoA for 90 min in 50 mM Tris-HCl buffer (pH 9.0) containing 0.3 mM EDTA. After 90 min, incubation mixtures were ultrafiltered using Centricon-10 filters. Filtrates were then analyzed by HPLC using an analytical Resolvex octadecylsilica column with a linear gradient formed from acetonitrile and 25 mM potassium phosphate (pH 5.3) as reported previously (*27*).

Elimination activity of the enoyl-CoA hydratase was also followed spectrophotometrically using BTTB-, NPTB-, and DNPTB-CoA (see above) using 1 μM enzyme with these chromophoric thioesters (50 μM) at pH 7.6. Steady state parameters for the elimination reaction with BTTB-CoA were determined using 220 nM ECH by following the formation of 2-mercaptobenzothiazole from BTTB-CoA ($\Delta\epsilon_{310}$ = 15.9 mM^{-1} cm^{-1}; *28*). The pH dependence on ECH-catalyzed thiolate elimination from BTTB-CoA was determined under similar conditions using the following buffers containing 0.3 mM EDTA: 50 mM potassium phosphate at pH 6.0 and 7.6 and 50 mM Tris-HCl at pH 7.1, 7.6, 8.0, and 9.0.

Enzyme Inactivation. Unless otherwise stated, all inactivations were performed at 25 °C in 50 mM Tris-HCl (pH 9.0) containing 0.3 mM EDTA. Samples were withdrawn and diluted with 50 mM potassium phosphate (pH 7.6) prior to being assayed. The rate constants of ECH inactivation for selected 4-thiaacyl-CoA thioesters were determined as described in the legend of Figure 1. The stoichiometry of DCTFTH-CoA inactivation was determined by incubating ECH (0.5, 10, and 50 μM) with increasing concentrations of the inhibitor for 90 min in 50 mM Tris-HCl (pH 9.0).

Table 1: Protection against DCTFTH-CoA Inactivation of Enoyl-CoA Hydratase[a]

	protectant	protectant concentration	DCTFTH-CoA concentration	percent activity remaining[b]
thiol	DTT	1 mM	20 μM	100
	NAC	1 mM	20 μM	100
	GSH	1 mM	20 μM	82.7
amino acids	lysine, histidine, tyrosine	1 mM each	25 μM	2.6
protein	BSA	50 μM	2.5 μM	93.5
	GAPDH	50 μM	2.5 μM	100
	RNase A	50 μM	2.5 μM	20.8
	aldolase	50 μM	2.5 μM	98.7
	NEM-aldolase[c]	50 μM	2.5 μM	76.0
vesicle	DMPC	1 mg/mL	2.5 μM	60.4
	DMPC	4 mg/mL	2.5 μM	70.8
DNA	adenosine, cytosine, guanosine, thymidine	1 mM each	2.5 μM	10.8[d]
	calf thymus	0.25 mg/mL	2.5 μM	11.7[d]
none		0	2.5 or 25 μM	4.0 ± 3.0

[a] ECH (0.4−0.5 μM) was incubated with DCTFTH-CoA at 25 °C in 50 mM Tris-HCl buffer (pH 9.0) in the presence or absence of protectant. [b] Activities were measured after a 30 min incubation period for vesicle protection, a 60 min incubation period for thiol protection, and a 90 min incubation period for amino acid, protein, and DNA protection. Activities at the end of the incubation period were compared to those of control mixtures containing similar protectant content and concentration without inhibitor, which were taken as 100% active. [c] NEM-aldolase was alkylated as described in Materials and Methods. [d] The control enzyme incubation with DCTFTH-CoA for the DNA protection experiments resulted in 10.5% activity remaining at 90 min due to the aged ECH stock that was used.

Coenzyme A thioester protection against DCTFTH-CoA inactivation was assessed using 0.4 μM ECH with 500 μM crotonyl-CoA or 250 μM DAC-CoA in the absence (taken as 100%) or in the presence of 20 μM DCTFTH-CoA. Protection of ECH against DCTFTH-CoA inactivation using small nucleophiles, proteins, vesicles, and DNA was performed as described in Table 1. Controls were performed to ensure that the inclusion of these protectants, after dilution, did not affect the crotonyl-CoA assay system. The NEM-alkylated aldolase, used as a protein protectant in Table 1, was prepared by incubating aldolase at 25 °C in 50 mM Tris-HCl (pH 9.0) containing 0.3 mM EDTA with a 2-fold excess of NEM over total thiols. After 30 min, excess NEM was removed by ultrafiltration. The concentration of thiols was measured before and after alkylation in 50 mM potassium phosphate buffer (pH 7.6) containing 500 μM DTNB. The vesicle protection experiments were performed using a stock solution of 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC), which was prepared in water (20 mg/mL) and frozen at −20 °C. Prior to use, the frozen DMPC stock was thawed in a bath-type sonicator and sonication was continued until the DMPC solution changed from turbid to clear (typically, 30 min). The DMPC stock was then diluted to

Table 2: Thiol Content Analysis of DCTFTH-CoA-Modified Enoyl-CoA Hydratase[a]

DCTFTH-CoA used to modify ECH (equiv)	observed amount of thiol[b]
0	5.0
1	3.4
2	3.0
10	2.4

[a] ECH (60 μM) was incubated without or with 1, 2, and 10 equiv of DCTFTH-CoA for 90 min at 25 °C in Tris-HCl (pH 9.0) containing 0.3 mM EDTA. After 90 min, the enzyme was washed by ultrafiltration and recovered in 50 mM potassium phosphate buffer (pH 7.6) containing 0.3 mM EDTA. The washed enzyme (2.6−3.9 μM final concentration) was added to 500 μM DTNB in 50 mM potassium phosphate (pH 7.6) containing 0.3 mM EDTA with 7.5 M guanidine HCl. [b] The thiol content was determined spectrophotometrically (ϵ_{412} = 13.6 mM^{-1} cm^{-1}; *47*).

the stated concentrations for the protection experiments described in Table 1.

Properties of DCTFTH-CoA-Modified ECH. Irreversible inactivation of the modified ECH was demonstrated by extensive washing with Tris-HCl buffer (pH 9.0) using a Centricon-10 ultrafiltration device. Reversal of DCTFTH-CoA inactivation by nucleophiles was tested for the modified ECH, prepared as described in Table 2. The dialyzed, unmodified, and 1, 2, and 10 equiv-of-DCTFTH-CoA-modified enoyl-CoA hydratases (0.5 μM) were then incubated with 1 mM DTT for 90 min at 25 °C in Tris-HCl (pH 9.0) containing 0.3 mM EDTA and assayed in duplicate as described earlier. Activities were compared to the unmodified control taken to be 100% active. Controls were also performed to ensure that modified ECH did not regain activity in the absence of DTT. The thiol content of DCTFTH-CoA-modified ECH was assessed as described in Table 2.

DCTFTH-CoA-modified enoyl-CoA hydratase was prepared for electrospray mass spectrometric analysis by incubating 5 μM ECH at 25 °C for 90 min with or without 1 equiv of DCTFTH-CoA in 50 mM Tris-HCl (pH 9.0). After 90 min, the pH was adjusted to 7.0 and the reaction was stopped by cooling the mixture to −20 °C. Controls were performed to ensure that this treatment did not affect activity. The protein samples were then injected onto a Vydac octadecylsilica column and were eluted isocratically at a rate of 180 μL/min with 50% methanol and 2% acetic acid. The eluent was directly analyzed by mass spectrometry.

Ligand Binding by the DCTFTH-CoA-Modified ECH. The dissociation constant for DAC-CoA binding was determined in 50 mM potassium phosphate buffer (pH 7.6) for both 2 equiv-modified (2% active, 5.8 μM) and native ECH (13.2 μM). Data were analyzed by following the absorbance changes at 496 nm.

Elimination activity of unmodified and 2, 5, and 50 equiv-of-DCTFTH-CoA-modified ECH was assessed spectrophotometrically by incubating 0.5 μM dialyzed enzyme (prepared as described in the legend of Figure 4) with 50 μM BTTB-CoA for 30 min in 50 mM phosphate buffer (pH 7.6). Data were analyzed as described in the legend of Figure 5. Additionally, 500 μM crotonyl-CoA was added to these assay mixtures to examine the effect of hydratase substrate on the elimination rate with BTTB-CoA. As a control, the 2 equiv-of-DCTFTH-CoA-modified ECH was assayed for hydration activity after the 30 min incubation with BTTB-CoA in the presence or absence of crotonyl-CoA to ensure that hydration activity was not recovered.

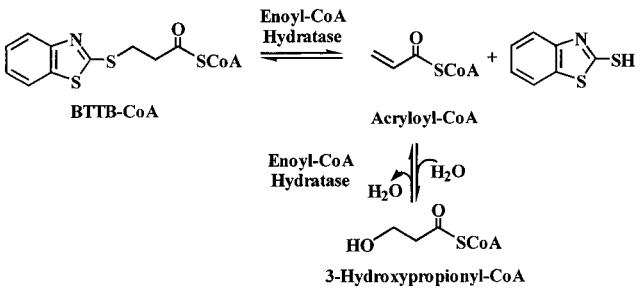
Enzyme Digests. The rat liver enoyl-CoA hydratase (40 μM) was incubated with and without 200 μM DCTFTH-CoA at 25 °C in 500 μL of 50 mM Tris-HCl buffer (pH 9.0) containing 0.3 mM EDTA. After 90 min, the incubation mixtures were washed and concentrated by ultrafiltration. The modified and native enzymes (30 μM) were mixed with 500 μM pyrene maleimide in 1.5 M urea. The solutions were placed in a boiling water bath for 5 min and then cooled on ice for 10 min. Excess pyrene maleimide was extracted with ether, and the enzyme samples were vacuum-centrifuged to a final volume of 200 μL. Ammonium bicarbonate (pH 7.5) was added to the samples prior to digestion, resulting in final bicarbonate and urea concentrations of 100 mM and 2 M, respectively. Sequencing grade modified trypsin (2.0% w/w aliquots) was added at 0 and 2 h, and digestion was continued with stirring for 24 h at 37 °C. Digestion was terminated by ultrafiltration, and samples were stored at −20 °C.

HPLC was performed on 100 μL of the digest using a Zorbax 300 Δ pore octadecylsilica reverse phase column at a flow rate of 1 mL/min. The gradient was formed from water (solvent A) and acetonitrile (solvent B) each containing 0.1% v/v TFA: 100% solvent A for 5 min, from 0 to 60% B in a linear gradient over the course of 120 min, and a linear gradient to 100% B over the course of 20 min. Individual peaks were collected manually on the basis of the 220 nm elution profile, and selected peaks were further analyzed by UV−vis, peptide sequencing, and MALDI-TOF-MS. Peptides selected for sequencing were first lyophilized to dryness and then redissolved in approximately 50 μL of a 50% acetonitrile solution containing 0.1% TFA. Typically, 1−5 nmol of peptide was used for analysis.

RESULTS AND DISCUSSION

Recombinant Rat Liver Enoyl-CoA Hydratase-Catalyzed β-Elimination of Activated Thiols. In the reverse of the physiological catalytic direction, ECH rapidly eliminates a hydroxyl group from 3-OH-acyl-CoA thioesters, yielding the corresponding unsaturated *trans*-2-enoyl-CoA. Similarly, elimination of a chloride ion from 3-chloropropionyl-CoA yields acryloyl-CoA, which is subsequently hydrated to 3-OH-propionyl-CoA (*6*). Recently, it has been shown that even relatively bulky thiolate species can be eliminated from the C-3 position by ECH (*12*, *29*). We have shown that enoyl-CoA hydratase releases 1 equiv of the chromophoric thiolate fragment, 2-mercaptobenzothiazole, from BTTB-CoA (Scheme 3; data not shown; *29*).

Scheme 3



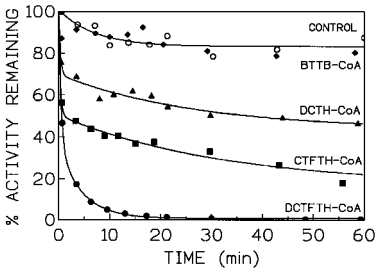


FIGURE 1: Inactivation of enoyl-CoA hydratase with BTTB-, DCTH-, CTFTH-, and DCTFTH-CoA. ECH (0.4 μM) was incubated at 25 °C with 50 μM BTTB-CoA (◆), DCTH-CoA (▲), CTFTH-CoA (■), and DCTFTH-CoA (●) in 50 mM Tris-HCl (pH 9.0) containing 0.3 mM EDTA. A control incubation with enzyme alone is shown (○). At specified times during the incubation, aliquots were removed, diluted with 50 mM potassium phosphate buffer (pH 7.6) containing 0.3 mM EDTA, and assayed using crotonyl-CoA as described in Materials and Methods. Solid lines are fit through the data points using a two-exponential decay equation: DCTH-CoA, rate constants of 3.1 and 0.04 min^{-1} with amplitudes of 30.2 and 26.2%, respectively; CTFTH-CoA, rate constants of 3.4 and 0.03 min^{-1} with amplitudes of 49.6 and 33.4%, respectively; and DCTFTH-CoA, rate constants of 2.0 and 0.22 min^{-1} with amplitudes of 63.5 and 35.7%, respectively.

Further, we have confirmed the presence of 3-OH-propionyl-CoA (Scheme 3) derived from the facile hydration of the liberated acryloyl-CoA with the HPLC retention time, UV absorbance, and mass spectrum (*29*; see Materials and Methods).

Initial rates of thiol release were plotted as a function of BTTB-CoA concentration, giving a TN_{max} of 7.0 min^{-1} and a K_M of 11 μM in 50 mM potassium phosphate buffer (pH 7.6) (*29*), similar to recently reported values (*12*). Comparable steady state parameters were obtained over the pH range of 6.0−9.0 using 50 mM potassium phosphate and Tris-HCl buffers (data not shown; *29*), indicating a pH independence of the elimination reaction using BTTB-CoA as a substrate (data not shown; see below).

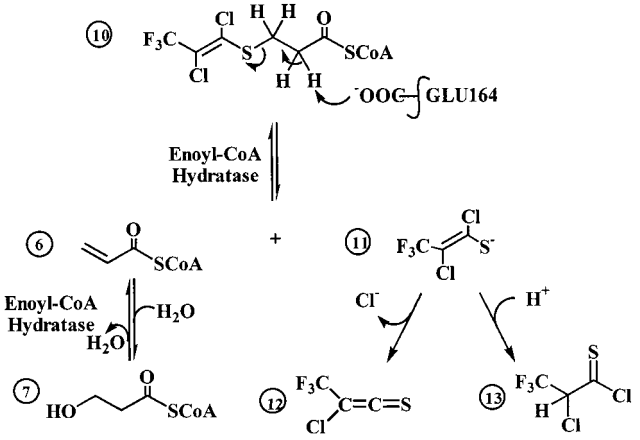
Two other compounds that would release chromophoric thiolates upon β-elimination were tested with ECH. 4-(2,4-Dinitrophenyl)-4-thiabutanoyl-CoA (DNPTB-CoA) and 4-(4-nitrophenyl)-4-thiabutanoyl-CoA (NPTB-CoA) were found to be poor elimination substrates of ECH, as compared to BTTB-CoA (with formation of only 3.5 and 0.5 μmol of the respective thiolate after incubation for 30 min with 1 μM ECH, data not shown; see Materials and Methods). 4-Thiaoctanoyl-CoA appeared to be insignificantly activated for detectable elimination as determined by HPLC (see Materials and Methods).

These data confirm that the enoyl-CoA hydratase catalyzes β-elimination of particular activated chromophoric thiolates. We then extended this investigation to CoA thioesters of the potentially cytotoxic halogenated fatty acid analogues shown in Chart 1. We have previously shown that DCTH-, DCTFTH-, and CTFTH-CoA are all irreversible, mechanism-based inactivators of the medium-chain acyl-CoA dehydrogenase in a reaction that is initiated by generation of a reactive thiolate fragment (*27, 28*).

Inactivation of Enoyl-CoA Hydratase by 4-Thiaacyl-CoA Analogues: Preliminary Studies. Figure 1 surveys the behavior of ECH (0.4 μM) toward the 4-thiaacyl-CoA analogues shown in Chart 1. As observed with the medium-chain acyl-CoA dehydrogenase (*28*), BTTB-CoA is not an inactivator of the enoyl-CoA hydratase (compare the diamonds with the white circles of the control incubation in Figure 1). This shows that elimination of a thiolate fragment, with creation of acryloyl-CoA in the active site of ECH, does not automatically lead to significant inactivation of the enzyme. However, when the incubation was repeated with DCTH-CoA, CTFTH-CoA, and DCTFTH-CoA (Chart 1), sizable decreases in activity were observed (Figure 1). In every case, the inactivation was biphasic, and the smooth curves are fit to a two-exponential process (see the legend of Figure 1). CTFTH-CoA is a slightly more potent inactivator than DCTH-CoA under these conditions, showing that the 5,6-double bond in DCTH-CoA is not a requirement for inactivation (Chart 1). However, of the compounds tested to date, DCTFTH-CoA is the most dramatic inactivator of the recombinant rat liver ECH (as well as the bovine liver enzyme; data not shown). The remainder of this investigation focuses on the DCTFTH-CoA inactivation of the rat liver enoyl-CoA hydratase.

Inactivation of the ECH by DCTFTH-CoA. Reaction of DCTFTH-CoA (**10**) with the enoyl-CoA hydratase (Scheme 4) would be expected to generate 1,2-dichloro-3,3,3-trifluoro-1-propenethiolate (**11**) and acryloyl-CoA (**6**), by analogy with earlier studies with BTTB and DCTH thioesters (*27*, *28*, *33*).

Scheme 4



Preliminary studies showed that inactivation with DCTFTH-CoA occurred over the pH range of 6.0−9.0, but was most rapid, and required the smallest ratio of thioester to ECH, at elevated pH. Using a single concentration of DCTFTH-CoA (25-fold molar excess incubated with 0.4 μM ECH for 90 min), the remaining activities were as follows: 0.5% at pH 9.0, 18% at pH 8.0, 37% at pH 7.6, 68% at pH 7.1 (all in 50 mM Tris buffer), and 77% at pH 6.0 in 50 mM phosphate buffer. Thus, Tris buffer at pH 9.0 was adopted for the studies described below (see Materials and Methods).

Unexpectedly, the stoichiometry of inactivation with DCTFTH-CoA is strongly dependent on the protein concentration, as shown in Figure 2. Extrapolation of the results with 10 and 50 μM ECH (circles, Figure 2) suggests that complete inactivation would be achieved with 1.1 equiv of DCTFTH-CoA. In contrast, 0.5 μM ECH (triangles) would require approximately 4 equiv. Note that these plots are significantly curved. For example, at 50 μM ECH, the inactivation observed with 0−0.3 equiv appears to extrapolate to a stoichiometry of about 0.5 equiv of DCTFTH-CoA per

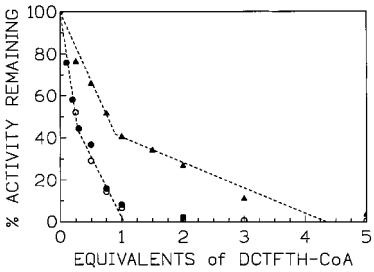


FIGURE 2: Inactivation stoichiometry of enoyl-CoA hydratase. ECH [0.5 (▲), 10 (●), and 50 μM (○)] was incubated at 25 °C for 90 min with increasing amounts of DCTFTH-CoA in 50 mM Tris-HCl (pH 9.0) containing 0.3 mM EDTA. After 90 min, the enzyme was assayed (as described earlier) in duplicate and averaged. A control incubation without inhibitor present was taken as 100% for each specific enzyme concentration.

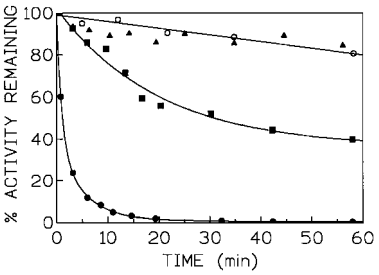


FIGURE 3: Substrate and substrate analogue protection of enoyl-CoA hydratase against DCTFTH-CoA inactivation. ECH (0.4 μM) was incubated at 25 °C with 20 μM DCTFTH-CoA in 50 mM Tris-HCl (pH 9.0) containing 0.3 mM EDTA in the absence (●) of added compounds or in the presence of either 500 μM crotonyl-CoA (▲) or 250 μM 4-(*N*,*N*-dimethylamino)cinnamoyl-CoA (■). A control incubation with enzyme alone is shown (○). At specified times during the incubation, aliquots were removed, diluted with 50 mM potassium phosphate buffer (pH 7.6) containing 0.3 mM EDTA, and assayed using crotonyl-CoA as described in Materials and Methods. Solid lines are fit to a linear equation (○), a two-exponential equation (●) with rate constants of 0.75 and 0.16 min^{-1} with amplitudes of 67.6 and 31.8%, respectively, and a single-exponential equation (■) with a rate constant of 0.05 min^{-1} and an amplitude of 66.3%.

enzyme subunit (Figure 2, dashed line). These data suggest some form of intersubunit communication, possibly promoted by covalent modification (see below). Preliminary stopped flow and static spectrophotometric studies also suggest binding inequivalence in the recombinant rat liver ECH (A. Conery and V. E. Anderson, unpublished observations).

Protection by CoA Thioesters against DCTFTH-CoA Inactivation. Figure 3 shows that 500 μM crotonyl-CoA, when added to ECH prior to the addition of a 50-fold excess (20 μM) of DCTFTH-CoA, almost completely protects the enzyme against inactivation. This behavior suggests that DCTFTH-CoA is a site-directed inactivator of ECH. The stable enoyl-CoA substrate analogue, 4-(*N*,*N*-dimethylamino)cinnamoyl-CoA (DAC-CoA; K_D of 0.5 μM; see below), at 250 μM was somewhat less effective as a protectant (Figure 3).

Effect of Endogenous Nucleophiles, Proteins, Vesicles, and DNA on the DCTFTH-CoA Inactivation of ECH. In mechanism-based inactivation (*46*), a reactive species generated during catalytic processing of the inhibitor must modify active site targets prior to dissociation from the enzyme surface. Inactivation is, thus, expected to be insensitive to nucleophiles dissolved in the bulk solvent (*46*). Thus, DCTH-CoA, CTFTH-CoA, and DCTFTH-CoA are all mechanism-based inactivators of the medium-chain dehydrogenase, and exogenous thiol nucleophiles have no effect on the course of inactivation of the enzyme (*28*). The behavior of enoyl-CoA hydratase is completely different; the effect of a range of external nucleophiles is summarized in Table 1. Thus, 1 mM DTT and *N*-acetylcysteamine (NAC) provide complete protection. GSH is somewhat less effective, but still provides substantial protection. In contrast, 1 mM histidine or lysine (not shown), or a mixture of histidine, lysine, and tyrosine (1 mM each), had no effect on the inactivation rate.

The strong protection observed with added thiols is consistent with a reactive species which diffuses into bulk solution. However, ECH has a mobile loop of residues which is believed to define the bottom of the active site for short-chain substrates but can open into a sizable solvent-filled central cavity to accommodate longer-chain analogues (*8*, *10*). Thus, it appeared to be conceivable that small exogenous thiols might attack the electrophilic inhibitory species before active site labeling had occurred. Alternatively, such access from the central cavity might intercept or redirect an intermediate species in the eventual irreversible modification of ECH (see below).

Experiments with bulky nucleophiles, and other trapping agents, make this possibility highly unlikely. For example, strong protection was observed with several exogenous proteins (Table 1). Nearly 100% activity remained in the presence of 50 μM aldolase, BSA, or GAPDH, whereas RNase A afforded comparatively little protection. As a control, we tested the possibility that the protection exerted by aldolase was due to aldolase-mediated destruction of DCTFTH-CoA, rather than it serving as a sacrificial nucleophile for the reactive species generated by ECH. HPLC analysis (see Materials and Methods) of 5 min incubation mixtures of 50 μM aldolase and 2.5 μM DCTFTH-CoA showed that 90−100% of the inhibitor remained in the absence of ECH (data not shown). Over this time interval, the hydratase activity would have declined to 31% without aldolase present. Thus, the protecting protein appears to provide a reservoir of competing nucleophilic residues which efficiently intercepts reactive species released from ECH into bulk solution. For this reason, DCTFTH-CoA does not fit the accepted criteria for a mechanism-based inactivator of enoyl-CoA hydratase (*46*).

The finding that low-molecular weight thiols exert efficient protection against inactivation of ECH and the observation that RNase A lacks free cysteine residues and is the weakest protector suggest a key role for thiol nucleophiles in protein protection. The situation is, however, more complex. Aldolase, modified with *N*-ethylmaleimide until all free thiol groups had been alkylated (see Materials and Methods), still exerted measurable protection (Table 1). This result may suggest that other nucleophilic amino acid residues could also be involved in the protection reaction. However, as described earlier, a simple mixture of lysine, histidine, and tyrosine failed to protect ECH against inactivation (Table 1). Second, strong protection was afforded by the addition of vesicles of the neutral phospholipid, DMPC (see Table 1 and Materials and Methods). Control experiments showed that the vesicles had no significant effect on the hydratase alone. Thus, in addition to thiol-mediated protection, se-

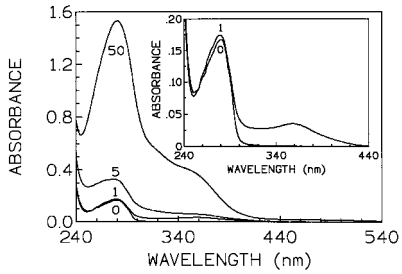


FIGURE 4: Spectra of dialyzed, DCTFTH-CoA-modified enoyl-CoA hydratase. ECH (10 μM) was incubated at 25 °C for 90 min with 0, 1, 5, and 50 equiv of DCTFTH-CoA in 50 mM Tris-HCl (pH 9.0) containing 0.3 mM EDTA. All incubations had less than 2% activity remaining at 90 min. The enzyme was then washed by ultrafiltration and recovered in Tris buffer (pH 9.0). All spectra were normalized to 10 μM on the basis of protein content as described in Materials and Methods. The inset shows spectra of 0 and 1 equiv-modified ECH on an enlarged scale.

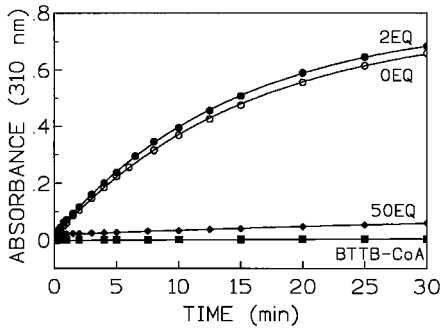


FIGURE 5: Elimination assays with BTTB-CoA of dialyzed, DCTFTH-CoA-modified enoyl-CoA hydratase. ECH was modified with DCTFTH-CoA and dialyzed with Tris (pH 9.0) as described in the legend of Figure 4. The 0 (○), 2 (●), and 50 (◆) equiv-of-DCTFTH-CoA-modified enoyl-CoA hydratases (0.5 μM) were incubated with 50 μM BTTB-CoA in 50 mM potassium phosphate buffer (pH 7.6) containing 0.3 mM EDTA. Spectra were taken at the specified time points and plotted against absorbance at 310 nm (after subtraction for BTTB-CoA absorbance) for the formation of the elimination product, 2-mercaptobenzothiazole. A control with BTTB-CoA alone in phosphate buffer (pH 7.6) is shown (■). Solid lines are fit to a single-exponential association equation: 0 equiv, rate constant of 0.064 min^{-1} and an amplitude of 0.75; and 2 equiv, rate of 0.069 min^{-1} and an amplitude of 0.76. A linear regression was used for 50 equiv and the BTTB-CoA control, corresponding to rate constants of 0.0015 and 0.0002 min^{-1}, respectively.

questration of a small neutral reactive species (e.g., such as **12** or **13** in Scheme 4) from solvent may occur in the hydrophobic interior of protein or membrane phases.

Because DNA bases may be targets of cytotoxic electrophilic sulfur species (*39*, *40*), the effect of a mixture of DNA bases (adenosine, cytosine, thymidine, and guanosine each at 1 mM) was examined. No protection was observed, and similarly, native calf thymus DNA (0.25 mg/mL) had no protective effect against the inactivation of 2.5 μM DCTFTH-CoA with 0.5 μM ECH. In all of these studies, control experiments showed that these nucleophiles, or sequestering agents, did not themselves interfere with the crotonyl-CoA assay system at the extreme dilutions used in the assay.

Properties of DCTFTH-CoA-Modified ECH. DCTFTH-CoA-treated enoyl-CoA hydratase, freed from excess reagents by extensive ultrafiltration, did not regain significant activity. Additional evidence for covalent modification is presented below. Although DTT can protect completely against loss of activity during DCTFTH-CoA inactivation experiments as described above, it is ineffective at reactivating the DCTFTH-CoA-modified enzyme. Thus, when ECH, modified with DCTFTH-CoA to residual activities of 24, 16, and 3% (see Materials and Methods), was incubated with 1 mM DTT, only slight increases in activity were observed after 60 min: to 29, 19, and 4%, respectively.

DCTFTH-CoA treatment also perturbs the UV−vis spectrum of the enoyl-CoA hydratase. Figure 4 compares the spectrum of the native enzyme with spectra of samples treated with 1, 5, and 50 equiv of DCTFTH-CoA (using 10 μM ECH). Control and treated samples were all washed extensively by ultrafiltration, so these spectral differences reflect covalent modification of ECH. The inset emphasizes the chromophore that develops upon addition of 1 equiv of DCTFTH-CoA (1.8% residual hydratase activity). Clearly, the adenine moiety of CoA is not retained during modification. Thus, the added absorbance (ϵ_{260} = 15.4 mM^{-1} cm^{-1}) would be a prominent addition to the unusually low extinction coefficient of ECH (ϵ_{280} = 14.6 mM^{-1} cm^{-1} and ϵ_{260} = 8.7 mM^{-1} cm^{-1}; Figure 4). This lack of bound CoA is confirmed by mass spectrometry (see below). Figure 4 does, however, contain an unusual feature. Since ECH is almost completely inactivated with 1 equiv of DCTFTH-CoA, why do 5 and, particularly, 50 equiv show further modification of the protein? Subsequent work will show that although the hydratase activity is almost completely suppressed by DCTFTH-CoA, the modified enzyme retains eliminase activity and continues to release reactive metabolites.

DCTFTH-CoA-Modified ECH Still Binds CoA Thioester Ligands. The ability of the washed, DCTFTH-CoA-modified enoyl-CoA hydratase to bind CoA thioesters was investigated with the nonhydratable substrate analogue, DAC-CoA (*13*), in 50 mM potassium phosphate buffer, containing 0.3 mM EDTA (pH 7.6). DAC-CoA binds tightly to ECH, and its absorbance spectrum is red-shifted 90 nm to 496 nm due to polarization of the bound ligand in the active site of the enzyme (*13*). Modified ECH (2% residual hydratase activity, modified with 2 equiv of DCTFTH-CoA; see Materials and Methods) gave a K_D of 2.4 μM compared to a value of 0.5 μM for the untreated enzyme. In addition to the slightly weaker binding of DAC-CoA, DCTFTH-CoA-modified ECH exhibits a lower extinction coefficient for the polarized DAC-CoA chromophore (22 vs 48 mM^{-1} cm^{-1}; not shown). Clearly, the modified enzyme retains a substantial ability to bind and polarize thioester ligands (see the next section).

DCTFTH-CoA-Modified ECH Retains β-Elimination Ability toward BTTB-CoA. The ability of DCTFTH-CoA-modified ECH to bind DAC-CoA suggested that it might bind BTTB-CoA whose bulky, aromatic nature is similar to that of DAC-CoA. Surprisingly, Figure 5 shows that 2 equiv-modified ECH (2% residual hydratase activity; see above) retains essentially complete β-eliminase activity using 50 μM BTTB-CoA in phosphate buffer (pH 7.6). Control experiments confirmed that the hydratase activity was not regained during the 30 min incubation with BTTB-CoA. These elimination assays with BTTB-CoA were also performed with 5 and 50 equiv-of-DCTFTH-CoA-modified ECH (Figure 5). The 5 equiv example is not shown in Figure 5, because it overlaps the control and 2 equiv cases. In contrast, modification with 50 equiv of DCTFTH-CoA leads to elimination rates that are only slightly higher than the

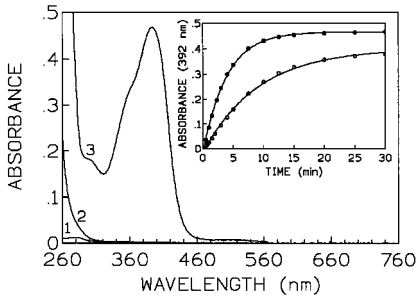


FIGURE 6: Formation of a DCTFTH-CoA-thiol chromophore by DCTFTH-CoA-modified enoyl-CoA hydratase. ECH (10 μM) was incubated at 25 °C for 90 min with or without 20 μM DCTFTH-CoA in 50 mM Tris-HCl (pH 9.0) containing 0.3 mM EDTA. ECH was then washed by ultrafiltration and recovered in Tris buffer (pH 9.0). DCTFTH-CoA-modified ECH (0.5 μM, 2% hydration activity) was incubated with 50 μM DCTFTH-CoA in the presence of 1 mM NAC in 50 mM Tris-HCl (pH 9.0) containing 0.3 mM EDTA. Spectra were taken at the specified time points and plotted against the absorbance at 392 nm. Curves 1 and 2 are the spectra of the enzyme before and after the addition of NAC, respectively. Curve 3 is a spectrum of the chromophore (approximate $\epsilon_{392} = 10$ mM^{-1} cm^{-1}) formed after a 30 min incubation of DCTFTH-CoA and NAC with unmodified control enzyme. The inset plots the formation of the chromophore over time at 392 nm for unmodified (●) and modified ECH (○). Solid lines are fit to a single-exponential association equation: unmodified ECH, rate constant of 0.26 min^{-1} and amplitude of 0.48; and 2 equiv-of-DCTFTH-CoA-modified ECH, rate constant of 0.11 min^{-1} and amplitude of 0.42.

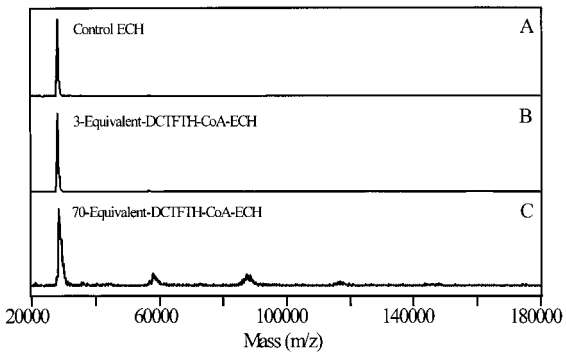


FIGURE 7: MALDI-TOF mass spectrometric analysis of DCTFTH-CoA-modified enoyl-CoA hydratase. ECH (6 μM) was incubated at 25 °C for 90 min with 0, 3, and 70 equiv of DCTFTH-CoA in 50 mM Tris-HCl (pH 9.0) containing 0.3 mM EDTA, except for the 70 equiv incubation performed at pH 8.0. The enzyme was then washed by ultrafiltration, recovered in Tris buffer at the specified pH value, and analyzed by MALDI-TOF mass spectrometry as described in Materials and Methods using 3,5-dimethoxy-4-hydroxycinnamic acid as the matrix. A calibration file was created using the singly and doubly charged species of the unmodified sample, and this was applied to the modified enzyme spectra. The data were smoothed using a 19-point Savitsky−Golay function.

background rate with BTTB-CoA alone. As described in detail later, the 50 equiv-treated enzyme is too heavily labeled to exhibit either significant hydratase or eliminase activity. The data described above show that the 2 equiv-modified enzyme can still bind DAC- and BTTB-CoA, suggesting that the failure to rapidly hydrate crotonyl-CoA is not simply a binding effect. Indeed, 500 μM crotonyl-CoA inhibits the BTTB-CoA elimination reaction of the treated enzyme by 39% compared to 91% for the native fully active hydratase.

Enoyl-CoA Hydratase Continues To Release Cytotoxic Metabolites after Modification by DCTFTH-CoA. Next, we addressed whether modified ECH could still activate DCTFTH-CoA (as it does with BTTB-CoA) after the hydratase activity had been reduced to <2% of that of an untreated control. First, we developed a continuous assay for release of cytotoxic thiolate species from DCTFTH-CoA. The main panel in Figure 6 shows the spectral changes observed when native ECH is incubated with DCTFTH-CoA in the presence of 1 mM *N*-acetylcysteamine (curve 3). Similar results were obtained with DTT and GSH (with new species, absorbing from 310 to 390 nm; data not shown). Comparable chromophores are not generated when thiols are substituted with either 1 mM lysine or histidine.

With NAC, the apparent extinction coefficient of the chromophore in Figure 6 is 9.3 mM^{-1} cm^{-1} at 392 nm. This species is stable to purification by HPLC (not shown), and its structure is a subject of continued investigation (J. F. Baker-Malcolm and C. Thorpe, unpublished data). However, it is clear that the chromophoric species cannot be due to an acryloyl-CoA-mediated reaction, since ECH does not generate the 392 nm chromophore with BTTB-CoA or DCTH-CoA (data not shown). Thus, the chromophore reflects the condensation of thiol with an electrophilic moiety, such as species **12** or **13** in Scheme 4. Whatever its structure, the strong new absorbance feature, shown in Figure 6, provides a useful method for monitoring elimination reactions with DCTFTH-CoA.

As described earlier, thiols cannot significantly reactivate the enoyl-CoA hydratase after inactivation by DCTFTH-CoA. Hence, we can incubate DCTFTH-CoA-modified ECH with NAC without significant recovery of native activity. Accordingly, the inset in Figure 6 compares the rate of elimination with DCTFTH-CoA of untreated (black circles) and enzyme pretreated with DCTFTH-CoA to 2% hydratase activity (white circles). Modified ECH shows 40% of the elimination rate of the control at pH 9.0. The elimination activity of the modified enzyme toward DCTFTH-CoA was also confirmed by HPLC (see Materials and Methods). Clearly, DCTFTH-CoA-modified ECH can catalyze thiolate elimination from DCTFTH-CoA.

The apparent reduction in the DCTFTH-CoA elimination rate of the modified enzyme (inset in Figure 6) deserves further comment. As mentioned earlier, the eliminase activity of *native* ECH toward BTTB-CoA is pH-independent, with little difference between pH 7.6 and 9.0 (not shown). However, the rate of 2-mercaptobenzothiazole elimination by DCTFTH-CoA-*treated* ECH is only 50% of that at pH 7.6. Thus, the apparent decrease in rate with DCTFTH-CoA as an elimination substrate at pH 9.0 (inset of Figure 6) likely reflects this additional pH dependence.

These data confirm that enoyl-CoA hydratase exposed to DCTFTH-CoA generates reactive electrophilic species even when the hydratase activity of the enzyme has been reduced to <2% of native activity. In the absence of competing nucleophiles, ECH continually accumulates modifications, as is evident in the spectrum of the modified enzyme labeled with 50 equiv of DCTFTH-CoA (Figure 4). In the presence of NAC, ECH retains hydratase activity (see above), is apparently unmodified by DCTFTH-CoA, and does not accumulate the additional absorbance shown in Figure 4 (data not shown).

Further insight into the course of inactivation is provided by the MALDI-TOF-MS experiments summarized in Figure 7. The native protein is a homohexamer, but only the

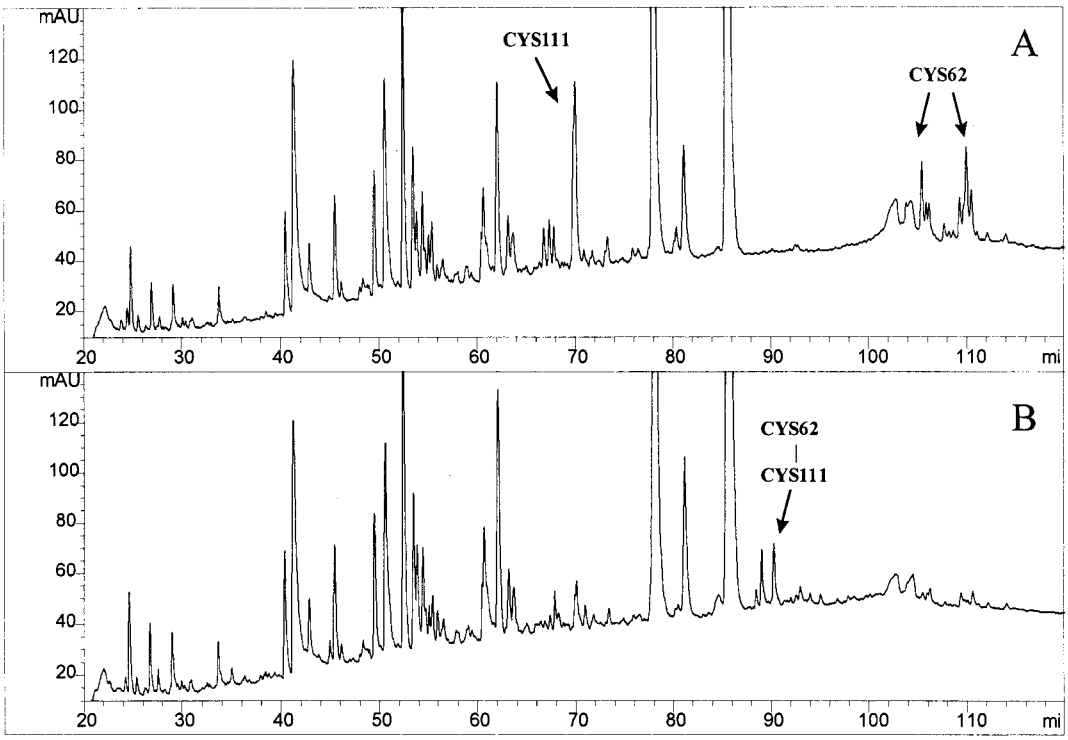


FIGURE 8: Peptide mapping of DCTFTH-CoA-modified and unmodified enoyl-CoA hydratase. Tryptic digests of unmodified and 5 equivalent-of-DCTFTH-CoA-modified ECH were prepared as described in Materials and Methods, and C_{18} HPLC elution profiles were monitored at 220 nm (panels A and B, respectively). Individual peaks were collected manually for analysis as described in Materials and Methods.

dissociated monomer is observed by MALDI-TOF-MS analysis (see panel A). ECH modified with 3 equiv of DCTFTH-CoA exhibits no distinguishable increase in the mass of the 28.3 kDa monomer compared to the unmodified enzyme using our MALDI instrument. Panel C shows that incubation with 70 equiv of DCTFTH-CoA leads to the accumulation of significant levels of dimer and trimer with traces of even higher-molecular weight aggregates. The relative prominence of the trimer is of interest, since ECH is considered a dimer of trimers (*8*, *10*). Thus, turnover of multiple DCTFTH-CoA molecules leads to a progressive derivatization of ECH, as judged both by UV−vis spectroscopy (Figure 4) and by the apparent cross-linking of protein subunits (Figure 7). At very high inhibitor levels, the modification of ECH appears to be so serious that it abolishes essentially all enzymatic activity (i.e., both hydratase and elimination reactivity, with BTTB-CoA; see Figure 5, 50 equiv). Further, multiply labeled enzyme is prone to precipitation (data not shown).

Electrospray mass spectrometric analysis of 1 equiv-of-DCTFTH-CoA-modified ECH showed only a modest increase in the native molecular weight of approximately 89 ± 5 mass units (see Materials and Methods). Thus, coenzyme A (767.5 g/mol) is not retained in the enzyme modification, consistent with the data described earlier. Further characterization of the modification reaction was based on thiol titrations and peptide mapping as described below.

Protein Chemistry of DCTFTH-CoA-Modified ECH. The finding that exogenous thiols afforded complete protection against DCTFTH-CoA inactivation suggested cysteine residues as possible targets. Further, the spectral changes observed upon modification of ECH (Figure 4) are also consistent with cysteine modification (see Figures 4 and 6). Table 2 summarizes the thiol content analysis determined using DTNB with 1, 2, and 10 equiv of DCTFTH-CoA-treated ECH. These results are consistent with the modification of one or two cysteine residues per monomer.

In attempts to identify the target of inactivation, tryptic digestions (Figure 8) were performed on ECH modified with 5 equiv of DCTFTH-CoA. Prior to digestion, the free cysteine residues of control and modified enzyme were alkylated with pyrene maleimide. After DCTFTH-CoA modification, several peaks had decreased intensities compared to that of the control peptide map at 220 nm (Figure 8A) and were identified by sequence and MALDI-TOF-MS analysis as the tryptic peptides Ala57−Lys92 (containing alkylated Cys62) and Thr107−Lys115 (containing alkylated Cys111). It is not known why the Cys62 peptide of the native enzyme emerges in two peaks as shown in panel A, but this situation is not uncommon in HPLC of peptides. The DCTFTH-CoA-treated enzyme digest (panel B) shows two prominent new peaks at 89 and 90.3 min. While we were unable to obtain sequence or mass spectra of the first peak, the second is a mixed disulfide between these two peptides, linked between Cys62 and Cys111. Peptide maps of 1 equiv-modified ECH also gave a new peak at 90.3 min with a mass corresponding to this mixed disulfide (4790 amu, not shown). Finally, HPLC analysis with a butyrylsilica column of 5 equiv-modified ECH digests showed a new peptide with a tandem sequence corresponding to the same mixed disulfide, Cys62−Cys111. These data clearly implicate one or both of these residues as participants in the original modification. However, the mechanism for this specific disulfide bridge formation remains uncertain. It might reflect migration or expulsion of the label during the denaturation and/or digestion conditions, since the two cysteine residues are 20 Å apart in the crystal structure of ECH complexed with octanoyl-CoA (*10*; see below). This disulfide bond formation is not

necessarily incompatible with the failure of the modified enzyme to recover hydratase activity upon DTT treatment. Thus, the original site of modification may be inaccessible and/or unreactive toward DTT prior to denaturation.

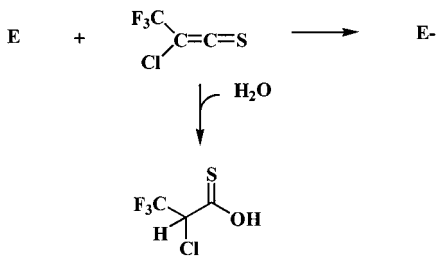
CONCLUSIONS

Previously, the role of enoyl-CoA hydratase in the bioactivation of 4-thia-fatty acids was believed to involve hydration of the corresponding *trans*-2-enoyl-CoA analogues generated by acyl-CoA dehydrogenase-mediated catalysis (Scheme 2). The work described here presents an additional dimension to the bioactivation of cytotoxic 4-thiaacyl-CoA compounds, with the identification of a dehydrogenase-independent route to the liberation of reactive thiolate species. The outcome of the interaction between DCTFTH-CoA and ECH is unusually complex. It depends, for example, on enzyme concentration, on inhibitor stoichiometry, on what activity of ECH is being monitored, and on whether exogenous nucleophiles or trapping agents are present in solution.

The wide range of protectants likely to be found in the mitochondrial matrix, from small thiols, such as glutathione, to large proteins and phospholipid bilayers, suggests that inactivation of the hydratase activity by DCTFTH-CoA would be hard to achieve in vivo. Even if such inactivation did occur, however, β-eliminase activity is retained and ECH would continue to generate cytotoxic species in the mitochondrion. This surprisingly pernicious behavior has been reproduced repeatedly with multiple preparations of pure ECH. These observations, together with the abundance of ECH in the mitochondrial matrix, suggest that this new pathway for the generation of cytotoxic species may be important in vivo.

Experiments with pure ECH suggest that the reactive entities generated from β-elimination can leave the active site and be sequestered by a range of exogenous species. However, in the absence of these external trapping agents, such species exhibit sufficient lifetimes to return to label a very limited range of susceptible positions on ECH. Thus, the stoichiometry of DCTFTH-CoA inactivation of the hydratase reaction approaches 1 when experiments are conducted at high enzyme concentrations. At lower protein concentrations, the bimolecular recombination of reactive species and enzyme is likely to compete less effectively with solvolysis of the reagent, as illustrated for the thioketene,

Scheme 5



leading to the increased stoichiometry seen in Figure 2. The fact that several exogenous nucleophiles provide complete protection against an otherwise almost stoichiometric inactivation also suggests that the reactive species generated after elimination have an appreciable lifetime at pH 9.0. This longevity and, particularly, the apparent ability of the reactive species to be sequestered in a membrane phase have important consequences for the effects of these compounds in vivo.

While we have strong spectroscopic and protein chemical evidence for a limited derivatization of cysteine residues in stoichiometrically modified ECH, a number of uncertainties remain in this novel inactivation mechanism. We do not yet understand how the hydratase activity can be strongly suppressed, while leaving the β-eliminase activity unaffected. Loss of hydratase activity does not simply reflect a failure of the DCTFTH-CoA-treated ECH to bind crotonyl-CoA. Thus, crotonyl-CoA (500 μM) reduces the elimination rate with BTTB-CoA of the modified enzyme in the standard assay by 40% (not shown; see Materials and Methods). One seemingly plausible explanation is that modification impacts Glu144 (Scheme 1) while leaving Glu164 (which serves to abstract an α-proton from 3-OH-acyl-CoA substrates in the reverse direction) unimpaired. However, recent studies of Glu164Gln and Glu144Gln mutants show that both carboxyl(ate)s are important to the elimination of 2-mercaptobenzothiazole from BTTB-CoA (*12*). The small size (about 89 amu) of the adduct(s) implies the loss of at least four of the five halogen atoms from a species such as compound **11** (Scheme 4) prior to, or during, its attachment to the enzyme. A further challenge is the elucidation of the nature of the pronounced chromophore observed both with the enzyme and with model compounds (Figures 4 and 6, respectively).

Our current experience with characterizing labile adducts formed upon incubation of the medium-chain acyl-CoA dehydrogenase with DCTH-CoA and DCTFTH-CoA (J. F. Baker-Malcolm, M. W. Anders, M. Wang, J. J.-P. Kim, and C. Thorpe, unpublished data) suggests that peptide mapping alone cannot provide an adequate characterization of these modifications. Accordingly, we intend to use X-ray crystallography and site-directed mutagenesis to further characterize the covalent and noncovalent changes that accompany the interaction between enoyl-CoA hydratase and DCTFTH-CoA.

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