Elimination Reactions in the Medium-Chain Acyl-CoA Dehydrogenase: Bioactivation of Cytotoxic 4-Thiaalkanoic Acids†

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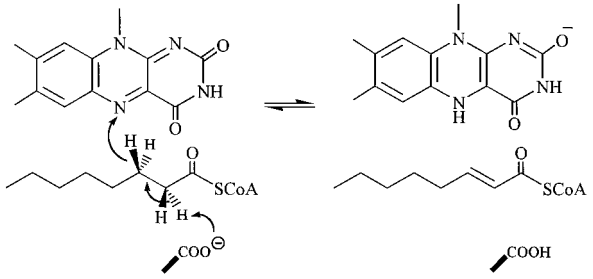
ABSTRACT: A range of 4-thiaacyl-CoA derivatives has been synthesized to study the bioactivation of cytotoxic fatty acids by the mitochondrial medium-chain acyl-CoA dehydrogenase and the peroxisomal acyl-CoA oxidase. Both enzymes catalyze α-proton abstraction from normal acyl-CoA substrates with elimination of a β-hydride equivalent to the FAD prosthetic group. In competition with this oxidation reaction, 4-thiaacyl-CoA thioesters undergo dehydrogenase-catalyzed β-elimination, providing that the corresponding thiolates are sufficiently good leaving groups and can be accommodated by the active site of the enzyme. Thus, the dehydrogenase catalyzes the elimination of 2-mercaptobenzothiazole and 4-nitrothiophenolate from 4-(2-benzothiazole)-4-thiabutanoyl-CoA and 4-(4-nitrophenyl)-4-thiabutanoyl-CoA, respectively. However, the 2,4-dinitrophenyl-analogue appears too bulky and the unsubstituted thiophenyl-derivative is insufficiently activated for significant elimination. Molecular modeling shows that steric interference from the flavin ring dictates a syn rather than an anti elimination. Acryloyl-CoA, the other product of 4-thiaacyl-CoA elimination reactions, is not a significant inactivator of the medium-chain dehydrogenase. In contrast, the irreversible inactivation observed during β-elimination using 5,6-dichloro-4-thia-5-hexenoyl-CoA (DCTH-CoA), 5,6-dichloro-7,7,7-trifluoro-4-thia-5-heptenoyl-CoA (DCTFTH-CoA), and 6-chloro-5,5,6-trifluoro-4-thiahexanoyl-CoA (CTFTH-CoA) is caused by release of cytotoxic thiolate products within the active site of the dehydrogenase. The double bond between C5 and C6 found in the vinylic analogues DCTH− and DCTFTH-CoA is not essential for enzyme inactivation, although CTFTH-CoA is a weaker inhibitor of the dehydrogenase. Mechanism-based inactivation with CTFTH-CoA requires elimination, is unaffected by exogenous nucleophiles, and is strongly protected by octanoyl-CoA. The peroxisomal acyl-CoA oxidase efficiently oxidizes 4-thiaacyl-CoA analogues, but is only rapidly inactivated by DCTFTH-CoA. The variable ratio of elimination to oxidation observed for DCTH-, DCTFTH-, and CTFTH-CoA may influence the metabolism of the corresponding cytotoxic alkanoic acids in vivo.

Normal substrates of the medium-chain acyl-CoA dehydrogenase undergo abstraction of the pro-R-α-proton by GLU376 (*1*−*5*) with the apparently concerted anti elimination of the pro-R-β-hydrogen as a hydride equivalent to the *N*-5-position of the flavin (*6*−*11*). This reductive half-reaction is depicted in Scheme 1 and is followed by reoxidation of the flavin in two 1-electron steps by electron-transferring flavoprotein (*12*−*14*) or by artificial oxidants such as the ferricenium ion (*15*).

Although the physiological role of the medium-chain acyl-CoA dehydrogenase is to catalyze the first step in mitochondrial fatty acid oxidation, recent work shows that it also catalyzes the bioactivation of certain cytotoxic 4-thia fatty acids. For example, 5,6-dichloro-4-thia-5-hexenoic acid (DCTH)[1] inhibits mitochondrial respiration in rat kidney and liver (*16*), is a potent hepatotoxin and nephrotoxin (*17*), and

Scheme 1



is cytotoxic when incubated with isolated rat hepatocytes (*18*). Synthesis of DCTH analogues of varying chain lengths

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[1] Abbreviations: CoA(SH), coenzyme 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; PTB-CoA, 4-phenyl-4-thiabutanoyl-CoA; NPTB-CoA, 4-(4-nitrophenyl)-4-thiabutanoyl-CoA; DNPTB-CoA, 4-(2,4-dinitrophenyl)-4-thiabutanoyl-CoA; HMG-CoA, 3-hydroxy-3-methylglutaryl-CoA; MCAD, medium-chain acyl-CoA dehydrogenase; ETF, electron-transferring flavoprotein; $Fc^+PF_6^-$, ferricenium hexafluorophosphate;

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Scheme 2

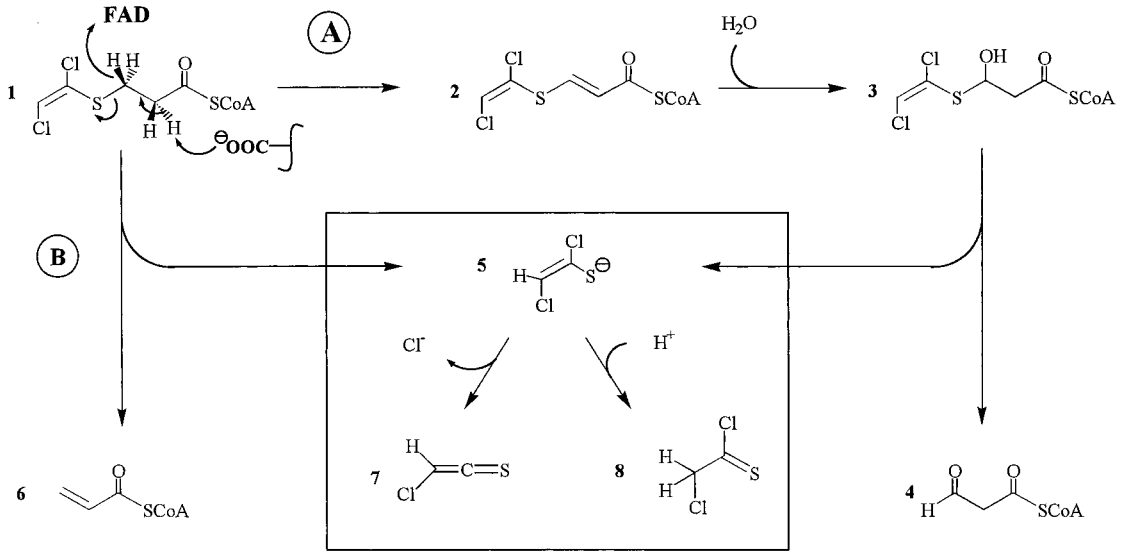
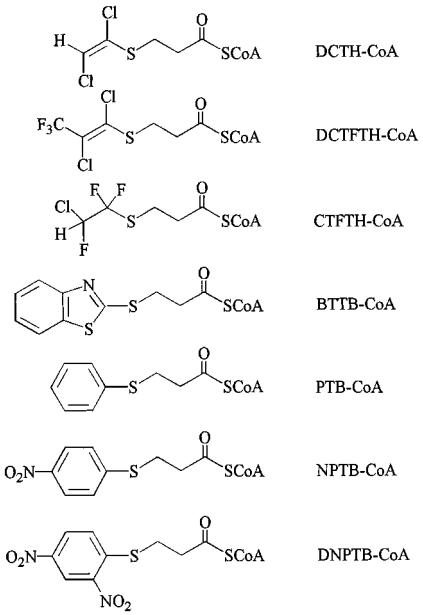


Chart 1: 4-Thiaacyl-CoA Analogues Used in This Work[a]



[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; PTB-CoA, 4-phenyl-4-thiabutanoyl-CoA; NPTB-CoA, 4-(4-nitrophenyl)-4-thiabutanoyl-CoA; and DNPTB-CoA, 4-(2,4-dinitrophenyl)-4-thiabutanoyl-CoA.

clearly implicates fatty acid oxidation in the bioactivation of these xenobiotics (*18*).

The biological effects of DCTH prompted the synthesis of the corresponding thioester, 5,6-dichloro-4-thia-5-hexenoyl-CoA (DCTH-CoA; Chart 1) and an initial characterization of its behavior toward the medium-chain acyl-CoA dehydrogenase (*19*). Two routes for the breakdown of DCTH-CoA were found (pathways A and B in Scheme 2).

Normal oxidation of DCTH-CoA to the corresponding *trans*-2-enoyl derivative (compound 2) provides a substrate for the next enzyme of mitochondrial β-oxidation, enoyl-CoA hydratase (pathway A). Hydration affords the thiohemiacetal (compound 3) leading to the observed formation of malonyl-semialdehyde-CoA and the elimination of 1,2-dichloroethenethiolate (compounds 4 and 5, respectively). This reaction was previously described with simpler 4-thia fatty acyl-CoA analogues (*20*). Model reactions, both in organic solvents and in the gas phase, provide support for the formation of the 1,2-dichloroethenethiolate anion (compound 5) as an immediate product of this elimination reaction (*21*). These studies also provided evidence for chlorothioketene formation (compound 7) as one route in the decomposition of compound 5 (*21*, *22*). The thionoacyl chloride (compound 8) is another likely participant in the decomposition of 1,2-dichloroethenethiolate (*23*−*26*). One or more of these reactive species is thought to acylate critical cellular targets leading to the cytotoxicity observed for DCTH.

A competing route for the decomposition of DCTH-CoA involves a direct base-catalyzed elimination of 1,2-dichloroethenethiolate by the acyl-CoA dehydrogenase (Scheme 2, pathway B). This elimination occurred in the absence of an electron acceptor and led to the inactivation of the acyl-CoA dehydrogenase (*19*). Neither the expected thioester product, acryloyl-CoA (compound 6), nor thiolate release was detected in this initial work. In addition, it was unclear which elimination product was responsible for enzyme inactivation. The present studies address these issues with the synthesis of a range of 4-thia analogues (Chart 1). Some of these derivatives contain a chromophoric moiety to allow direct observation of thiolate release. In all cases, elimination leads to the formation of acryloyl-CoA, but only the highly reactive α-halo-thiolate species are responsible for enzyme inactivation. Finally, the present work documents the competition between oxidation and elimination pathways, indicating that the cytotoxic effects of these compounds may be markedly influenced by the metabolic state of the cell.

MATERIALS AND METHODS

Materials. Medium-chain acyl-CoA dehydrogenase was purified from pig kidney as described previously (*14*, *27*). 8-Cl-FAD-substituted enzyme was prepared according to

DCIP, 2,6-dichlorophenolindophenol; PMS, phenazine methosulfate; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); EDTA, ethylenediaminetetraacetic acid; TFA, trifluoroacetic acid; DTT, dithiothreitol; GSH, glutathione; and NEM, *N*-ethyl maleimide.

Thorpe and Massey (*28*). Pig liver electron-transferring flavoprotein was partially purified from isolated mitochondria (*29*). Acyl-CoA oxidase from Candida species, bovine liver enoyl-CoA hydratase (crotonase), CoASH (lithium salt), octanoyl-CoA, 2,6-dichlorophenolindophenol, phenazine methosulfate, *N*-ethyl maleimide, and glutathione were purchased from Sigma. Hydroxylamine hydrochloride, and HPLC-grade acetonitrile, methanol, and monobasic potassium phosphate were purchased from Fisher. Ferricenium hexafluorophosphate, dithiothreitol, and 2-mercaptobenzothiazole were purchased from Aldrich.

General Methods. Unless stated otherwise, all buffers were 50 mM potassium phosphate, pH 7.6, containing 0.3 mM EDTA and incubations were performed at 25 °C. HPLC separations were performed on a Perkin-Elmer Series 400 liquid chromatograph equipped with a Dynamax UV-C detector (Rainin). Samples were filtered using either microfilterfuge tubes (Rainin; 0.2 micron) or Centricon 30 microconcentrators (Amicon). Static absorbance measurements were recorded on a Hewlett-Packard 8452A diode array spectrophotometer, and where noted, turbidity corrections were made using the scatter correct routine included in the 8452A software program. Concentrations of flavoproteins were determined with the following extinction coefficients: native dehydrogenase, 15.4 mM^{-1} cm^{-1} at 446 nm (*30*) and 8-Cl-FAD-reconstituted dehydrogenase, 14.6 mM^{-1} cm^{-1} at 442 nm (*28*); ETF, 13.3 mM^{-1} cm^{-1} at 436 nm (*29*); and acyl-CoA oxidase, 14.2 mM^{-1} cm^{-1} at 446 nm (*31*). Anaerobic titrations were performed as described previously (*32*).

Preparation of 4-Thia CoA Compounds. The following acids were synthesized as described previously: 5,6-dichloro-4-thia-5-hexenoic acid (DCTH; *33*), 6-chloro-5,5,6-trifluoro-4-thiahexanoic acid (CTFTH; *18*), 4-(2-benzothiazole)-4-thiabutanoic acid (BTTB; *18*), and 4-phenyl-4-thiabutanoic acid (PTB; *20*). 5,6-Dichloro-7,7,7-trifluoro-4-thia-5-heptenoic acid (DCTFTH) was synthesized from 3-mercaptopropionic acid and 1,1,2-trichloro-3,3,3-trifluoro-1-propene: 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 room temperature. 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 overnight at room temperature. Water was added to the reaction mixture, and the aqueous layer was brought to pH 1 with 1 N HCl. The reaction mixture was extracted with ether, and the organic layer was separated and dried over anhydrous sodium sulfate. The ether was removed under reduced pressure at 40 °C. The product was purified by flash chromatography on silica gel with hexane followed by ether as the eluents. ^{1}H NMR ($CDCl_3$): 2.8 (t, 2H), 3.3 (t, 2H). A sample of the product was treated with diazomethane to give the methyl ester; GC/MS analysis gave a single peak corresponding to methyl 5,6-dichloro-7,7,7-trifluoro-4-thia-5-heptenoate and a molecular ion at *m*/*z* 282 and an isotopic cluster indicating the presence of 2 chlorine atoms. 4-(4-Nitrophenyl)-4-thiabutanoic acid (NPTB) and 4-(2,4-dinitrophenyl)-4-thiabutanoic acid (DNPTB) were synthesized from their respective fluorobenzene derivatives: 3-mercaptopropionic acid (10 mmol) was added dropwise to a mixture of 40 mL of dry dimethylformamide and K_2CO_3 (20 mmol). 1-Fluoro-4-nitrobenzene or 1-fluoro-2,4-dinitrobenzene (10 mmol) was then added, and the solution was stirred overnight at room temperature. The reaction mixture was diluted with 100 mL of water, adjusted to pH 2 with concentrated HCl, and filtered. The filtrate was then extracted twice with ether to recover the 4-thiaalkanoic acid.

All of the 4-thia acids were converted to their corresponding CoA thioesters by the mixed anhydride method (*34*). Purification and desalting procedures utilized a semipreparative octadecyl silica column (Zorbax) with a methanol gradient as described previously (*35*). All thioesters were detected at 260 nm and were further characterized by NMR. An extinction coefficient value of 16.0 mM^{-1} cm^{-1} at 260 nm was used to determine the concentration of DCTH-, DCTFTH-, CTFTH-, and PTB-CoA, whereas 21.7, 18.0, and 21.1 mM^{-1} cm^{-1} were used for BTTB-, NPTB-, and DNPTB-CoA, respectively. An extinction coefficient of 20.0 mM^{-1} cm^{-1} at 260 nm was used to determine the concentration of the corresponding enoyl-CoA derivatives, except for the BTTB-, NPTB-, and DNPTB-enoyl-CoA compounds in which values of 25.3, 22.0, and 25.1 mM^{-1} cm^{-1} were used. The extinction coefficients at 310 nm for BTTB-CoA and commercial 2-mercaptobenzothiazole were determined to be 5.1 and 21.0 mM^{-1} cm^{-1}, respectively. Concentrations of the thiolate elimination products of NPTB-CoA and DNPTB-CoA were determined at 412 nm from extinction values of 13.6 and 16.8 mM^{-1} cm^{-1}, respectively.

Enzyme Assays. Unless otherwise stated, assay mixtures contained 50 μM substrate and 10 nM to 10 μM acyl-CoA dehydrogenase and were performed at 25 °C using Perkin-Elmer 552A or Cary 219 spectrophotometers. Three assay systems were used to evaluate the effects of redox potential of the primary oxidant on the turnover of thioester ligands by the acyl-CoA dehydrogenase. The ferricenium assay was followed at either 300 or 618 nm as appropriate (*15*). Assays using DCIP as the terminal electron acceptor used either PMS (*30*) or 1 μM ETF as mediators and were followed at 600 nm (*36*). When ETF was used, assays included 1 mM NEM to suppress the background reduction of DCIP (*30*). Acyl-CoA oxidase assays followed the increase in absorbance either at 312 nm when 4-thiatridecanoyl-CoA or PTB-CoA was converted to its *trans*-2-enoyl-product ($\epsilon = 22.0$ mM^{-1} cm^{-1}; *20*) or at 260 nm for the conversion of all other saturated CoA thioesters to the unsaturated derivatives ($\Delta\epsilon = 4.0$ mM^{-1} cm^{-1}).

Enzyme Inactivation. Medium-chain acyl-CoA dehydrogenase inactivation studies were performed as described in the legend to Figure 1. Protection against DCTH-CoA-, DCTFTH-CoA-, and CTFTH-CoA-inactivation was assessed with 50 μM octanoyl-CoA. Control reaction mixtures contained no octanoyl-CoA with and without inhibitor present. The effect of GSH on the inactivation of the dehydrogenase by CTFTH-CoA used 5 μM enzyme and 0.1 or 0.5 mM inhibitor in the presence or absence of 0.2 or 1 mM GSH, respectively. After 30 min, excess reagents were removed by ultrafiltration, and the enzyme was assayed using the ferricenium method. Acyl-CoA oxidase inactivation was evaluated aerobically over 1 h with 100 μM of each of the 4-thiaacyl-CoA analogues and either 70 nM enzyme for DCTFTH-CoA or 200 nM enzyme for the remaining compounds.

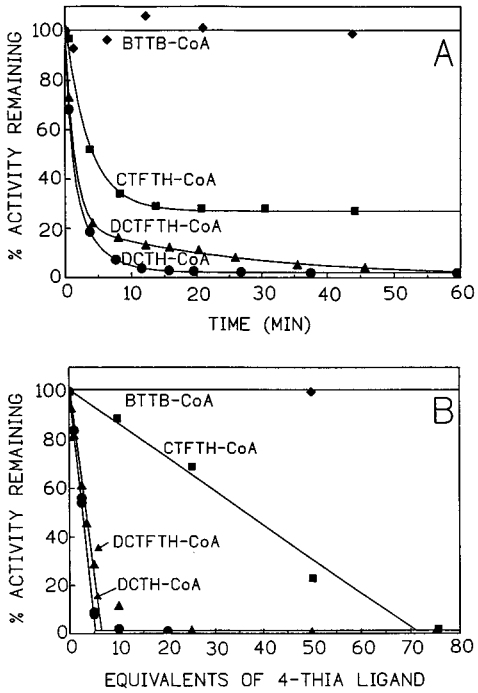


FIGURE 1: Inactivation time courses and stoichiometries for the medium-chain acyl-CoA dehydrogenase incubated with a series of 4-thiaacyl-CoA analogues. Thioesters shown here are DCTH-CoA (●), DCTFTH-CoA (▲), CTFTH-CoA (■), and BTTB-CoA (◆). (A) The medium-chain acyl-CoA dehydrogenase (2 μM in 50 mM phosphate buffer, pH 7.6, 25 °C) was incubated with 100 μM of each 4-thiaacyl-CoA analogue. The solid lines are fit through the data points using one or two exponential decays: DCTH-CoA, rates of 1.1 and 0.28/min with amplitudes of 54 and 43%, respectively; DCTFTH-CoA, 0.68 and 0.03/min with amplitudes of 80 and 20%, respectively; and CTFTH-CoA, 0.33/min with an amplitude of 72%. (B) The dehydrogenase (2 μM) was incubated with increasing concentrations of 4-thiaacyl-CoA thioesters for 90 min in 50 mM phosphate buffer, pH 7.6, at 25 °C. Enzyme activity was measured using the standard ferricenium method (see Results).

Reversal of CTFTH-CoA inactivation by nucleophiles was tested for the dehydrogenase by mixing the ultrafiltered enzyme (3.5 μM) with either 0.7 mM GSH, 0.7 mM DTT, or 10 mM hydroxylamine at 25 °C. Samples were ultrafiltered after 90 min to remove excess reagents and the enzyme was assayed as described above. Activities were compared to native enzyme controls treated with thiol or hydroxylamine reagents as described above.

HPLC Partition Studies. HPLC analyses of enzyme incubations used an analytical Resolvex octadecyl column with a linear gradient formed from acetonitrile and 25 mM potassium phosphate, pH 5.3 (*19*). Retention times are given in Table 1. The dehydrogenase was incubated with 100 μM ligand in the presence or absence of a saturating concentration of ferricenium hexafluorophosphate (1.2 mM). Concentrations of enzyme were adjusted from 2 to 22 μM to ensure that all inhibitory thioesters were converted to products before enzyme inactivation occurred. Similar incubations were performed with 50 nM acyl-CoA oxidase in aerobic solution. The reactions were stopped by ultrafiltration using Centricon 30 filters. Filtrates from experiments using the ferricenium ion were extracted twice with diethyl ether to remove the lipophilic ferrocene. Samples of 200 μL of the filtrate were used for HPLC analysis. The remainder was incubated with 5 units of crotonase for 30 min, ultrafiltered, and analyzed by HPLC. The areas of the peaks were scaled using an extinction coefficient of 22.6 mM^{-1} cm^{-1} for acryloyl-CoA and extinction values as reported earlier for the other 4-thia-*trans*-2-enoyl-CoA derivatives.

Table 1: HPLC Retention Times of 4-Thiaacyl-CoA Thioesters and Partition Ratios of Acryloyl-CoA to 4-Thia-2-enoyl-CoA in the Presence of the Ferricenium Ion

	retention time (min)		
	substrate	2-enoyl product	acryloyl-CoA/ 4-thia-2-enoyl-CoA
DCTH-CoA	20.5	21.3	0.02
DCTFTH-CoA	24.0	24.2	1.5
CTFTH-CoA	20.5	21.1	0.09
BTTB-CoA	21.7	22.4	0.63
PTB-CoA	21.6	22.0	<0.01
NPTB-CoA	23.2	23.6	<0.01
DNPTB-CoA	21.9	22.5	0.30

[a] Medium-chain acyl-CoA dehydrogenase was incubated with 1.2 mM of ferricenium hexafluorophosphate with 100 μM of the indicated ligands at 25 °C in phosphate buffer, pH 7.6. Concentrations of enzyme were adjusted to ensure that essentially all inhibitors were transformed to products prior to complete enzyme inactivation. Products were analyzed by HPLC as described in Materials and Methods. Coenzyme A, malonyl-semialdehyde-CoA, 3-hydroxypropionyl-CoA, and acryloyl-CoA eluted at 14.2, 14.4, 14.6, and 15.7 min, respectively. The first two columns are the elution times for the indicated thioesters and their corresponding *trans*-2-enoyl-CoA products.

Peak identification was supported by absorbance spectra. Selected peaks were collected, dried in vacuo, and redissolved in water. Treatment of 4-thia-*trans*-2-enoyl-CoA derivatives with crotonase yielded malonyl-semialdehyde-CoA, whose formation was confirmed by the pH dependence of its absorbance spectrum (*37*, *38*). Hydration of acryloyl-CoA (15.7 min) yielded 3-hydroxypropionyl-CoA, which eluted at 14.6 min.

RESULTS AND DISCUSSION

The first three 4-thiaacyl-CoA analogues depicted in Chart 1 were selected for study because the corresponding alkanoic acids or cysteine S-conjugates are cytotoxic (*18*, *39*, *40*). The remaining 4-thiaacyl-CoA compounds were synthesized because they would yield a chromophoric thiolate group on elimination. The effect of selected thioesters (100 μM) on the medium-chain acyl-CoA dehydrogenase (2 μM at pH 7.6) in the absence of an electron acceptor is depicted in Figure 1A. DCTH-CoA (Chart 1) is a known inhibitor of the medium-chain dehydrogenase (*19*). Under the present conditions, a rapid biphasic loss of enzyme activity occurred which was half-complete in about 1.1 min. Such behavior is widely encountered; a biphasic or multiphasic response is observed during the interaction between the medium-chain dehydrogenase and a wide range of thioester ligands (*8*, *20*, *41*, *42*). A 50-fold excess of DCTFTH-CoA (Chart 1) inactivated the medium-chain dehydrogenase with 2% activity remaining after 60 min (Figure 1A). This inactivation was also biphasic with a fast phase (0.68/min) accounting for about 80% of the activity loss and a slow phase with a rate constant of 0.03/min. The loss of activity with CTFTH-CoA (Chart 1) under the same conditions was slower and resulted in a residual activity of 27% (Figure 1A) which did not decline further over 2.5 h (data not shown). Thus,

inactivation of the medium-chain acyl-CoA dehydrogenase was observed with both vinylic (DCTH-CoA and DCTFTH-CoA) and saturated (CTFTH-CoA) 4-thiaacyl-CoA analogues, although the vinylic compounds were more potent inactivators than the saturated compound. Enzyme inactivated with these halogenated compounds failed to regain significant activity after removal of excess reagents by ultrafiltration (data not shown; see Materials and Methods).

In contrast to the results obtained with the halogenated 4-thiaacyl-CoA analogues, the remaining 4-thiaacyl-CoA thioesters (BTTB-CoA, PTB-CoA, NPTB-CoA, and DNPTB-CoA; Chart 1) were not significantly inhibitory under these conditions over 1 h. Of these, BTTB-CoA is shown as a representative example in Figure 1A. Clearly only some 4-thia analogues are inhibitory, although all but PTB-CoA (*20*) eliminate acryloyl-CoA to some extent (see later).

Exploratory experiments showed that inactivation of the dehydrogenase by DCTH-CoA was slightly faster at both pH 6.0 and 8.5 (with the times required for 50% inactivation of 0.76 and 0.86 min, respectively) compared with 1.1 min at pH 7.6. However, these differences did not appear to be of sufficient magnitude to require detailed pH studies. Hence, a pH of 7.6 was adopted for most of the experiments to be described in this paper.

Figure 1B shows the stoichiometry of inactivation for the inhibitors identified in Figure 1A. The vinylic CoA thioesters, DCTH− and DCTFTH-CoA, showed similar stoichiometries of 5 and 6, respectively, whereas approximately 70 equiv of CTFTH-CoA was required to completely inactivate the medium-chain acyl-CoA dehydrogenase. This explains why the 50-fold molar ratio of CTFTH-CoA used in Figure 1A only effected partial inactivation of the enzyme. In contrast, ratios of BTTB-CoA to enzyme subunit from 50 (Figure 1B) to 1000 (data not shown) did not significantly inactivate the medium-chain dehydrogenase. This chromophoric thioester provides insight into the interaction of 4-thiaacyl-CoA analogues with the medium-chain acyl-CoA dehydrogenase and is described first.

BTTB-CoA. Figure 2A shows the spectral changes observed when BTTB-CoA was incubated with the medium-chain acyl-CoA dehydrogenase in aerobic solution. The addition of BTTB-CoA leads initially to a small (2−3 nm) red shift in the main absorbance peak of the flavin chromophore (inset, Figure 2A). These changes are smaller than, but qualitatively similar to, those generated by ligands, such as octyl-SCoA or *trans*-2-octenoyl-CoA (*43*). Thus, despite its steric bulk (Chart 1; see later), BTTB-CoA binds within the active site of the dehydrogenase. The observed small decrease in absorbance at 446 nm (5%; Table 2) includes that expected for the red-shift induced on ligand binding (*43*) together with a small contribution from reduction of the flavin by BTTB-CoA substrate. This latter contribution is minor, and correspondingly, no significant reduced flavin-enoyl-CoA charge-transfer complex (*9*, *28*, *44*) is observed (Figure 2A). However, substitution of the normal FAD by the more oxidizing 8-Cl-FAD analogue (*28*, *45*) leads to substantial bleaching of the enzyme flavin (Figure 2B; Table 2). A weak long-wavelength band accompanies reduction of the 8-Cl-FAD-substituted enzyme (centered about 665 nm) consistent with the formation of a reduced enzyme·4-thia-2-enoyl-CoA complex. These data indicate that the internal equilibrium between oxidized and reduced enzyme forms lies strongly to the left with native enzyme·BTTB-CoA complex but substantially to the right on 8-Cl-FAD replacement (Scheme 3). BTTB-CoA, thus, is a poor reductant of the

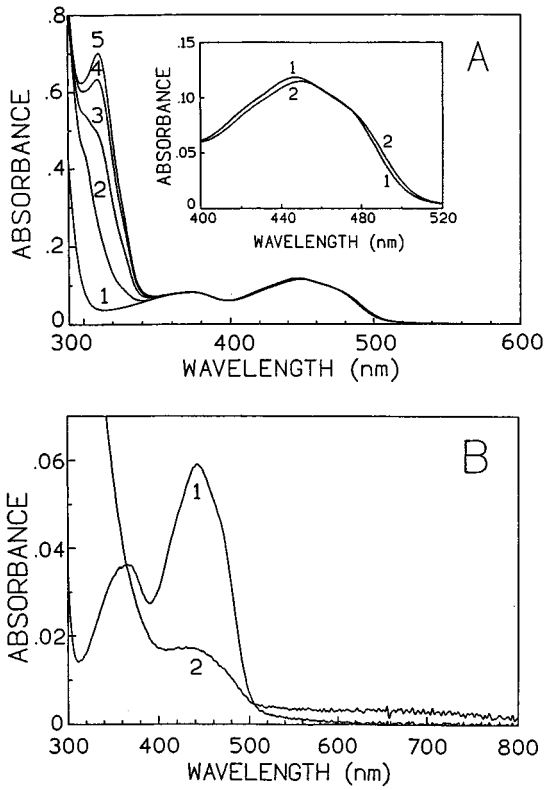


FIGURE 2: Incubation of medium-chain acyl-CoA dehydrogenase with BTTB-CoA. (A) The medium-chain dehydrogenase (curve 1, 7.7 μM in 50 mM phosphate buffer, pH 7.6, 25 °C) was mixed with 33 μM of BTTB-CoA under aerobic conditions and spectra were recorded after 11, 45, 90, and 1200 s (curves 2−5, respectively). The same changes were obtained anaerobically (not shown). The inset is an enlargement of the flavin absorbance envelope before and 11 s after the addition of BTTB-CoA (curves 1 and 2, respectively). (B) The spectrum of 8-Cl-FAD-medium-chain acyl-CoA dehydrogenase (4 μM in 50 mM phosphate buffer) was recorded before and after the addition of 100 μM BTTB-CoA (curves 1 and 2, respectively).

Table 2: Spectral Effects of 4-Thiaacyl-CoA Analogues on Native and 8-Cl−FAD-substituted Medium-Chain Acyl-CoA Dehydrogenase[a]

	percent decrease of flavin		charge transfer band (λ_{max})	
substrate	MCAD	8-Cl-MCAD	MCAD	8-Cl-MCAD
octanoyl-CoA	84	100	570	550
DCTH-CoA	36	100	645	615
DCTFTH-CoA	11	26	—[b]	660
CTFTH-CoA	14	92	670	670
BBTB-CoA	5	84	−	665
PTB-CoA	66	ND[c]	−	ND
NPTB-CoA	9	71	670	670
DNPTB-CoA	***[d]	***	−	670

[a] Each of the 4-thiaacyl-CoA analogues was incubated with either native or 8-Cl−FAD substituted medium-chain acyl-CoA dehydrogenase and observed spectrophotometrically over time. Percent decrease in flavin was calculated from the lowest observed absorbance at 446 and 442 nm for the native and 8-Cl−FAD-substituted enzymes, respectively. The bleaching of 8-Cl−FAD-substituted MCAD incubated with octanoyl-CoA was set as 100%. [b] (−) No charge-transfer band observed. [c] ND = not determined. [d] (***) Any decrease in flavin observed would be obscured by the elimination product absorbance in this region.

Scheme 3

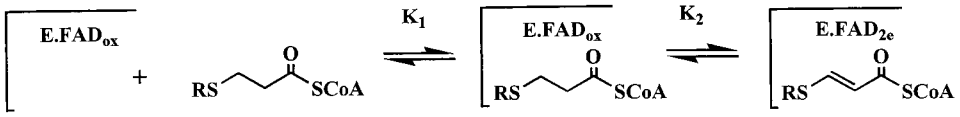
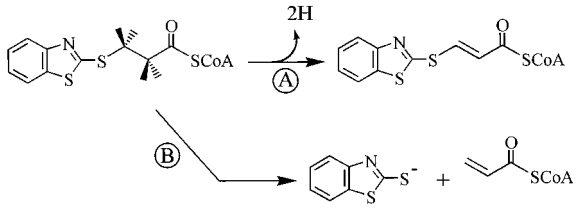


Table 3: 4-Thiaacyl-CoA Thioesters as Oxidative Substrates of the Medium-Chain Acyl-CoA Dehydrogenase[a]

CoA thioester	percent activity $Fc^+PF_6^-$ (618 nm)	DCIP/PMS (600 nm)	DCIP/ETF (600 nm)
octanoyl-CoA	100	100	100
DCTH-CoA	4.3	7.3	10.6
DCTFTH-CoA	0.04	0.3	<0.01
CTFTH-CoA	8.9	1.5	4.3
BTTB-CoA	0.3	0.08	<0.01
PTB-CoA	10.6	12.9	13.8
NPTB-CoA	16.5	3.2	9.2
DNPTB-CoA	0.01	0.02	<0.01

[a] Each of the 4-thia-analogues (50 μM) was tested as a substrate with the medium-chain acyl-CoA dehydrogenase using ferricenium hexafluorophosphate, DCIP/PMS, or DCIP/ETF as electron acceptors (see Materials and Methods). Activities are expressed as a percentage compared to the turnover of the preferred substrate, octanoyl-CoA (turnover numbers of 1100, 175, and 225/min for each assay system, respectively).

Scheme 4



native enzyme and a weak substrate (0.30, 0.08, and <0.01% of the turnover number using octanoyl-CoA in three standard assays of the medium-chain dehydrogenase; Table 3).

The rise in absorbance at 310 nm shown in Figure 2A is not due to an oxidase activity of the dehydrogenase such as that documented for furylpropionyl-CoA (*46*) and for indolepropionyl-CoA (*47*), because the same absorbance changes were generated under anaerobic conditions (see Materials and Methods; not shown). If such oxidation of BTTB-CoA were to occur, it would yield the 4-thia-*trans*-2-enoyl-CoA derivative with an absorbance maximum at 324 nm not 310 nm (see Materials and Methods). The products from a larger scale incubation, formed under the same conditions as shown in Figure 2A, were recovered by ultrafiltration, and the 310 nm chromophore was found to have a spectrum, p*K*, and HPLC retention time consistent with an authentic sample of 2-mercaptobenzothiazole (apparent p*K* 6.8; isosbestic point at 310 nm; retention time 31.8 min; see Materials and Methods). The increase observed at 310 nm in Figure 2 indicates an essentially stoichiometric release of thiolate: 0.95 mol of 2-mercaptobenzothiazole/mol of BTTB-CoA (Scheme 4; step B). Steady-state analysis of this elimination reaction following the release of 2-mercaptobenzothiazole at 310 nm gave a V_{max} of 6.9/min and a K_m of 6 μM for BTTB-CoA (data not shown).

The other product of the elimination reaction would be expected to be acryloyl-CoA (Scheme 4; step B). Indeed,

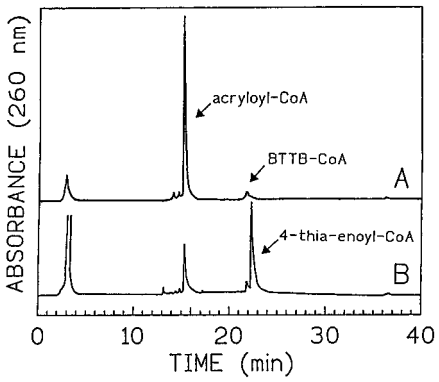


FIGURE 3: HPLC analysis of supernatants after incubation of the medium-chain acyl-CoA dehydrogenase with BTTB-CoA. The dehydrogenase (3.2 μM) was incubated with 100 μM BTTB-CoA in 50 mM phosphate buffer, pH 7.6, 25 °C for 11 min in the absence or presence of 1.2 mM ferricenium hexafluorophosphate (traces A and B, respectively). Peaks were identified by elution time and spectrum. The large early peak in trace B is due to the ferricenium ion.

Figure 3 shows that the disappearance of BTTB-CoA in the presence of the medium-chain acyl-CoA dehydrogenase is accompanied by the appearance of a more polar component with a retention time corresponding to authentic acryloyl-CoA (15.7 min; Table 1). Further, addition of enoyl-CoA hydratase to either a synthetic sample of acryloyl-CoA (*48*; see Materials and Methods) or to ultrafiltrates of enzyme incubation mixtures converted both to the corresponding 3-hydroxypropionyl-CoA eluting at 14.6 min (Table 1). Thus, the medium-chain dehydrogenase catalyzes the elimination of 2-mercaptobenzothiazole from BTTB-CoA leading to the release of acryloyl-CoA (Scheme 4). This is apparently the first direct demonstration of the elimination of a thiolate fragment in the acyl-CoA dehydrogenases. These data show clearly that the elimination of acryloyl-CoA per se does not lead to appreciable inactivation of the dehydrogenase: no significant inactivation occurs even after 1000 turnovers (see above). Thus, the inactivations encountered in Figure 1 derive ultimately from the reactivity of the eliminated thiolate fragments.

In addition to elimination, Scheme 4 depicts oxidation of BTTB-CoA (step A) with the ultimate oxidant being the facile 1-electron oxidant ferricenium hexafluorophosphate (*15*). Figure 3 shows that the inclusion of the ferricenium ion leads to the formation of a peak eluting 0.7 min later than the substrate. All 4-thia-*trans*-2-enoyl-CoA derivatives thus far examined (both simple straight-chained and those listed in Table 1) elute after their corresponding saturated analogues, in marked distinction to the behavior observed with normal substrates in which the enoyl-derivatives appear more polar on reverse-phase chromatography (*20*). The spectrum of oxidized BTTB-CoA (absorbance maxima at 324 and 260 nm; not shown) is also consistent with the appearance of a 4-thia-*trans*-2-enoyl-CoA chromophore (*20*). Finally, crotonase treatment of the material leads to the disappearance of the product peak and the formation of

malonyl-semialdehyde-CoA via nonenzymatic decomposition of the resulting thiohemiacetal, as observed for 4-thia-*trans*-2-octenoyl-CoA (*20*). The analogous reactions for DCTH-CoA (Scheme 2) involve conversion of compound 3 into species 4 and 5.

Table 1 summarizes the ratio between elimination and oxidation observed at 1.2 mM ferricenium ion estimated from the areas of the acryloyl-CoA/4-thia-*trans*-2-enoyl-CoA peaks observed in Figure 3. This relatively high concentration of oxidant should be compared to a K_m for ferricenium of 55 μM in the presence of a preferred substrate, octanoyl-CoA (*15*). However, high concentrations of this facile, kinetic, and potent thermodynamic oxidant of the dehydrogenase (*15*, *49*) do not completely prevent the elimination reaction of BTTB-CoA leading to acryloyl-CoA formation (Figure 3, curve B; acryloyl-CoA/oxidized product ratio of 0.63, Table 1).

Dehydrogenase Catalyzed Elimination of 4-Phenyl-4-thiabutanoyl-CoA, 4-(4-Nitrophenyl)-4-thiabutanoyl-CoA, and 4-(2,4-Dinitrophenyl)-4-thiabutanoyl-CoA. The partition between elimination and oxidation would be expected to depend, in part, on the leaving group ability of the thiolate fragment (*50*). Thus, we synthesized 4-phenyl-4-thiabutanoyl-CoA (PTB-CoA; Chart 1), 4-(4-nitrophenyl)-4-thiabutanoyl-CoA (NPTB-CoA), and 4-(2,4-dinitrophenyl)-4-thiabutanoyl-CoA (DNPTB-CoA) with increasing leaving group abilities of their thiolate fragments (*50*). PTB-CoA is dehydrogenated by the enzyme with the production of the corresponding 4-thia-*trans*-2-enoyl-CoA analogue (Table 1) at rates between 11 and 14% that shown by octanoyl-CoA in three different assay systems (Table 3). In the absence of electron acceptor, no significant elimination of acryloyl-CoA was observed by HPLC (over 1 h, see Materials and Methods) presumably because thiophenol is a poor leaving group. The corresponding mononitro and dinitro analogues shown in Chart 1 eliminate at very slow rates (0.039/min and 0.044/min, respectively; see Materials and Methods). Whereas the mononitro analogue, NPTB-CoA, is a significant substrate of the dehydrogenase in the ferricenium assay (17% of that of octanoyl-CoA), an additional ortho-nitro substituent drastically reduces activity (to 0.01%; Table 3). The steric bulk, polarity, and electron-withdrawing character of the dinitrophenyl substituent is likely to suppress normal oxidative turnover (see later).

CTFTH-CoA. 6-Chloro-5,5,6-trifluoro-4-thiahexanoyl-CoA (CTFTH-CoA; Chart 1) at 50 equiv partially inactivates the dehydrogenase with a half-time of 2.1 min and requires a stoichiometry of 72 mol of inhibitor for complete inactivation of the enzyme (Figure 1). This finding is significant, because this analogue lacks the 5,6-double bond found in DCTH-CoA and DCTFTH-CoA (Chart 1) and, therefore, could not generate the chlorothioketene moiety suggested as one cause for the cytotoxicity of DCTH (see later).

The extent of inactivation of the dehydrogenase by CTFTH-CoA at pH 7.6 was unaffected by the inclusion of up to 1 mM GSH in the incubation medium (see Materials and Methods). Thus, it is likely that inactivation of the dehydrogenase occurs before release of reactive species into bulk solution where they could be trapped by glutathione or solvent water (see later). Inactivation of the dehydrogenase (2 μM) by CTFTH-CoA (150 μM) was strongly protected by 50 μM octanoyl-CoA (giving 89% residual activity compared to only 2% residual activity after a 1 h incubation; see Materials and Methods). Enzyme inactivated with CTFTH-CoA does not regain significant activity after removal of excess reagents by ultrafiltration or upon prolonged storage at −20 °C (see Materials and Methods). In addition, activity is not recovered upon incubation of the treated enzyme (3.5 μM in 50 mM phosphate buffer, pH 7.6) with either 0.7 mM GSH, 0.7 mM DTT, or 10 mM hydroxylamine for 90 min at 25 °C (see Materials and Methods). These findings, and the spectral data to be reported later, suggest that CTFTH-CoA is a mechanism-based inactivator (*51*) of the medium-chain acyl-CoA dehydrogenase.

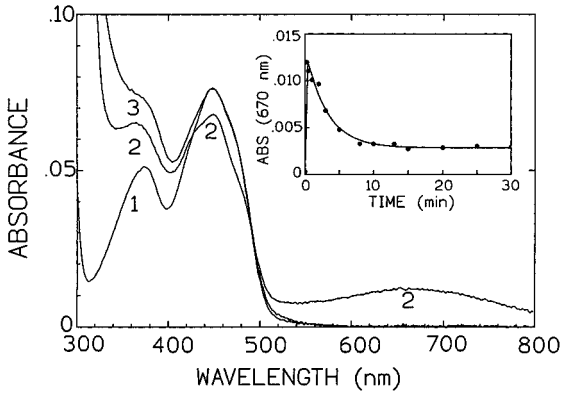


FIGURE 4: Spectral changes on the addition of CTFTH-CoA to the medium-chain acyl-CoA dehydrogenase. The medium-chain dehydrogenase (curve 1, 5 μM in 50 mM phosphate buffer, pH 7.6, 25 °C) was mixed with 350 μM CTFTH-CoA and the spectrum was recorded after 16 s (curve 2, corresponding to maximal development of the charge transfer band) and 30 min (in which the band has essentially disappeared). At 30 min, turbidity in the sample (corresponding to an absorbance between 0.003 to 0.004 at 820 nm) was consistently observed. For comparison, the spectrum at 30 min was corrected to minimize this light scattering contribution (see Materials and Methods). The inset plots the uncorrected absorbance at 670 nm with time. The solid line is fit to two exponentials with a rapid phase greater than 10/min and a slow phase of 0.32/min.

Figure 4 shows the spectral changes observed during inactivation of the medium-chain acyl-CoA dehydrogenase by CTFTH-CoA. Curve 2 was recorded 16 s after the addition of 70 equiv of the inhibitor and shows a prominent, long-wavelength band (λ_{max} about 670 nm; apparent extinction coefficient of about 2 mM^{-1} cm^{-1}). The flavin peak is decreased by about 16% with the appearance of a noticeable shoulder at 432 nm leading to pronounced skewing of the main absorbance envelope. Subsequent scans show loss of the long-wavelength feature ($t_{1/2}$ = 2.2 min; inset, Figure 4) with a return of the original flavin absorbance. Loss of activity in Figure 1A shows essentially the same half-time, suggesting that inactivation is associated with loss of the charge-transfer complex.

The spectral change that occurs immediately after mixing in Figure 4 (curve 2) shows comparatively little decrease in the flavin absorbance at 446 nm, indicating that CTFTH-CoA was a thermodynamically poor reductant of the medium-chain dehydrogenase. The substitution of the more oxidizing 8-Cl-FAD (*45*) allows essentially complete reduction of the flavin chromophore with appearance of a strong charge-transfer band at 670 nm (Figure 5) showing that, as in the case of BTTB-CoA (Scheme 3), the equilibrium K_2 is

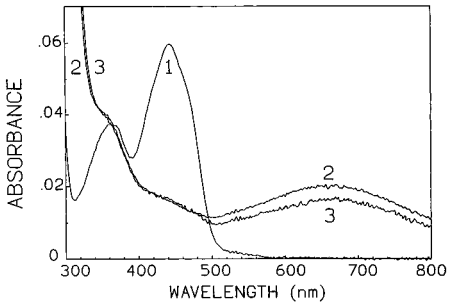


FIGURE 5: Reduction of 8-Cl-FAD-substituted dehydrogenase by CTFTH-CoA. The 8-Cl-FAD-substituted dehydrogenase was diluted to 4.2 μM in 50 mM phosphate buffer, pH 7.6, 25 °C (curve 1), and the spectrum was recorded 30 s and 30 min after the addition of 100 μM of CTFTH-CoA (curves 2 and 3, respectively).

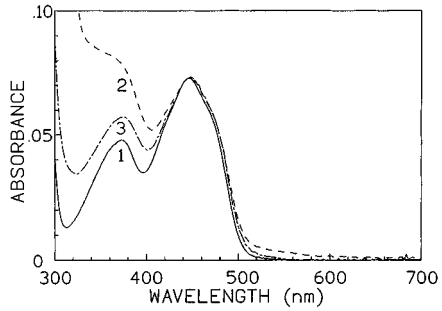


FIGURE 6: The spectrum of the native dehydrogenase after treatment with CTFTH-CoA. The dehydrogenase was diluted to 5 μM in 50 mM phosphate buffer pH 7.6, 25 °C (curve 1), and the spectrum was recorded 30 min after the addition of 350 μM of CTFTH-CoA (curve 2, corrected for turbidity as before). The CTFTH-CoA-incubated enzyme was washed with buffer by ultrafiltration and the spectrum of the recovered enzyme was recorded (curve 3; see Materials and Methods).

readily shifted to the right. The long-wavelength band observed upon 8-Cl−FAD substitution of the medium-chain acyl-CoA dehydrogenase is comparatively stable declining by only about 20% over 30 min when 25 equivalents of CTFTH-CoA is used. Importantly, a 1 h incubation of 50 equiv leads to an only modest (23%) inactivation of the substituted dehydrogenase (data not shown), in marked contrast to the substantial inactivation observed with normal enzyme (Figure 1A). These data suggest that inactivation proceeds from the oxidized enzyme·4-thiaacyl-CoA substrate complex and not from the corresponding reduced flavin·4-thia-2-enoyl-CoA charge transfer species (Scheme 3). Clearly the bound enoyl-CoA is not the inactivating species.

The results outlined above suggest that, like BTTB-CoA, elimination and oxidation pathways should compete for the available CTFTH-CoA (Scheme 5). In the absence of the ferricenium ion, CTFTH-CoA is converted to acryloyl-CoA (via pathway B) with the release of the reactive thiolate fragment (compound 4; see later). Acryloyl-CoA formation is strongly suppressed in the presence of 1.2 mM ferricenium ion with the generation of a new peak at 21.1 min (Table 1) with an absorbance spectrum characteristic of the 4-thia-2-enoyl-CoA analogue (compound 2, Scheme 5). In standard assay systems, CTFTH-CoA is between 8.9 and 1.3% as effective as octanoyl-CoA as a conventional substrate of the medium-chain dehydrogenase (Table 3).

Figure 6 shows the spectrum of the native dehydrogenase after treatment with CTFTH-CoA and removal of excess reagents by ultrafiltration. Unlike other thioester inactivators of the medium-chain dehydrogenase, such as 2- and 3-octynoyl-CoA (*2*, *52*), only slight changes in the main flavin envelope are seen without the appearance of significant shoulders on either side of the peak. These data suggest that inactivation is associated with relatively small changes in the polarity of the active site. The modified enzyme cannot be reduced by excess octanoyl-CoA and no significant spectral changes to the flavin chromophore were observed with the charge transfer donors acetoacetyl-CoA (*43*, *53*, *54*) and CoA-persulfide (*55*; data not shown).

DCTFTH-CoA. The second vinylic compound depicted in Chart 1, 5,6-dichloro-7,7,7-trifluoro-4-thia-5-heptenoyl-CoA, differs from DCTH-CoA by the presence of a terminal trifluoromethyl group. It is the poorest reductant of the enzyme flavin even after reconstitution with 8-Cl-FAD (Table 2). DCTFTH-CoA is a very weak substrate of the enzyme in all three assay systems with a maximal activity of 0.3% of that shown by octanoyl-CoA (Table 3). In keeping with these observations, even the facile oxidant, ferricenium, can only divert a relatively small amount of this inhibitor to the corresponding 4-thia-2-enoyl-CoA. Thus, the ratio of acryloyl-CoA to 2-enoyl-CoA product remains high for DCTFTH-CoA (1.5; Table 1), whereas it is almost totally suppressed in favor of the oxidation product with DCTH-CoA (0.02).

Scheme 5

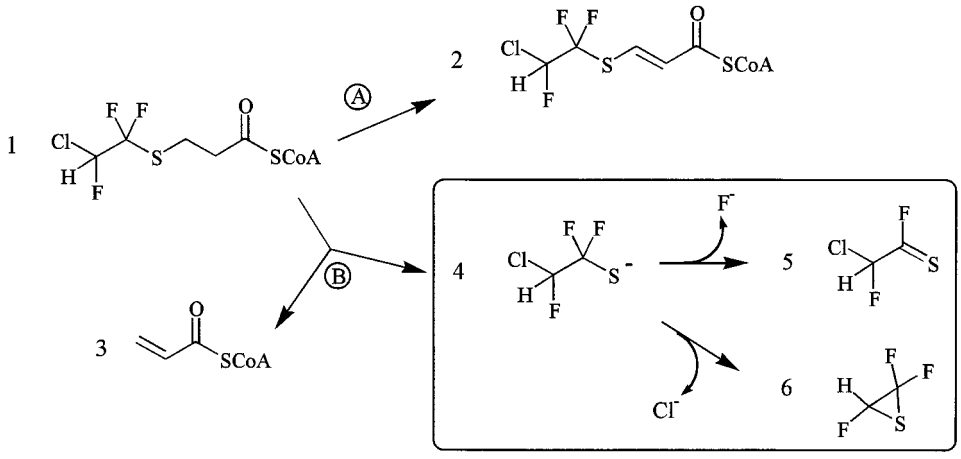


Table 4: 4-Thiaacyl-CoA Thioesters as Substrates of the Acyl-CoA Oxidase: Activity and HPLC Partition Ratios of Acryloyl-CoA to 4-Thia-2-enoyl-CoA[a]

substrate	turnover (min^{-1})	acryloyl-CoA/ 2-enoyl-CoA
DCTH-CoA	184	0.01
DCTFTH-CoA	286[b]	0.40
CTFTH-CoA	279	0.12
BTTB-CoA	178	0.16
PTB-CoA	30	<0.01
NPTB-CoA	513	<0.01
DNPTB-CoA	60	0.03

[a] Each of the 4-thiaacyl-CoA analogues (50 μM) was tested as a substrate in assays with acyl-CoA oxidase at 25 °C in phosphate buffer, pH 7.6, following the increase in 260 nm absorbance, except for PTB-CoA in which 312 nm was used (see Materials and Methods). Products of aerobic enzyme incubation mixtures with each of the 4-thiaacyl-CoA thioesters were analyzed by HPLC as described in Materials and Methods. [b] Assays were substantially curved due to rapid inactivation of the enzyme (see Results and Discussion).

Interaction of Acyl-CoA Oxidase with the 4-Thiaacyl-CoA Analogues. Although the peroxisomal acyl-CoA oxidases use molecular oxygen as an electron acceptor instead of ETF, their reductive half-reactions share many similarities with the acyl-CoA dehydrogenases (*31*, *56*). For example, both the dehydrogenase and oxidase are inhibited by the site-directed inactivators 3-octynoyl-CoA, 3,4-pentadienoyl-CoA, and methylenecyclopropylacetyl-CoA (*31*, *56*, *57*). Thus, it was of interest to examine the behavior of the oxidase toward the thioesters shown in Chart 1.

All of the 4-thiaacyl-CoA thioesters are relatively good substrates of the oxidase when tested aerobically at 50 μM ligand (with initial rates corresponding to turnover numbers ranging from 60 to 513/min; Table 4). HPLC analysis of aerobic reaction mixtures of the oxidase with each of the 4-thia analogues showed the formation of variable amounts of acryloyl-CoA (with acryloyl-CoA/4-thia-2-enoyl-CoA ratios of <0.01 to 0.40; Table 4). DCTFTH-CoA yielded the most acryloyl-CoA (Table 4) with the concurrent formation of the corresponding thiolate elimination product, 1,2-dichloro-3,3,3-trifluoropropenethiolate. DCTFTH-CoA was the only thioester which fully inactivated the oxidase (with a $t_{1/2} = 0.61$ min and a residual activity of 3% at 60 min with 100 μM inhibitor; data not shown; see Materials and Methods). In contrast, DCTH-CoA eliminated poorly (ratio = 0.01; Table 4) and, consequently, did not significantly inactivate the oxidase (data not shown; see Materials and Methods).

In the medium-chain acyl-CoA dehydrogenase, inactivation by DCTH-, DCTFTH-, and CTFTH-CoA is caused by the release of reactive thiolate fragments and not by acryloyl-CoA (see earlier). Similarly, inactivation of the oxidase by DCTFTH-CoA presumably results from the formation of the elimination product, 1,2-dichloro-3,3,3-trifluoropropenethiolate. Accordingly, multiple turnovers of BTTB-CoA generated substantial levels of acryloyl-CoA in the active center of the acyl-CoA oxidase (Table 4) without significant inactivation of the enzyme (data not shown).

Mode of Inactivation of the Medium-Chain Acyl-CoA Dehydrogenase. This work shows conclusively that acryloyl-CoA is a primary product of the elimination reaction of DCTH-CoA (Table 1). The previous observation of 3-hydroxypropionyl-CoA and not acryloyl-CoA from DCTH-CoA (*19*) presumably arose because of traces of enoyl-CoA hydratase in the enzyme used for those experiments. Indeed, all the activated thioesters described here eliminate acryloyl-CoA (Table 1). Acryloyl-CoA has been suggested as the inactivating species during 3-chloropropionyl-CoA incubation with HMG-CoA synthase (*58*) and fatty acid synthase (*59*). In addition, *S*-acryloyl-*N*-acetylcysteamine was shown to inhibit HMG-CoA synthase and lyase and fatty acid synthase (*60*). However, the data with BTTB-, NPTB-, and DNPTB-CoA clearly show that multiple cycles of generation of this Michael acceptor within the active site of the dehydrogenase does not lead to significant inactivation. Further, acryloyl-CoA does not appear to inactivate the bacterial butyryl-CoA dehydrogenase (*61*, *62*) or the acyl-CoA oxidase (this work).

For elimination to compete significantly with normal oxidative turnover of a 4-thiaacyl-CoA analogue, several requirements must be met. First, the thiolate to be eliminated must be a sufficiently good leaving group (*50*). In a nonenzymatic model system, utilizing 3-quinuclidinone as the catalyst (*63*, *64*), the order of elimination rates was BTTB-CoA > DNPTB-CoA > NPTB-CoA $\gg$ PTB-CoA (J. F. Baker-Malcolm, unpublished observations), consistent with the electron-withdrawing character of the substituents (*50*). On the enzyme, neither 4-thiaoctanoyl-CoA nor PTB-CoA appear sufficiently activated to be significant elimination substrates of the medium-chain dehydrogenase (*20*; this work), whereas BTTB-CoA, DNPTB-CoA, and NPTB-CoA all undergo elimination (see earlier).

Second, β-elimination reactions require the attainment of an appropriate geometry that brings the leaving group anti or syn to the abstracted proton (*50*). In normal catalysis, the hydride eliminated from C-3 of acyl-CoA thioesters is anti to the pro-R-α-proton (Scheme 1; *1*, *65*). This hydride is transferred to the *N*-5 position of the flavin ring (*6*, *61*). The corresponding anti elimination of a thiolate fragment at this same locus appears to be sterically improbable. Thus, a minimized computer model (*66*, *67*) of the BTTB-CoA·enzyme complex suggests that anti elimination of 2-mercaptobenzothiazole would encounter prohibitive steric interference from the flavin ring (not shown). Modeling of complexes with all others analogues is also consistent with the syn elimination of thiolate fragments. Syn elimination occurs during the dehydration of 3-hydroxyacyl-CoA analogues by enoyl-CoA hydratase (*68*−*70*).

A third factor influencing rates of elimination is the equilibrium between oxidized and reduced enzyme forms (K_2; Scheme 3). The elimination reaction would only be expected to proceed at the oxidized enzyme·4-thiaacyl-CoA substrate level because the 4-thia-*trans*-2-enoyl-CoA derivatives lack a sufficiently acidic α-proton. Thus, 4-thia analogues in which this redox equilibrium lies far to the right would not be expected to undergo rapid elimination of their thiolate fragments. Manipulation of the internal equilibrium depicted in Scheme 3 by substitution of the normal flavin by the more oxidizing 8-Cl-FAD analogue supports this expectation. Thus, the data with 8-Cl-FAD show that substitution of the enzyme (see Materials and Methods) leads to a 5-fold slowing of the elimination turnover number with BTTB-CoA (data not shown).

Thus, we show in this paper that a range of 4-thiaacyl-CoA analogues eliminate thiolate fragments at a significant

rate. Although it can be definitively concluded that the acryloyl-CoA released during these β-elimination reactions is not responsible for the inactivation of the enzyme encountered by DCTH-, CTFTH-, and DCTFTH-CoA, the exact nature of the thiolate-derived attacking species and the amino acid target(s) responsible for loss of enzyme activity remain to be established. In the case of DCTH-CoA, chemical model studies, both in solution and in the gas phase, suggest that the immediate elimination product (compound 5; Scheme 2) would rapidly yield the electrophilic thioketene (compound 7). DCTFTH-CoA, a DCTH-CoA analogue containing an additional trifluoromethyl group, appears at least as potent with the medium-chain dehydrogenase and is the only compound that inhibits acyl-CoA oxidase (see above). The mode of inactivation of DCTH-CoA and DCTFTH-CoA would be expected to be similar with the release of even more highly electrophilic species analogous to compounds 5, 7, and 8 in Scheme 2. Perhaps this increased reactivity explains the effectiveness of DCTFTH-CoA toward acyl-CoA oxidase.

CTFTH-CoA is the least potent of the three inhibitors toward the acyl-CoA dehydrogenase and potential modes of decomposition of the corresponding thiolate are shown in Scheme 5. Both the thionoacyl fluoride (compound 5) and the thiirane (compound 6) have been implicated in the decomposition of the thiolate (compound 4; *71*−*77*). Model studies in the gas phase are consistent with the formation of thiirane (compound 6) as a predominant product from the propyl thioester of CTFTH in the presence of the hydroxide ion (Zhang, T.-L., Wang, L., Anders, M. C., Thorpe, C., and Ridge, D. P., unpublished observations).

The present studies suggest that the partition between oxidation and elimination pathways (e.g., pathways A and B, Scheme 2) in the mitochondrion is strongly influenced by the presence of an efficient electron acceptor for the dehydrogenase and also the nature of the thioester ligand. Thus, for example, the physiological electron acceptor ETF is a good oxidant of DCTH-CoA-reduced enzyme (Table 3), and significant flux of electrophilic species will be generated via the oxidation pathway A (Scheme 2). In contrast, DCTFTH-CoA is a weak reductant of the enzyme (Table 2) and a very poor oxidizable substrate in all of the assay systems tested (e.g., the rate with ETF is undetectable, Table 3). Thus, the elimination pathway would be expected to predominate in the mitochondrion with DCTFTH-CoA. Finally, the relative efficiency of several of these 4-thia analogues as substrates of the yeast acyl-CoA oxidase suggests that the contribution of the mammalian peroxisome to the metabolism of these cytotoxic fatty acids needs to be explored.

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