Medium-Chain Acyl-CoA Dehydrogenase- and Enoyl-CoA Hydratase-Dependent Bioactivation of 5,6-Dichloro-4-thia-5-hexenoyl-CoA†

Michael E. Fitzsimmons,‡,§ Colin Thorpe,|| and M. W. Anders*,§

Department of Pharmacology, School of Medicine and Dentistry, University of Rochester, 601 Elmwood Avenue, Rochester, New York 14642, and Department of Chemistry and Biochemistry, University of Delaware, Newark, Delaware 19716

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ABSTRACT: 5,6-Dichloro-4-thia-5-hexenoic acid (DCTH) is a potent hepato- and nephrotoxin that induces mitochondrial dysfunction in rat liver and kidney. Previous studies indicate that DCTH undergoes fatty acid β-oxidation-dependent bioactivation. The objectives of the present experiments were to elaborate the bioactivation mechanism of DCTH and to examine the interaction of the coenzyme A thioester of DCTH (DCTH-CoA) with the medium-chain acyl-CoA dehydrogenase. In the presence of the terminal electron acceptor ferricenium hexafluorophosphate ($FcPF_6$), DCTH-CoA was oxidized by the medium-chain acyl-CoA dehydrogenase to give 5,6-dichloro-4-thia-*trans*-2,5-hexadienoyl-CoA. Enoyl-CoA hydratase catalyzed the conversion of 5,6-dichloro-4-thia-*trans*-2,5-hexadienoyl-CoA to 5,6-dichloro-4-thia-3-hydroxy-5-hexenoyl-CoA, which eliminated 1,2-dichloroethenethiol and gave malonyl-CoA semialdehyde as a product. Chloroacetic acid was detected as a terminal product derived from 1,2-dichloroethenethiol. Incubation of DCTH-CoA with the medium-chain acyl-CoA dehydrogenase in the absence of $FcPF_6$ gave 3-hydroxypropionyl-CoA as the major product and resulted in the irreversible inactivation of the enzyme. Under these conditions, DCTH-CoA apparently undergoes a β-elimination reaction to give 1,2-dichloroethenethiol and acryloyl-CoA, which is hydrated to give 3-hydroxypropionyl-CoA as the terminal product. The β-elimination product 1,2-dichloroethenethiol may yield reactive intermediates that inactivate the dehydrogenase. Enzyme inactivation was rapid, DCTH-CoA concentration-dependent, and blocked by octanoyl-CoA, but not by glutathione. The medium-chain acyl-CoA dehydrogenase was not inactivated by acryloyl-CoA, and little inactivation was observed in the presence of $FcPF_6$. These results show that DCTH-CoA is bioactivated by the mitochondrial fatty acid β-oxidation system to reactive intermediates. This bioactivation mechanism may account for the observed toxicity of DCTH *in vivo* and *in vitro*.

The mitochondrial acyl-CoA dehydrogenases are a family of flavin-dependent enzymes that catalyze the first oxidative step in the catabolism of fatty acids. Four immunologically distinct acyl-CoA dehydrogenases have been identified that metabolize straight-chain acyl-CoA substrates: short-chain or butyryl-CoA dehydrogenase catalyzes the oxidation of C_4−C_6 acyl-CoA substrates (Green et al., 1954); medium-chain acyl-CoA dehydrogenase catalyzes the oxidation of C_4−C_{16} acyl-CoA substrates and shows maximum activity with C_{10} acyl-CoA substrates (Crane et al., 1956; Hall & Kamin, 1975; Thorpe et al., 1979); long-chain acyl-CoA dehydrogenase catalyzes the oxidation of C_8−C_{18} acyl-CoA substrates with maximum activity with C_{12} acyl-CoA substrates (Hauge et al., 1956); and the very long-chain acyl-CoA dehydrogenase shows maximum activity with C_{16} acyl-CoA substrates (Izai et al., 1992). Oxidation of acyl-CoA substrates by acyl-CoA dehydrogenases produces *trans*-2-enoyl-CoA metabolites with concomitant reduction of the bound flavin (Beinert, 1963). Substrate-reduced acyl-CoA dehydrogenases are oxidized by the electron-transferring flavoprotein in two successive one-electron steps (Crane & Beinert, 1956; Hall & Kamin, 1975; Gorelick et al., 1985). The electron-transferring flavoprotein ubiquinone oxidoreductase transfers electrons from the electron-transferring flavoprotein to the electron-transport chain at the level of coenzyme Q (Ruzicka & Beinert, 1977; Beckmann & Frerman, 1985; Ramsay et al., 1987).

The acyl-CoA dehydrogenase-catalyzed oxidation of fatty acids is initiated by abstraction of the acidic *pro-R*-α-hydrogen as a proton by an active-site basic amino acid (Biellmann & Hirth, 1970; Frerman et al., 1980; Fendrich & Abeles, 1982; Gomes et al., 1981; Ghisla et al., 1984). The *pro-R*-β-hydrogen is transferred as a hydride ion to the oxidized flavin to give the *trans*-enoyl-CoA product (Ghisla et al., 1984). With most substrates, α-proton abstraction and β-hydride transfer appear to be concerted, with no accumulation of α-carbanion or enolate intermediates (Ghisla et al., 1984; Pohl et al., 1986; Schopfer et al., 1988). Studies with 3-thia- and 3-oxaoctanoyl-CoA show, however, that α-proton abstraction occurs in the absence of β-hydride transfer because these analogs lack β-hydrogens (Lau et al., 1988). Moreover, 3-thia- and 3-oxaoctanoyl-CoA form charge-transfer complexes with the medium-chain acyl-CoA dehydrogenase that have been assigned to an enolate as the charge-transfer complex donor and an oxidized flavin as the charge-transfer acceptor. In contrast to 3-thiaoctanoyl-CoA, 4-thiaoctanoyl-CoA is oxidized by the medium-chain acyl-CoA dehydrogenase to give 4-thia-*trans*-2-octenoyl-CoA (Lau et al., 1988). 4-Thia-*trans*-2-octenoyl-CoA is hydrated

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* Author to whom correspondence and reprint requests should be addressed: Telephone, 716-275-1681; Fax, 716-244-9283.

‡ Present address: Division of Clinical Pharmacology, FDA Center for Drug Evaluation and Research, Laurel, MD 20708.

§ University of Rochester.

|| University of Delaware.

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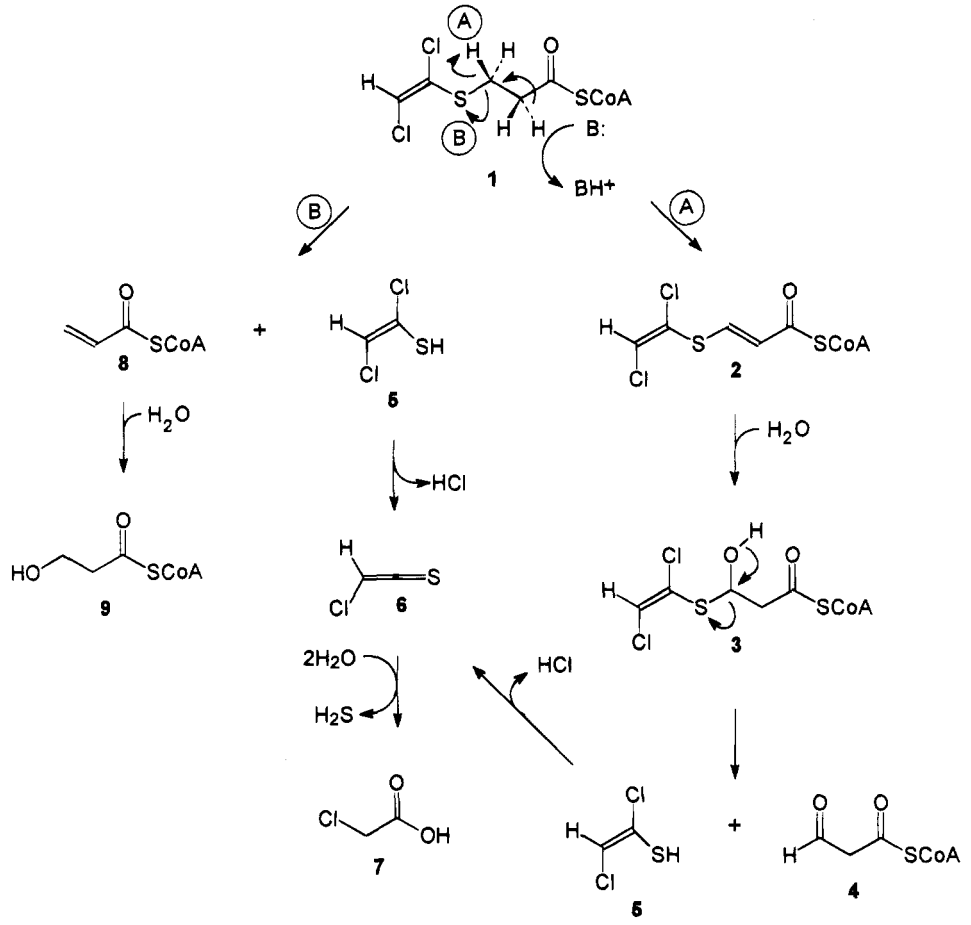


FIGURE 1: Proposed bioactivation pathway of 5,6-dichloro-4thia-5-hexenoyl-CoA: **1**, 5,6-dichloro-4-thia-5-hexenoyl-CoA; **2**, 5,6-dichloro-4-thia-*trans*-2,5-hexadienoyl-CoA; **3**, 5,6-dichloro-4-thia-3-hydroxy-5-hexenoyl-CoA; **4**, malonyl-CoA semialdehyde; **5**, 1,2-dichloroethenethiol; **6**, chlorothioketene; **7**, chloroacetic acid; **8**, acryloyl-CoA; **9**, 3-hydroxypropionyl-CoA.

by enoyl-CoA hydratase to the presumed thiohemiacetal 4-thia-3-hydroxyoctanoyl-CoA, which eliminates butanethiol to give malonyl-CoA semialdehyde (Lau et al., 1989).

In addition to catalyzing dehydrogenation reactions, the short-chain acyl-CoA dehydrogenase catalyzes the elimination of HF from 3-fluoropropionyl-CoA to yield acryloyl-CoA (Fendrich & Abeles, 1982). Similarly, lactate oxidase catalyzes the elimination of chloride from β-chlorolactate (Walsh et al., 1973). Other flavoproteins also catalyze elimination reactions with substrates possessing good leaving groups in the β-position (Walsh et al., 1971).

5,6-Dichloro-4-thia-5-hexenoic acid (DCTH,[1] see Figure 1, **1**, for the structure of the CoA thioester) is the desamino analog of the nephrotoxic *S*-conjugate, *S*-(1,2-dichlorovinyl)-L-cysteine, which is bioactivated by cysteine conjugate β-lyase to give 1,2-dichloroethenethiol as a product (Dekant et al., 1988). DCTH is cytotoxic in isolated rat hepatocytes (Fitzsimmons & Anders, 1993), an inhibitor of rat liver and kidney mitochondrial respiration (Stonard & Parker, 1971), and a potent hepatotoxin and nephrotoxin in rats (Fitzsimmons et al., 1994). Chain-length analog studies showed that the 6-thia analog of DCTH, 7,8-dichloro-6-thia-7-octenoic acid, was as toxic as DCTH, whereas the 3-, 5-, and 7-thia analogs 4,5-dichloro-3-thia-4-pentenoic acid, 6,7-dichloro-5-thia-6-heptenoic acid, and 8,9-dichloro-7-thia-8-nonenoic acid, respectively, were not toxic (Fitzsimmons & Anders, 1993; Koechel et al., 1991). These studies indicate that DCTH is bioactivated by a fatty acid β-oxidation-dependent mechanism.

The objectives of the present investigation were to study the interaction of DCTH-CoA with the medium-chain acyl-CoA dehydrogenase and to elucidate the bioactivation mechanism of DCTH-CoA (Figure 1). Two bioactivation mechanisms may be involved: the medium-chain acyl-CoA dehydrogenase may catalyze α-proton abstraction from DCTH-CoA (**1**), with transfer of the β-hydrogen as a hydride ion to the oxidized flavin to give 5,6-dichloro-4-thia-*trans*-2,5-hexadienoyl-CoA (**2**) (pathway A). Enoyl-CoA **2** may be hydrated by enoyl-CoA hydratase to afford 5,6-dichloro-4-thia-3-hydroxy-5-hexenoyl-CoA (**3**), which would be expected to eliminate 1,2-dichloroethenethiol (**5**) and yield malonyl-CoA semialdehyde (**4**). Loss of HCl from 1,2-dichloroethenethiol (**5**) would give the electrophilic chlorothioketene (**6**), which may be hydrolyzed to chloroacetic acid (**7**). Alternatively, α-proton abstraction from DCTH-CoA may result in the elimination of 1,2-dichloroethenethiol (**5**) and give acryloyl-CoA (**8**) as a product (pathway B).

The data presented herein show that the medium-chain acyl-CoA dehydrogenase catalyzes both β-oxidation and

[1] Abbreviations: DCTH, 5,6-dichloro-4-thia-5-hexenoic acid; DCTH-CoA, 5,6-dichloro-4-thia-5-hexenoyl-CoA; DCTHD-CoA, 5,6-dichloro-4-thia-*trans*-2,5-hexadienoyl-CoA; FAD, flavin adenine dinucleotide; 8-Cl-FAD, 8-chloroflavin adenine dinucleotide; $FcPF_6$, ferricenium hexafluorophosphate; HPLC, high-performance liquid chromatography; GC/MS, gas chromatography/mass spectrometry; NMR, nuclear magnetic resonance; EDTA, ethylenediaminetetraacetic acid; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid.

β-elimination reactions with DCTH-CoA as the substrate. Both bioactivation pathways yield the unstable metabolite 1,2-dichloroethenethiol. The present work provides additional insight into reactions in which 1,2-dichloro ethenethiol is generated *in vivo*. This paper also introduces a new mode of inactivation of the acyl-CoA dehydrogenase, in which a highly reactive acylating agent is formed within the active site of the enzyme. Presumably, an electrophilic product derived from 1,2-dichloroethenethiol reacts with cellular nucleophiles, and its formation and reactions may account for the inactivation of the medium-chain acyl-CoA dehydrogenase and the observed toxicity of DCTH *in vivo* and *in vitro*. Finally, the elimination reactions from 4-thiaalkanoates may provide a strategy for the development of prodrugs that selectively deliver thiol-based agents to cells that exhibit an active β-oxidation system.

MATERIALS AND METHODS

Materials. Medium-chain acyl-CoA dehydrogenase was purified from pig kidney as described previously (Gorelick et al., 1985; Lau et al., 1986). 8-Cl-FAD-substituted medium-chain acyl-CoA dehydrogenase was prepared as described earlier (Thorpe & Massey, 1983). $FcPF_6$ was prepared as described by Lehman and Thorpe (1990). Coenzyme A, octanoyl-CoA, enoyl-CoA hydratase (EC 4.2.1.17), and 5,5′-dithiobis(2-nitrobenzoic acid) were purchased from Sigma Chemical Co. (St. Louis, MO). $NaBH_4$, tetrabutylammonium hydrogen sulfate, pentafluorobenzyl bromide, dicyclohexylcarbodiimide, and potassium nitroferricyanide were purchased from Aldrich Chemical Co. (Milwaukee, WI).

Instrumental Analyses. ^{1}H NMR spectra were acquired with a Bruker WP270 spectrometer that operated at 270.13 MHz and are reported in ppm downfield from tetramethylsilane. Electron-impact mass spectra were acquired with a Hewlett-Packard 5880A gas chromatograph equipped with a 25 m × 0.02 cm i.d. fused-silica capillary column coated with cross-linked methyl silicone and connected to a Hewlett-Packard 5970 mass-selective detector. For the GC/MS detection of fatty acid methyl esters, the initial column temperature was held at 50 °C for 3 min and was then increased at 10 °C/min to 250 °C; the column temperature was held at 250 °C for 10 min. For GC/MS analysis of pentafluorobenzyl chloroacetate, the initial column temperature was held at 50 °C for 1 min, the column temperature was then increased at 10 °C/min to 250 °C, and the temperature was held at 250 °C for 1 min.

HPLC analyses of coenzyme A thioester standards and enzyme incubation samples were conducted with a Hewlett-Packard 1090 solvent delivery system coupled to a Waters NOVA-PAK C_{18} reversed-phase column. Samples from incubation mixtures and coenzyme A thioester standards were eluted with 25 mM potassium phosphate buffer (pH 5.3) and acetonitrile as follows: the initial eluant profile was held at 100% 25 mM potassium phosphate buffer (pH 5.3) for 5 min; the acetonitrile content of the eluant was then increased linearly to 50% over 25 min; the eluant was held at 50% acetonitrile/50% 25 mM potassium phosphate buffer (pH 5.3) for 5 min; the acetonitrile content was then decreased linearly to 0% in 5 min; and the column was equilibrated with 100% 25 mM potassium phosphate buffer (pH 5.3) for 5 min. Samples that contained malonyl-CoA semialdehyde were analyzed similarly except that 25 mM potassium phosphate buffer (pH 7.6) was used as an eluant instead of 25 mM potassium phosphate buffer (pH 5.3). The absorbance of the eluate was recorded at 260 nm with a Hewlett-Packard 1040A diode array detector, and the electronic absorption spectra of selected peaks were recorded from 220 to 600 nm. The electronic absorption spectra of the medium-chain acyl-CoA dehydrogenase and 8-Cl-FAD-substituted medium-chain acyl-CoA dehydrogenase were recorded from 200 to 800 nm on a Hewlett-Packard 8452A diode array spectrophotometer. The absorption spectrum of malonyl-CoA semialdehyde was recorded at pH 7.6 and 3.0 from 220 to 400 nm with a Perkin-Elmer Lambda 3A spectrophotometer connected to a Perkin-Elmer R100A recorder.

Preparation of DCTH-CoA. DCTH (296 μmol) was dissolved in 3 mL of dry acetone cooled on ice, and dicyclohexylcarbodiimide (326 μmol) was added with stirring. After 30 min, coenzyme A (29.6 μmol), dissolved in 2 mL of 0.2 M $KHCO_3$, was added, and the reaction mixture was stirred at 0 °C for 15 min. The reaction mixture was warmed to room temperature and brought to pH 3; 1 mL of 1 M KCl was then added. DCTH-CoA was purified and desalted with a Waters C_{18} Sep-Pak column that had been equilibrated with 1 mM HCl: the DCTH-CoA solution was added to the Sep-Pak, 5 mL of 1 mM HCl was passed through the Sep-Pak, and DCTH-CoA was eluted with 5 mL of a solution that contained 25% methanol/75% 1 mM HCl; the methanol was removed *in vacuo*. Unreacted DCTH was removed from the eluate by extraction with three 1 mL portions of diethyl ether. Thin-layer chromatography (silica gel with fluorescent indicator: *n*-butanol/water/acetic acid (5:3:2)) showed one spot ($R_f = 0.6$) that was nitroferricyanide positive only after hydrolysis of the thioester with a KOH/methanol solution. HPLC analysis showed a single peak (t_R = 19.2 min) that exhibited an absorption maximum at 260 nm (Table 1). The DCTH-CoA solution was lyophilized, and a sample was removed for ^{1}H NMR analysis. For ^{1}H NMR analysis, DCTH-CoA was dissolved in D_2O and lyophilized three times to remove exchangeable protons. DCTH-CoA was then dissolved in 25 mM potassium phosphate buffer (pH 5.3) that was prepared in D_2O and then lyophilized. Coenzyme A was prepared for ^{1}H NMR analysis by an identical procedure. DCTH-CoA ^{1}H NMR (D_2O): δ 0.6 (s, 3H); 0.7 (s, 3H); 2.3 (t, 2H); 2.7 (t, 2H); 2.8 (t, 2H); 3.0 (t, 2H); 3.2 (t, 2H); 3.3 (t, 2H); 3.4 (m, 1H); 3.7 (m, 1H); 3.9 (s, 1H); 4.1 (s, 2H); 4.4 (s, 1H); 4.5 (s, 1H); 6.0 (d, 1H); 6.5 (s, 1H); 8.1 (s, 1H); 8.4 (s, 1H). The ^{1}H NMR spectrum of DCTH-CoA showed resonances that were assigned to the coenzyme A moiety, along with a singlet at δ 6.5 and triplets at δ 2.7 and 3.0 that were assigned to the vinylic proton and methylene protons, respectively, of the 5,6-dichloro-4-thia-5-hexenoyl side chain. The triplet at δ 2.8 was shifted downfield from the corresponding resonance at δ 2.4 assigned to the terminal methylene group of pantetheine in coenzyme A (Fung et al., 1976).

Preparation of Acryloyl-CoA. Acryloyl-CoA was synthesized by the method of Kucka and Abeles (1985). Acryloyl-CoA ($\epsilon = 22.6$ mM^{-1} cm^{-1} at 260 nm) concentrations were determined spectrophotometrically. The product was 65% pure as determined by HPLC. HPLC analysis of the reaction mixture gave two major peaks: $t_R \approx 11.9$ min and $t_R \approx 13.6$ min. The peak at 13.6 min was assigned to acryloyl-CoA on the basis of its absorption spectrum, polarity, and hydration to 3-hydroxypropionyl-CoA by enoyl-

CoA hydratase (Dixon & Webb, 1979; see the following). The major impurity was apparently the bis adduct, which may be formed by the 1,4-addition of coenzyme A to acryloyl-CoA. Because of the instability of acryloyl-CoA, solutions were used within 5 h of preparation without further purification.

Preparation of 3-Hydroxypropionyl-CoA. Acryloyl-CoA (65 μM) was incubated with enoyl-CoA hydratase (12 units) in 50 mM potassium phosphate buffer (pH 7.6) at 25 °C for 10 min in a final volume of 1 mL. The incubation mixture was diluted with 4 mL of water, and a 100 μL portion was analyzed by HPLC. Hydration resulted in the loss of acryloyl-CoA ($t_R \approx 13.5$ min) and the formation of a single peak ($t_R \approx 12.4$ min) that was assigned to 3-hydroxypropionyl-CoA. 3-Hydroxypropionyl-CoA was purified by HPLC as described earlier for the analysis of coenzyme A thioesters, acetonitrile was removed *in vacuo*, and the sample was lyophilized. The white solid obtained was dissolved in D_2O and lyophilized three times to remove exchangeable protons. The thioester was dissolved in 500 μL of 25 mM phosphate buffer (pH 5.3) that was prepared in D_2O and then lyophilized, and the NMR spectrum was recorded. ^{1}H NMR (D_2O): δ 0.6 (s, 3H); 0.8 (s, 3H); 2.3 (t, 2H); 2.7 (t, 2H); 2.9 (t, 2H); 3.2 (t, 2H); 3.3 (t, 2H); 3.4 (m, 1H); 3.7 (t, 2H); 3.9 (s, 1H); 4.1 (s, 2H); 4.4 (s, 1H); 4.5 (s, 1H); 6.0 (d, 1H); 6.5 (s, 1H); 8.1 (s, 1H); 8.4 (s, 1H). The ^{1}H NMR spectrum of 3-hydroxypropionyl-CoA showed resonances that were assigned to the coenzyme A moiety and triplets at δ 2.9 and 3.7 that were assigned to the methylene protons of the 3-hydroxypropionyl-CoA side chain.

GC/MS Analyses. Acyl-CoA thioesters were converted to their methyl esters prior to GC/MS analysis: an aqueous solution of the acyl-CoA thioester was adjusted to pH 11 with 1 M KOH and was heated for 30 min at 37 °C. The aqueous solution was adjusted to pH 2.0 with 1 M HCl, and the acidified solution was extracted with three 1 mL portions of diethyl ether. The ether extracts were pooled, dried over Na_2SO_4, and filtered. The ether was removed with a stream of nitrogen, and the carboxylic acids were converted to their methyl esters by the addition of 30 μL of diazomethane in ether. A 10 μL sample was analyzed by GC/MS.

Determination of Molar Absorption Coefficients of DCTHD-CoA. The molar absorption coefficients of DCTHD-CoA were determined after reductive cleavage of the coenzyme A thioester (Lau et al., 1989). $NaBH_4$ (20 mg) was added to approximately 35 nmol of DCTHD-CoA in 0.7 mL of 50 mM potassium phosphate buffer (pH 7.6). The reaction mixture was stirred for 1 h, and 0.15 mL of 4 M HCl was added to destroy excess $NaBH_4$. The reaction mixture was neutralized with 5 M NaOH, 35 μL of 10 mM 5,5′-dithiobis-(2-nitrobenzoic acid) was added, and the reaction mixture was kept at room temperature for 5 min. The concentration of coenzyme A, and therefore that of DCTHD-CoA, was determined spectrophotometrically (ϵ = 14.1 mM^{-1} cm^{-1} at 412 nm). Coenzyme A was not detected prior to $NaBH_4$ treatment. The molar absorption coefficients for DCTHD-CoA were as follows: ϵ = 20.2 mM^{-1} cm^{-1} at 260 nm; ϵ = 16.1 mM^{-1} cm^{-1} at 300 nm; and ϵ = 4.7 mM^{-1} cm^{-1} at 335 nm.

Enzyme Assays. Medium-chain acyl-CoA dehydrogenase activity was determined by measuring the decrease in absorbance at 300 nm (ϵ = 4.3 mM^{-1} cm^{-1}) or 617 nm (ϵ = 410 M^{-1} cm^{-1}) due to the reduction of $FcPF_6$, as described previously (Lehman & Thorpe, 1990). Enoyl-CoA hydratase activity was measured with crotonyl-CoA (10−200 μM) or DCTHD-CoA (20−150 μM) as substrate in incubation mixtures that contained 0.15 mg of fatty acid-free bovine serum albumin and 0.3 mM EDTA in 700 μL of 50 mM potassium phosphate buffer (pH 7.6). The reaction was initiated by the addition of 16.6 or 0.7 μg of enoyl-CoA hydratase to incubation mixtures that contained crotonyl-CoA or DCTHD-CoA, respectively. Enoyl-CoA hydratase activity was determined by measuring the decrease in absorbance at 260 nm ($\Delta\epsilon$ = 6.22 mM^{-1} cm^{-1}) or 335 nm ($\Delta\epsilon$ = 4.7 mM^{-1} cm^{-1}) with crotonyl-CoA or DCTHD-CoA, respectively, as substrate (see above). Kinetic constants for enoyl-CoA hydratase-catalyzed hydration of crotonyl-CoA and DCTHD-CoA were determined with a Lineweaver−Burk plot.

Enzyme Incubations. All enzyme incubations were conducted in 50 mM potassium phosphate buffer (pH 7.6) and 0.3 mM EDTA and were incubated at 25 °C. Concentrations of medium-chain acyl-CoA dehydrogenase and 8-Cl-FAD-substituted medium-chain acyl-CoA dehydrogenase were determined spectrophotometrically with molar absorption coefficients of 15.4 mM^{-1} cm^{-1} at 446 nm (Thorpe et al., 1979) and 14.0 mM^{-1} cm^{-1} at 440 nm (Thorpe & Massey, 1983), respectively. Medium-chain acyl-CoA dehydrogenase and enoyl-CoA hydratase were removed from incubation mixtures with a Centricon-10 microconcentrator (Amicon, Danvers, MA).

Enoyl-CoA Hydratase-Catalyzed Hydration of DCTHD-CoA; Derivatization and Identification of Chloroacetic Acid. DCTHD-CoA (0.67 μmol) was incubated with enoyl-CoA hydratase (16.6 μg) for 30 min at 25 °C in a final volume of 1.25 mL of incubation buffer. Protein was removed from the incubation mixture with a Centricon-10 microconcentrator, and chloroacetic acid concentrations were determined after esterification with pentafluorobenzyl bromide (Ehrsson, 1971). One milliliter of chloroacetic acid standard (5−250 μM) or incubation sample was added to 2 mL of a solution that contained 1 mL of methylene chloride, 1 mL of 0.2 M NaOH and 0.2 M tetrabutylammonium hydrogen sulfate, and 20 μL of pentafluorobenzyl bromide. The reaction mixture was shaken for 3 h at room temperature, the organic and aqueous layers were separated, and the organic layer was dried over Na_2SO_4. After the removal of Na_2SO_4, methylene chloride was evaporated with a stream of nitrogen. The residue was dissolved in 50 μL of methylene chloride, and a 10 μL sample was analyzed by GC/MS. Pentafluorobenzyl chloroacetate concentrations were quantified by monitoring the GC eluate for *m/z* 274 (M^+) and 276 (M^{2+}) and by comparing with pentafluorobenzyl chloroacetate standards.

Inactivation of Medium-Chain Acyl-CoA Dehydrogenase. DCTH-CoA-induced inactivation of medium-chain acyl-CoA dehydrogenase was measured in the absence and presence of $FcPF_6$. The medium-chain acyl-CoA dehydrogenase (0.3 μM) was incubated with 0, 2.5, 5, 10, or 50 μM DCTH-CoA, with both 10 μM DCTH-CoA and 100 μM octanoyl-CoA, or with $FcPF_6$ (500 μM) and 10, 25, 50, or 100 μM DCTH-CoA for 30 min. At 5 min intervals, a 7 μL sample of the incubation mixture was added to 700 μL of 50 mM phosphate buffer (pH 7.6) that contained 0.3 mM EDTA, 100 μM octanoyl-CoA, and 200 μM $FcPF_6$. Medium-chain acyl-CoA dehydrogenase activity was determined as described previously (Lehman & Thorpe, 1990).

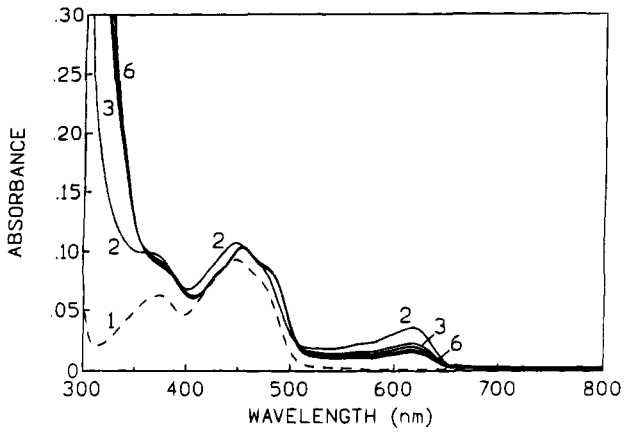


FIGURE 2: Spectral changes observed after the addition of DCTH-CoA to medium-chain acyl-CoA dehydrogenase in the presence of $FcPF_6$. Curves 1 and 2 show the absorption spectra of 6.1 μM medium-chain acyl-CoA dehydrogenase before and after the addition of $FcPF_6$ (97 μM), respectively, in 50 mM potassium phosphate buffer (pH 7.6, 25 °C). DCTH-CoA was added to the incubation mixture to give a final concentration of 24 μM, and curves 3–6 were recorded at 13, 25, and 120 s, respectively, after the addition of substrate. No further changes in the absorption spectra were observed after 120 s.

RESULTS

Oxidation of DCTH-CoA by the Medium-Chain Acyl-CoA Dehydrogenase in the Presence of $FcPF_6$: Identification of DCTHD-CoA as a Product. Figure 2 illustrates the oxidative turnover of DCTH-CoA catalyzed by the medium-chain acyl-CoA dehydrogenase in the presence of the facile one-electron oxidant, $FcPF_6$. Curve 1 shows the spectrum of the dehydrogenase, and curve 2 represents the added contribution of $FcPF_6$. The absorption band at 618 nm was due to $FcPF_6$, whereas the absorption maximum at 446 nm was dominated by the oxidized flavin chromophore. After the addition of a limiting concentration of DCTH-CoA, the absorbance at 618 nm decreased with a concomitant increase in the absorbance in the near-UV, which reflects the accumulation of the enoyl-CoA product (see the following). The decrease in absorbance at 618 nm corresponded to the reduction of 2.2 mol of $FcPF_6$ per mole of DCTH-CoA and is in agreement with the expected stoichiometry for a substrate such as octanoyl-CoA (Lehman & Thorpe, 1990). Reduction of the flavin chromophore at 450 nm was not evident during turnover in the presence of the $FcPF_6$ (curves 3–6). The flavin envelope was, however, red shifted from 446 to 450 nm with a pronounced shoulder at about 480 nm. Similar changes are seen with binding of a range of nonreducing ligands, such as *trans*-2-octenoyl-CoA, to the medium-chain dehydrogenase (Powell et al., 1987).

A decrease in absorbance at 618 nm was observed after the addition of medium-chain acyl-CoA dehydrogenase to a solution that contained DCTH-CoA and $FcPF_6$ (data not shown). The absorbance changes over 10 min corresponded to a total of 300 turnovers of $FcPF_6$ per active site. The average turnover within the first 20 s of the reaction was about 130 min^{-1} and decreased to 11 min^{-1} at 600 s. No significant decrease in absorbance was observed in the absence of enzyme.

HPLC analysis of an incubation mixture that contained 40 μM DCTH-CoA, 4.6 μM medium-chain acyl-CoA dehydrogenase, and 160 μM $FcPF_6$ showed the loss of DCTH-CoA ($t_R \approx 19.0$ min) and the appearance of a peak that

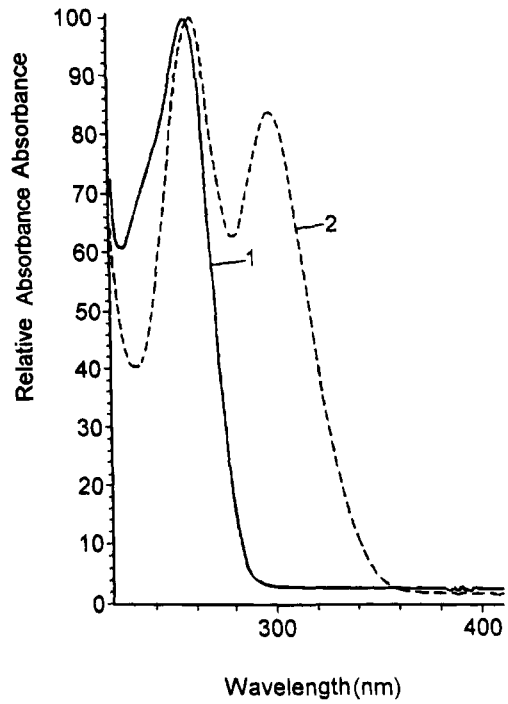


FIGURE 3: Electronic absorption spectra of DCTH-CoA and its medium-chain acyl-CoA dehydrogenase-catalyzed product. Curve 1 shows the spectrum of DCTH-CoA ($t_R \approx 19.0$ min). Curve 2 shows the spectrum of the oxidized metabolite ($t_R \approx 19.4$ min) obtained from the incubation of 4.6 μM medium-chain acyl-CoA dehydrogenase, 160 μM $FcPF_6$, and 40 μM DCTH-CoA. No other metabolites were detected by HPLC.

contained chromophores that indicated the presence of a coenzyme A moiety ($t_R \approx 19.4$ min). The absorption spectra of DCTH-CoA and the oxidized product are compared in Figure 3. The absorption spectrum of the metabolite showed a decrease in absorbance in the 230–250 nm region relative to DCTH-CoA and the appearance of a new absorption band at 300 nm that was absent from the spectrum of DCTH-CoA. These spectral changes are consistent with the formation of a 4-thia-2-enoyl-CoA chromophore (Silverstein et al., 1981; Lau et al., 1989).

After hydrolysis of the thioester, the mass spectrum of the methyl ester of the oxidized metabolite showed fragments at *m/z* 212 (M^+), *m/z* 214 (M + 2), and *m/z* 216 (M + 4) and an isotope cluster characteristic of the presence of two chlorine atoms (Figure 4). The molecular ion at *m/z* 212 and the fragmentation pattern agree with the expected mass spectrum of methyl 5,6-dichloro-4-thia-2,5-hexadienoate. The ^1H NMR spectrum of the metabolite showed the formation of a *trans*-enoyl-CoA product. ^1H NMR (D_2O): δ 0.6 (s, 3H); 0.7 (s, 3H); 2.3 (t, 2H); 2.9 (t, 2H); 3.2 (t, 2H); 3.3 (t, 2H); 3.4 (m, 1H); 3.7 (m, 1H); 3.8 (s, 1H); 4.0 (s, 2H); 4.4 (s, 1H); 4.5 (s, 1H); 6.0 (d, 1H); 6.1 (d, 1H); 6.5 (s, 1H); 6.8 (s, 1H); 7.4 (d, 1H); 8.1 (s, 1H); 8.4 (s, 1H). The loss of triplets at δ 2.7 and 3.0 and the appearance of doublets at δ 6.1 (J = 16.9 Hz) and 7.4 (J = 16.9 Hz) indicate the oxidation of DCTH-CoA to 5,6-dichloro-4-thia-*trans*-2,5-hexadienoyl-CoA (DCTHD-CoA).

Hydration of DCTHD-CoA by Enoyl-CoA Hydratase: Formation of Malonyl-CoA Semialdehyde and Chloroacetic Acid as Products. Hydration of DCTHD-CoA by enoyl-CoA hydratase would be expected to yield the thiohemiacetal 5,6-dichloro-4-thia-3-hydroxy-5-hexenoyl-CoA, which may eliminate 1,2-dichloroethenethiol (**5**) and give malonyl-CoA

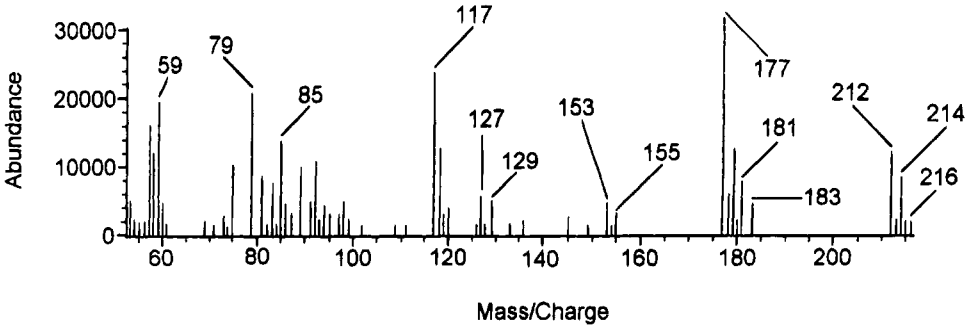


FIGURE 4: Mass spectrum of the oxidized product produced by medium-chain acyl-CoA dehydrogenase-dependent oxidation of DCTH-CoA. The coenzyme A-containing metabolite ($t_R \approx 19.1$ min) was purified by HPLC after incubation of 115 μM medium-chain acyl-CoA dehydrogenase, 2.79 mM $FcPF_6$, and 500 μM DCTH-CoA. The coenzyme A thioester of the metabolite was converted to its methyl ester with diazomethane, and the methyl ester was analyzed by GC/MS, as described in Materials and Methods.

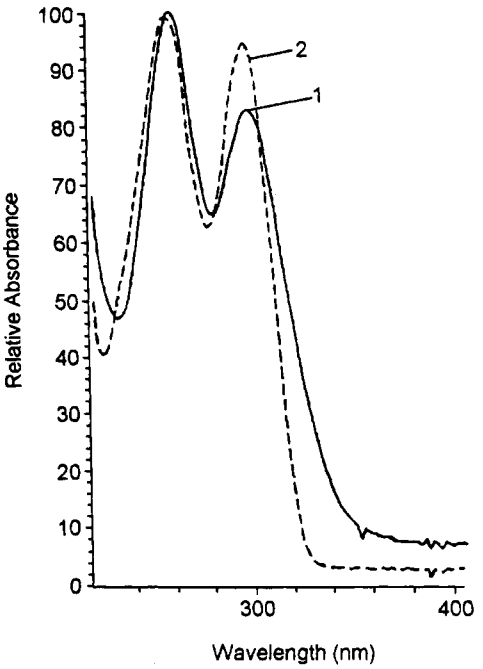


FIGURE 5: Electronic absorption spectra of DCTHD-CoA and its enoyl-CoA hydratase-catalyzed product. DCTH-CoA (40 μM) was incubated with 160 μM $FcPF_6$ and 4.6 μM medium-chain acyl-CoA dehydrogenase for 10 min. Medium-chain acyl-CoA dehydrogenase was removed from the incubation mixture with a Centricon-10 microconcentrator, and the incubation mixture was analyzed by HPLC, as described in Materials and Methods. The absorption spectrum of the product DCTHD-CoA ($t_R \approx 19.0$ min, curve 1) was recorded. Enoyl-CoA hydratase (0.25 unit) was added to the DCTHD-CoA-containing incubation mixture, the mixture was incubated at 25 °C for 10 min, and the incubation mixture was analyzed by HPLC, as described in Materials and Methods. Curve 2 shows the absorption spectrum of the enoyl-CoA hydratase-catalyzed product ($t_R \approx 11.7$ min).

semialdehyde (**4**) as a product (Figure 1), analogous to the transformation of 4-thia-*trans*-2-octenoyl-CoA to butanethiol and malonyl-CoA semialdehyde (Lau et al., 1989). HPLC analysis of the incubation mixture that contained enoyl-CoA hydratase and DCTHD-CoA showed a peak that contained the coenzyme A moiety ($t_R \approx 11.7$ min at pH 7.6) in addition to unreacted DCTHD-CoA ($t_R \approx 19.0$ min at pH 7.6). Figure 5 shows the absorption spectra of DCTHD-CoA (curve 1) and of the product formed after incubation with enoyl-CoA hydratase (curve 2). Both compounds exhibited intense absorption bands at 260 and 300 nm. The spectrum of the hydrated metabolite was sensitive to changes in pH: acidification to pH 3 resulted in the disappearance of the absorption band at 300 nm (curve 2), whereas the absorption band at 260 nm was unchanged (data not shown). The absorption spectra of the metabolite recorded at pH 7.6 and 3.0 were identical with published spectra of malonyl-CoA semialdehyde (Vagelos & Earl, 1959). The absorption spectrum of DCTHD-CoA was not sensitive to changes in pH (data not shown).

Table 1: Kinetic Constants for the Enoyl-CoA Hydratase-Catalyzed Hydration of Crotonyl-CoA and DCTHD-CoA[a]

substrate	K_m (μM)	V_{max} (μmol min^{-1} mg^{-1})	turnover (min^{-1})	PFBCA (mol/mol of DCTHD-CoA hydrated)
crotonyl-CoA	42	303.0	3.0×10^5	ND
DCTHD-CoA	18	1.6	1.6×10^3	0.12

[a] Crotonyl-CoA (10−200 μM) or DCTHD-CoA (20−150 μM) was incubated with 16.6 or 0.7 μg of enoyl-CoA hydratase, respectively, in 50 mM potassium phosphate buffer (pH 7.6). Enoyl-CoA hydratase activity was determined by measuring the decrease in absorbance at 260 nm ($\Delta\epsilon = 6.22$ mM^{-1} cm^{-1}) or 335 nm ($\Delta\epsilon = 4.69$ mM^{-1} cm^{-1}) with crotonyl-CoA or DCTHD-CoA, respectively, as substrates. Kinetic constants were determined from a Lineweaver−Burk plot. Pentafluorobenzyl chloroacetate formation was quantified as described in Materials and Methods: PFBCA, pentafluorobenzyl chloroacetate; ND, not determined.

The thiohemiacetal 5,6-dichloro-4-thia-3-hydroxy-5-hexenoyl-CoA (Figure 1, **3**) would be expected to eliminate 1,2-dichloroethenethiol (**5**). 1,2-Dichloroethenethiol is highly reactive, however, and attempts to trap this intermediate have been unsuccessful (Dekant et al., 1988). 1,2-Dichloroethenethiol may eliminate HCl to give chlorothioketene or may tautomerize to give chlorothioacetyl chloride. Reaction of either chlorothioketene or chlorothioacetyl chloride with water would give chloroacetic acid as the terminal product. Therefore, the incubation mixture that contained DCTHD-CoA and enoyl-CoA hydratase was assayed for the presence of chloroacetic acid. GC/MS analysis of the incubation mixture that contained 0.67 μmol of DCTHD-CoA and 16.6 μg of enoyl-CoA hydratase showed the formation of 0.12 mol of chloroacetic acid per mole of DCTHD-CoA hydrated, as determined by the formation of pentafluorobenzyl chloroacetate (Table 1). The ratio of *m/z* 274 (M^+) to *m/z* 276 (M + 2) for pentafluorobenzyl chloroacetate from the incubation samples indicated the presence of one chlorine atom.

Table 1 shows the kinetic constants for the enoyl-CoA hydratase-catalyzed hydration of crotonyl-CoA and DCTHD-CoA. The turnover number for the enoyl-CoA hydratase-catalyzed hydration of DCTHD-CoA was 0.5% of that for crotonyl-CoA.

Table 2: HPLC Retention Times and Absorbance Maxima for DCTH-CoA Medium-Chain Acyl-CoA Dehydrogenase-Generated Metabolites of DCTH-CoA and Related Coenzyme A-Containing Compounds

compound	retention time (min)	absorbance maximum (nm)
CoASH	12.1	260
$(CoAS)_2$	12.4	260
DCTH-CoA	19.2	260
acryloyl-CoA	13.6	260
peak I	12.1	260
peak II	12.4	260
peak III	13.1	260
FAD[a]	14.1	260, 450

[a] FAD released from the medium-chain acyl-CoA dehydrogenase.

Turnover of DCTH-CoA by the Medium-Chain Acyl-CoA Dehydrogenase in the Absence of $FcPF_6$: Identification of 3-Hydroxypropionyl-CoA and Coenzyme A as Products. HPLC analysis of an incubation mixture that contained 10 μM DCTH-CoA and 10 μM medium-chain acyl-CoA dehydrogenase, but lacked a terminal electron acceptor, showed the disappearance of DCTH-CoA ($t_R \approx 19.2$ min) and the appearance of three products that contained a coenzyme A moiety. The retention times and absorbance maxima of metabolites I, II, and III and related products that contained a coenzyme A moiety are shown in Table 2. Metabolites I–III eluted earlier than both DCTH-CoA and DCTHD-CoA and are, therefore, more polar. In addition, metabolites I–III lacked absorption bands near 300 nm, which are present in the absorption spectra of 4-thia-2-alkenoyl-CoA compounds (Figure 3; Lau et al., 1989). The absorption spectrum of metabolite I showed little absorption in the 230 nm region, which indicated the absence of a coenzyme A thioester; coenzyme A thioesters exhibit an absorptivity of 8.7 mM^{-1} cm^{-1} at 232 nm (Stadtman, 1957). The absorption spectra and retention times for metabolite I and coenzyme A were identical (Table 2), and coenzyme A and metabolite I coeluted.

Retention times for coenzyme A disulfide and metabolite II were similar ($t_R \approx 12.4$ min; Table 2), but their absorption spectra were different, which indicates that metabolite II is not coenzyme A disulfide (data not shown). The spectrum of metabolite II had a prominent absorption band in the 230 nm region, which is consistent with that of a coenzyme A thioester. Moreover, the spectra and retention times of metabolite II and acryloyl-CoA were different, which indicates that metabolite II is not acryloyl-CoA (Table 2). The retention time and absorption spectrum of metabolite II were identical with that of synthetic 3-hydroxypropionyl-CoA (Table 2; see Materials and Methods), the hydration product of acryloyl-CoA (Dixon & Webb, 1979). Synthetic 3-hydroxypropionyl-CoA coeluted with metabolite II when added to an incubation mixture that contained the medium-chain acyl-CoA dehydrogenase/DCTH-CoA (data not shown). Insufficient amounts of metabolite III were available for characterization, and the generation of sufficient quantities of metabolites I and II for ^1H NMR analyses was unsuccessful due to the inactivation of medium-chain acyl-CoA dehydrogenase by DCTH-CoA (see the following).

Electronic Absorption Spectra of Medium-Chain Acyl-CoA Dehydrogenase after Incubation with DCTH-CoA in the Absence of $FcPF_6$. In the presence of $FcPF_6$, medium-chain acyl-CoA dehydrogenase catalyzed the oxidation of DCTH-CoA to the corresponding *trans*-2-enoyl-CoA product. In the absence of a suitable oxidant (i.e., $FcPF_6$), however, elimination of 1,2-dichloroethenethiol appears to occur with the concomitant generation of acryloyl-CoA. Accordingly, the kinetics of the reaction of DCTH-CoA with the medium-chain acyl-CoA dehydrogenase in the absence of $FcPF_6$ was studied (Figure 6). Curve 2 was recorded 55 s after the addition of an 18-fold excess of DCTH-CoA to medium-chain acyl-CoA dehydrogenase (curve 1) and showed a 25% bleaching of the flavin chromophore at 446 nm, with resolution of the residual flavin chromophore. In addition, a prominent long-wavelength absorption band centered at 680 nm appeared and exhibited an apparent molar absorption coefficient of 1.95 mM^{-1} cm^{-1}. The inset shows that the long-wavelength absorption band required 50 s for full development and then decayed with a first-order rate constant of 0.4 min^{-1}. The final spectrum (curve 3) was recorded after 50 min and shows the loss of the long-wavelength absorption band and the appearance of a red-shifted, oxidized flavin spectral envelope ($\lambda_{max} = 450$ nm). Identical absorption spectra were observed in experiments conducted in the absence of oxygen, which indicates that oxygen was not involved in the decay of the long-wavelength absorption band seen in Figure 6 (data not shown). A second addition of DCTH-CoA did not alter the absorption spectrum of the flavin chromophore observed in curve 3, and no bleaching of the flavin chromophore was observed upon the addition of octanoyl-CoA (data not shown). These data indicate that DCTH-CoA irreversibly inactivates the medium-chain acyl-CoA dehydrogenase.

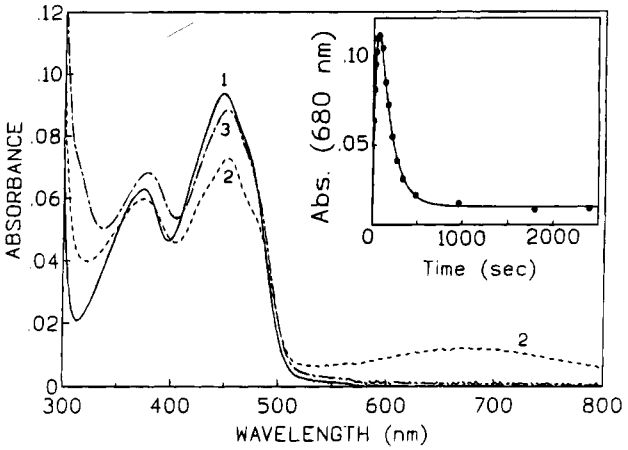


FIGURE 6: Spectral changes observed after the addition of DCTH-CoA to medium-chain dehydrogenase in the absence of $FcPF_6$. Curve 1 shows the absorption spectrum of 6.1 μM medium-chain acyl-CoA dehydrogenase in 50 mM phosphate buffer (pH 7.6). The dehydrogenase was then incubated with 118 μM DCTH-CoA at 25 °C, and curves 2 and 3 were recorded at 55 s and 50 min, respectively, after the addition of DCTH-CoA. The appearance and decay of the long-wavelength band are shown in the inset. Intermediate spectra have been omitted for clarity. The curve is a nonlinear least-squares fit for the exponential rise and decay of an intermediate with rate constants of 2.0 and 0.44 min^{-1}, respectively.

Although excess DCTH-CoA (19 equiv per flavin) was added (Figure 6), only partial bleaching of the flavin chromophore was observed (curve 2) before the oxidized flavin spectrum appeared (curve 3). In contrast, the addition of 1 equiv of octanoyl-CoA gave approximately 90% reduction of the enzyme under comparable conditions (Thorpe & Massey, 1983). The partial bleaching of the medium-chain acyl-CoA dehydrogenase by DCTH-CoA does

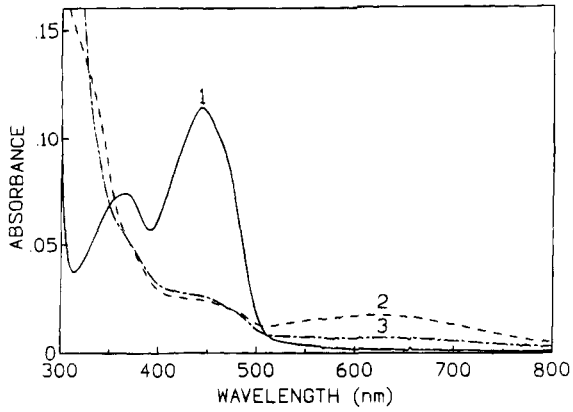


FIGURE 7: Reduction of 8-Cl-FAD-substituted medium-chain acyl-CoA dehydrogenase with DCTH-CoA. The dehydrogenase apoprotein was reconstituted with 8-Cl-FAD as described previously (Thorpe & Massey, 1983), and the absorption spectrum of the enzyme (8 μM) in 50 mM phosphate buffer (pH 7.6) was recorded (curve 1). The substituted dehydrogenase was then incubated with 118 μM DCTH-CoA at 25 °C, and curves 2 and 3 were recorded 11 s and 20 min after the addition of DCTH-CoA, respectively.

not reflect a failure to saturate the dehydrogenase because higher concentrations of DCTH-CoA produced the same spectral changes (data not shown).

To define more clearly the nature of the long-wavelength absorption band, 8-Cl-FAD was substituted for FAD in the medium-chain acyl-CoA dehydrogenase (Thorpe & Massey, 1983). 8-Cl-FAD has a redox potential about 60 mV more positive than that of FAD (Moore et al., 1978) and is, therefore, a thermodynamically more potent oxidant. Incubation of 8-Cl-FAD-substituted medium-chain acyl-CoA dehydrogenase with DCTH-CoA yielded almost complete bleaching of the flavin chromophore, the formation of a prominent absorption band in the 330−340 nm region, and the appearance of a long-wavelength absorption band centered at 630 nm (Figure 7). The long-wavelength absorption band was blue-shifted from 680 nm with native medium-chain acyl-CoA dehydrogenase to 630 nm with 8-Cl-FAD-substituted medium-chain acyl-CoA dehydrogenase. These data indicate that the long-wavelength absorption band is a charge-transfer complex formed between reduced medium-chain acyl-CoA dehydrogenase and the unsaturated metabolite DCTHD-CoA (see Discussion).

Inactivation of the Medium-Chain Acyl-CoA Dehydrogenase by DCTH-CoA. *In vivo* administration of DCTH decreases fatty acid β-oxidation by about 75% in rat liver mitochondria. Furthermore, oxidation of octanoyl-CoA is irreversibly inhibited in rat liver mitochondria incubated with DCTH (Fitzsimmons et al., 1994), which indicates that inhibition of fatty acid β-oxidation occurs at the level of the medium-chain acyl-CoA dehydrogenase. Incubation of the medium-chain acyl-CoA dehydrogenase with increasing concentrations of DCTH-CoA in the absence of $FcPF_6$ resulted in increasing losses of enzyme activity (Figure 8A). Inactivation was not blocked when glutathione was added along with DCTH-CoA (data not shown), whereas octanoyl-CoA afforded complete protection (Figure 8A). Moreover, enzyme incubated with DCTH-CoA until 10% activity remained did not regain activity after the removal of excess reagent by ultrafiltration. When the medium-chain acyl-CoA dehydrogenase was incubated with DCTH-CoA in the presence of $FcPF_6$, little loss of enzyme activity was observed with the inhibitor concentrations used in the previous

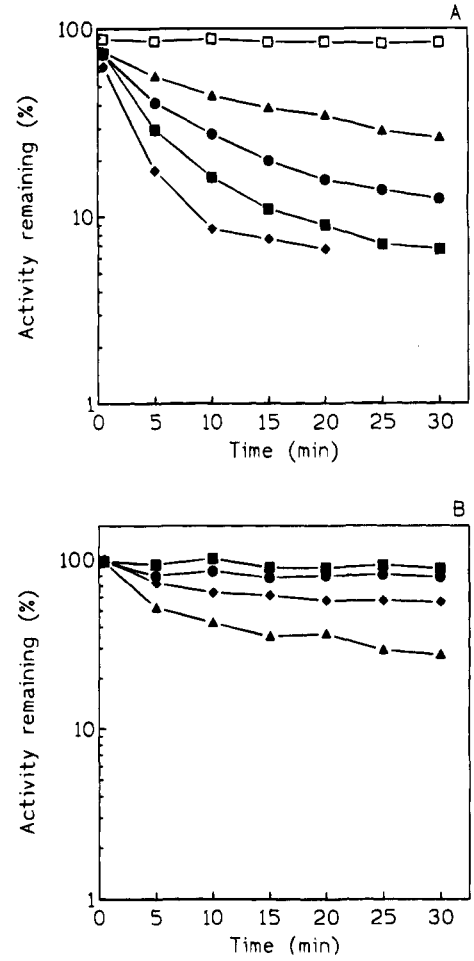


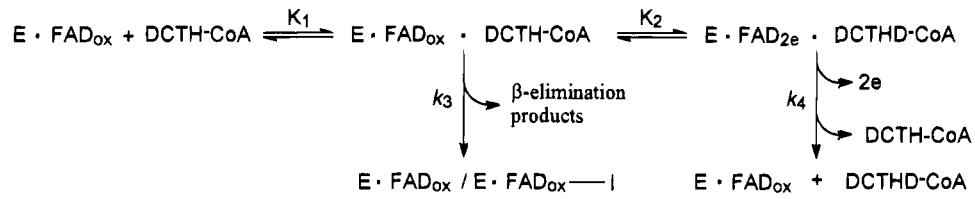
FIGURE 8: Inactivation of medium-chain acyl-CoA dehydrogenase by DCTH-CoA in the absence (A) or presence (B) of 500 μM $FcPF_6$. (A) Medium-chain acyl-CoA dehydrogenase (0.3 μM) was incubated with 2.5 (▲), 5 (●), 10 (■), or 50 μM (◆) DCTH-CoA or with both 10 μM DCTH-CoA and 100 μM octanoyl-CoA (□). (B) Medium-chain acyl-CoA dehydrogenase (0.3 μM) was incubated with 500 μM $FcPF_6$ and 10 (■), 25 (●), 50 (◆), or 100 μM (▲) DCTH-CoA. A 7 μL sample was removed and medium-chain acyl-CoA dehydrogenase activity was determined at the indicated times, as described in Materials and Methods.

experiment (Figure 8B). With 100 μM DCTH-CoA, however, 70% of the enzyme activity was lost over a 30 min period. These data indicate that DCTH-CoA, rather than its enoyl-CoA product DCTHD-CoA, was responsible for the observed inactivation (see Discussion).

DISCUSSION

Previous studies indicate that the fatty acid β-oxidation system is involved in the *in vivo* and *in vitro* toxicity of DCTH (Fitzsimmons et al., 1994; Fitzsimmons & Anders, 1993). A role for the fatty acid β-oxidation system in the bioactivation of DCTH was established in the present investigation. Two bioactivation pathways for DCTH-CoA were elucidated (Figure 1): in the presence of a terminal electron acceptor, the medium-chain acyl-CoA dehydrogenase catalyzed the oxidation of DCTH-CoA to DCTHD-CoA, which was hydrated by enoyl-CoA hydratase (pathway A) with the subsequent generation of potentially cytotoxic metabolites. In the absence of a terminal electron acceptor, however, the medium-chain acyl-CoA dehydrogenase catalyzed the elimination of the 1,2-dichloroethenethiol moiety of DCTH-CoA (pathway B) and was rapidly inactivated.

Scheme 1



The medium-chain acyl-CoA dehydrogenase-catalyzed oxidation of DCTH-CoA gave DCTHD-CoA (**2**, Figure 1), which was identified by its electronic, mass, and ^{1}H NMR spectra. The related 4-thia analog, 4-thiaoctanoyl-CoA, is converted to 4-thia-*trans*-2-octenoyl-CoA by the medium-chain acyl-CoA dehydrogenase (Lau et al., 1988, 1989). Both DCTHD-CoA and 4-thia-*trans*-2-octenoyl-CoA show an additional peak each in their absorption spectra that is characteristic of a delocalized 4-thia enone chromophore. With DCTHD-CoA, an absorption band is present at 300 nm (ϵ = 16.2 mM^{-1} cm^{-1}; Figure 3, curve 2), compared with the absorption band at 312 nm in 4-thiaoctenoyl-CoA (ϵ = 22 mM^{-1} cm^{-1}; Lau et al., 1989). The wavelength shift and the decreased molar absorption coefficient are attributed to the inductive effect of the 1,2-dichlorovinyl group, which would be expected to decrease electron contribution to the conjugated enone (Silverstein et al., 1981).

DCTH-CoA was a substrate for the medium-chain acyl-CoA dehydrogenase and exhibited an initial turnover number of approximately 130 min^{-1}, compared with 1100 min^{-1} with octanoyl-CoA as the substrate (Lehman & Thorpe, 1990). In the absence of an electron acceptor, substrates for the acyl-CoA dehydrogenases are expected to effect substantial reduction of the enzyme flavin. DCTH-CoA, however, reduced the dehydrogenase flavin by about 25% of that observed after the addition of octanoyl-CoA (Thorpe et al., 1979). Weak binding of DCTH-CoA is not the cause of the partial reduction the because the addition of higher concentrations of DCTH-CoA does not induce greater spectral changes. Incubation of DCTH-CoA with the more oxidizing 8-Cl-FAD-substituted dehydrogenase (Moore et al., 1979), however, effected complete reduction of the enzyme (Figure 7). The partial reduction of native dehydrogenase reflects the thermodynamic disadvantage associated with the presence of the electron-withdrawing 1,2-dichlorovinyl group in DCTH-CoA. Moreover, DCTH-CoA appears to be a thermodynamically weaker reductant of the dehydrogenase than either octanoyl-CoA or 4-thiaoctanoyl-CoA (Lau et al., 1989). Thus, the equilibrium K_2 in Scheme 1 lies to the left with DCTH-CoA, but to the right with octanoyl-CoA (Thorpe et al., 1979; Cummings et al., 1992). A similar manipulation of the internal equilibrium K_2 between oxidized and reduced complexes of the medium-chain acyl-CoA dehydrogenase has been reported earlier (Thorpe & Massey, 1983; Cummings et al., 1992).

Incubation of the medium-chain-acyl-CoA dehydrogenase with octanoyl-CoA or 4-thiaoctenoyl-CoA results in the appearance of stable, long-wavelength absorption bands centered at 570 and 585 nm, respectively, which have been assigned to charge-transfer complexes formed with the reduced flavin as the donor and the corresponding enoyl-CoA's as the acceptors (Lau et al., 1988; Thorpe & Massey, 1983). The long-wavelength absorption bands that formed after incubation of DCTH-CoA with the medium-chain acyl-CoA dehydrogenase (Figure 6) or with the 8-Cl-FAD-substituted dehydrogenase (Figure 7) in the absence of an electron acceptor were centered at 680 and 630 nm, respectively, and are characteristic of a charge-transfer complex between reduced flavin as the donor and DCTHD-CoA as the acceptor (E·FAD$_{ox}$ DCTHD-CoA, Scheme 1; Engel & Massey, 1971; Massey & Ghisla, 1974). The electron-withdrawing effect of the 1,2-dichlorovinyl group makes DCTHD-CoA a better electron acceptor and a less efficient electron donor than octenoyl-CoA or 4-thiaoctenoyl-CoA. The absorption maxima of the charge-transfer complexes that formed after incubation of the medium-chain acyl-CoA dehydrogenase with DCTH-CoA, 4-thiaoctanoyl-CoA, or octanoyl-CoA (680, 585, and 570 nm, respectively; Lau et al., 1988; Thorpe & Massey, 1983) reflect the electron-withdrawing effect of the 1,2-dichlorovinyl group.

Hydration of DCTHD-CoA by enoyl-CoA hydratase was about 0.5% of that observed for crotonoyl-CoA, but still exhibited a turnover number of 1600 min^{-1} (Table 1). The putative hydration product 5,6-dichloro-4-thia-3-hydroxy-5-hexenoyl-CoA (**3**) may eliminate 1,2-dichloroethenethiol (**5**) to give malonyl-CoA semialdehyde (**4**). The identification of chloroacetic acid (**7**) provides evidence that 1,2-dichloroethenethiol is eliminated from the thiohemiacetal. 1,2-Dichloroethenethiol is the putative cytotoxic metabolite of the cysteine *S*-conjugate, *S*-(1,2-dichlorovinyl)-L-cysteine, but previous attempts to trap 1,2-dichloroethenethiol were unsuccessful (Dekant et al., 1988). 1,2-Dichloroethenethiol is highly unstable and may eliminate HCl to give chlorothioketene. Evidence for thioketene formation from α-chloroalkenethiolates has been presented (Dekant et al., 1991). Chlorothioketene may react with protein amino or sulfhydryl groups to yield the corresponding thioamides or dithiocarboxylates, respectively, or it may be hydrolyzed to give chlorothionoacetic acid or chloroacetic acid as products (Dekant et al., 1988). Covalent binding of chlorothioketene to protein nucleophiles may account for the low yield of chloroacetic acid detected after the hydration of DCTHD-CoA by enoyl-CoA hydratase. Moreover, chlorothionoacetic acid formation was not quantified, and its formation may partially account for the low chloroacetic acid concentrations detected.

Pathway B (Figure 1) leads to the elimination of 1,2-dichloroethenethiol rather than a hydride equivalent in the active site of the acyl-CoA dehydrogenase. The competition between β-oxidation and β-elimination finds precedent in the work of Fendrich and Abeles (1982). The short-chain acyl-CoA dehydrogenase from *Megasphaera elsdenii* catalyzes the elimination of HF from 3-fluoropropionyl-CoA and the generation of acryloyl-CoA at more than 100-fold the rate of dehydrogenation. In the present work, 3-hydroxypropionyl-CoA rather than acryloyl-CoA was formed after incubation of the medium-chain acyl-CoA dehydrogenase with DCTH-CoA. 3-Hydroxypropionyl-CoA (**9**) formation

may result from the hydration of acryloyl-CoA (**8**) by the intrinsic hydratase activity of the medium-chain acyl-CoA dehydrogenase (Lau et al., 1986) or by contaminating enoyl-CoA hydratase, which hydrates acryloyl-CoA (Dixon & Webb, 1979). Although the mechanism of formation of coenzyme A and coenzyme A disulfide as metabolites of DCTH-CoA has not been established, solutions of 3-hydroxypropionyl-CoA kept at room temperature released coenzyme A as a product (data not shown). Hydrolysis of 3-hydroxypropionyl-CoA or lactonization to β-propiolactone may account for the formation of the observed products.

Incubation of DCTH-CoA with oxidized medium-chain acyl-CoA dehydrogenase in the absence of $FcPF_6$ resulted in a rapid, concentration-dependent inactivation of the enzyme (Figure 8A). Although elucidation of the mechanism of inactivation of the medium-chain acyl-CoA dehydrogenase by DCTH-CoA was not an objective of the present studies, several points were established. Enzyme inactivation was blocked by coincubation with octanoyl-CoA, but was not affected by glutathione. Excess octanoyl-CoA, however, failed to reduce the medium-chain acyl-CoA dehydrogenase previously incubated with DCTH-CoA, which indicates that enzyme inactivation was irreversible. The flavin chromophore of the DCTH-CoA-inactivated dehydrogenase exhibited a red shift when compared with that of the untreated enzyme, and this red shift may indicate a modification of the flavin environment (Palmer & Massey, 1968). In the absence of $FcPF_6$, the equilibrium K_2 is perturbed by the elimination reaction k_3 (Scheme 1; Figure 1, pathway B) and the generation of acryloyl-CoA. This elimination is associated with the irreversible inactivation of enzyme activity and loss of the charge-transfer complex seen in Figure 6. Addition of exogenous acryloyl-CoA to the medium-chain acyl-CoA dehydrogenase, however, had no effect on enzyme activity (data not shown). Moreover, acryloyl-CoA does not inhibit the short-chain acyl-CoA dehydrogenase (Shaw & Engel, 1984). Incubation of the medium-chain acyl-CoA dehydrogenase with DCTH-CoA in the presence of $FcPF_6$ resulted in little enzyme inactivation (Figure 8B). Hence, in the presence of $FcPF_6$, the fate of DCTH-CoA is governed by competition between oxidation, k_4, and β-elimination, k_3 (Scheme 1). Because k_4 is relatively rapid [about 130 min^{-1} when 77 nmol of medium-chain acyl-CoA dehydrogenase is mixed with 49 μM DCTH-CoA and 200 μM $FcPF_6$ in 50 mM phosphate buffer (pH 7.6) at 25 °C], oxidation of DCTH-CoA to DCTHD-CoA may spare the dehydrogenase from inactivation via k_3.

Two modes of inactivation of the medium-chain acyl-CoA dehydrogenase by DCTH-CoA can be envisaged. In the first, acryloyl-CoA generated *in situ* may react with a target within the active site of the dehydrogenase. A precedent is found in the generation of an acryloyl moiety from 3-chloropropionyl-CoA during the inactivation of fatty acid synthase and HMG-CoA synthase (Miziorko & Behnke, 1985; Miziorko et al., 1986). Neither the medium-chain enzyme (this work, not shown) nor the short-chain dehydrogenase (Shaw & Engel, 1984) is significantly inactivated by exogenously added acryloyl-CoA. A more likely possibility is that the loss of activity (Figure 8A) stems from covalent binding of a reactive metabolite derived from 1,2-dichloroethenethiol to the enzyme (e.g., Figure 1, **6**).

Two bioactivation mechanisms for DCTH-CoA have been described in the present work: β-oxidation, which provides a substrate for enoyl-CoA hydratase, and β-elimination, which is associated with inactivation of the dehydrogenase. The present results indicate that the partition between these pathways will depend on the efficiency of the transfer of reducing equivalents to electron-transferring flavoproteins. Inactivation of the medium-chain acyl-CoA dehydrogenase may explain the inhibition of fatty acid oxidation and the disruption of lipid homeostasis observed in DCTH-treated rats (Fitzsimmons et al., 1994). The cytotoxicity of DCTH is associated with bioactivation by a β-elimination-dependent mechanism that involves little inactivation of the medium-chain acyl-CoA dehydrogenase. Inhibition of fatty acid β-oxidation cannot, however, account for the potent nephrotoxicity of DCTH *in vivo*. Methylenecyclopropylacetyl-CoA is a more potent inhibitor of β-oxidation than is DCTH-CoA (Tserng et al., 1991; Ikeda & Tanaka, 1990), but is not cytotoxic. These data indicate that the cytotoxicity of DCTH is not a consequence of a β-elimination-dependent mechanism, but is due to a β-oxidation-dependent mechanism with the production of electrophilic intermediates within the mitochondrial matrix. The metabolites of DCTH-CoA that cause cytotoxicity or their cellular targets have not been identified. Future studies to explore the mechanism of the cytotoxic effects of DCTH and the details of the interaction of DCTH-CoA with the acyl-CoA dehydrogenases are warranted.

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