3-Methyleneoctanoyl-CoA and 3-Methyl-*trans*-2-octenoyl-CoA: Two New Mechanism-Based Inhibitors of Medium Chain Acyl-CoA Dehydrogenase from Pig Kidney†

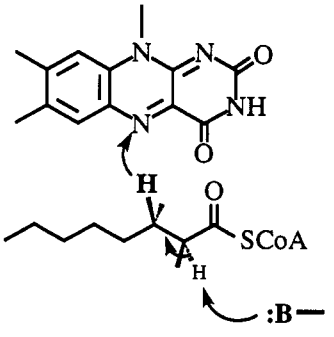
John G. Cummings and Colin Thorpe*

Department of Chemistry and Biochemistry, University of Delaware, Newark, Delaware 19716

Received August 23, 1993; Revised Manuscript Received October 29, 1993•

ABSTRACT: The medium chain acyl-CoA dehydrogenase catalyzes the FAD-dependent oxidation of a variety of acyl-CoA substrates to the corresponding *trans*-2-enoyl-CoA thioesters. This work identifies 3-methyleneoctanoyl-CoA and 3-methyl-*trans*-2-octenoyl-CoA as representatives of a new class of mechanism-based inhibitor of the dehydrogenase. One equivalent of either compound generates an inactive reduced flavin species with low absorption at 450 nm and a shoulder at 320 nm suggestive of an N-5 adduct. Reduction is rapid with the 3-methylene analogue (10/s at 1 °C), but comparatively slow for 3-methyl-*trans*-2-octenoyl-CoA (1.1×10^{-4}/s, under the same conditions). The reduced species is very stable, but the adduct can be slowly displaced with a large excess of octanoyl-CoA. The reduced adduct resists oxidation by the facile one-electron oxidant of the dehydrogenase, ferricenium hexafluorophosphate. Evidence that both isomeric inhibitors generate the same reduced flavin species includes an essentially identical visible spectrum, the same kinetics of displacement using octanoyl-CoA, and the same mixture of products on HPLC after denaturation of the treated enzyme with trichloroacetic acid, methanol, or by boiling. Experiments with the corresponding shorter analogues of these inhibitors, 3-methylenebutanoyl-CoA and 3-methyl-2-butenoyl-CoA confirm and extend these findings. These reduced adducts are less stable, allowing the dehydrogenase to catalyze the interconversion of the unconjugated 3-methylenebutanoyl-CoA to the more stable conjugated 3-methyl-2-butenoyl-CoA thioester (K_{eq} ca. 150). These data suggest that α-proton abstraction from the 3-methylene derivatives or γ-proton removal from the 3-methyl-2-enoyl analogues generates a common carbanionic intermediate which attacks oxidized flavin. As would be expected, the unconjugated 3-methylene derivatives are more effective inhibitors of the dehydrogenase than the thermodynamically more stable 3-methylenoyl analogues.

Catalysis by the acyl-CoA dehydrogenases is initiated by abstraction of the *pro-R* α-proton of a thioester substrate with elimination of a hydride equivalent from the *pro-R* β-position to the N-5 locus of the isoalloxazine ring (Biellman & Hirth, 1970a,b; Bücklers et al., 1970; Ghisla et al., 1984):



Proton abstraction and hydride transfer have been shown to be concerted when the medium chain dehydrogenase is reduced by a nonoptimal substrate (such as butyryl-CoA or dihydrocinnamoyl-CoA; Murfin, 1974; Reinsch et al., 1980; Pohl et al., 1986; Schopfer et al., 1988). However, proton abstraction and hydride transfer can be completely uncoupled with a number of C-3 substituted acyl-CoA analogues yielding acyl-CoA enol(ate) species which bind very tightly to the medium chain enzyme (Powell et al., 1987: Lau et al., 1988).

At least four different types of mechanism-based inactivators of the acyl-CoA dehydrogenases have been described (Frerman et al., 1980; Gomes et al., 1981; Wenz et al., 1981, 1985; Freund et al., 1985; Powell & Thorpe, 1988; Shaw & Engel, 1984). 3-Alkynoyl-CoA thioesters (e.g., compound **1** in Table 1) were the first mechanism-based inactivators of the acyl-CoA dehydrogenases to be reported. Covalent modification of the protein moiety probably occurs after base-catalyzed isomerization to the corresponding 2,3-allenic thioesters (Frerman et al., 1980; Gomes et al., 1981; Fendrich & Abeles, 1982). Fendrich and Abeles identified a glutamate residue as the target of this modification reaction using the short chain acyl-CoA dehydrogenase and suggested that this residue may be the active site base. Somewhat surprisingly, 2-alkynoyl-CoA derivatives (compound **2**, Table 1) were also found to be mechanism-based inactivators of both the short (Lundberg & Thorpe, 1993) and the medium chain acyl-CoA dehydrogenase (Freund et al., 1985; Powell & Thorpe, 1988). In these cases, the thioester is activated by a rate-limiting γ-proton abstraction (Powell & Thorpe, 1988) with the eventual covalent modification of glutamate-376. X-ray diffraction (Kim et al., 1988, 1992; Kim, 1991) and mutagenesis studies (Bross et al., 1990) of the medium chain acyl-CoA dehydrogenase have provided strong support for the role of glutamate-376 as the catalytic base in the medium chain enzyme.

Methylenecyclopropylacetyl-CoA (compound **3**, Table 1), the toxic metabolite generated on ingestion of hypoglycin (Hassall & Reyle, 1955), is also activated by α-proton

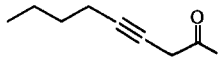
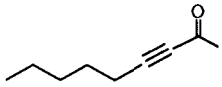
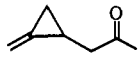
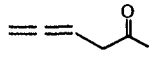
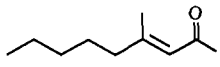
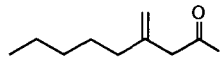
† This work was supported by a grant from the U. S. Public Health Service (GM 26643).

* Author to whom correspondence should be addressed. Telephone, 302-831-2689; fax, 302-831-6335.

• Abstract published in *Advance ACS Abstracts*, January 1, 1994.

0006-2960/94/0433-788$04.50/0

Table 1: Mechanism-Based Inhibitors of the Medium Chain Acyl-CoA Dehydrogenase

1		3-octynoyl-CoA
2		2-octynoyl-CoA
3		methylenecyclopropyl-acetyl-CoA
4		3,4-pentadienoyl-CoA
5		3-methyl-*trans*-2-octenoyl-CoA
6		3-methylene-octanoyl-CoA

abstraction, but with the eventual formation of a reduced flavin adduct (Wenz et al., 1981). Ghisla and colleagues suggested that a carbanion mediated ring-opening preceded modification of the flavin (Wenz et al., 1981). In contrast, Liu and co-workers favor one-electron oxidation of the initial carbanion followed by radical mediated opening of the cyclopropyl ring prior to flavin attack (Lai et al., 1993).

The irreversible inactivation of the dehydrogenase with methylenecyclopropylacetyl-CoA contrasts with the reversible inactivation seen with 3,4-pentadienoyl-CoA (compound **4**, Table 1; Wenz et al., 1985). Two modes of recovery of activity have been found. First, the allene can be displaced by a large excess of the tightly binding substrate, octanoyl-CoA. Second, the reduced flavin adduct slowly liberates the more stable 2,4-pentadienoyl-CoA with recovery of oxidized enzyme, even in the absence of a displacing ligand (Wenz et al., 1985).

A fourth type of mechanism-based inhibition involves incubation of the short chain dehydrogenase with the very poor substrate, propionyl-CoA (Shaw & Engel, 1985). Inactivation is associated with the formation of an N-5 reduced flavin adduct which is released upon denaturation of the enzyme (Shaw & Engel, 1984, 1985; George et al., 1984). The mechanism of inactivation is unclear, although the expected product of dehydrogenation, acryloyl-CoA, is not believed to be a direct participant in adduct formation (Shaw & Engel, 1984).

The present work describes a characterization of a new class of mechanism-based inactivators of the acyl-CoA dehydrogenases (compounds **5** and **6**, Table 1). These isomeric inhibitors appear much less activated than the methylenecyclopropyl moiety of compound **3** or the allene functionality of compound **4** (Table 1). In fact, 3-methyl-*trans*-2-octenoyl-CoA (compound **5**, Table 1) is the normal dehydrogenation product of *S*-(−)-3-methyloctanoyl-CoA, an apparently innocuous weak substrate of the medium chain dehydrogenase. These results provide new insights into the reactivity of enol(ate) species generated within the active site of the acyl-CoA dehydrogenases.

MATERIALS AND METHODS

Materials. Medium chain acyl-CoA dehydrogenase was isolated from pig kidney as described previously (Gorelick et al., 1985) and was quantitated using an extinction coefficient of 15.4 mM^{-1} cm^{-1} at 446 nm (Thorpe et al., 1979). *R*-(+)- and *S*-(−)-3-methyloctanoic acids were generous gifts from Dr. Philip Sonnet (USDA Agricultural Research Service, Philadelphia, PA), with each isomer having >97% configurational purity (Sonnet & Gazzillo, 1990; Sonnet & Baillargeon, 1989). Ferricenium hexafluorophosphate was a gift from Ms. Ruth A. Schaller (Lehman et al., 1990). Coenzyme A (lithium salt), 3-methyl-2-butenoyl-CoA, DCIP,[1] PMS, octanoic acid, and crotonase (EC 4.2.1.17) were purchased from Sigma. 2-Heptanone, 3-methyl-3-buten-1-ol, and methyl diethylphosphonoacetate were from Aldrich. TCA was obtained from Fisher.

Synthesis and Characterization of 3-Methyl-2-octenoic Acid. The preparation of a mixture of *cis*- and *trans*-3-methyl-2-octenoic acid was essentially as described by Ogura et al. (1970). Sodium methoxide was prepared from 1.15 g of sodium and 15 mL of methanol, mixed with 9.2 mL of methyl diethylphosphonoacetate followed by the dropwise addition of 7.0 mL of 2-heptanone over 30 min with stirring. After an additional hour, 30 mL of H_2O was added and the solution extracted three times with ether. The combined extracts were dried over anhydrous sodium sulfate and the residue distilled under reduced pressure to give a mixture of *cis* and *trans* methyl 3-methyl-2-octenoate, 4.1 g (Ogura et al., 1970). The esters were hydrolyzed at 70 °C for 12 h using 1.2 equiv NaOH in methanol. After acidification, the free acids were extracted with diethyl ether and dried over anhydrous sodium sulfate. Both the methyl ester and the corresponding free acid showed a 2-fold preponderance of the *trans* isomer as judged by ^1H NMR spectra ($CDCl_3$) recorded using a Bruker 250-MHz spectrometer with an Aspect 3000 computer system.

Synthesis of 3-Methylene-butanoic Acid.[2] Jones reagent (Bowden et al., 1946) was added dropwise to 1.17 g of 3-methyl-3-buten-1-ol in 50 mL of acetone and cooled in an ice/salt bath, until a distinct orange coloration persisted. Stirring was continued for 1 h at 0 °C, followed by the addition of 1 mL of isopropanol and 40 mL of cold H_2O. The solution was extracted three times with diethyl ether and washed with 10% aqueous sodium hydroxide. The acid was recovered from the acidified solution with diethyl ether, dried over magnesium sulfate, and collected by rotary evaporation.

Preparation of CoA Thioesters. The CoA thioesters of all carboxylic acids were prepared by the mixed anhydride method of Bernert and Sprecher (1977). The designation butanoyl- and octanoyl- refer to unbranched acyl chains to which named substituents are appended. All thioesters were purified by HPLC with a Perkin-Elmer Series 400 liquid chromatograph on a semipreparative octadecylsilica column (Vydac) using a

[1] Abbreviations: DCIP, 2,6-dichlorophenolindophenol; PMS, phenazine methosulfate; TCA, trichloroacetic acid; HPLC, high-performance liquid chromatography; FAD, flavin adenine dinucleotide; GC/MS, gas chromatography/mass spectrometry; MCAD and SCAD, medium and short chain acyl-CoA dehydrogenase; Tris, Tris(hydroxymethyl)aminomethane; MCPA-CoA, methylenecyclopropylacetyl-CoA.

[2] For clarity a consistent set of names for the inhibitors are used (alternative names are listed in parentheses): 3-Methyl-*trans*-2-octenoyl-CoA; 3-methyleneoctanoyl-CoA; 3-methyl-2-butenoyl-CoA (3-methylcrotonyl-CoA); 3-methylenebutanoyl-CoA (3-methylene-butyryl-CoA, 3-methyl-3-butenoyl-CoA).

gradient of methanol and 25 mM phosphate, pH 5.3 (Corkey et al., 1981). Thioesters were detected at 260 nm using a Dynamax Model UV-C detector, from Rainin. A typical elution program for octanoyl-CoA derivatives would be 5 min, 30% methanol; 5–20 min, linear gradient to 80% methanol; 20–25 min linear gradient to 30% methanol. Where appropriate, gradients were modified depending upon the chain length of the ligand being purified and the degree of separation from contaminating peaks. For example, maximal separation of 3-methyleneoctanoyl-CoA and 3-methyl-*trans*-2-octenoyl-CoA was achieved using the following gradient: 5 min, 30% methanol; 5–45 min, linear gradient to 75% methanol; 45–50 min linear gradient to 30% methanol. All purified CoA thioesters were desalted by HPLC using a linear gradient of methanol and water and were stored as lyophilized powders. Thioesters were quantitated using extinction coefficients of 22 mM^{-1} cm^{-1} at 260 nm for 3-methyl-2-butenoyl-CoA and 3-methyl-*trans*-2-octenoyl-CoA and 16 mM^{-1} cm^{-1} at 260 nm for 3-methylenebutanoyl-CoA and 3-methyleneoctanoyl-CoA (Stadtman, 1957).

^1H NMR Assignments of 3-Methyl-trans-2-octenoyl-CoA, 3-Methyleneoctanoyl-CoA, and 3-Methylenebutanoyl-CoA. All synthesized CoA thioesters in this study were characterized by ^1H NMR in D_2O. 3-Methyl-*trans*-2-octenoyl-CoA was further subjected to 2D NMR (COSY). The ^1H NMR resonances for the acyl moiety of 3-methyl-*trans*-2-octenoyl-CoA are listed below referenced to the HOD peak at 4.60 ppm: δ 0.7 (t, 3 H, CH_3), δ 1.1 (m, 4 H, CH_2), δ 1.3 (m, 2 H, CH_2), δ 1.9 (t, 2 H, CH_2), δ 1.9 (s, 3 H, CH_3), δ 5.9 (s, 1 H, CH). Resonances for the acyl moiety of 3-methylene-octanoyl-CoA are δ 0.7 (t, 3 H, CH_3), δ 1.1 (m, 4 H, CH_2), δ 1.2 (m, 2 H, CH_2), δ 1.8 (t, 2 H, CH_2), δ 3.1 (s, 2 H, CH_2), δ 4.7 (s, 1 H, CH_2), δ 4.8 (s, 1 H, CH_2). 3-Methylenebutanoyl-CoA showed the following resonances: δ 1.5 (s, 3 H, CH_3), δ 3.1 (s, 2 H, CH_2), δ 4.7 (s, 1 H, CH_2), δ 4.8 (s, 1 H, CH_2).

Transmethylation of Acyl-CoA Derivatives. Transmethylation in methanolic HCl (Kates, 1986) was used for characterization of the acyl moiety of CoA thioester derivatives. Lyophilized thioester derivatives (150 nmol in 400 μL of 5% concentrated HCl in methanol) were incubated at 75 °C for 10 h in small screw capped vials. The vials were cooled, 100 μL of H_2O was added, and the methyl ester was extracted with two 200-μL aliquots of pentane. An additional 100 μL of H_2O was added before a final extraction with pentane. A portion of the combined pentane extracts was then examined by GC/MS using a Hewlett-Packard 5890GC/5970MSD spectrometer equipped with a 30-m Alltech SE-54 column.

Assays for Medium Chain Acyl-CoA Dehydrogenase. Medium chain acyl-CoA dehydrogenase was routinely assayed using either 200 μM ferricenium hexafluorophosphate as the electron acceptor (extinction coefficient at 300 nm, 4.3 mM^{-1} cm^{-1}; Lehman & Thorpe, 1990) or the DCIP/PMS system (extinction coefficient for DCIP at 600 nm, 21.0 mM^{-1} cm^{-1}; Thorpe et al., 1979). Typically, assays included 50 μM substrate and 10 nM to 1.3 μM enzyme. The potency of 3-methyleneoctanoyl-CoA and 3-methyl-*trans*-2-octenoyl-CoA was evaluated including 0–100 μM of these inhibitors in the standard assay. The percent residual activity was calculated from a control assay with no inhibitor present.

Spectrophotometric Titrations of Medium Chain Acyl-CoA Dehydrogenase. Spectrophotometric titrations were performed on a Cary 219 or an HP8452A instrument, using 50 mM phosphate buffer and 0.3 mM EDTA, pH 7.6, at 20 or 25 °C, as previously described (Gorelick et al., 1985).

Stopped-Flow Analysis of the Inactivation of Medium Chain Acyl-CoA Dehydrogenase. Rapid reaction studies used a Kinetic Instruments Stopped Flow fitted with a 2-cm flow cell and with peripherals and software from Online Instrument Systems. Experiments were performed aerobically in 50 mM phosphate buffer, pH 7.6, at 1 °C.

Crotonase Activity. Crotonase, 0.4 unit in 2 μL of 50 mM Tris, pH 8.9, was added to 10 μM ligand in 700 μL of 50 mM phosphate, pH 7.6. Crotonase activity was followed by a decline in absorbance of the enone at 260 nm.

Purification of the Turnover Product of Medium Chain Acyl-CoA Dehydrogenase with S-(-)-3-Methyloctanoyl-CoA. Medium chain acyl-CoA dehydrogenase, 10 μM, was incubated aerobically for 90 min with 100 μM *S*-(–)-3-methyloctanoyl-CoA and 600 μM ferricenium hexafluorophosphate, in 50 mM phosphate, pH 7.6. The solution was then ultrafiltered and the filtrate applied to a reverse-phase C_{18} column. The methanol/phosphate buffer system (see earlier) allowed baseline separation between *S*-(–)-3-methyloctanoyl-CoA and the corresponding product.

Denaturation of Reduced Flavin Adducts. Medium chain acyl-CoA dehydrogenase, 11–30 μM, was incubated aerobically with a 3-fold excess of 3-methyl-*trans*-2-octenoyl-CoA or 3-methyleneoctanoyl-CoA for 12 h in 50 mM phosphate buffer, pH 7.6, at 25 °C. After ultrafiltration, the enzyme was brought to 5% trichloroacetic acid at 0 °C, and the precipitate was removed by centrifugation. The supernatant was repeatedly extracted with ether, and residual ether was removed with a stream of nitrogen. The enzyme adduct was also denatured using 4 volumes of vigorously stirred methanol at room temperature. After 20 min on ice, the solution was centrifuged at 12 000 rpm for 10 min, and methanol was evaporated with a stream of nitrogen. The reduced enzyme adducts were also denatured by boiling for 5 min in dim light. Protein precipitates were removed by centrifugation for 10 min at 12 000 rpm and subsequently dissolved in 6 M guanidine HCl for spectrophotometric analysis. All three methods yielded protein precipitates with no detectable flavin absorbance.

HPLC Analysis of the Reduced Flavin Adduct of Medium Chain Acyl-CoA Dehydrogenase and Several Inhibitors. After denaturation, protein-free supernatants from treated and free medium chain dehydrogenase were analyzed by reverse-phase HPLC. The chromatograms were monitored at 254 nm and developed at 2 mL/min with the following gradient: 5 min, 5% methanol in 5 mM phosphate buffer, pH 7.3; 5–25 min, linear gradient to 50% methanol; 25–30 min, linear gradient to 100% methanol; 30–35 min, 100% methanol; 35–40 min, linear gradient to 5% methanol. In some cases acetonitrile was used instead of methanol. Peaks were collected manually and analyzed spectrophotometrically.

RESULTS

A New Inhibitor of the Acyl-CoA Dehydrogenases. Both *R*- and *S*-isomers of 3-methyloctanoyl-CoA are very weak substrates of medium chain acyl-CoA dehydrogenase. Both enantiomers show 0.5% of the activity seen with octanoyl-CoA in either the ferricenium or the DCIP/PMS assay systems. This lack of discrimination is notable in light of the highly stereoselective transfer of the *pro-R* β-hydrogen of normal substrates to the N-5 position of the enzyme flavin (Biellman & Hirth, 1970a; Bücklers et al., 1970; Ghisla et al., 1984). Incubation of the dehydrogenase with 3 equiv of *S*-(–)-3-methyloctanoyl-CoA, under aerobic conditions, led to the slow accumulation of a form of the enzyme which showed

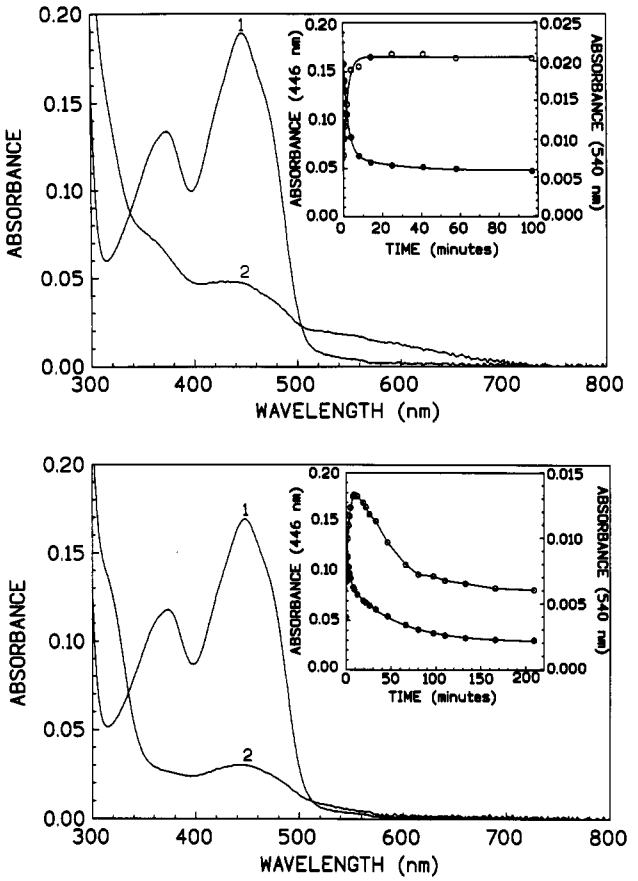


FIGURE 1: Anaerobic and aerobic reduction of pig kidney medium chain acyl-CoA dehydrogenase by *S*-(−)-3-methyl-octanoyl-CoA. (A, top) Medium chain acyl-CoA dehydrogenase was deoxygenated (curve 1; 12 μM enzyme in 50 mM phosphate buffer, pH 7.6, 25 °C) and reduced with 36 μM *S*-(−)-3-methyloctanoyl-CoA (curve 2). The spectrum shown in curve 2 was recorded 97 min after ligand addition. The inset displays the decrease in absorbance at 446 nm (●, left axis) and the increase at 540 nm (○, right axis) versus time over the course of the reduction. (B, bottom) Medium chain acyl-CoA dehydrogenase (curve 1, 11 μM enzyme in 50 mM phosphate buffer, pH 7.6, 25 °C) was reduced with 33 μM *S*-(−)-3-methyloctanoyl-CoA under aerobic conditions (curve 2, 209 min after addition). The inset shows the decrease in absorbance at 446 nm(●, left axis) over the course of the reaction and the formation and decay of a long wavelength band at 540 nm (○, right axis).

very low residual activity in the standard assay using octanoyl-CoA as substrate (not shown). Under comparable conditions the *R*-(+)-isomer was a much less effective inhibitor of the dehydrogenase, and so the *S*-isomer formed the focus of further work.

Reduction of Medium Chain Acyl-CoA Dehydrogenase by S-(−)-3-Methyloctanoyl-CoA under Anaerobic and Aerobic Conditions. Figure 1A shows the reduction of the dehydrogenase with a 3-fold excess of *S*-(−)-3-methyloctanoyl-CoA under anaerobic conditions. A slow bleaching of the oxidized flavin spectrum at 446 nm is accompanied by the appearance of a characteristic charge transfer complex between reduced enzyme flavin and oxidized product (curve 2; Engel & Massey, 1971; Massey & Ghisla, 1974; Thorpe & Massey, 1983). This long wavelength band is stable over 100 min under anaerobic conditions (see inset, Figure 1A). These spectral changes show biphasic kinetics with 85% of the total amplitude occurring with a rate constant of 0.43 min^{-1} and the remainder showing a rate of 0.04 min^{-1}. The fast phase in Figure 1A is some 10^5-fold slower than the corresponding rate observed with a preferred substrate of the enzyme, octanoyl-CoA (Hall et al., 1979; Gorelick et al., 1985; Lau et al., 1989). Clearly a methyl substituent at the C-3 position of octanoyl-CoA is very poorly tolerated in the reductive half-reaction of the medium chain enzyme. The corresponding *R*-(+)-3-methyl analogue is an even worse reductant of the dehydrogenase, with only about 20% bleaching of the flavin chromophore over 4 h under the same conditions as used in Figure 1A.

Figure 1B shows the aerobic incubation of 3 equiv of *S*-3-methyloctanoyl-CoA with the medium chain dehydrogenase. As observed anaerobically (Figure 1A), reduction is biphasic with rates of 0.42 and 0.02 min^{-1}; however, the amplitudes of each phase are now comparable (see inset, Figure 1B). Two additional differences between anaerobic and aerobic experiments are of note. First, the long wavelength band is unstable under aerobic conditions decaying to near baseline levels with a rate constant of 0.024 min^{-1} (see inset, Figure 1B). Second, the final spectrum aerobically shows markedly lower absorbance at 360 nm with a shoulder appearing at 316 nm. In comparison, no difference in behavior is observed when the medium chain dehydrogenase is reduced with octanoyl-CoA under anaerobic or aerobic conditions. The charge transfer complex between the enzyme and *trans*-2-octenoyl-CoA (Beinert, 1963; Engel & Massey, 1971; Massey & Ghisla, 1974; Thorpe & Massey, 1983) is very stable in air, and no inactivation of the enzyme is encountered as above.

This paper will show that the spectral changes in Figure 1B reflect the reversible inactivation of the dehydrogenase via the accumulation of a reduced flavin adduct. The difference in the final spectra observed between Figure 1 panels A and B could have two simple explanations. First, oxygen could be directly and intimately involved in the formation of the reduced flavin adduct. Alternatively, oxygen could be serving as an electron acceptor for the reduced enzyme allowing the accumulation of the true thioester inhibitor. An obligatory involvement of oxygen in adduct formation was excluded by repeating Figure 1A in an anaerobic cuvette containing ferricenium hexafluorophosphate in a side arm (Gorelick et al., 1985; Lehman & Thorpe, 1990). After reduction was complete, the oxidant was tipped into the main space of the cuvette to give a final concentration of 68 μM ferricenium ion. A rapid return of the oxidized dehydrogenase was observed (data not shown), followed by the slow accumulation of the same reduced flavin species seen in Figure 1B. The transitory reappearance of oxidized flavin is not seen in Figure 1B because reoxidation by molecular oxygen is slower than the subsequent rate of adduct formation. Thus, inhibition of the enzyme probably involves an interaction between the oxidized enzyme and a product of the turnover of *S*-(−)-3-methyloctanoyl-CoA by the medium chain enzyme (logically, 3-methyl-*trans*-2-octenoyl-CoA; compound **5**, Table 1).

The dehydrogenation product was generated on a larger scale by enzyme-catalyzed oxidation in the presence of excess ferricenium hexafluorophosphate and purified by HPLC (see Materials and Methods). Addition of this pure thioester to the oxidized enzyme under either anaerobic or aerobic conditions gave the bleached flavin chromophore shown in curve 6 of Figure 2. This spectrum is essentially identical to that seen in curve 2 of Figure 1B. Reduction with the purified product has a first-order rate constant of 0.085 min^{-1} (see inset, Figure 2). Bleaching is preceded by the formation of an oxidized enzyme–ligand complex judged by the 6-nm red-shift of the main flavin peak and the pronounced shoulder at 480 nm evident in curve 2 of Figure 2. No long wavelength band or other intermediates are evident in the approach to curve 6 of Figure 2.

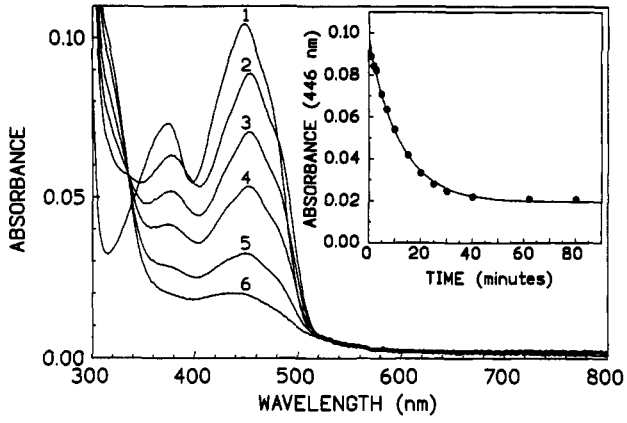


FIGURE 2: Anaerobic reduction of pig kidney medium chain acyl-CoA dehydrogenase by the dehydrogenation product of *S*-(−)-3-methyloctanoyl-CoA. Medium chain acyl-CoA dehydrogenase was deoxygenated (curve 1; 7 μM enzyme in 50 mM phosphate buffer, pH 7.6, 25 °C) and reduced with an excess of the product of incubations of *S*-(−)-3-methyloctanoyl-CoA and the enzyme (see Materials and Methods). Spectra shown were recorded at 1, 5, 10, 20, and 80 min (curves 2–6, respectively). The inset displays the decrease in absorbance at 446 nm over time and is fit to a first-order decrease of 0.085/min.

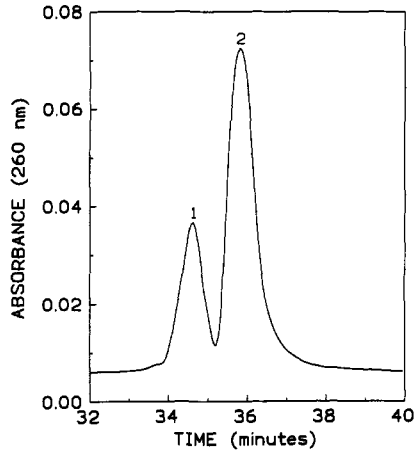


FIGURE 3: HPLC profile of the products from the chemical synthesis of 3-methyl-2-octenoyl-CoA. The reaction mixture from the synthesis of 3-methyl-2-octenoyl-CoA was applied to an octadecylsilica semipreparative column and developed as described under Materials and Methods. Peaks 1 and 2, eluting at 34.7 min and 35.8 min, respectively, were collected from repeated injections.

Two observations argue for a conjugated enoyl-CoA structure for the inhibitory thioester. First, the UV spectrum of the purified product is typical of an α–β unsaturated thioester (Stadtman, 1957), with increased absorption at the long wavelength edge of the 260-nm peak compared to the saturated substrate, 3-methyloctanoyl-CoA (see later). Second, incubation of the inhibitor with enoyl-CoA hydratase (crotonase; EC 4.2.1.17; Stern, 1961; Waterson & Hill, 1972) leads to rapid loss of this additional enone absorbance (data not shown). Structural proof was obtained by synthesis.

Synthesis and Characterization of 3-Methyl-trans-2-octenoyl-CoA. A mixture of *cis*- and *trans*-3-methyl-2-octenoic acids (in a 1:2 ratio, respectively) was synthesized essentially according to Ogura et al. (1970) and characterized by ^{1}H NMR. The mixture was thioesterified using the standard mixed anhydride method of Bernert and Sprecher (1976), and the resulting thioesters were purified by HPLC. Figure 3 presents the relevant portion of the chromatogram of an analytical injection monitored at 260 nm. Both peaks proved to be inhibitors of the dehydrogenase, and both required

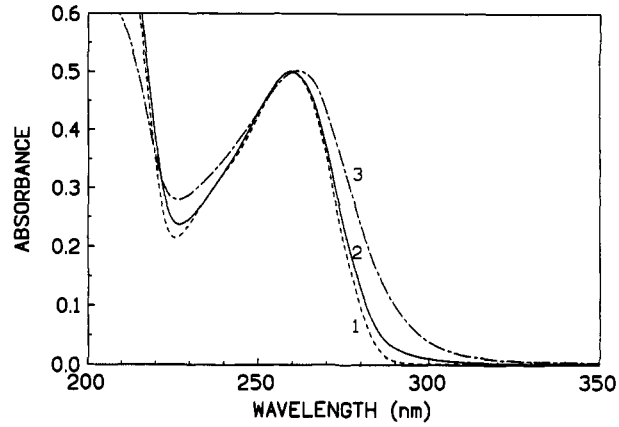


FIGURE 4: UV spectra of three octanoyl-CoA analogues. UV spectra were recorded, in water, of *S*-(−)-3-methyloctanoyl-CoA (31 μM; curve 1, – – –) and peaks 1 (31 μM; curve 2, —) and 2 (23 μM; curve 3, – · –) from the chemical synthesis of 3-methyl-2-octenoyl-CoA.

multiple semipreparative chromatographic injections to provide sufficient material for an unambiguous structural analysis.

One- and two-dimensional (COSY) ^{1}H NMR of peak 2 clearly identified it to be 3-methyl-*trans*-2-octenoyl-CoA (compound **5**, Table 1; see Materials and Methods). In addition, transmethylation of the thioester (see Materials and Methods) followed by GC/MS gave a mass spectrum consistent with methyl 3-methyl-*trans*-2-octenoate. Peak 2 shows a UV spectrum (curve 3, Figure 4) which is superimposable on the enzymatically generated material. This spectrum is consistent with a conjugated enone of a CoA thioester (Stadtman, 1957) and is clearly different from that of the original *S*-3-methyloctanoyl-CoA (curve 1, Figure 4). Both chemically and enzymatically synthesized materials behave identically toward the medium chain dehydrogenase in all aspects tested and upon treatment with enoyl-CoA hydratase (see Materials and Methods). Thus, the inhibitory thioester formed in Figure 1B is 3-methyl-*trans*-2-octenoyl-CoA (compound **5**, Table 1), the expected product for the dehydrogenation of *S*-(−)-3-methyloctanoyl-CoA by the medium chain enzyme (Biellman & Hirth, 1970a,b; Bücklers et al., 1970).

Unexpectedly, peak 1 of Figure 3 is not the corresponding *cis* isomer of 3-methyl-2-octenoyl-CoA, which was absent from the entire chromatogram, but proved to be an extremely potent inhibitor of the medium chain dehydrogenase. The identification and evaluation of this smaller, and more variable, component of the thioesterification mixture is summarized below.

3-Methylene-octanoyl-CoA as an Inhibitor of the Medium Chain Dehydrogenase. Peak 1 in Figure 3 showed a spectrum more typical of a saturated or unconjugated enoyl-CoA thioester (curve 2, Figure 4; without the additional absorbance from 260 to 300 nm which is characteristic of conjugated enones). GC/MS of the transmethylated product of peak 1 gave a molecular weight identical to the corresponding ester from peak 2 (molecular ion of 170), suggesting that they represent structural isomers. Consistent with these observations, ^{1}H NMR characterization of peak 1 showed it to be 3-methyleneoctanoyl-CoA (see Materials and Methods; compound **6**, Table 1).

Figure 5 shows that full reduction of the oxidized enzyme requires 1.0 equiv of 3-methyleneoctanoyl-CoA yielding a spectrum essentially identical with that obtained with 3-methyl-*trans*-2-octenoyl-CoA (curve 6, Figure 2). A similar stoichiometry of 1.3 equiv/flavin was also obtained with 3-methyl-

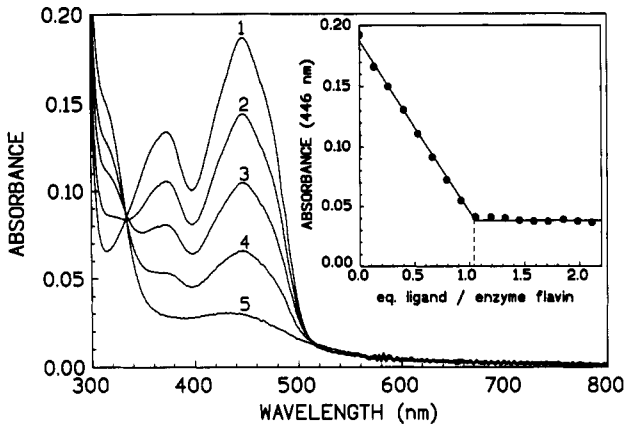


FIGURE 5: Anaerobic titration of pig kidney medium chain acyl-CoA dehydrogenase by 3-methyleneoctanoyl-CoA. Medium chain acyl-CoA dehydrogenase was deoxygenated (curve 1; 12 μM enzyme in 50 mM phosphate buffer, pH 7.6, 25 °C) and reduced with 3.1, 6.4, 9.5, and 25 μM 3-methyleneoctanoyl-CoA (curves 2–5, respectively). The inset shows the stoichiometry of reduction evaluated at 446 nm.

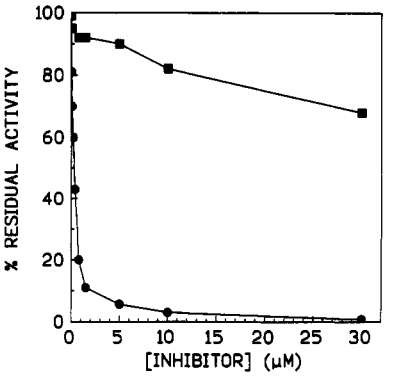


FIGURE 6: Potency of 3-methyl-*trans*-2-octenoyl-CoA and 3-methyleneoctanoyl-CoA as inhibitors of medium chain acyl-CoA dehydrogenase. Ferricenium assays were performed using 50 μM octanoyl-CoA and 10 nM enzyme (see Materials and Methods), with the addition of various concentrations of 3-methyl-*trans*-2-octenoyl-CoA (■) or 3-methylene-octanoyl-CoA (●). Rates were recorded 4–5 min after assay inception and were then converted to percent residual activity by comparison with the corresponding values for a control assay with no inhibitor present.

trans-2-octenoyl-CoA if sufficient time is allowed to complete the slow spectral changes between additions of titrant. In contrast to the sluggish reaction seen with the conjugated analogue, reduction of the medium chain enzyme by 3-methyleneoctanoyl-CoA is rapid. Stopped-flow experiments performed by mixing equal volumes of 12 μM dehydrogenase and 36 μM of the 3-methylene analogue at 1 °C gave a biphasic rate of reduction of 10/s and 1.8/s with amplitudes of 82 and 16% of the total change, respectively. For comparison, the conjugated analogue gave a reaction which was half-complete in approximately 100 min at 1 °C (10^5-fold slower than the predominant phase with 3-methyleneoctanoyl-CoA). These stopped-flow studies showed no evidence for an intermediate in the reduction of the flavin by 3-methyleneoctanoyl-CoA when the reaction was monitored from 320 to 700 nm.

Comparison of 3-Methyl-trans-2-octenoyl-CoA and 3-Methyleneoctanoyl-CoA as Inhibitors of the Medium Chain Dehydrogenase. The effectiveness of both thioesters as inhibitors of the medium chain dehydrogenase was qualitatively compared by incorporating increasing concentrations of the analogues into the standard assay system using 50 μM octanoyl-CoA as substrate. Assays were initiated by the addition of enzyme, and the final steady-state rate was expressed as a percentage of control values recorded in the absence of inhibitor (Figure 6). Under these conditions, half-inactivation requires approximately 0.3 μM 3-methyleneoctanoyl-CoA but needs about 80 μM of the 3-methyl-*trans*-2-octenoyl-CoA analogue. Although these two isomers differ widely in their effectiveness as inhibitors of the medium chain enzyme when competing with the tightly binding substrate octanoyl-CoA (K_D apparent about 20 nM; Thorpe et al., 1981), the evidence summarized below suggests that they apparently form the same reduced flavin species with the dehydrogenase.

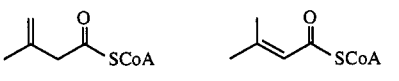
Behavior of Reduced Enzyme Species formed from 3-Methyl-trans-2-octenoyl- and 3-Methyleneoctanoyl-CoA. Adducts were formed with both inhibitors and freed from excess reagents by ultrafiltration and washing with 50 mM phosphate buffer, pH 7.6. Both species showed essentially the same UV–visible spectrum (data not shown) and could be stored for periods of a month at –20 °C without significant changes. In addition to their stability in aerobic solutions, neither adduct could be reoxidized in the presence of up to a 30-fold excess of ferricenium hexafluorophosphate (a facile oxidant of substrate reduced enzyme; Lehman & Thorpe, 1990; Lehman et al., 1990). Ultrafiltered samples of both adducts showed initial rates in standard assays of close to zero when diluted to nanomolar concentrations in the presence of 50 μM octanoyl-CoA, and both regained complete activity with a half-time of 2 min.

Enzyme reduced with either 3-methyl-*trans*-2-octenoyl-CoA or 3-methyleneoctanoyl-CoA also show identical behavior on denaturation by boiling or after treatment with methanol or 5% trichloroacetic acid (see Materials and Methods). Supernatants were examined by HPLC after centrifugation to remove precipitated protein. Boiling and methanol treatment release substantially oxidized FAD into the supernatant from both adducts (data not shown). In contrast, trichloroacetic acid precipitation leads to the destruction of the primary flavin species and to the appearance of multiple peaks on HPLC (not shown). Both adducts gave virtually superimposable HPLC profiles, and none of the breakdown products showed significant absorbance beyond 300 nm. Thus a variety of lines of evidence suggest that both inhibitors form a common reduced flavin adduct (see Discussion).

If both 3-methylene and 3-methyl-2-enoyl thioesters form the same reversible adduct with the dehydrogenase, then the enzyme would be expected to catalyze the thermodynamically favorable isomerization:

$$\text{3-methyleneoctanoyl-CoA} + \text{E·FAD} \rightleftharpoons \text{E·FAD-I} \rightleftharpoons \text{3-methyl-}\textit{trans}\text{-2-octenoyl-CoA} + \text{E·FAD}$$

This reaction, monitored by an increase in absorbance at 260 nm ($\Delta\epsilon = 6.0\ \text{mM}^{-1}\ \text{cm}^{-1}$), is extremely slow with these medium chain derivatives (with a turnover number of 0.015 min^{-1}). Their shorter chain counterparts, 3-methylene-butanoyl-CoA and 3-methyl-2-butenoyl-CoA, are more rapidly isomerized



3-methylene-butanoyl-CoA 3-methyl-2-butenoyl-CoA

by the enzyme and are thus more amenable to study.

Medium Chain Acyl-CoA Dehydrogenase Catalyzes the Isomerization of 3-Methylenebutanoyl-CoA to 3-Methyl-2-butenoyl-CoA. 3-Methylenebutanoic acid was synthesized by Jones oxidation of the corresponding alcohol (see Materials

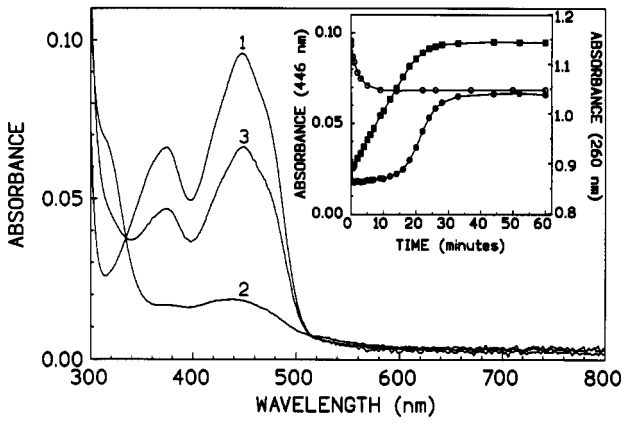


FIGURE 7: Anaerobic reduction of pig kidney medium chain acyl-CoA dehydrogenase by 3-methylenebutanoyl-CoA and 3-methyl-2-butenoyl-CoA. Medium chain acyl-CoA dehydrogenase was deoxygenated (curve 1; 5.8 μM enzyme in 50 mM phosphate buffer, pH 7.6, 25 °C) and reduced with 29 μM 3-methylenebutanoyl-CoA. Spectra shown in the main panel were recorded at 10 s and 60 min after the addition of ligand (curves 2 and 3, respectively). Several intermediate spectra have been omitted for clarity. The inset displays the absorbance at 446 nm (●, left axis) and at 260 nm (■, right axis) over the entire anaerobic incubation. In addition, the inset shows the decrease in absorbance at 446 nm after the addition of 29 μM 3-methyl-2-butenoyl-CoA to 5.8 μM medium chain acyl-CoA dehydrogenase (○, left axis), under identical conditions.

and Methods; Bowden et al., 1946), and the CoA thioester was purified by HPLC and characterized by ^{1}H NMR. The corresponding conjugated isomer, 3-methyl-2-butenoyl-CoA, is available commercially. Figure 7 shows the addition of a 5-fold excess of 3-methylenebutanoyl-CoA to the acyl-CoA dehydrogenase under anaerobic conditions. Initially a rapid drop in absorbance is seen at 446 nm (curve 2 and inset, filled circles, Figure 7) leading to a reduced flavin adduct with a spectrum very similar to that seen in Figure 1B. However, unlike the adduct formed with 3-methyleneoctanoyl-CoA, this species is unstable under anaerobic (or aerobic) conditions. Thus, after a pronounced lag phase (inset Figure 7), a partial recovery of oxidized flavin absorbance yields curve 3. Turnover of the inhibitor during the lag phase is evident by a steady increase in absorbance at 260 nm (filled squares, inset Figure 7), accompanying isomerization of the inhibitor to the conjugated analogue. As expected, the length of the lag phase is increased with increasing inhibitor concentration (not shown). Both the duration of the lag phase and the increase in 260 nm absorbance are consistent with a turnover number of about 0.3 min^{-1} for the isomerization of 3-methylenebutanoyl-CoA to 3-methyl-2-butenoyl-CoA. The product of turnover coelutes with authentic 3-methyl-2-butenoyl-CoA on HPLC (see Materials and Methods) and has a visible spectrum identical to that of this conjugated thioester (not shown). HPLC analyses of ultrafiltered supernatants from these experiments suggest that the equilibrium constant for the isomerization

3-methylenebutanoyl-CoA $\rightleftharpoons$ 3-methyl-2-butenoyl-CoA

is approximately 150 (see Materials and Methods; not shown). If the experiment in Figure 7 is repeated using the 3-methyl-2-butenoyl-CoA, a slow (0.43 min^{-1}) partial reduction of the oxidized enzyme occurs (open circles, inset Figure 7). The resulting spectrum is superimposable on curve 3 of Figure 7.

The data with these shorter analogues confirm that the 3-methylene analogues are much more facile reductants of the dehydrogenase. Further, these experiments lend additional support for the interconversion of the two isomeric inhibitors via a common reduced flavin species (see Discussion).

DISCUSSION

This investigation set out to characterize the inhibitory product of the oxidation of the weak substrate *S*-(−)-3-methyloctanoyl-CoA by the medium chain dehydrogenase. During chemical synthesis of the enoyl-CoA product of this reaction (compound **5**, Table 1), an additional, and much more effective, inhibitor was isolated and characterized (compound **6**). 3-Methyleneoctanoic acid, is a trace (3%) contaminant of the 3-methyl-2-octenoic acids used in the thioesterification reaction analyzed in Figure 3. Since unconjugated fatty acids are both more readily thioesterified than their conjugated counterparts, and are subject to fewer side reactions (Stadtman, 1957), 3-methylene-octanoyl-CoA becomes a significant proportion of the total thioester pool recovered in Figure 3.

This study provides a fourth general class of mechanism-based inhibitors which target the isoalloxazine moiety of the medium chain acyl-CoA dehydrogenase. A summary comparison of the best characterized inhibitors, MCPA-CoA, 3,4-pentadienoyl-CoA, 3-methyl-*trans*-2-octenoyl-CoA and 3-methyleneoctanoyl-CoA is presented in Table 2. Clearly there are differences in the rates of formation and stability of the adducts, but marked similarities exist in both their spectral properties and in their resistance to oxidation by one-electron acceptors. At the outset, neither 3-methyl-2-enoyl- nor 3-methyleneacyl-CoA analogues would be considered as likely mechanism-based inhibitors of the dehydrogenase, since they lack the latently reactive methylenecyclopropyl or 3,4-allenic moieties of the other inhibitors (Table 1). However, both isomeric inhibitors form stoichiometric reduced flavin adducts with the dehydrogenase which are not on the pathway for normal substrates. Adduct formation clearly requires activation of both thioesters by the dehydrogenase (see later). The resulting reduced enzyme species are inactive but can be reactivated completely upon prolonged incubation with a 1500-fold excess of octanoyl-CoA. Hence, 3-methyl-*trans*-2-octenoyl-CoA and 3-methyleneoctanoyl-CoA fulfill the general criteria for mechanism-based inhibitors of the medium chain acyl-CoA dehydrogenase (Silverman, 1988).

Scheme 1 presents a proposed mechanism for adduct formation for these inhibitors. Like MCPA-CoA and 3,4-pentadienoyl-CoA (Wenz et al., 1981, 1985), 3-methyleneoctanoyl-CoA is presumably activated by α-proton abstraction (step 1, Scheme 1). Again by analogy with the earlier studies (Wenz et al., 1981, 1985), step 3 involves nucleophilic attack of the resulting resonance stabilized carbanion/enolate at the N-5 position of the isoalloxazine ring. In normal catalysis this locus receives the hydride equivalent eliminated from the β-position by the developing enolate at C-1 (Ghisla et al., 1984; Schopfer et al., 1988). Recently, Liu and coworkers have proposed that inactivation of the dehydrogenase with MCPA-CoA involves a radical-mediated opening of the strained methylenecyclopropyl ring (Lai et al., 1991, 1993). While the unusual structural features of MCPA-CoA sustain a radical involvement with the acyl-CoA dehydrogenases, there are no indications for such a mechanism with 3-methyleneoctanoyl-CoA and related inhibitors.

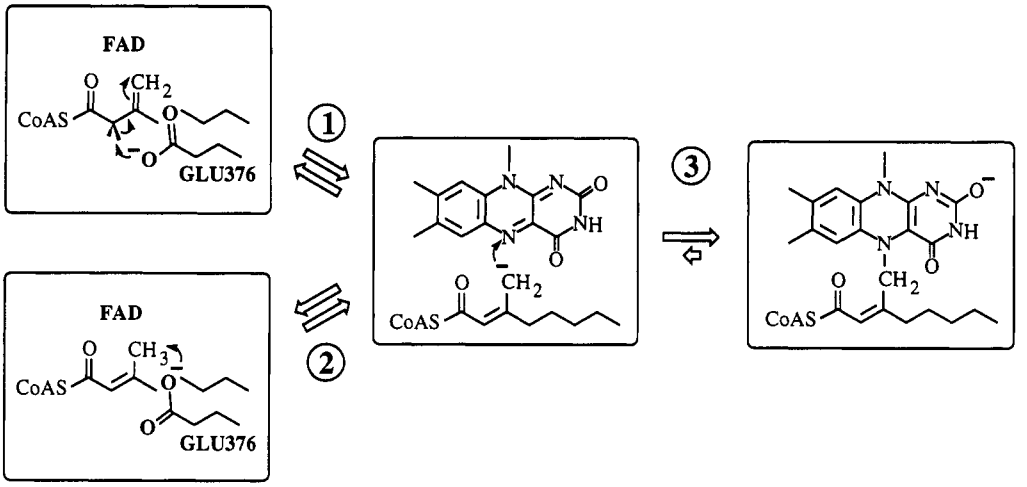
The choice of an N-5 flavin adduct is made in Scheme 1 because the reduced species shows a prominent shoulder at 320 nm, where such adducts should absorb strongly (Brüstlein et al., 1971; Ghisla & Massey, 1975; Wenz et al., 1985), but comparatively little absorbance in the 350–390 nm region, where C-4a adducts would be expected to absorb (Ghisla et al., 1973, 1974). A definitive assignment of the structure of the adduct will probably require X-ray diffraction studies of the reduced enzyme species in view of the multiplicity of

Table 2: Comparison of Several Flavin Adducts of Medium Chain Acyl-CoA Dehydrogenase from Pig Kidney

property	mechanism-based inhibitors			
	MCPA-CoA[a]	3,4-pentadienoyl-CoA[b]	3-methyleneoctanoyl-CoA	3-methyl-*trans*-2-octenoyl-CoA
type of adduct	reduced flavin	reduced flavin	reduced flavin	reduced flavin
reversibility versus octanoyl-CoA	no	yes	yes	yes
half-time for adduct formation (s)	10–60[c]	0.02[d]	0.07[d]	6×10^3 [d]
flavin derived products on TCA denaturation	multiple	oxidized FAD	multiple	multiple
reoxidation with one-electron acceptors	no	no	no	no

[a] Compound 3, Table 1 (Wenz et al., 1981; Lai et al., 1991; Baldwin et al., 1990). [b] Compound 4, Table 1 (Wenz et al., 1985). [c] Determined at 25°C. [d] Determined at 1°C.

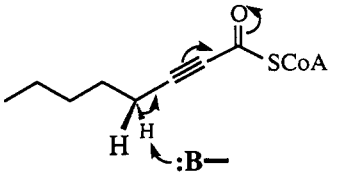
Scheme 1: Proposed Mode of Inactivation of the Medium Chain Acyl-CoA Dehydrogenase by 3-Methyleneoctanoyl-CoA and 3-Methyl-*trans*-2-octenoyl-CoA



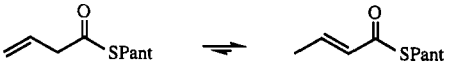
products generated on denaturation of the modified dehydrogenase (see earlier). Similarly, crystallographic methods will likely prove necessary to define the nature of the primary reduced enzyme species generated with MCPA-CoA and 3,4-pentadienoyl-CoA.

Step 2 in Scheme 1 depicts γ-proton abstraction from the methyl group of 3-methyl-*trans*-2-octenoyl-CoA generating the same resonance stabilized carbanion seen with the more potent inhibitor. An alternative would involve abstraction of a proton from the γ-secondary carbon atom in the acyl chain. However, this would generate a different flavin adduct from that derived from 3-methyleneoctanoyl-CoA. Structural differences between the two adducts would be expected to lead to significant differences in their thermodynamic and/or kinetic stability. Because no such differences have been observed, we suggest that this alternative pathway is less likely. This issue can be resolved with the synthesis of isotopically labeled inhibitors and the use of X-ray crystallography.

Two examples of γ-proton abstractions in the acyl-CoA dehydrogenases provide precedent for step 2 in Scheme 1, in which the same flavin adduct is generated from 3-methyl-*trans*-2-octenoyl-CoA. Inactivation of the medium chain dehydrogenase by 2-octynoyl-CoA involves an initial rate-limiting γ-proton abstraction with a half-time of about 0.7 min (Freund et al., 1985; Powell & Thorpe, 1988):



A second example involves the isomerization of vinylacetylpantetheine to crotonylpantetheine by the butyryl-CoA dehydrogenase from *Megasphaera elsdenii* (Fendrich & Abeles, 1982):



Microscopic reversibility requires that the dehydrogenase abstract a γ-proton from the methyl group of crotonyl-CoA to generate the less stable unconjugated isomer. In the case of glutaryl-CoA dehydrogenase from *Pseudomonas fluorescens*, proton transfer between α- and γ-positions occurs without substantial exchange with solvent, suggesting that a monoprotic base communicates between these positions (Gomes et al., 1981). A glutamate residue, thought to be the base in a number of the acyl-CoA dehydrogenases (Fendrich & Abeles, 1982; Powell & Thorpe, 1988; Bross et al., 1990; Kim, 1991; Kim et al., 1992; Lundberg & Thorpe, 1993), is well suited for this role, because rotation of the carboxyl moiety could deliver a proton to either position without major conformational adjustments to the active center. Thus, although we have no direct evidence for the activation of both 3-methylene and 3-methyl inhibitors by glutamate-376 of the medium chain enzyme, the precedents outlined above suggest that this would be feasible.

The data with the short chain derivatives clearly show that the dehydrogenase catalyzes the thermodynamically favorable (Pollack et al., 1989) isomerization of 3-methylenebutanoyl-CoA to 3-methyl-2-butenoyl-CoA. Although isomerization is accompanied by flavin adduct formation, there are many examples where cofactors are not employed in allylic rearrangements (Schwab & Henderson, 1990), and there is no *a priori* reason for the involvement of the prosthetic group in this case. When such adducts intervene, they presumably

reflect a more efficient capture of the carbanion by the oxidized flavin compared to a direct protonation at the α- or γ-positions. The unit stoichiometries for adduct formation reported in this paper support a highly effective trapping reaction and hence an initial partition between turnover and inhibition (Walsh, 1978; Silverman, 1988) of close to zero. A similarly efficient trapping of the enolate of 3,4-pentadienoyl-CoA has been reported prior to the formation of the conjugated 2,4-diene (Wenz et al., 1985). It is uncertain whether the isomerization of vinylacetyl-CoA to crotonyl-CoA catalyzed by the short chain acyl-CoA dehydrogenase from *M. elsdenii* (Fendrich & Abeles, 1982) results in the accumulation of reduced flavin. In general, partition between a direct isomerization and covalent adduct formation is likely to be strongly dependent on steric differences between potential isomerase substrates.

The substantially greater inhibitory potency of 3-methyleneoctanoyl-CoA compared to 3-methyl-*trans*-2-octenoyl-CoA ultimately reflects the greater thermodynamic stability of the conjugated isomer (Pollack et al., 1989) and the consequently decreased acidity of the γ-protons in 3-methyl-*trans*-2-octenoyl-CoA compared to the α-protons in 3-methyleneoctanoyl-CoA. In addition to these thermodynamic arguments, the methyl group at C-3 is likely to be unfavorably placed with respect to the catalytic base normally functioning in α-proton abstraction. In aggregate, these factors make 3-methyleneoctanoyl-CoA a far superior inhibitor than its conjugated isomer (Figure 6), both kinetically and thermodynamically. Thus, the 3-methylene analogue is a faster reductant than the corresponding 3-methyl-2-enoyl isomer by a factor of approximately 10^5 and can compete effectively with octanoyl-CoA in assay mixtures.

These studies have identified a new class of mechanism-based inhibitor of the medium chain acyl-CoA dehydrogenase. Preliminary experiments show that the short chain dehydrogenase from pig liver (Lundberg & Thorpe, 1993) is bleached by 3-methylenebutanoyl-CoA. Perhaps this type of inactivation may prove generally applicable to the acyl-CoA dehydrogenase family. One might therefore expect that fatty acids which carry a methylene branch point at odd numbered carbon atoms might be effective inhibitors of fatty acid oxidation. Clearly, the metabolism and possible pharmacological effects of these simple compounds merit investigation.

ACKNOWLEDGMENT

We thank Drs. Keith Chenault and Douglass Taber for helpful advice, Dr. Philip Sonnet for a gift of *R*- and *S*-3-methyl-octanoic acids, and Drs. Martha Bruch and Gordon Nicol for outstanding help with the acquisitions and interpretation of NMR and mass spectral data.

REFERENCES

Baldwin, J. E., Ostrander, R. L., Simon, C. D., & Widdison, W. C. (1990) *J. Am. Chem. Soc. 112*, 2021–2022.

Beinert, H. (1963) *The Enzymes*, 2nd ed., Vol. 7, pp 447–466, Academic Press, New York.

Bernert, J. T., & Sprecher, H. (1977) *J. Biol. Chem. 252*, 6736–6744.

Biellman, J. F., & Hirth, C. G. (1970a) *FEBS Lett. 8*, 55–56.

Biellman, J. F., & Hirth, C. G. (1970b) *FEBS Lett. 9*, 335–336.

Bowden, K., Heilbron, I. M., Jones, E. R. H., & Weedon, B. C. L. (1946) *J. Chem. Soc.*, 39.

Bross, P., Engst, S., Strauss, A. W., Kelley, D. P., Rasched, I., & Ghisla, S. (1990) *J. Biol. Chem. 265*, 7116–7119.

Brüstlein, M., Knappe, W.-R., & Hemmerich, P. (1971) *Angew. Chem., Int. Ed. Engl. 10*, 804–806.

Bücklers, L., Umani-Ronchi, A., Rétey, J., & Arigoni, D. (1970) *Experimentia 26*, 931–933.

Corkey, B. E., Brandt, M., Williams, R. J., & Williamson, J. R. (1981) *Anal. Biochem. 118*, 30–41.

Engel, P. C., & Massey, V. (1971) *Biochem. J. 125*, 879–887.

Fendrich, G., & Abeles, R. H. (1982) *Biochemistry 21*, 6685–6695.

Frerman, F. E., Miziorki, H. M., & Beckmann, J. D. (1980) *J. Biol. Chem. 25*, 11192–11198.

Freund, K., Mizzer, J. P., Dick, W., & Thorpe, C. (1985) *Biochemistry 24*, 5996–6002.

George, G. N., Shaw, L., Bray, R. C., & Engel, P. C. (1984) in *Flavins and Flavoproteins* (Bray, R. C., Engel, P. C., & Mayhew, S. G., Eds.) pp 421–433, Walter de Gruyter, Berlin.

Ghisla, S., & Massey, V. (1975) *J. Biol. Chem. 250*, 577–584.

Ghisla, S., Hartmann, U., Hemmerich, P., & Müller, F. (1973) *Justus Liebigs Ann. Chem.* 1388–1415.

Ghisla, S., Massey, V., Lhoste, J.-M., & Mayhew,S.G. (1974) *Biochemistry 13*, 589–597.

Ghisla, S., Thorpe, C., & Massey, V. (1984) *Biochemistry 23*, 3154–3160.

Gomes, B., Fendrich, G., & Abeles, R. H. (1981) *Biochemistry 20*, 1481–1490.

Gorelick, R. J., Schopfer, L. M., Ballou, D. P., Massey, V., & Thorpe, C. (1985) *Biochemistry 24*, 6830–6839.

Hall, C. L., Lambeth, J. D., & Kamin, H. (1979) *J. Biol. Chem. 254*, 2023–2031.

Hassall, C. H., & Reyle, K. (1955) *Biochem. J. 60*, 334–339.

Kates, M. (1986) in *Techniques of Lipidology* (Burdon, R. H., & van Knippenberg, P. H., Eds.) 2nd ed., pp 124–125, Elsevier, New York.

Kim, J. J. P. (1991) in *Flavins and Flavoproteins*, pp 291–298, Walter de Gruyter, Berlin.

Kim, J. J. P., & Wu, J. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 6677–6681.

Kim, J. J. P., Wang, M., Djordjevik, S., & Paschke, R. (1992) in *New Developments in Fatty Acid Oxidation* (Coates, P. M., & Tanaka, K., Eds.) pp 111–126, Wiley-Liss Inc., New York.

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

Lai, M.-t., Li, D., Oh, E., & Liu, H.-w. (1993) *J. Am. Chem. Soc. 115*, 1620–1628.

Lau, S.-M., Brantley, R. K., & Thorpe, C. (1988) *Biochemistry 27*, 5089–5095.

Lau, S.-M., Brantley, R. K., & Thorpe, C. (1989) *Biochemistry 28*, 8255–8262.

Lehman, T. C., & Thorpe, C. (1990) *Biochemistry 29*, 10594–10602.

Lehman, T. C., Hale, D. E., Bhala, A., & Thorpe, C. (1990) *Anal. Biochem. 186*, 280–284.

Lundberg, N. N., & Thorpe, C. (1993) *Archives Biochem. Biophys.* (in press).

Massey, V., & Ghisla, S. (1974) *Ann. N.Y. Acad. Sci. 227*, 446–465.

Murfin, W. W. (1974) Ph.D. Thesis, Washington University, St. Louis, MO.

Ogura, K., Nishino, T., Koyama, T., & Seto, S. (1970) *J. Am. Chem. Soc. 92*, 6036–6041.

Pohl, B., Raichle, T., & Ghisla, S. (1986) *Eur. J. Biochem. 160*, 109–115.

Pollack, R. M., Bounds, P. L., & Bevins, C. L. (1989) in *The Chemistry of Functional Groups: The Chemistry of Enones, Part 1* (Patai, S., & Rappoport, Z., Eds.) pp 559–597, John Wiley & Sons, New York.

Powell, P. J., & Thorpe, C. (1988) *Biochemistry 27*, 8022–8028.

Powell, P. J., Lau, S.-M., Killian, D., & Thorpe, C. (1987) *Biochemistry 26*, 3704–3710.

Reinsch, J., Katz, A., Wean, J., Aprahamian, G., & McFarland, J. T. (1980) *J. Biol. Chem. 255*, 9093–9097.

Schopfer, L. Massey, V., Ghisla, S., & Thorpe, C. (1988) *Biochemistry 27*, 6599–6611.

Schwab, J. M., & Henderson, B. S. (1990) *Chem. Rev. 90*, 1203–1245.
Shaw, L., & Engel, P. C. (1984) in *Flavins and Flavoproteins* (Bray, R. C., Engel, P.C., & Mayhew, S. G. Eds) pp 417–420, Walter de Gruyter, Berlin.
Shaw, L., & Engel, P. C. (1985) *Biochem. J. 239*, 723–731.
Silverman, R. B. (1988) in *Mechanism-Based Enzyme Inactivation: Chemistry and Enzymology*, pp 3–30, CRC Press, Inc., Boca Raton, FL.
Sonnet, P. E., & Baillargeon, M. W. (1989) *Lipids 24*, 434.
Sonnet, P. E., & Gazzillo, J. (1990) *Org. Prep. Proced. Int. 22*, 203–208.
Stadtman, E. R. (1957) *Methods Enzymol. 3*, 931–941.
Stern, J. R. (1961) *Enzymes, 2nd Ed., 5*, 511–529.
Thorpe, C., & Massey, V. (1983) *Biochemistry 22*, 2972–2978.
Thorpe, C., Matthews, R. G., & Williams, C. H. (1979) *Biochemistry 18*, 331–337.
Thorpe, C., Ciardelli, T. L., Stewart, C. J., & Wieland, T. L. (1981) *Eur. J. Biochem. 147*, 553–560.
Walsh, C. T. (1978) *Annu. Rev. Biochem. 47*, 881–893.
Waterson, R. M., & Hill, R. L. (1972) *J. Biol. Chem. 247*, 5258–5265.
Wenz, A., Thorpe, C., & Ghisla, S. (1981) *J. Biol. Chem. 256*, 9809–9812.
Wenz, A., Ghisla, S., & Thorpe, C. (1985) *Eur. J. Biochem. 147*, 553–560.