Interaction of Acyl Coenzyme A Substrates and Analogues with Pig Kidney Medium-Chain Acyl-CoA Dehydrogenase†

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ABSTRACT: Several alkylthio coenzyme A (CoA) derivatives (from ethyl- to hexadecyl-SCoA) have been synthesized to probe the substrate binding site in the flavoprotein medium-chain acyl-CoA dehydrogenase from pig kidney. All bind to apparently equivalent sites with a stoichiometry of four per tetramer. A plot of log K_d vs. hydrocarbon chain length is linear from 2 to 16 carbons with a free energy of binding of 390 cal/methylene group. These data suggest an acyl-binding site of moderate hydrophobicity and imply that the observed substrate specificity of the medium-chain dehydrogenase is not achieved simply by the length of the hydrocarbon binding pocket. Extrapolation of the graph to zero chain length predicts a K_d of 1 mM for the CoA moiety. The difference between this value and the experimentally determined value of 206 μM may be attributed to a contribution from the ionization of the sulfhydryl group in CoASH. The interaction of several eight-carbon intermediates of β-oxidation (*trans*-2- and *trans*-3-octenoyl-CoA and L-3-hydroxy- and 3-ketooctanoyl-CoA) with the dehydrogenase has also been studied. All but the L-3-OH derivative bind tightly to the enzyme (with K_d values in the 50–90 nM range) and are very effective inhibitors of the dehydrogenation of octanoyl-CoA. The *trans*-3-enoyl analogue produces an immediate, intense, long-wavelength band (λ_{max} = 820 nm), which probably represents a charge-transfer interaction between the delocalized α-carbanion donor and oxidized flavin as the acceptor. The L-3-OH analogue is a reductant of the flavin, yielding 3-ketooctanoyl-CoA. The results of these studies suggest that all of the 28 intermediates of palmitoyl-CoA oxidation plus those derived from the degradation of unsaturated fatty acid chains could bind to the medium chain length dehydrogenase from pig kidney. Possible metabolic consequences of this are discussed.

Mitochondrial β-oxidation involves a large number of acyl-CoA[1] intermediates and a considerable number of distinct enzymes. For example, the oxidation of palmitoyl-CoA to eight acetyl-CoA units involves 28 even chain length intermediates grouped into saturated acyl-CoA and *trans*-2-enoyl-, 3-hydroxyacyl-, and 3-ketoacyl-CoA categories. The utilization of unsaturated fatty acids such as oleic or linoleic acids results in the formation of additional intermediates containing both cis and trans double bonds. In mammals, the enzymes representing the central activities in mitochondrial β-oxidation (namely, acyl-CoA dehydrogenase, enoyl-CoA hydratase, 3-OH-acyl-CoA dehydrogenase, and thiolase) exist in at least two forms to complement the array of acyl chain lengths encountered. In addition to these activities, ancillary enzymes such as a *cis*-3-/*trans*-2-isomerase (Stoffel et al., 1964; Miesowicz & Bloch, 1979) and a NADPH-linked 2,4-dienoyl-CoA reductase (Dommes & Kunau, 1984a; Cuebas & Schulz, 1982; Mizugaki et al., 1984) are required for the oxidation of unsaturated chains in mitochondria. This multiplicity of enzymes and acyl-CoA intermediates in the mitochondrial matrix poses problems of organization and coordination, and these aspects of fatty acid oxidation are poorly understood.

This paper examines the ability of the medium-chain acyl-CoA dehydrogenase (Crane et al., 1956; Beinert, 1963) to discriminate between a variety of CoA thio ester analogues in vitro. This flavoenzyme, isolated from a variety of sources, shows an optimal activity toward medium-chain acyl-CoA derivatives and exhibits lower activities toward butyryl- and palmitoyl-CoA (Crane et al., 1956; Hall & Kamin, 1975; Thorpe et al., 1979; Frerman et al., 1980a; Furuta et al., 1981; Davidson & Schultz, 1982; Dommes & Kunau, 1984b; Ikeda et al., 1985a; Finocchiaro et al., 1986). In this work, the chain length discrimination (from C-2 to C-16) of the pig kidney medium-chain acyl-CoA dehydrogenase has been assessed at the level of the Michaelis complex. In addition, the effect of several C-8 intermediates (*trans*-2-enoyl, *trans*-3-enoyl, L-3-hydroxyacyl, and 3-ketoacyl) has been tested on the pure dehydrogenase. This study in substrate discrimination reveals some surprising features. A number of these thio esters bind very tightly to the acyl-CoA dehydrogenase, suggesting that their accumulation in vivo might severely limit the flux through the β-oxidation pathway.

Materials and Methods

Materials. Medium-chain acyl-CoA dehydrogenase was isolated from pig kidneys as described previously (Gorelick et al., 1985). An additional octyl-Sepharose step was used to remove traces of a contaminating enoyl-hydratase activity (Lau et al., 1986). Concentrations of holoenzyme and apoprotein were expressed spectrophotometrically with extinction coefficients of 15.4 mM^{-1} cm^{-1} at 446 nm (Thorpe et al., 1979) and 63 mM^{-1} cm^{-1} at 280 nm (Mayer, 1981) respectively. Bovine liver crotonase and 3-hydroxyacyl-CoA dehydrogenase were from Sigma. 8-Cl-FAD was prepared by the method of Spencer et al. (1976) from 8-chlororiboflavin (a generous gift

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[1] Abbreviations: CoA, coenzyme A; EDTA, ethylenediaminetetraacetic acid; FAD, flavin adenine dinucleotide; HPLC, high-performance liquid chromatography.

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from Dr. John Lambooy, University of Maryland). Crotonyl-, octanoyl-, acetyl-, and acetoacetyl-CoA and CoASH (lithium salt) were from P-L Biochemicals. NAD^+ and NADH were from Sigma Chemical Co. All alkyl iodides used in this work were from Aldrich. Hexadecyl iodide was recrystallized from ethanol before use. *trans*-2-Octenoic acid was obtained from Pfaltz and Bauer. *cis*-2-Octenoic acid was prepared by catalytic hydrogenation of 2-octynoic acid. *trans*-3-Octenoic acid was synthesized by the method of Linstead and Noble (1933).

Preparations of CoA Thioether and Sulfonate Analogues. CoA thioethers were prepared from ethyl, butyl, octyl, dodecyl, and hexadecyl iodide essentially as described by Ciardelli et al. (1981). Ethyl-SCoA was purified by HPLC with a Perkin-Elmer Series 400 liquid chromatograph on a semipreparative octadecylsilica column (Zorbax) with a gradient of 50 mM phosphate, pH 5.3, and methanol (Corkey et al., 1981). Hexadecyl-SCoA was made 2 M in LiCl and applied to an octyl-Sepharose column equilibrated with 1 M LiCl. The column was then washed with a decreasing gradient from 1 M to water (Merrill et al., 1982), and the thioether was eluted with 50% aqueous ethanol. The remaining CoA thioethers were purified by anion exchange on DEAE-cellulose, with a LiCl gradient in 1 mM HCl (Lau et al., 1977). The purified derivatives were concentrated by lyophilization and desalted on Bio-Gel P-2. They were stored as lyophilized powders, and solutions in water were standardized spectrophotometrically with an extinction coefficient of 15.4 mM^{-1} cm^{-1} (Stadtman, 1957). All thioethers appeared greater than 95% pure as judged by HPLC monitored at 260 nm. CoA sulfonate was prepared as described by Nishimura et al. (1982) and purified by DEAE-cellulose as described above.

Preparation of CoA Thio Esters. *trans*-2- and *trans*-3-octenoyl-CoA were prepared by the mixed anhydride procedure of Bernert and Sprecher (1977). They were purified by HPLC as described above. In our hands, *trans*-2-octenoyl-CoA prepared in this way from commercial 2-octenoic acid contains a few percent of *trans*-3-octenoyl-CoA. This is evident by the immediate small increase in the absorbance at 820 nm when a 10-fold excess of *trans*-2-octenoyl-CoA is added to the medium-chain acyl-CoA dehydrogenase (see later). These isomers may be separated on the Zorbax reverse-phase column with a shallow methanol gradient (see earlier). *cis*-2-Octenoyl-CoA was prepared by the method of Mizugaki et al. (1982). L-3-hydroxyoctanoyl-CoA was prepared enzymatically by the addition of 75 μg of crotonase to 1 mL of 4 mM *trans*-2-octenoyl-CoA in 50 mM phosphate buffer pH 7.6, 25 °C. The reaction was followed by the decrease in absorbance at 302 nm. After 1 h, the mixture was brought to pH 5 with HCl and ultrafiltered, and the filtrate was purified by HPLC as described earlier. D-3-hydroxyoctanoyl-CoA was prepared and purified as above from *cis*-2-octenoyl-CoA. 3-Ketooctanoyl-CoA was prepared and standardized as in Thorpe (1986).

Spectrophotometric Titrations. Unless otherwise stated, titrations were performed in 50 mM potassium phosphate buffer, pH 7.6, containing 0.3 mM EDTA at 25 °C. Anaerobic titrations were performed as described previously (Gorelick et al., 1982). Spectra were recorded on a Cary 219 spectrophotometer interfaced to a microcomputer. Spectra could be stored on diskettes for subsequent generation of difference spectra and for other manipulations. Dissociation constants and the stoichiometry of binding (n ligands per flavin) were obtained by a nonlinear regression analysis program (Jain et al., 1982) that varies K_d, n, and the maximal absorbance change. In some cases, dissociation constants were obtained by visual comparison of real and simulated curves, assuming a stoichiometry of one ligand per flavin. Both approaches gave very similar results. Most experiments were conducted in 1-cm path-length cells, but for compounds that bind very tightly, low enzyme concentrations (about 1 μM) and 5-cm cells were used.

Results and Discussion

Interaction of Acyl-CoA Thio Esters with Pig Kidney Medium-Chain Acyl-CoA Dehydrogenase. The medium (or general) acyl-CoA dehydrogenase is reduced by stoichiometric levels of acyl-CoA substrates to extents that depend on the chain length of the thio esters used (Crane et al., 1956; Hall & Kamin, 1975; Thorpe et al., 1979; Ikeda et al., 1985b). These data have been interpreted in terms of the following scheme in which the equilibrium K_2 is chain length dependent (Beinert & Page, 1957; Thorpe et al., 1979; Thorpe & Massey, 1983):

$$E\cdot FAD + SH_2 \overset{K_1}{\rightleftharpoons} E\cdot FAD\cdot SH_2 \overset{K_2}{\rightleftharpoons}$$

$$E\cdot FADH_2\cdot S \overset{K_3}{\rightleftharpoons} E\cdot FADH_2 + S$$

The variable contribution of K_2, together with uncertainty as to the nature and spectral characteristics of the reduced enzyme forms, makes analysis of the binding of thio ester substrates of different chain lengths very difficult. We have therefore selected alkyl-SCoA thioethers to estimate the contribution of the hydrocarbon chain length to K_1 above. These derivatives are competitive inhibitors of the medium chain length dehydrogenase, and lacking acidic α-protons, they are not oxidized via step K_2 (Frerman et al., 1980b; Thorpe et al., 1981).

Binding of CoA Thioethers to the Medium Chain Length Dehydrogenase. Ethyl-, butyl-, octyl-, dodecyl-, and hexadecyl-SCoA were synthesized (see Materials and Methods), and their interaction with the dehydrogenase was studied by visible spectroscopy. Binding leads to the appearance of shoulders at 435 and 480 nm and a red shift of the major absorbance band: these changes are similar to those reported earlier for the binding of octyl-SCoA (Frerman et al., 1980b) and heptadecyl-SCoA (Thorpe et al., 1981) to the medium chain length acyl-CoA dehydrogenase. A comparison of the spectral changes generated by saturating levels of each thioether is shown in Figure 1. All yield qualitatively similar difference spectra at wavelengths greater than 380 nm. Below 380 nm, the negative displacements seen with ethyl- and butyl-SCoA reflect end absorption from the relatively high concentration of unbound ligand (see legend to Figure 1). Ethyl- and butyl-SCoA show only about 15 and 25% of the spectral change shown by the longer chain derivatives (octyl-, dodecyl-, and hexadecyl-SCoA). The spectral changes observed with these alkyl-SCoA derivatives (namely, increased resolution and red shift of the flavin chromophore) are generally associated with a decreased polarity of the flavin environment (Palmer & Massey, 1968). These effects could reflect a close proximity of a portion of the hydrocarbon chain to the chromophore, or possibly they might represent a progressive exclusion of water from the flavin binding site as the chain extends.

Spectrophotometric titrations were conducted for each thioether analogue, adjusting the enzyme concentration to allow accurate determination of the dissociation constants (see Materials and Methods). In all cases (except ethyl-SCoA, where the dissociation constant is too large to permit accurate estimation) a stoichiometry of one ligand per flavin was observed. Figure 2 plots the logarithm of the K_d values obtained

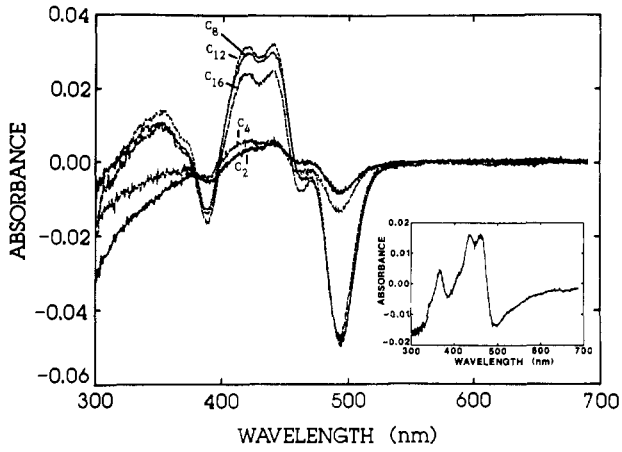


FIGURE 1: Extrapolated maximal difference spectra for the binding of ethyl-, butyl-, octyl-, dodecyl-, and hexadecyl-CoA to the medium-chain acyl-CoA dehydrogenase. Difference spectra were constructed by subtraction of the spectral file recorded at the highest ligand concentration from that of the native enzyme and scaling the difference to reflect the degree of saturation attained in the titration. Difference spectra are normalized to a concentration of 10.6 μM enzyme. The additional negative displacement seen with ethyl- and butyl-SCoA at 300 nm reflects end absorbance from the high concentration of unbound ligand. The inset shows a difference spectrum of the native enzyme (54 μM) minus the same concentration of enzyme in the presence of 245 μM CoASH.

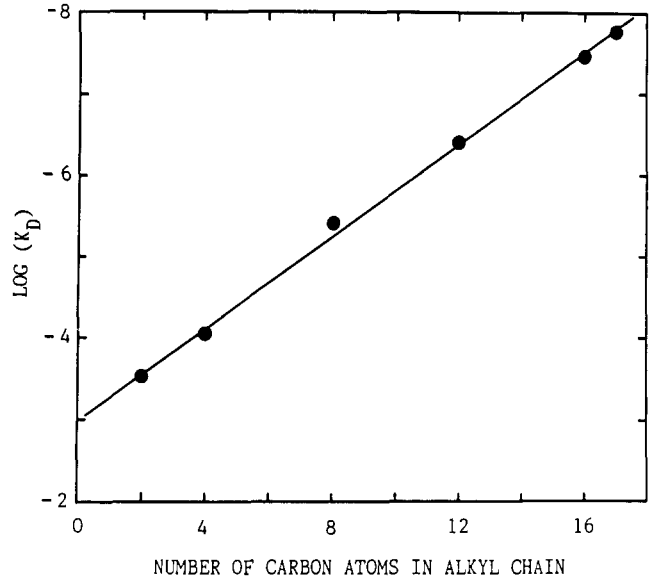


FIGURE 2: Logarithm of the dissociation constants for the binding of a series of alkyl-SCoA derivatives to the medium-chain acyl-CoA dehydrogenase as a function of chain length. Titrations were performed with ethyl- to hexadecyl-SCoA with concentrations of enzyme adjusted to allow adequate estimations of the K_d (see Materials and Methods). The value for heptadecyl-SCoA is taken from Thorpe et al. (1982). The K_d for octyl-SCoA in this system (4 μM) compares favorably with the K_i of 6.5 μM reported by Frerman et al. (1980b).

from these titrations vs. the chain length of the CoA analogue used. Binding becomes tighter by approximately 390 cal/mol for each methylene group added to the chain (see legend). Various experimental approaches indicate that a value of about 800 cal/mol is expected for the transfer of a $-CH_2-$ group in a linear hydrocarbon from water to a hydrocarbon solvent (Tanford, 1980). Thus, the hydrocarbon binding site in the dehydrogenase appears to be of moderate hydrophobicity. Further, the absence of either discontinuities or downward curvature in Figure 2 argues against a binding site that is tailored to fit saturated hydrocarbon chains of medium length optimally. Thus, the chain length discrimination shown by the medium-chain acyl-CoA dehydrogenase is probably not expressed upon the initial encounter between acyl-CoA substrate and oxidized enzyme, i.e., at the level of K_i above (see later).

The above conclusions rest upon the use of nonoxidizable thioether derivatives, and it is clearly important to estimate the contribution of the thio ester carbonyl group to substrate binding. We therefore chose the thio ester acetyl-CoA, since it lacks a β-carbon and is therefore not a substrate of the dehydrogenase. The spectral changes observed with this thio ester were very similar to those seen with the thioethers (Figure 1) but with an extrapolated maximum absorbance change of 0.01 at 492 nm. This value is slightly greater than that seen with ethyl-SCoA but smaller than that produced by butyl-SCoA (data not shown). Acetyl-SCoA binds with a K_d of 450 μM as compared to 300 μM for ethyl-SCoA. Thus, the carbonyl group does not appear to contribute significantly to the binding energies in these two examples, nor does it exert any particular spectral effects. These data suggest that thioethers represent reasonable models for substrate binding in the medium-chain acyl-CoA dehydrogenases.

pH, Buffer, and Temperature Effects. Titrations performed with octyl-SCoA in 50 mM potassium phosphate buffer at pH 6, 7.6, and 8 gave very similar spectral changes without large changes in K_d (12, 4, and 9 μM, respectively). Replacing phosphate buffer by 50 mM morpholinopropanesulfonate at pH 7.6 gave a K_d of 2.5 μM. For consistency, the bulk of the titrations described here have been conducted at pH 7.6 in 50 mM phosphate.

It has been suggested that increasing temperature weakens the interaction between the bound hydrocarbon chain of acyl-CoA substrates and the pig liver medium-chain acyl-CoA dehydrogenase (Hall et al., 1979). This is not the expected behavior for hydrophobic interactions, and thus the binding of dodecyl-SCoA was measured at 25 and 40 °C. In this analogue, the binding energy is about equally divided between alkyl chain and CoA moiety (see later). The K_d at the higher temperature was not significantly different from that at 25 °C. It should be noted that the initial suggestion was made to explain the temperature dependence of the spectrum of octanoyl-CoA-reduced enzyme (Hall et al., 1979). As noted earlier, the extent of reduction may be influenced by several equilibria, and unless their separate temperature dependencies are known, meaningful conclusions cannot be drawn from overall temperature effects.

Binding of CoASH and Its Derivatives. Extrapolation of the data in Figure 2 to the vertical axis suggest that the CoA moiety per se confers approximately 4.1 kcal of binding energy (corresponding to a K_d of about 1 mM). A titration of the pig kidney dehydrogenase with CoASH gave small spectral changes (see inset to Figure 1), allowing a K_d of 206 μM to be calculated. Note there is a very slight long-wavelength tail extending into the flavin envelope: this feature does not represent turbidity and is largely abolished when the pH of the solution is adjusted to 6.0 by the addition of 250 mM potassium phosphate, pH 5.3 (data not shown). In view of the tight binding of $CoASS^-$ (Williamson et al., 1982) and the prominent charge-transfer band observed with this persulfide, it seemed plausible that the results shown in the inset to Figure 1 represent a contribution of the thiolate of CoA to the observed binding. To test this hypothesis, the binding of $CoASO_3^-$ (Nishimura et al., 1982) was examined, since the sulfonate moiety would be expected to be completely ionized at physiological pH values. A spectrophotometric titration showed a K_d of 22 μM for the sulfonate (data not shown)—

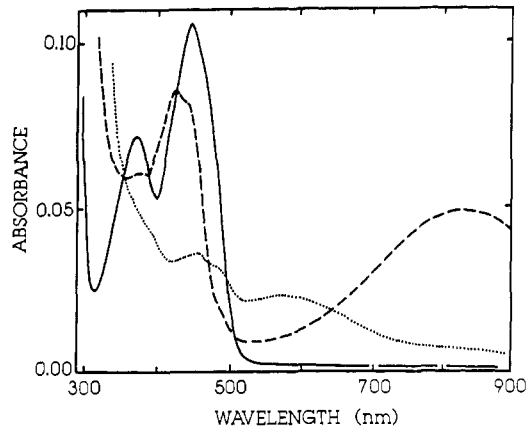


FIGURE 3: Effect of *trans*-3-octenoyl-CoA on pig kidney medium-chain acyl-CoA dehydrogenase. The spectrum of 6.5 μM enzyme in 50 mM phosphate, pH 7.6, at 25 °C was recorded before (—), immediately after (– –), and 3 h after (···) the addition of 1.3 equiv of *trans*-3-octenoyl-CoA.

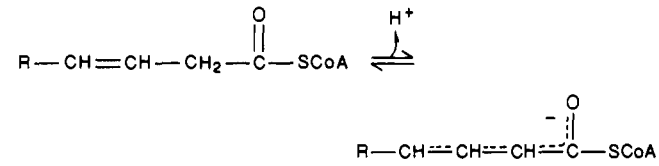
some 50-fold tighter than the value predicted for CoA alone. Binding of the sulfonate was accompanied by an approximately 5-nm blue shift of the flavin absorbance bands without any increased resolution of the spectrum (data not shown). As expected, no long-wavelength band was observed because of the unfavorable ionization potential for sulfonate compared to thiolate. These data provide another example of the preferential binding of anionic species within the active center of the dehydrogenase (see later).

Binding of trans-2-Octenoyl-CoA. *trans*-2-Octenoyl-CoA binds tightly to pig kidney medium-chain acyl-CoA dehydrogenase with sizable red shifts of the major absorbance peaks (446 to 455 nm and 373 to 388 nm) and the appearance of a prominent shoulder at 480 nm. These spectral changes yield a K_d of 90 nM with a stoichiometry of 1.0 mol of octenoyl-CoA bound per FAD (in 50 mM phosphate buffer, pH 7.6, at 25 °C; results not shown). This K_d should be compared to K_i values for the pig liver enzyme of about 10^{-7} M (Beinert, 1963) and 3.4 μM (Frerman et al., 1980b).

Interaction of trans-3-Octenoyl-CoA with Medium-Chain Acyl-CoA Dehydrogenase. Figure 3 shows the striking spectral changes observed upon the addition of 1.3 equiv of *trans*-3-octenoyl-CoA (see Materials and Methods) to the pig kidney enzyme at pH 7.6. The immediate formation of an intense long-wavelength absorbance (λ_{max} 820 nm) is followed by a slow decline ($t_{1/2}$ = 23 min) with apparent reduction of the bound flavin (Figure 3). At pH 6.0, the decline is more rapid ($t_{1/2}$ about 10 min) whereas at pH 8.0 it is slightly slower than that observed at pH 7.6 (data not shown). Spectrophotometric titration at 4 °C with 1.6 μM enzyme (see Materials and Methods) suggests a K_d of approximately 70 nM. This value is subject to some uncertainty owing to the time dependence of the changes, but it is clear that the *trans*-3-octenoyl analogue binds tightly and can serve as an effective inhibitor of the enzyme (see later).

The spectrum of the 820-nm species resembles a charge-transfer complex between a donor ligand and the oxidized flavin chromophore (Massey & Ghisla, 1974). Evidence supporting this assignment comes from repeating the experiment shown in Figure 3 with enzyme substituted with the more oxidizing analogue 8-Cl-FAD [E_0' = −152 mV (Moore et al., 1978) vs. −208 mV for FAD (Draper & Ingraham, 1968)]. As would be expected if the oxidized flavin serves as a charge-transfer acceptor (Abramovitz & Massey, 1976; Massey & Ghisla, 1974), this substitution results in a red shift in the charge-transfer band to about 850 nm (data not shown).

A logical candidate for the charge-transfer donor in these complexes is the delocalized carbanion:



Accordingly, the exchange of the α-protons was examined by NMR (Ghisla et al., 1984). Studies with normal substrates have shown that the mammalian dehydrogenase catalyze the exchange of the *pro-R* α-proton (Biellman & Hirth, 1970a,b; Murfin, 1974; Ghisla et al., 1984; Ikeda et al., 1985c). In the absence of enzyme, the incorporation of deuterium into *trans*-3-octenoyl-CoA was insignificant over 26 h. Upon the addition of enzyme, a rapid exchange of one α-proton was observed that was complete in less than 10 min (using 10 μM enzyme and 4 mM *trans*-3-octenoyl-CoA at 20 °C in 50 mM phosphate buffer in D_2O with an apparent pH of 7.6). No significant isomerization of *trans*-3- to *trans*-2-octenoyl was observed by NMR. In contrast, Engel and Massey (1971) and Fendrich and Abeles (1982) have reported the isomerization of vinylacetyl-CoA or pantetheine to their corresponding trans-2 thio esters by butyryl-CoA dehydrogenase from *Megasphaera elsdenii*. Possibly the medium-chain dehydrogenase fails to catalyze isomerization of the trans-3 to the trans-2 analogue because reprotonation of the delocalized carbanion at the γ-position (see above) is considerably slower than the dissociation of the trans-3 thio ester. Thus, the anion decays within the active center by reducing the bound flavin (Figure 3), not by isomerization. This reaction will be addressed in a separate paper.

The effects of *trans*-3-octenoyl-CoA were discovered when *trans*-2-octenoyl-CoA titrations were attempted with medium-chain dehydrogenase preparations later found to contain traces of enoyl-CoA hydratase activity (Lau et al., 1986). The short-chain hydratase has a secondary trans-2/trans-3-isomerase activity (Stern, 1961), leading to a slow appearance of long-wavelength absorbance as the trans-3 isomer binds to the dehydrogenase. Removal of this contaminating enoyl-CoA hydratase (see Materials and Methods) yields dehydrogenase that shows essentially no long-wavelength band on the addition of pure *trans*-2-octenoyl-CoA (data not shown).

Since preparations of butyryl-CoA dehydrogenase from *M. elsdenii* are also prone to crotonase contamination (Engel & Massey, 1971; Ellison & Engel, 1986; P.A. Ellison and P. C. Engel, unpublished data), the long-wavelength bands previously ascribed to the interaction of straight-chain and alicyclic *trans*-2-enoyl-CoA species with the oxidized dehydrogenase (Engel & Massey, 1971; Engel, 1972; Williamson & Engel, 1984) may actually represent complexes with the corresponding trans-3 species.

The tight binding of *trans*-3-octenoyl-CoA may have physiological significance in that *trans*-3-enoyl-CoA thio esters are intermediate in the metabolism of unsaturated fatty acids with cis double bonds at even-numbered carbon atoms (Dommes & Kunau, 1984a; Cuebas & Schulz, 1982; Mizugaki et al., 1984). Furthermore, the hypoglycemic agent 4-pentenoic acid (Billington et al., 1978) is metabolized to the corresponding 2,4-dienoyl-CoA species (Holland et al., 1973) and then largely reduced to *trans*-3-pentenoyl-CoA (Schulz, 1983) by 2,4-dienoyl-CoA reductase (Dommes & Kunau, 1984a). The accumulation of this metabolite within the mitochondrial matrix might reversibly inhibit the acyl-CoA dehydrogenase (particularly the short-chain enzyme) in ad-

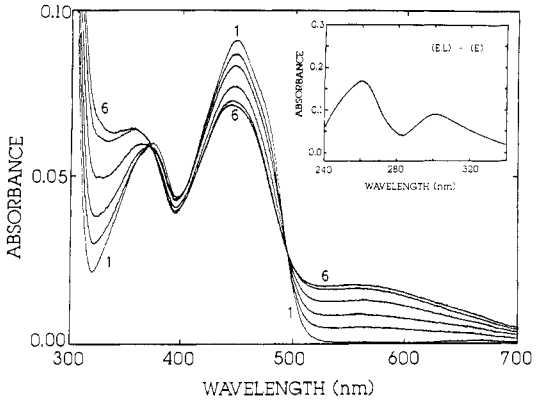


FIGURE 4: Spectral changes on the addition of 3-ketooctanoyl-CoA to the medium-chain acyl-CoA dehydrogenase. The dehydrogenase (curve 1; 1.2 μM in 13.8 mL of 50 mM phosphate, pH 7.6, 25 °C, in a 5-cm path-length cell) was titrated with 3-ketooctanoyl-CoA (curves 2–6; 0.3, 0.6, 1.05, 1.8, and 3.6 μM ligand, respectively). Absorbance changes at 552 nm best fit a K_d of 55 nM and a stoichiometry of 1.05 per FAD. The inset represents subtraction of the spectrum of 10.4 μM enzyme in 50 mM phosphate buffer, pH 7.6, 25 °C, containing 10 μM 3-ketooctanoyl-CoA from that of the enzyme alone. Under these conditions about 95% of the ligand is enzyme bound.

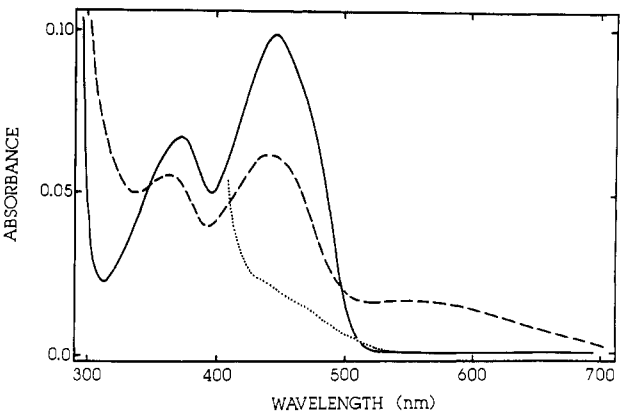


FIGURE 5: Reduction of pig kidney medium-chain acyl-CoA dehydrogenase by L-3-hydroxyoctanoyl-CoA. The native enzyme was made anaerobic (6.4 μM FAD in 50 mM phosphate, pH 7.6, at 25 °C and the initial spectrum recorded (—). L-3-Hydroxyoctanoyl-CoA (1.3 equiv) was then introduced from a side arm and the spectrum recorded after 15 min (– –). No further reduction occurred. The dotted curve is the spectrum at 8 h of an analogous incubation mixture supplemented with 6.5 μg of 3-OH-acyl-CoA dehydrogenase and 100 μM NADH in the main space of the cuvette.

dition to the other suggested modes of action of this acid (Billington et al., 1978, Schulz, 1983). This hypothesis is being tested further.

3-Ketooctanoyl-CoA Binding. Figure 4 shows a titration of the pig kidney medium-chain dehydrogenase with 3-ketooctanoyl-CoA. Binding is very tight (approximately 55 nM, necessitating the use of low enzyme concentrations) and shows a stoichiometry of 4.2 molecules per tetramer (data not shown). This tight binding enables an estimate to be made of the spectrum of the bound ligand (Figure 4: inset and legend). Up to 1 equiv of 3-ketooctanoyl-CoA added per flavin, the apparent spectrum of the bound ligand shows a constant ratio of 300/260-nm absorbance (0.54). Above 1 equiv, the absorbance ratio falls as free 3-keto analogue (300/260-nm ratio of 0.05 at pH 7.6; Thorpe, 1986) accumulates in solution (data not shown). The inset approximates the spectrum of the bound ligand provided that there are not appreciable changes in the spectrum of the enzyme itself upon binding. Certainly no appreciable shoulders develop in the 280-nm region of the spectrum, which might be indicative of significant perturbation of tryptophan residues. The absorbance maximum at 300 nm is characteristic of the enolate of 3-ketooctanoyl-CoA (extinction coefficient 21 mM^{-1} cm^{-1}; Thorpe, 1986). This enolate is believed to be the charge-transfer donor to oxidized flavin yielding the long-wavelength band at 560 nm (Engel & Massey, 1971; Massey & Ghisla, 1974; McKean et al., 1979; Benecky et al., 1979; Auer & Frerman, 1980; Williamson et al., 1982b; Thorpe & Massey, 1983).

The binding of acetoacetyl-CoA to the pig liver medium-chain dehydrogenase has been reported to exhibit half-site reactivity with stoichiometries of 1.8 per tetramer (McKean et al., 1979). In contrast, a value of 4 per tetramer has been reported for both the ox liver (Shaw & Engel, 1984) and the rat liver (Ikeda et al., 1985b) short-chain dehydrogenase. Accordingly, we have examined the behavior of the kidney medium-chain enzyme toward this ligand. Spectrophotometric experiments were conducted at 25 °C with either 10 or 41 μM enzyme and gave spectra very similar to those shown in Figure 4. Data were analyzed at both 446 and 560 nm by a variety of methods, all of which gave a stoichiometry of close to 4 per tetramer and no convincing evidence for two classes of binding site. Thus, an excellent fit was obtained for $n = 0.96$ per flavin, with a K_d of 12 μM and a maximal extinction coefficient change of 3.3 mM^{-1} cm^{-1} (using 41 μM enzyme; data not shown). The reason for the difference in behavior of the liver and kidney enzyme is not yet apparent. It should be noted, however, that the partial bleaching of the pig liver medium chain length dehydrogenase upon the addition of 1 equiv of butyryl-CoA cannot be cited as evidence for half-site reactivity (McKean et al., 1979). This is because the extent of reduction largely reflects an internal equilibrium between oxidized and reduced flavin forms (see earlier). This equilibrium is dependent both on the chain length of the substrate used and on the redox potential of the flavin prosthetic group (Beinert & Page, 1957; Thorpe et al., 1979; Thorpe & Massey, 1983).

Reduction of the Enzyme by L-3-Hydroxyoctanoyl-CoA. The addition of 1.3 equiv of L-3-hydroxyoctanoyl-CoA (See Materials and Methods) to the oxidized medium-chain acyl-CoA dehydrogenase leads to partial reduction of the enzyme with concomitant rise in absorbance at 570 nm ($t_{1/2}$ = 45 s; Figure 5). This incomplete reduction is surprising, since the L-3-OH analogue would be expected to be a thermodynamically stronger reductant than octanoyl-CoA, a favored substrate of the dehydrogenase. The experiments described below show that this partial reduction reflects formation of the corresponding 3-keto analogue in reaction sequence 1, followed by sequestration of the remaining free oxidized enzyme via equilibrium 2:

$$E\cdot FAD + 3\text{–}OH \rightleftharpoons E\cdot FAD\cdot 3\text{–}OH \rightleftharpoons E\cdot FAD_{2e}\cdot 3{=}O \rightleftharpoons E\cdot FAD_{2e} + 3{=}O \quad (1)$$

$$E\cdot FAD + 3{=}O \rightleftharpoons E\cdot FAD\cdot 3{=}O \quad (2)$$

When the experiment shown in Figure 5 is repeated in the presence of 1.3 equiv of 3-ketooctanoyl-CoA, essentially no reduction is observed on the addition of 3-hydroxyoctanoyl-CoA (data not shown). In contrast, the presence of NADH and catalytic levels of 3-OH-acyl-CoA dehydrogenase to reduce the 3-ketooctanoyl-CoA as it is being formed leads to complete reduction of the flavin (see Figure 5).

The reduction of the dehydrogenase by L-3-hydroxyoctanoyl-CoA is consistent with the known stereochemistry of the acyl-CoA dehydrogenase (see Scheme I). The direct transfer of the *pro-R* C-3 hydrogen to the 5-position of the flavin ring has been demonstrated with 5-deazaflavin-substituted acyl-CoA dehydrogenase (left panel, Scheme I; Ghisla

Scheme I

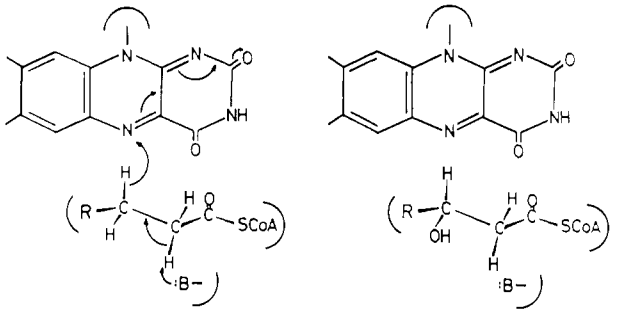


Table I: Effect of Various Thio Esters on the Activity of Medium-Chain Acyl-CoA Dehydrogenase from Pig Kidney

thio ester	K_d	% activity
none		100[a]
trans-2-octenoyl-CoA	90 nM	38[a]
trans-3-octenoyl-CoA	70 nM	33[a]
L-3-hydroxyoctanoyl-CoA	nd[c]	93[a]
3-ketooctanoyl-CoA	55 nM	24[b]
acetoacetyl-CoA	12 μM	99[b]

[a] Activities were determined in the phenazine methosulfate/2,6-dichlorophenolindophenol assay system with 30 μM thio ester in addition to 30 μM octanoyl-CoA (Thorpe, 1981). In this system the background rates with the 3-keto derivatives were unacceptably high. [b] Activities determined as above but replacing phenazine methosulfate by electron-transferring flavoprotein (Gorelick et al., 1982). [c] Not determined.

et al., 1984). The equivalent hydrogen is available for abstraction in the L-3-hydroxy thio ester (right panel, Scheme I). As would be expected, D-3-hydroxyoctanoyl-CoA is not a reductant for the medium-chain enzyme under the conditions of Figure 5 (data not shown, see Materials and Methods). Whether reduction of the flavin proceeds via abstraction of the *pro-R* α-proton, as in normal catalysis, followed by tautomerization of the enol to the 3-keto derivative or is initiated by removal of the hydroxyl proton must await further investigation. This issue does not appear to have been resolved with the NAD^+-linked L-3-OH-acyl-CoA dehydrogenase. Finally, it should be noted that butyryl-CoA dehydrogenase from *M. elsdenii* has been reported to show a very small dehydrogenase activity toward 3-hydroxybutyryl-CoA (Engel & Massey, 1971).

General Conclusions. Combining the results from the binding of thioether analogues of increasing chain lengths with those using various eight-carbon acyl-CoA thio esters suggests that all of the 28 intermediates in the catabolism of palmitoyl-CoA will bind to the medium chain length dehydrogenase from pig kidney. This enzyme is also apparently involved in the metabolism of unsaturated fatty acids (Dommes & Kunau, 1984b), and this will greatly increase the number of potential ligands for the enzyme in vivo. Some of the thio esters investigated here bind sufficiently tightly (with K_d values in the 10–100 nM range) to warrant consideration of the metabolic consequences should they accumulate in vivo. While it is not the object of this work to investigate the kinetic behavior of these potential inhibitors of the dehydrogenase, Table I summarizes the effects of a number of the thio esters studied. For comparison, potential inhibitors are present at equimolar levels with octanoyl-CoA, a preferred substrate of the medium-chain acyl-CoA dehydrogenase. *trans*-2-Enoyl, *trans*-3-enoyl, and 3-ketooctanoyl-CoA are very effective inhibitors of the dehydrogenase, whereas L-3-hydroxyoctanoyl- and acetoacetyl-CoA are not. These results are qualitatively similar to those of Davidson and Schulz (1982), who found that 3-ketodecanoyl-CoA was a potent inhibitor of partially purified bovine medium-chain acyl-CoA dehydrogenase. *trans*-2-Decanoyl-CoA was somewhat less effective, whereas acetoacetyl- and DL-3-hydroxydecanoyl-CoA were ineffective (Davidson & Schulz, 1982).

These studies add support to the concept that 3-keto derivatives of suitable chain length may function as feed-back inhibitors of the acyl-CoA dehydrogenases (Engel & Massey, 1971; McKean et al., 1979; Davidson & Schulz, 1982; Shaw & Engel, 1984). They also identify *trans*-3-octenoyl-CoA as another potential inhibitor of the medium-chain acyl-CoA dehydrogenase in addition to the corresponding trans-2 isomer. Thus a number of medium-chain length intermediates of the β-oxidation of saturated and unsaturated fatty acids are plausible inhibitors of the medium-chain acyl-CoA dehydrogenase. Such species might attain inhibitory concentrations as a consequence of impairment in one of the steps toward the center of the β-oxidation spiral. Inhibition might be propagated to earlier turns of the pathway as thio esters of longer chain length begin to accumulate. β-Oxidation might therefore be regulated by product accumulation at multiple sites, including the short, medium, and long-chain acyl-CoA dehydrogenases.

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Registry No. *trans*-2-Octenoyl-CoA, 10018-94-7; *trans*-3-octenoyl-CoA, 6375-77-5; L-3-hydroxyoctanoyl-CoA, 79171-48-5; 3-ketooctanoyl-CoA, 54684-64-9; acetoacetyl-CoA, 1420-36-6; ethyl-CoA, 70019-68-0; butyl-CoA, 70019-70-4; octyl-CoA, 75501-78-9; dodecyl-CoA, 107913-46-2; hexadecyl-CoA, 71425-89-3; acyl-CoA dehydrogenase, 9027-65-0.

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