# Spectroscopic Analysis of the Interaction of Rat Liver Short-Chain, Medium-Chain, and Long-Chain Acyl Coenzyme A Dehydrogenases with Acyl Coenzyme A Substrates†

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ABSTRACT: We systematically studied the visual spectral changes of short-chain, medium-chain, and long-chain acyl coenzyme A (acyl-CoA) dehydrogenases, purified from rat liver mitochondria, that occur upon reaction with acyl-CoA in the absence of an electron acceptor (half-reaction). Acyl-CoA esters having various chain lengths were tested, and changes in the steady-state spectral parameters were correlated with the turnover number in the complete reaction, which represented the ability of an enzyme/substrate combination to produce an enoyl-CoA. The long-wavelength absorbance, centered around 580 nm, was observed only in the enzyme/substrate combinations in which enoyl-CoA product was produced at a significant rate in the complete reaction. There was a good correlation between the magnitudes of the long-wavelength absorbance and the turnover numbers. In contrast, the bleaching of the flavin chromophore at 450 nm was observed not only in the titration with preferred substrates but also in that with unfavorable substrates, which were shorter than favorable substrates. In the interaction with the shorter than favorable substrates, however, enoyl-CoA was not produced, nor did long-wavelength absorbance occur. When short-chain and medium-chain acyl-CoA dehydrogenases were reacted with longer than favorable substrate from which no enoyl-CoA was produced, neither the appearance of the long-wavelength absorbance nor bleaching of flavin chromophore was observed. These data suggest that the catalytic base, which abstracts $\alpha$-proton, and flavin adenine dinucleotide are internally located, and the region containing these two sites may physically be in the form of crevice or pocket.

Mammalian fatty acyl coenzyme A (acyl-CoA) dehydrogenases are mitochondrial flavoproteins, originally isolated from beef and pig livers. They are butyryl-CoA (short-chain acyl-CoA) (Green et al., 1954), general acyl-CoA (medium-chain acyl-CoA) (Crane et al., 1956), and palmitoyl-CoA (long-chain acyl-CoA) dehydrogenases (Hauge et al., 1956). The complete reaction of these acyl-CoA dehydrogenases requires a ternary system that consists of the enzyme, substrate, and ETF[1] (Crane & Beinert, 1956).

The spectral changes of the enzyme-bound flavin, which occur upon interaction of acyl-CoA dehydrogenases with acyl-CoA substrates in the absence of electron acceptor (half-reaction), were investigated mainly by using pig general acyl-CoA dehydrogenase (Crane et al., 1956; Beinert, 1957; Hall et al., 1979; Thorpe et al., 1979; Auer & Frerman, 1980). In general, acyl-CoA dehydrogenase and a suitable substrate were considered to form a tightly bound, stable intermediate complex, which was spectrally characterized by the decrease of FAD absorbance at 450 nm and the appearance of long-wavelength absorbance centered at around 580 nm. On the basis of these spectral characteristics, this enzyme/substrate complex has been suggested to be a charge-transfer complex formed between the reduced enzyme and enoyl-CoA (Massey & Ghisla, 1974). Several groups of workers observed that pig general acyl-CoA dehydrogenase formed this intermediate complex with a wide spectrum of substrates having $C_4$–$C_{16}$ chain lengths (Beinert, 1957; Hall et al., 1975; Thorpe et al., 1979).

Little information is available, however, in regard to the spectral properties of short-chain and long-chain acyl-CoA dehydrogenases. Also, due to the uncertain purity of the enzyme preparations used in the previous studies, the accuracy of the kinetic parameters and spectral properties of acyl-CoA dehydrogenases needs to be reexamined with preparations of rigorously ascertained purity. For instance, the substrate specificities of previous general and long-chain acyl-CoA dehydrogenases largely overlapped each other, each dehydrogenating a broad spectrum of substrates ranging from $C_4$ to $C_{16}$. This large overlap of the substrate specificities of these two enzymes caused a suspicion that the previous preparations of general and long-chain acyl-CoA dehydrogenases may have been cross-contaminated. Recent studies on the steady-state kinetics of general acyl-CoA dehydrogenase from rat and pig livers (Furuta et al., 1981; Davidson & Schulz, 1982; Dommes & Kunau, 1984; Hall et al., 1979) also showed turnover numbers for a broad range of substrates although their substrate specificities were narrower than those previously reported by Hauge (1956).

We have recently purified to homogeneity from rat liver mitochondria and physically and immunologically characterized five distinct acyl-CoA dehydrogenases (Ikeda et al., 1983) including short-chain, medium-chain, and long-chain acyl-CoA (Ikeda et al., 1985a), isovaleryl-CoA (Ikeda & Tanaka, 1983a), and 2-methyl-branched-chain acyl-CoA dehydrogenases (Ikeda & Tanaka, 1983b). The purities and the

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[1] Abbreviations: DCIP, 2,6-dichloroindophenol; EDTA, ethylenediaminetetraacetic acid; ETF, electron-transfer flavoprotein; FA-CoA, [3-(2-furyl)acryloyl]-CoA; FAD, flavin adenine dinucleotide; FP-CoA, [3-(2-furyl)propionyl]-CoA; GC, gas chromatography; GC/MS/COM, gas chromatograph/mass spectrometer/computer; PMS, phenazine methosulfate; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

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absence of cross-contamination of our purified enzymes were ascertained by several lines of evidence. These include the absence of cross-contamination ascertained by monitoring activities of the five acyl-CoA dehydrogenases at each fractionation step, the homogeneity on SDS–PAGE, and the immunological monospecificity of each final preparation.

In this study, we systematically investigated the spectral changes of enzyme-bound FAD in the pure short-chain, medium-chain, and long-chain acyl-CoA dehydrogenases in the half-reaction using a wide spectrum of substrates having varying chain length for each of the three acyl-CoA dehydrogenases.

## Experimental Procedures

*Materials and Syntheses.* Free coenzyme A lithium salt and all acyl-CoA esters were purchased from P-L Biochemicals (Milwaukee, WI) unless otherwise mentioned. The octanoyl-CoA (lot no. 015846) obtained from this source contained 15% palmitoyl-CoA, as analyzed by GC/MS as its hydrolysis product. For this reason, we synthesized octanoyl-CoA from pure octanoic acid in our laboratory by the mixed anhydride synthesis (Wieland & Rueff, 1953). *trans*-2-Octenoic and *trans*-2-hexadecenoic acids were obtained from K and K Rare Chemicals (Cleveland, OH). Their coenzyme A thio esters were synthesized by the same method in our laboratory. These synthetic acyl-CoA's were purified by paper chromatography using ethanol/0.1 M potassium acetate (1:1), pH 4.5, as a developing solvent to remove unreacted coenzyme A and other reagents. The purities of all of the synthesized acyl-CoA's were confirmed to be at least 95% pure by GC/MS analysis of the acyl moiety as a methyl ester. Two packings for gas chromatographic column, 10% SP-1200/1% $H_3PO_4$ on 80/100 Chromosorb WAW and 10% OV-17 on 80/90 Chromosorb WAW, were procured from Supelco (Bellefonte, PA).

*Preparation of Acyl-CoA Dehydrogenases and ETF.* Short-chain, medium-chain, and long-chain acyl-CoA dehydrogenases were purified to homogeneity from rat liver mitochondria as previously described (Ikeda et al., 1985a). Short-chain acyl-CoA dehydrogenase was obtained only in the yellow form. The final preparations of the three enzymes were free of crotonase. In order to compute the amount of the enzyme-bound FAD, the extinction coefficient of FAD (11.3 $mM^{-1}$ $cm^{-1}$) at 450 nm was used.

*Assay of Acyl-CoA Dehydrogenases.* Assays for the various acyl-CoA dehydrogenase activities were performed spectrophotometrically with PMS and DCIP as intermediate and terminal electron acceptors, respectively, according to the method described previously (Ikeda et al., 1983). A unit (U) of enzyme activity was expressed as micromoles of DCIP reduced per minute. Apparent $V_{max}$ values in the steady-state kinetic studies were expressed as turnover number ($S^{-1}$).

*Identification and Quantitation of Reaction Products Using GC and GC/MS.* The reaction mixture contained 100 mM potassium phosphate buffer (pH 8.0), 3 mM PMS, 0.1 mM FAD, 0.3 mM substrate, and an appropriate enzyme. No DCIP was added unless otherwise mentioned. Total volume was 0.5 mL. The mixture was incubated at 37 °C for the period indicated. At the end of the reaction, 0.05 mL of 3.5 M KOH was added, and the mixture was further incubated at 37 °C for 30 min in order to hydrolyze the acyl-CoA. In the case of the short-chain acyl-CoA dehydrogenase reaction, the hydrolyzed sample was acidified with 0.1 mL of 5 M $H_3PO_4$, steam-distilled in a small Markham still, and analyzed, without derivatization, by GC according to the method previously described (Ikeda et al., 1983). A glass gas chromatographic column (2 mm × 1.8 m) packed with SP-1200 was used. The temperature of the column oven was 110 °C, and nitrogen gas was used as a carrier gas. In the case of medium-chain and long-chain acyl-CoA dehydrogenase reactions, the hydrolyzed sample was first extracted, methylated with gaseous diazomethane, and analyzed by GC according to the method previously described (Ikeda et al., 1985a). One microliter of the sample was injected into a glass gas chromatographic column (2 mm × 1.8 m) packed with 10% OV-17. The temperature of the column was 120 °C for octanoate/octenoate and 220 °C for palmitate/hexadecenoate. A Hewlett-Packard 5840A gas chromatograph equipped with dual flame ionization detectors and a 18850A terminal/data system was utilized for analysis.

*Mass Spectrometric Analysis of Reaction Products.* For mass spectral identification, the methylated sample was analyzed with the electron impact ionization mode by using a Finnigan 4510 automated gas chromatograph/mass spectrometer/computer system (GC/MS/COM). The ionizing voltage was 70 eV, and a 3% OV-1 column was the inlet gas chromatographic column. The oven temperature was programmed from 40 °C at a rate of 6 °C/min.

*Titration of Acyl-CoA Dehydrogenases with Various Substrates.* Titrations were performed anaerobically at 25 °C by adding increasing amounts of acyl-CoA to 0.5 mL of acyl-CoA dehydrogenase solution in 10 mM potassium phosphate (pH 8.0)/10% glycerol/0.1 mM EDTA buffer in a cuvette. Each UV/visible spectrum was recorded by using a Beckman DU-7 spectrophotometer with microprocessor control, immediately after the addition of substrate. Each scan was completed within 40 s after the substrate addition. The entire experiment on an enzyme/substrate combination was completed within 15 min. Under these conditions, all absorbances were basically stable, but some changes were observed along the time course. For instance, the long-wavelength band, produced by the addition of a stoichiometric amount of octanoyl-CoA to medium-chain acyl-CoA dehydrogenase, decreased 10% after 30 min. The long-wavelength band, produced by the addition of a 10 times molar excess of butyryl-CoA to the same enzyme, decreased 17% after 15 min and 33% after 30 min. These decreases are not nearly as large as those reported by McFarland et al. (1982).

## Results

*Spectral Analysis of the Interaction of Acyl-CoA Dehydrogenases with Acyl Coenzyme A Esters Having Various Chain Lengths in the Half-Reaction.* Anaerobic titrations of short-chain, medium-chain, and long-chain acyl-CoA dehydrogenases with eight even-numbered acyl-CoA substrates having $C_4$–$C_{18}$ carbon chains were studied. Spectral changes of these three acyl-CoA dehydrogenases due to butyryl-CoA, octanoyl-CoA, and palmitoyl-CoA are shown in Figures 1–3, as typical representatives for those due to favorable or unfavorable substrates. The addition of butyryl-CoA to the short-chain acyl-CoA dehydrogenase preparation caused a decrease in absorbance at 450 nm and the appearance of a broad, long-wavelength band centered at 572 nm with absorbance extending to 700 nm (Figure 1A). The isosbestic points at 343 and 497 nm were retained throughout the titration. Extrapolation of the linear portion of the titration curve for the decrease in 450-nm absorbance intersects the extension of the plateau line at approximately 1 mol of butyryl-CoA/mol of enzyme-bound FAD (see inset in Figure 1A). In contrast, that of the long-wavelength-band increase intersects at approximately 0.7 mol of butyryl-CoA/mol of enzyme-bound FAD. Upon titration with octanoyl-CoA, the flavin chromophore was bleached to small degrees with the

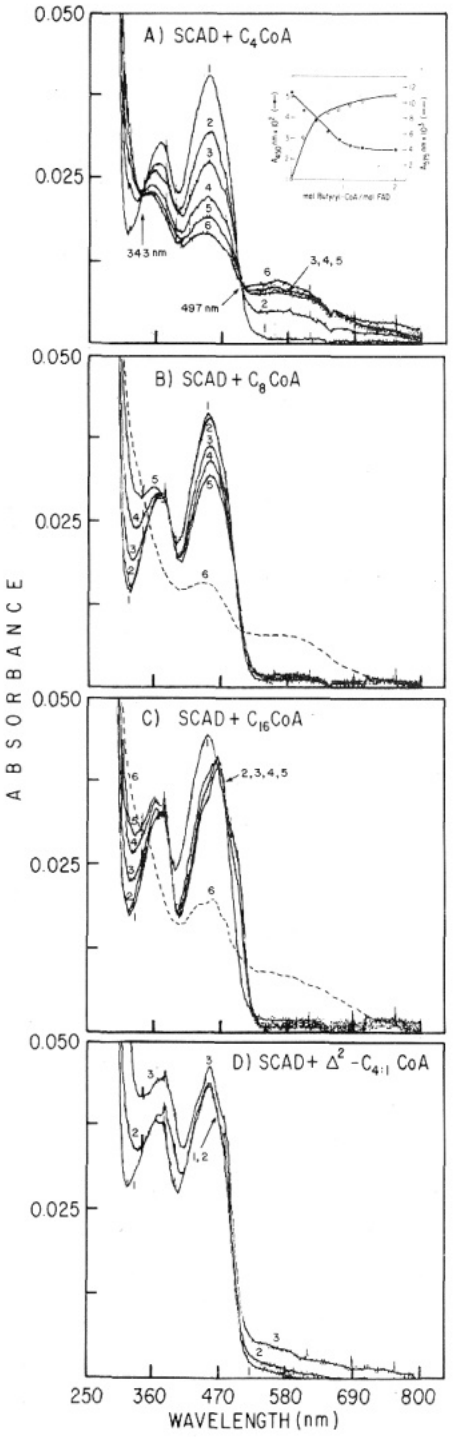



FIGURE 1: Spectrophotometric titration of short-chain acyl-CoA dehydrogenase with butyryl-CoA (A), octanoyl-CoA (B), palmitoyl-CoA (C), and crotonyl-CoA (D). The enzyme (3.7 $\mu$M bound FAD, $A_{450}/A_{271}$ = 6.3) in 0.5 mL of 10 mM potassium phosphate buffer, pH 7.8, was titrated anaerobically at 25 °C with the increments of the acyl-CoA's indicated. (A) 0 (curve 1), 0.23 (curve 2), 0.46 (curve 3), 0.69 (curve 4), 0.91 (curve 5), and 1.14 (curve 6) mol of butyryl-CoA/mol of enzyme-bound FAD. Inset: The intensities of the 450-nm (●) and 575-nm bands (○) at varying butyryl-CoA concentrations are plotted. (B) 0 (curve 1), 1.0 (curve 2), 4.57 (curve 3), 9.2 (curve 4), and 13.7 (curve 5) mol of octanoyl-CoA/mol of FAD. Curve 6 (dashed line) shows the spectrum of the enzyme with 13.7 equiv of octanoyl-CoA (curve 5) upon further addition of 1.0 mol of butyryl-CoA/mol of FAD. (C) 0 (curve 1), 1.0 (curve 2), 4.4 (curve 3), 8.7 (curve 4), and 13.1 (curve 5) mol of palmitoyl-CoA/mol of FAD. Curve 6 (dashed line) shows the spectral change of the enzyme with 13.1 equiv of palmitoyl-CoA (curve 5) upon further addition of 2.3 mol of butyryl-CoA/mol of FAD. (D) 0 (curve 1), 1.0 (curve 2), and 5.0 (curve 3) mol of crotonyl-CoA/mol of FAD.

addition of large excesses of octanoyl-CoA (Figure 1B). In the titration with palmitoyl-CoA, even the large molar excesses of this substrate bleached the flavin chromophore only slightly, and a 7–14-nm red shift of the 450-nm absorbance was observed (Figure 1C). No long-wavelength absorbance was observed at all in the titration with either octanoyl-CoA or palmitoyl-CoA. In both cases, when 2 times molar excesses of butyryl-CoA were further added, bleaching of the FAD chromophore and appearance of the long-wavelength band, both of the maximum degree, were observed [Figure 1B,C, dashed line (curve 6)]. The percent bleachings of the 450-nm absorbance caused by the addition of 1 equiv of butyryl-CoA, octanoyl-CoA, and palmitoyl-CoA per mole of enzyme-bound FAD were 60, 2, and 7, respectively. At a 10 times molar excess of these substrates, the percent bleachings were 63, 17, and 7.0, respectively.

When medium-chain acyl-CoA dehydrogenase was titrated with octanoyl-CoA, spectral changes similar to those observed in the titration of short-chain acyl-CoA dehydrogenase with butyryl-CoA, namely, bleaching of the 445-nm absorance and appearance of the 580-nm absorbance, were observed (Figure 2). The observed changes were similar to those reported by others (Beinert, 1957; Hall et al., 1979; Thorpe et al., 1979; Auer & Frerman, 1980), using pig general acyl-CoA dehydrogenase and octanoyl-CoA. Upon titration with equimolar butyryl-CoA, only a weak bleaching of the 445-nm absorbance was observed. However, the bleaching of the 445-nm band significantly intensified when molar excesses of butyryl-CoA were added. In contrast, the magnitude of the 580-nm absorbance was small, even when a 10 times molar excess of butyryl-CoA was added: it was only 20% of that caused by the same amount of octanoyl-CoA (Figure 2A,B). The addition of a 10 times molar excess of palmitoyl-CoA to medium-chain acyl-CoA dehydrogenase resulted only in slight bleaching of the 445-nm band, which was red-shifted by 7 nm (Figure 2C). No absorbance at 580 nm was observed. When a 7 times molar excess of octanoyl-CoA was further added, strong bleaching of the FAD chromophore and the long-wavelength band were observed [Figure 2C, dashed line (curve 7)]. The percent bleachings of the 445-nm absorbance caused by the addition of 1 mol of butyryl-CoA, octanoyl-CoA, and palmitoyl-CoA per mole of enzyme-bound FAD were 8, 63, and 7, respectively. The percent bleachings due to a 10 times molar excess of these substrates were 45, 77 and 12, respectively. These results of our medium-chain acyl-CoA dehydrogenase titration significantly differ from those on pig general acyl-CoA dehydrogenase previously observed by Thorpe et al. (1979). They observed considerable bleaching (20%) of the 446-nm absorbance and a weak long-wavelength band with the addition of equimolar palmitoyl-CoA, and the bleaching further intensified as molar excesses (40% at a 12.9 times molar excess) of palmitoyl-CoA were added. Also, they observed no 570-nm absorbance upon addition of a 10 times molar excess of butyryl-CoA.

Titration of long-chain acyl-CoA dehydrogenase with palmitoyl-CoA resulted in strong bleaching of the 443-nm absorbance accompanied by the appearance of a broad, long-wavelength absorbance centered around 560 nm (Figure 3C). The shape, position, and intensity of the long-wavelength band in this case were slightly different from those observed in the titration of short-chain acyl-CoA dehydrogenase with butyryl-CoA and that of medium-chain acyl-CoA dehydrogenase with octanoyl-CoA. The extrapolation of the linear portion of the curve for the decrease of the 443-nm absorbance intersects the extension of the ultimate plateau at

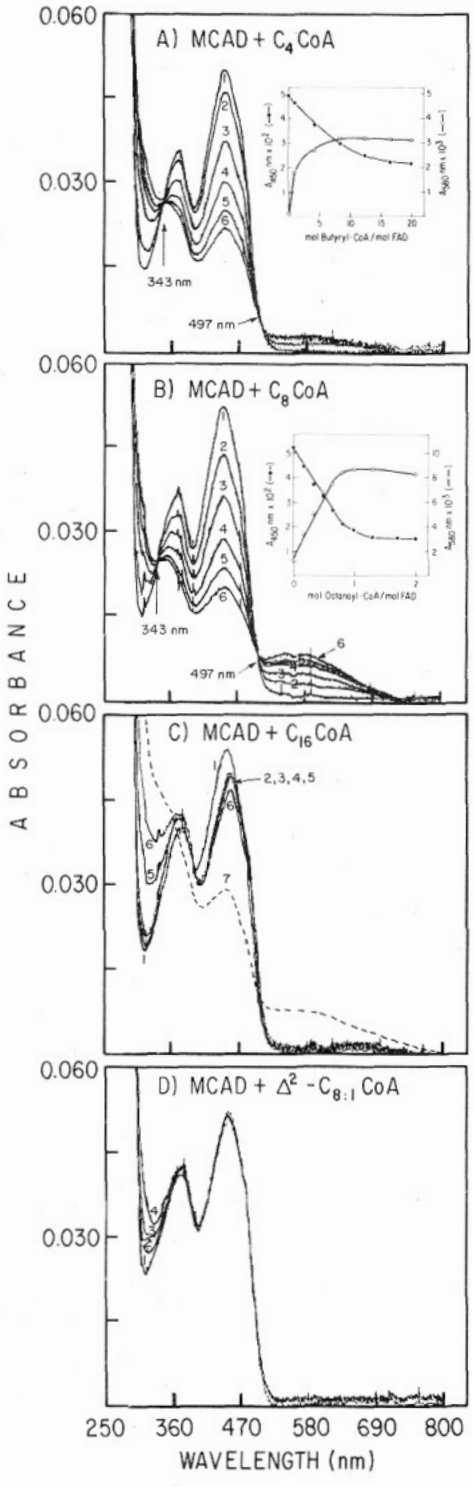



FIGURE 2: Spectrophotometric titration of medium-chain acyl-CoA dehydrogenase with butyryl-CoA (A), octanoyl-CoA (B), palmitoyl-CoA (C), and 2-octenoyl-CoA (D). The enzyme (4.6 $\mu$M bound FAD, $A_{450}/A_{271}$ = 5.96) was titrated anaerobically under the conditions described in Figure 1. (A) 0 (curve 1), 1.0 (curve 2), 4.2 (curve 3), 8.3 (curve 4), 12.4 (curve 5), and 16.4 (curve 6) mol of butyryl-CoA/mol of enzyme-bound FAD. Inset: The intensities of the 450-nm (●) and 580-nm bands (○) at varying butyryl-CoA concentrations are plotted. (B) 0 (curve 1), 0.16 (curve 2), 0.32 (curve 3), 0.48 (curve 4), 0.64 (curve 5), and 0.98 (curve 6) mol of octanoyl-CoA/mol of FAD. Inset: The intensities of the 450-nm (●) and 580-nm bands (○) at varying octanoyl-CoA concentrations are plotted. (C) 0 (curve 1), 1.0 (curve 2), 3.3 (curve 3), 6.7 (curve 4), 10.0 (curve 5), and 13.0 (curve 6) mol of palmitoyl-CoA/mol of FAD. Curve 7 (dashed line) shows the spectral change of the enzyme with 13.0 equiv of palmitoyl-CoA (curve 6) upon further addition of 7.4 mol of octanoyl-CoA/mol of FAD after curve 6. (D) 0 (curve 1), 1.0 (curve 2), 2.0 (curve 3), and 4.0 (curve 4) mol of 2-octenoyl-CoA/mol of FAD.

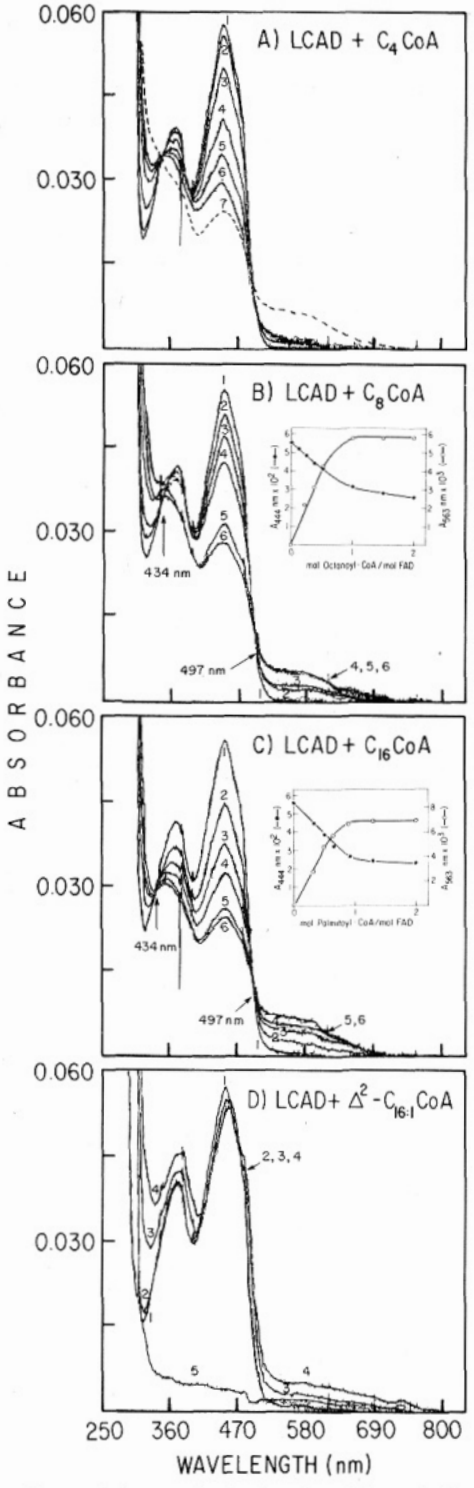



FIGURE 3: Spectral changes in the titration of long-chain acyl-CoA dehydrogenase with butyryl-CoA (A), octanoyl-CoA (B), palmitoyl-CoA (C), and 2-hexadecenoyl-CoA (D). The enzyme (5.1 $\mu$M bound FAD, $A_{444}/A_{275}$ = 9.2) was titrated anaerobically under the conditions described in Figure 1. (A) 0 (curve 1), 1.0 (curve 2), 3.3 (curve 3), 6.7 (curve 4), 10.0 (curve 5), and 13.3 (curve 6) mol of butyryl-CoA/mol of enzyme-bound FAD. Curve 7 (dashed line) shows the spectral change of the enzyme with 13.3 equiv of butyryl-CoA (curve 6) upon further addition of 1.0 mol of palmitoyl-CoA. (B) 0 (curve 1), 0.13 (curve 2), 0.26 (curve 3), 0.51 (curve 4), 1.02 (curve 5), and 1.53 (curve 6) mol of octanoyl-CoA/mol of FAD. Inset: The intensities of the 444-nm (●) and 563-nm bands (○) at varying octanoyl-CoA concentrations are plotted. (C) 0 (curve 1), 0.33 (curve 2), 0.49 (curve 3), 0.65 (curve 4), 1.31 (curve 5), and 2.3 (curve 6) mol of palmitoyl-CoA/mol of FAD. Inset: The intensities of the 444-nm (●) and 563-nm bands (○) at varying palmitoyl-CoA concentrations are plotted. (D) 0 (curve 1), 1.0 (curve 2), 4.8 (curve 3), and 9.7 (curve 4) mol of 2-hexadecenoyl-CoA/mol of FAD. Curve 5 shows the spectrum of 2-hexadecenoyl-CoA (48.8 $\mu$M) alone.

approximately 1 mol of palmitoyl-CoA/mol of enzyme-bound FAD. In contrast, the intersection of that of the long-wavelength band was observed at 0.7 mol of palmitoyl-CoA/mol of enzyme-bound FAD. The bleaching of the 443-nm absorbance, due to the addition of an equimolar amount of octanoyl-CoA, was not as strong as that seen with the addition of the same amount of palmitoyl-CoA, but bleaching further intensified with molar excesses of octanoyl-CoA. The long-wavelength band also intensified. Upon titration with butyryl-CoA, significant bleaching of the FAD chromophore occurred, particularly with considerable molar excesses of butyryl-CoA, but no long-wavelength band was observed (Figure 3A). When a 5 times molar excess of palmitoyl-CoA was further added, the flavin chromophore was slightly further bleached and the strong long-wavelength band was observed [Figure 3A, dashed line (curve 7)]. The percent bleachings of the 443-nm absorbance due to the addition of 1 equiv each of butyryl-CoA, octanoyl-CoA, and palmitoyl-CoA were 3, 42, and 50, respectively, and those caused by a 10 times molar excess of each of the same substrates were 38, 47, and 62, respectively.

We titrated short-chain, medium-chain, and long-chain acyl-CoA dehydrogenases with crotonyl-CoA, 2-octenoyl-CoA, and 2-hexadecenoyl-CoA, respectively (Figures 1D, 2D, and 3D). In no case was significant bleaching of flavin chromophore observed. The responses of the three enzymes were somewhat different in regard to the appearance of the long-wavelength band. There was no change in the case of medium-chain acyl-CoA dehydrogenase/2-octenoyl-CoA (Figure 2D). In the case of short-chain acyl-CoA dehydrogenase/crotonyl-CoA, a featureless elevation in the long-wavelength region was observed. This is mainly due to a nonspecific elevation of the base line resulting from the addition of crotonyl-CoA since curve 3 is elevated from curve 1 at all points of wavelength (Figure 1D). In contrast, the addition of large molar excesses of 2-hexadecenoyl-CoA to long-chain acyl-CoA dehydrogenase resulted in the appearance of a featureless, long-wavelength absorbance extending to 800 nm (Figure 3D). However, this band differed from those observed when octanoyl- or palmitoyl-CoA was added to the same enzyme (panels B and C, respectively, of Figure 3) in its degree, shape, and range of wavelength.

We also carried out titrations of the three dehdyrogenases with acetoacetyl-CoA. The spectral changes of short-chain and medium-chain acyl-CoA dehydrogenases upon addition of acetoacetyl-CoA were similar. Both exhibited a slightly decreased flavin chromophore at 450 nm accompanied by a blue shift of 7 nm and the appearance of a strong, long-wavelength band, centered at 575 nm (data not shown). These spectral changes are essentially identical with those reported by others with bacterial butyryl-CoA dehydrogenase (yellow form) (Engel & Massey, 1971) and pig general acyl-CoA dehydrogenase (McKean et al., 1979). The extrapolation of the linear portions of the curve for the appearance of the long-wavelength band intersects at approximately 1 mol of acetoacetyl-CoA/mol of FAD in the case of short-chain acyl-CoA dehydrogenase, but it occurred at 3 mol of acetoacetyl-CoA/mol of FAD in the case of medium-chain acyl-CoA dehydrogenase. In contrast, when long-chain acyl-CoA dehydrogenase was titrated with acetoacetyl-CoA, only a very weak absorbance at 580 nm appeared with a large molar excess of acetoacetyl-CoA, while the 450-nm absorbance slightly decreased.

*Identification and Quantitation of Product from the Complete Reactions Carried Out in the Presence of PMS.* The reaction mixture, which contained a catalytic amount of an enzyme, was incubated at 37 °C in the presence of 3 mM PMS as an electron acceptor. The reaction products were identified by gas chromatographic and GC/MS/COM analysis of the acyl moiety after hydrolysis of coenzyme A esters.

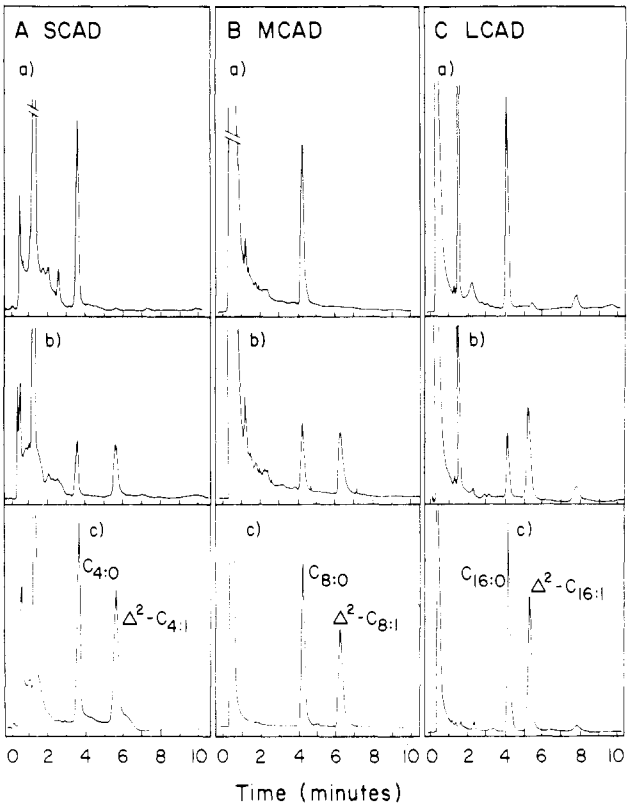



FIGURE 4: Identification of the reaction products of short-chain (A), medium-chain (B), and long-chain acyl-CoA dehydrogenases (C) by gas chromatography. (A) Purified short-chain acyl-CoA dehydrogenase (1.7 $\mu$g of protein) was reacted with *n*-butyryl-CoA (0.3 mM) for 0 (a) and 10 min (b). The reaction mixture was analyzed as a free acid by gas chromatography after alkaline hydrolysis and steam distillation, as described under Experimental Procedures. An SP-1200 (1.8 m × 2 mm) column was utilized for analysis. The pattern of analysis of a standard mixture that contained butyric ($C_{4:0}$) and crotonic acids ($\Delta^2$-$C_{4:1}$) is shown in (c). (B) Purified medium-chain acyl-CoA dehydrogenase (1.4 $\mu$g of protein) was reacted with *n*-octanoyl-CoA (0.3 mM) for 0 (a) and 10 min (b). (C) Purified long-chain acyl-CoA dehydrogenase (5 $\mu$g of protein) was reacted with palmitoyl-CoA (0.3 mM) for 0 (a) and 10 min (b). Reaction mixtures for the last two enzymes were analyzed by gas chromatography after alkaline hydrolysis and methylation as described under Experimental Procedures. A 10% OV-17 (1.8 m × 2 mm) column was utilized for analysis. The pattern of analysis of a standard mixture that contained octanoic ($C_{8:0}$) and *trans*-2-octenoic acids ($\Delta^2$-$C_{8:1}$) or palmitic ($C_{16:0}$) and *trans*-2-hexadecenoic acids ($\Delta^2$-$C_{16:1}$) is shown in panels Bc and Cc, respectively.

All substrates and products were well separated under the chromatographic conditions employed (Figure 4, panels Ac, Bc, and Cc). The products from the reaction of short-chain acyl-CoA dehydrogenase/*n*-butyryl-CoA, that of medium-chain acyl-CoA dehydrogenase/octanoyl-CoA, and that of long-chain acyl-CoA dehydrogenase/palmitoyl-CoA (each at 10 min) were identified as crotonyl-CoA, 2-octenoyl-CoA, and 2-hexadecenoyl-CoA, respectively, by identity of the retention time of the hydrolysis products with those of authentic crotonic, *trans*-2-octenoic, and *trans*-2-hexadecenoic acids (Figure 4, panels Aa, Bb, and Cb). These identifications were further confirmed by GC/MS analysis. No product was detected in the control reaction media to which boiled enzyme was added (Figure 4, panels Aa, Ba, and Ca). The reaction product from each enzyme/substrate combination was produced linearly

with time for at least 5–10 min, but after this point, the decrease of substrate and the increase of product both slowed down, reaching an equilibrium after 15–30 min (Ikeda et al., 1985a).

The reaction rates as assessed by measuring the amount of product formed in the first 5 min by gas chromatographic analysis were 9.5, 10.6, and 2.0 μmol of enoyl-CoA formed/(min·mg of protein), respectively, for short-chain, medium-chain, and long-chain acyl-CoA dehydrogenase reductions. These values were in good agreement with the corresponding values of 9.6, 10.7, and 2.3 μmol of DCIP reduced/(min·mg of protein) obtained from the dye-reduction assay using the same amount of the corresponding enzyme preparations.

*Correlation of Turnover Numbers in the Complete Reactions to Results from Spectrophotometric Titrations in the Half-Reactions.* The spectral changes, which were observed in the half-reactions, are graphically shown in comparison to the turnover numbers ($V_{max}^{app}$) in the complete reactions with various acyl-CoA's (Ikeda et al., 1985a) for the three dehydrogenases. The two spectral parameters used are the bleaching of flavin chromophore at 450 nm (Figure 5C) and the long-wavelength absorbance at 580 nm (Figure 5B). These two parameters were recorded at two different substrate concentrations (equimolar and a 10 times molar excess).

The appearance of the long-wavelength band was observed only in the enzyme-substrate combinations in which an enoyl-CoA product was produced at a signifcant rate in the complete reaction (Figure 5A,B). Good correlations were observed between the turnover numbers and the magnitudes of the 580-nm band. In contrast, the spectrum of the substrate causing FAD bleaching was broader than those for the turnover number and the appearance of the long-wavelength band even when the half-reaction was carried out at an equimolar concentration of the enzyme and substrate. For instance, significant bleaching of long-chain acyl-CoA dehydrogenase was observed with hexanoyl-CoA as substrate, but no significant activity was observed in the complete reaction with this combination. The magnitude of the long-wavelength absorbance was small. Some bleaching of medium-chain acyl-CoA dehydrogenase was observed when titrated with equilmolar myristoyl- or palmitoyl-CoA, but no activity was observed in the complete reaction, and the long-wavelength band was not observed with these combinations.

When these dehydrogenases were titrated with a 10 times molar excess of substrate, the spectrum of the substrates causing the FAD bleaching became much wider and the degree of bleaching by unfavorable substrates greatly increased. Long-chain acyl-CoA dehydrogenase was bleached by any acyl-CoA with a chain length from four to eighteen carbons. In contrast, the magnitude of the long-wavelength absorbance upon addition of molar excesses of substrate did not significantly differ from that observed with an equimolar amount of substrate (data not shown). It should also be noted that bleaching of the flavin chromophore by an unfavorable substrate was observed only when the molecular size of the substrate was smaller or slightly larger than that of the favorable substrate. For instance, the addition of acyl-CoA longer than decanoyl-CoA to short-chain acyl-CoA dehydrogenase did not cause any flavin bleaching. Medium-chain acyl-CoA dehydrogenase was bleached considerably with large molar excesses of myristoyl-CoA and moderately with palmitoyl-CoA, but it was not bleached at all with stearoyl-CoA in any amount.

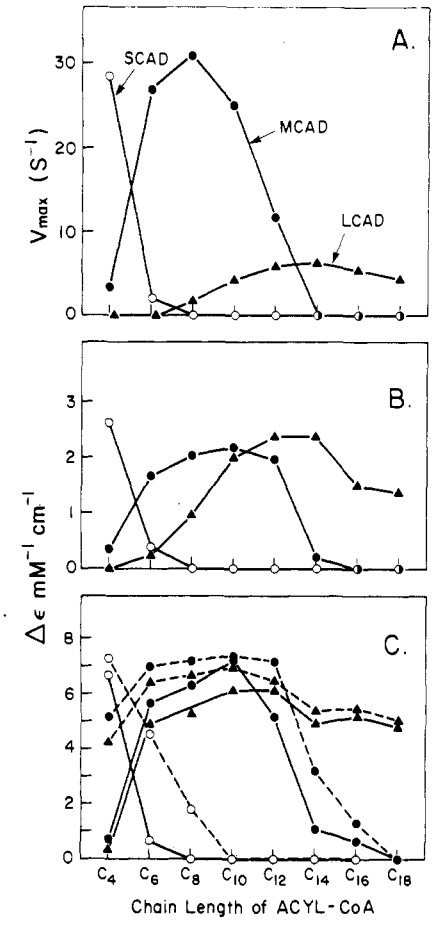


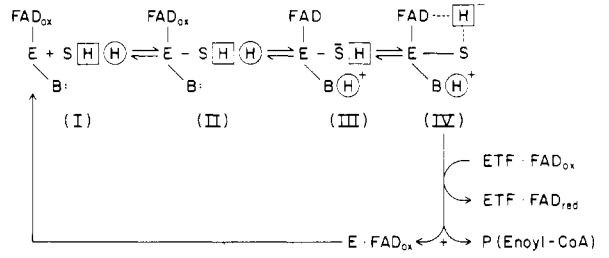

FIGURE 5: Steady-state turnover numbers (A), extinction of the long-wavelength absorbance band (B), and bleaching of the enzyme-bound FAD (C) of short-chain (SCAD), medium-chain (MCAD), and long-chain acyl-CoA dehydrogenase (LCAD) for various acyl-CoA's. (A) The turnover numbers presented in our previous paper (Ikeda et al., 1985a) are used for the graphic presentation. (B) Extinction coefficients for the long-wavelength absorbance that appeared in the stoichiometric titrations with various acyl-CoA's. (C) Extinction coefficients for bleaching of FAD that was induced by titration with a stoichiometric amount (solid line) or a 10 times molar excess (dashed line) of the acyl-CoA's indicated: (○) SCAD; (●) MCAD; (▲) LCAD. The titrations of the enzymes with acyl-CoA's were done under the same conditions as those described in Figures 1–3. The computation of extinction coefficients for the absorbance changes was done on the basis of $A_{450} = 11.3 \times 10^3$ $M^{-1}$ $cm^{-1}$ for the oxidized enzymes.

## DISCUSSION

Our systematic titration study of the three acyl-CoA dehydrogenases accurately defined their spectral changes upon interaction with various substrates. We first demonstrated that the amount of enoyl-CoA produced, as measured by gas chromatographic analysis, agreed well with the amount of DCIP reduced in the PMS/DCIP assay. The stable long-wavelength band was observed only in those in which enoyl-CoA product was produced at a significant rate in the complete reaction. A good correlation was observed between the steady-state turnover numbers measured by the PMS/DCIP assay and the magnitudes of the long-wavelength absorbance in the half-reaction.

It had previously been hypothesized that the long-wavelength absorbance is due to the charge-transfer complex presumably formed between the reduced enzyme and enoyl-CoA product after the initial C-2 proton abstraction by a base in the enzyme (Engel & Massey, 1971; Massey & Ghisla, 1974; Hall et al., 1979; Thorpe et al., 1979; Auer & Frerman, 1980). Although the experiments using alternate substrates, dead-end inhibitors, and enzyme-activated inhibitors (Murfin, 1974; Reinsch et al., 1980; Frerman et al., 1980; Thorpe et

Scheme I




al., 1981; Fendrich & Abeles, 1982) and those using radioactivity labeling (Bielmann & Hirth, 1970b; Gomes et al., 1981) were all supportive of the initial C-2 proton abstraction, direct evidence for the hypothesis was lacking. In regard to the nature of the charge-transfer complex, McFarland et al. (1982) showed using pig general acyl-CoA dehydrogenase and a novel pseudosubstrate, [3-(2-furyl)propionyl]-CoA (FP-CoA), that this substrate was dehydrogenated to [3-(2-furyl)acryloyl]-CoA (FA-CoA) in the half-reaction. Absorbance at 340 nm due to FA-CoA appeared at the same rate as that of the long-wavelength band, indicating that FA-CoA was produced in the charge-transfer complex. However, this pseudosubstrate has an unusual structure. Its C-3 carbon is $\alpha$ to one of the double bonds of the furyl moiety. It would contain four conjugated double bonds when it is dehydrogenated at C-2 and C-3. Therefore, unlike those of fatty acyl-CoA's, C-3 hydrogens of this pseudosubstrate are highly activated. The behavior of this pseudosubstrate in the half-reaction may not be a good model for that of the natural substrates. Thus, the nature of the charge-transfer complex of the natural substrate also remained to be studied.

Recently, we have shown using GC/MS and proton NMR that when the half-reaction was carried out in $D_2O$ with a catalytic amount of an acyl-CoA dehydrogenase and a large excess of optimal substrate, extensive $\alpha$-monodeuteration of the substrate occurred in the absence of $\beta$-deuteration or $\alpha,\alpha$-dideuteration, giving the first direct experimental evidence for the initial C-2 proton abstraction (Ikeda et al., 1985b). Also, our results clearly indicated that although the enzyme and the substrate form a spectrophotometrically stable complex, the substrate in the complex turns over very fast, indicating that the charge-transfer complex is not a rigid static one, but it is in a dynamic equilibrium. Furthermore, we have shown that when a large amount of acyl-CoA dehydrogenase was reacted with a moderate excess of favorable substrate in $D_2O$, the stable charge-transfer complex was formed, but the substrate remained unoxidized in the complex. Enoyl-CoA was produced either not at all or in an amount that represented only a minor fraction of the amount of enzyme added as analyzed by GC and GC/MS, while the substrates in the enzyme–substrate complex rapidly turned over as indicated by the extensive monodeuteration (Ikeda et al., 1985b). This is a sharp contrast to the observation by McFarland et al. (1982) using the pseudosubstrate FP-CoA, which was dehydrogenated to FA-CoA by pig general acyl-CoA dehydrogenase as it formed the charge-transfer complex with the enzyme. This difference between the natural fatty acyl-CoA substrates and FP-CoA is probably due to the unusual structure of the latter, which renders a high reactivity to its C-3 hydrogen.

Scheme I summarizes the sequence of events in the acyl-CoA dehydrogenase reaction based on the available evidence from us and others. First, acyl-CoA dehydrogenase binds acyl-CoA substrate forming a simple enzyme/substrate complex (II). In the second stage, a base in the enzyme abstracts *pro-R* C-2 hydrogen as a proton (Ikeda et al., 1985b; Bielman & Hirth, 1970b), producing the third species (III), in which the substrate is transformed into a carbanion. The carbanion in the complex interacts with the enzyme via flavin N-5 (Schmidt et al., 1981; Ghisla et al., 1984) and *pro-R* C-3 hydrogen of the substrate (Bielmann & Hirth, 1980a). The transfer of *pro-R* C-3 hydrogen to flavin N-5 is not yet completed in the enzyme/carbanion complex in the absence of an electron acceptor since enoyl-CoA was not formed in the complex (Ikeda et al., 1985b). Thus, we proposed that the enzyme and the carbanion form a highly resonant donor/acceptor hybrid (IV) (charge-transfer complex) with *pro-R* C-3 hydrogen of the substrate intervening as a hydride ion between enzyme-bound FAD and the carbanion (Ikeda et al., 1985b). It is likely that this resonant donor/acceptor hybrid species (IV) is responsible for the long-wavelength absorbance that is characteristic for the charge-transfer complex. N-5 of the enzyme-bound FAD accepts the hydride ion only in the presence of an electron acceptor (Ikeda et al., 1985b).

The most unexpected result from this study was the finding that the bleaching of the flavin chromophore occurred not only in the combination of an acyl-CoA dehydrogenase and an optimal substrate (product-producing combination) but also in that of an acyl-CoA dehydrogenase with an unfavorable substrate from which no 2-enoyl-CoA product was formed in the complete reaction, although with an unfavorable substrate, higher concentration was needed than with a favorable substrate to achieve the same degree of bleaching. The significant bleaching of flavin chromophore with an unfavorable substrate occurred only when the molecular size of the substrate was either smaller or only slightly larger than that of favorable substrates. Thus, no clear correlation was observed between the degrees of bleaching and the turnover numbers. This observation was suprising in view of the fact that the degree of bleaching in the titration with various substrates has been used by several workers to express the substrate specificity of pig general acyl-CoA dehydrogenase (Crane et al., 1956; Hall et al., 1979; McKean et al., 1979; Thorpe et al., 1979).

Our present data suggest that unfavorable substrates with a shorter acyl chain can gain access to the region in which active sites are located. Since FAD interacts with the C-3 methylene (Schmidt et al., 1981; Ghisla et al., 1984), the spatial arrangement of the C-2 proton abstracting base and the FAD in the active site is such that it fits to the physical configuration of the coenzyme A head and C-1 through C-3 of acyl-CoA's. Since the configurations of all straight-chain acyl-CoA's tested in this study are identical in this region but differ in the length of hydrocarbon chain, the factor that distinguishes the favorable substrates from the unfavorable ones for a given acyl-CoA dehydrogenase is not located in the vicinity of the carboxyl region but rather lies in the hydrophobic part of the hydrocarbon chain. It has been shown by Hall et al. (1979) and Frerman et al. (1980) that the hydrophobic hydrocarbon chain provides acyl-CoA's with one of the sites that bind to the acyl-CoA dehydrogenase. These considerations seem to suggest that the binding of shorter than optimal substrate to the enzyme is labile and of short duration, causing only weak FAD bleaching at an equimolar concentration. FAD bleaching is greatly enhanced by mass action by the increasing concentration of the substrate. However, binding of the substrate to the enzyme for a finite period of time via the hydrocarbon chain at stage II appears to be a prerequisite for C-2 proton abstraction by the catalytic base. Unfavorable substrates with a shorter acyl chain can interact with the enzyme-bound FAD or the adjacent structures,

causing FAD bleaching, but cannot interact with the base, due to their short hydrocarbon chain length. Therefore, the stable long-wavelength absorbance does not appear, and 2-enoyl-CoA product is not formed. In this case, although flavin bleaching is observed, this does not represent the chemical reduction or the redox state of the enzyme-bound FAD from which electrons can be transferred to ETF or other electron acceptors.

Unfavorable substrates with acyl chains considerably longer than those of the favorable substrates hardly gain access to the active site region due to their large size: thus, neither significant FAD bleaching nor appearance of the long-wavelength absorbance was detected as seen in the titration of short-chain and medium-chain acyl-CoA dehydrogenases with palmitoyl-CoA. From this observation, it may be considered that the C-2 proton abstracting base and FAD are internally located and that the region containing the two sites may physically be in the form of a crevice or pocket. The crevice would be of the size that allows the entry of optimal substrates or those with shorter acyl chains but does not allow the entry of the whole molecule of those that are too long.

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