Role of the Carbonyl Group in Thioester Chain Length Recognition by the Medium Chain Acyl-CoA Dehydrogenase†

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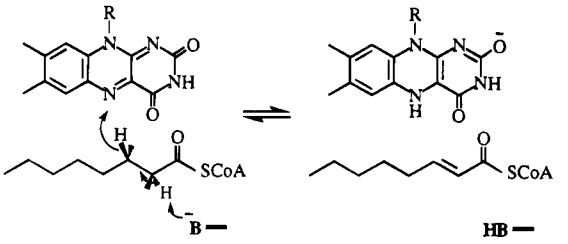
ABSTRACT: Medium chain acyl-CoA dehydrogenase from pig kidney catalyzes the oxidation of acyl-CoA thioesters to *trans*-2-enoyl-CoA derivatives with an optimal chain length of about C-8. The binding energy for alkyl-SCoA thioethers shows no such optimum but increases linearly from C-2 to C-16 with a slope of about 390 cal/-CH_2 group. In contrast, four types of CoA-thioester analogues (2-aza-acyl-, 3-thia-acyl-, 3-keto-acyl-, and *trans*-2-enoyl-) yield an incremental binding energy of about 800 cal/-CH_2 group until a chain length of about C-8 is reached. The observed binding energy then decreases, or remains constant, with increasing chain length. Studies with dithiooctanoyl-CoA and 2-azadithiooctanoyl-CoA show that the C=S moiety is accommodated poorly by the medium chain dehydrogenase. A model for chain length discrimination, based on the crystal structure of the enzyme [Kim, J. J. P., Wang, M., & Paschke, R. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90,* 7523−7527], is proposed in which hydrogen-bonding interactions between enzyme and thioester carbonyl oxygen atom are maximized at optimal chain lengths. Oversized chains decrease the frequency of effective alignment between enzyme and the C-1 to C-3 region of thioester ligands. Thus the extent of polarization of bound 4-thia-*trans*-2-enoyl-CoA thioesters decreases sharply with chains longer than C-12. Both the intensity of the charge transfer bands formed with 3-thiaacyl-CoA analogues and the extent of reduction of the enzyme using thioester substrates show a similar chain length dependence, suggesting that polarization of the thioester carbonyl group is of central importance to the reductive half-reaction. The spectral effects of indoleacryloyl-CoA on the medium chain dehydrogenase are reinterpreted in light of the polarization observed with 4-thia-*trans*-2-enoyl-CoA analogues.

Many aspects of the reductive half-reaction in the acyl-CoA dehydrogenases can be explained in terms of an initial binding of substrate (K_1), followed by a chain length dependent redox equilibration (K_2) between bound flavin and substrate moieties (Beinert, 1963; Beinert & Page, 1957; Thorpe et al., 1979; Schopfer et al., 1988):

$$E{\cdot}FAD_{ox} + SH_2 \overset{K_1}{\rightleftharpoons} E{\cdot}FAD_{ox}{\cdot}SH_2 \overset{K_2}{\rightleftharpoons} E{\cdot}FAD_{2e}{\cdot}P$$

Studies with the medium chain dehydrogenase, the best understood member of the acyl-CoA dehydrogenase enzyme family, suggest that dehydrogenation, step K_2, involves removal of the *pro-R*-α-proton with a concerted *anti* elimination of the *pro-R*-β-hydrogen as a hydride equivalent to the N-5 position of the isoalloxazine ring (Murfin, 1974; Reinsch et al., 1980; Ghisla et al., 1984; Pohl et al., 1986; Schopfer et al., 1988; Scheme 1). The base in Scheme 1, which plays a central role in substrate activation, is Glu376 in the medium chain acyl-CoA dehydrogenase (Powell & Thorpe, 1988; Bross et al., 1990; Kim, 1991; Kim et al., 1993).

Scheme 1



The active site of the medium chain acyl-CoA dehydrogenase was originally probed using alkyl-SCoA derivatives (Frerman et al., 1980: Powell et al., 1987). These thioethers (such as octyl-SCoA, compound 3, Chart 1) lack the acidic α-protons of normal thioester substrates, allowing the substrate binding site of the dehydrogenase to be probed uncomplicated by subsequent reduction of the bound flavin cofactor (i.e., without the involvement of K_2; Frerman et al., 1980; Powell et al., 1987). Somewhat surprisingly, binding measurements with these thioethers did not show the chain length dependent optimum expected for a medium chain acyl-CoA dehydrogenase. Instead, the binding energy increased approximately linearly as the chain length is increased from C2 to C16 with a slope of 390 cal/methylene group (Powell et al., 1987). These data suggested that the binding site was not only of moderate hydrophobicity but also extensive and not tailored to fit saturated hydrocarbon chains of medium length optimally (Powell et al., 1987). This

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[1] Abbreviations: $E{\cdot}FAD_{ox}$ and $E{\cdot}FAD_{2e}$, oxidized and two-electron-reduced forms of acyl-CoA dehydrogenase without regard to protonation state of the flavin prosthetic group; P, *trans*-2-enoyl-CoA product.

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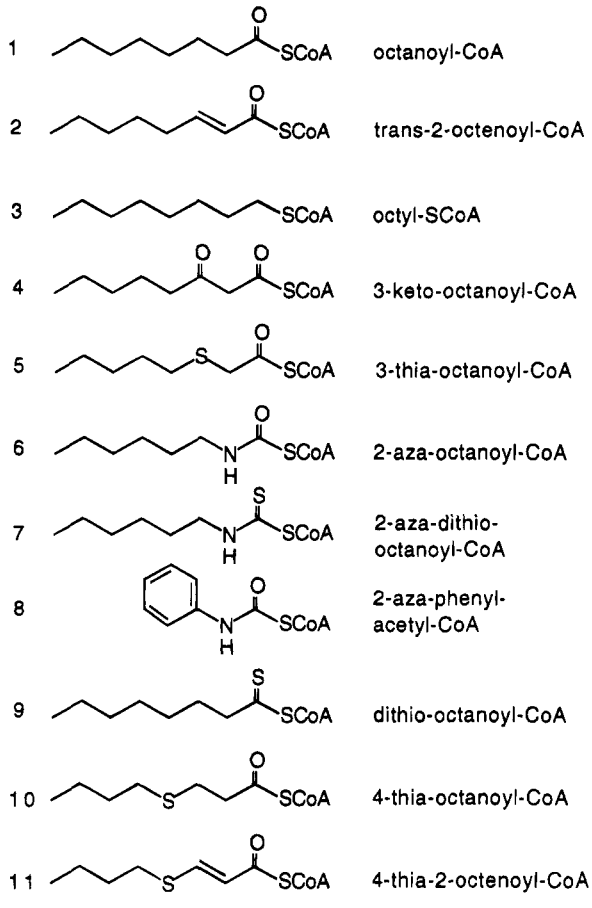
Chart 1. Some Acyl-CoA Analogues Used in This Work



latter conclusion is in apparent contradiction with the recent 2.4 Å crystal structure of the medium chain acyl-CoA dehydrogenase (Kim et al., 1993).

The present work employs a series of redox inactive thioester analogues to show that chain length discrimination requires the presence of the carbonyl oxygen atom found in normal thioester substrates but absent in alkyl-SCoA thioethers previously used as active site probes. The binding contributions of carbonyl oxygen and hydrocarbon chain prove to be distinctly nonadditive. The work started using thioesters in which dehydrogenation is blocked by derivatization of the β-methylene group (e.g., the 3-keto-, or the 3-thiaacyl-CoA derivatives; compounds 4 and 5, Chart 1; Thorpe, 1986; Lau et al., 1988). This paper also characterizes a series of analogues in which substitution of an -NH- group at the α-position (e.g., 2-azaoctanoyl-CoA, compound 6, Chart 1) prevents the normal dehydrogenation reaction depicted in Scheme 1. Finally, utilizing a number of chromophoric enoyl-CoA analogues (such as compound 11, Chart 1), we show that polarization of the enoyl moiety is, itself, chain length dependent. These data, taken with the crystal structure of the medium chain acyl-CoA dehydrogenase (Kim et al., 1993, 1994), provide a clear demonstration of the importance of the thioester carbonyl in substrate recognition and catalysis. It will be interesting to see whether the thioester carbonyl group is of similar importance to chain length discrimination in other acyl-CoA dependent enzymes.

MATERIALS AND METHODS

Materials. Medium chain acyl-CoA dehydrogenase was isolated from pig kidneys as described previously (Lau et al., 1989). Butyl, octyl, and phenyl isocyanate, ethyl, butyl, and phenyl isothiocyanate, octanoyl chloride, thiophenol, and Lawesson's reagent were from Aldrich. Ethyl isocyanate and hexyl isothiocyanate were purchased from Lancaster Synthesis. Hexyl and dodecyl isocyanate were obtained from Kodak. CoASH (lithium salt) and pyrophosphatase (nucleotide, type III from *Crotalus atrox*) were from Sigma.

General. Unless otherwise stated all spectrophotometric titrations were performed at 25 °C in 50 mM phosphate buffer, pH 7.6, containing 0.3 mM EDTA. The dissociation constants were obtained for strongly binding ligands using approximately 1 μM enzyme in 5 cm path-length cylindrical quartz cells. Ligand binding data were examined using Enzfitter (Elsevier BioSoft). The concentration of pig kidney medium chain acyl-CoA dehydrogenase was measured using an extinction coefficient of 15.4 mM^{-1} cm^{-1} at 446 nm (Thorpe et al., 1979) using Cary 219 or Hewlett Packard 8452A spectrophotometers. Fluorescence spectra were collected using a Perkin Elmer 650-10S instrument. Anaerobic techniques were as described earlier (Gorelick et al., 1985). The dehydrogenase was assayed using ferricenium hexafluorophosphate as electron acceptor (Lehmann et al., 1990).

Preparation and Purification of 2-Azaacyl-CoA Derivatives. Acyl thiocarbamates were synthesized by reacting CoASH with the corresponding alkyl isocyanates (Crosby & Niemann, 1954; Wang, 1992). Those alkyl isocyanates which were not available commercially were synthesized via the Curtius rearrangement from the corresponding acyl azides (Allen & Bell, 1955). Preparation of the 2-azathioesters is illustrated below for 2-azahexanoyl-CoA. Butyl isocyanate (15 mg) was added to a stirred solution containing 40 mg of freshly dissolved CoASH in 1 mL of 0.25 M sodium bicarbonate. The reaction was quenched after 30 min by adjusting the pH to about 5.5 with acetic acid. Dithiocarbamates were prepared in the same way: e.g., for the preparation of 2-azadithiohexanoyl-CoA, 30 mg of butyl isothiocyanate and 40 mg of CoASH were stirred for 2.5 h and treated as before.

Both thiocarbamates and dithiocarbamates were purified on a semipreparative octadecylsilica HPLC column developed with an increasing gradient of methanol in 50 mM phosphate buffer, pH 5.3, formed using a Perkin-Elmer Series 400 liquid chromatograph. Long chain derivatives were more conveniently eluted by replacing methanol by 1-propanol. All the analogues emerged as sharp symmetrical peaks without undue trailing when monitored at 254 nm. The analogues were concentrated by rotary evaporation, desalted on a Bio-Gel P-2 column, and stored as lyophilized powders at −20 °C. The isolated yields for 2-azahexanoyl- and 2-azadithiohexanoyl-CoA were about 65% and 60%, respectively.

NMR Spectra of 2-Azaacyl-CoA Derivatives. NMR spectra of CoA analogues were recorded at 20 mg/mL in D_2O using a Bruker 250-MHz spectrometer with an Aspect 3000 computer system. The terminal methylene group of pantetheine (C9; Fung et al., 1976) shifts to 2.70 ppm (triplet; two protons) on formation of 2-azahexanoyl-CoA. In addition, the following resonances for the 2-azahexanoyl moiety were observed: C3 methylene group, 2.95 ppm (triplet; two protons); C4 methylene group, 1.18 ppm (multiplet, two protons); C5 methylene group, 1.03 ppm (multiplet, two protons); C6 methyl group, 0.60 ppm (triplet, three protons). Resonances for the corresponding 2-azadithiohexanoyl-CoA derivatives were as follows: terminal methylene group of pantetheine (C9) shifts to 3.15 ppm

(multiplet, two protons); C3 methylene, 3.32 ppm (triplet, two protons); C4 methylene group, 1.39 ppm (multiplet, two protons); C5 methylene group, 1.10 ppm (multiplet, two protons); C6 methyl group, 0.66 ppm (triplet, three protons). Structures of all the other CoA derivatives were checked by ^{1}H NMR.

Stability of 2-Azaacyl-CoA Analogues. Lyophilized powders were stable for months without detectable decomposition. Stock solutions were prepared in water at about 10 mM and proved stable for weeks at −20 °C. Solutions of this concentration showed no significant decomposition after 20 h incubation at 20 °C as judged by HPLC and proton NMR spectroscopy. The stability of dilute solutions was evaluated following the release of CoASH. When 600 μM DTNB is added to a freshly diluted solution of 37 μM 2-azadecanoyl-CoA in 50 mM phosphate buffer, pH 7.6, decomposition was undetectable over 10 min. However, solutions of the analogue stored overnight at pH 7.6 (37 μM, at 25 °C; under anaerobic conditions to minimize oxidation of released CoASH) decomposed by 20 and 30% for 2-azadecanoyl-CoA and 2-azadithiohexanoyl-CoA respectively.

UV Spectrum and Extinction Coefficients of 2-Aza- and 2-Azadithioacyl-CoA Analogues. Whereas 2-azaoctanoyl-CoA shows a spectrum typical of a simple acyl-CoA thioester, the dithio counterpart shows the additional spectral characteristic of the dithiocarbamate moiety, with additional absorbance at 260 nm and a very weak band at 320 nm (not shown; Djerassi et al., 1962; Toniolo, 1973). The extinction coefficient of 2-azahexanoyl-CoA was determined as 16 mM^{-1} cm^{-1} at 260 nm by weighing the lyophilized analogue (see above) and measuring the absorbance of its solution in water. Two methods were used to determine the extinction coefficient of the analogous dithiocarbamate derivative. Samples of the 2-azadithiocarbamate were cleaved with pyrophosphatase (0.5 mg/mL pyrophosphatase in phosphate buffer, pH 7.6, at 25 °C for 5 h) and the resulting 3′,5′-ADP was quantitated by HPLC using an extinction coefficient of 15.4 mM^{-1} cm^{-1} (Dawson et al., 1986). In this system 3′,5′-ADP, 2-azadithiohexanoyl-CoA, and the 2-azadithiohexanoylpantetheine phosphate elute at 3, 15, and 19 min, respectively. The pantetheine analogue shows a spectrum very similar to that of a thiocarbamate model compound (Djerassi et al., 1962). The extinction coefficient obtained by this method was 23 mM^{-1} cm^{-1} compared with 22 mM^{-1} cm^{-1} determined by dry weight.

Preparation of Other Acyl-CoA Analogues. The following thioester analogues were synthesized in a range of chain lengths (see Results) and characterized as described previously: 3-thiaacyl- (Lau et al., 1988); 3-ketoacyl- (Thorpe, 1986); *trans*-2-enoyl- (Powell et al., 1987); and 4-thia-*trans*-2-enoyl-CoA (Lau et al., 1989). Octanoyldithio-CoA was synthesized from *S*-phenyldithiooctanoate and CoA as described by Wlassics et al. (1988). *S*-Phenyldithiooctanoate was prepared from the corresponding thiooctanoate with Lawesson's reagent and was purified by flash chromatography on silica gel. ^{1}H NMR indicated that less than 2% of the thiolester was present. *S*-Phenyldithiooctanoate (80 μmol) was added to a solution of CoA (10 μmol; Li^+ salt) in 4 mL of a 1:1 mixture of 0.1 M sodium carbonate (pH 8.5) and ethanol. Ethyl acetate was added dropwise until a homogenous mixture was obtained and the solution maintained at room temperature for 20 min. The mixture was then acidified with a few drops of phosphoric acid to a pH of 4 and the aqueous solution extracted three times with ethyl acetate. Residual ethyl acetate was evaporated with a stream of nitrogen and the octanoyldithio-CoA, containing less than 5% free CoA, was lyophilized and stored at −20 °C.

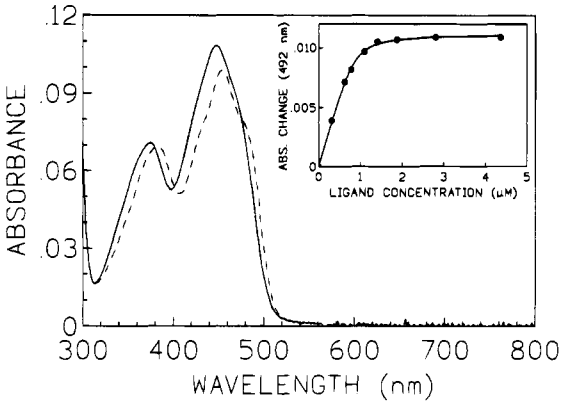


FIGURE 1: Spectral changes on the addition of 2-azaoctanoyl-CoA to the medium chain acyl-CoA dehydrogenase. The dehydrogenase (7.1 μM in 0.8 mL of 50 mM phosphate buffer, pH 7.6, 25 °C; —) was mixed with 1 equiv of 2-azaoctanoyl-CoA (– – –). The inset records a titration performed with 0.85 μM enzyme in the same buffer using a 5 cm path length cell. The line corresponds to a K_d of 0.051 μM. In all cases spectral changes were completed before measurement could be made (see text).

Circular Dichroism Studies. CD spectra were acquired using a Jasco 710 circular dichroism spectrometer scanning from 300 to 180 nm at 50 nm/min. Three scans were averaged and reliable data to 185 nm was obtained using 10 mM phosphate buffer with the nitrogen purging flow rates used. Enzyme was diluted to a concentration of 2 μM in 10 mM potassium phosphate buffer, pH 7.6, 25 °C, in the presence or absence of 4 and 8 μM concentrations of the following ligands: 2-azaoctanoyl-CoA, octanoyl-CoA, and octyl-SCoA. Spectra were recorded using 200 μL of each solution in 1 mm path-length cylindrical cells.

Molecular Modeling. The crystal structure of the pig medium chain acyl-CoA dehydrogenase complexed reduced with octanoyl-CoA was obtained from the Brookhaven Protein Data Bank (3MDE). Modeling was performed with Insight II (Biosym) on a Silicon Graphics Indigo 2 workstation. Water molecules 801H to 998H were removed, and hydrogens were added to the structure at pH 7.00. Dithiooctanoyl-CoA was modeled into the active site of the B subunit replacing the thioester carbonyl oxygen of the ligand by a sulfur atom.

RESULTS

Interaction of 2-Azaacyl-CoA Thioesters with the Medium Chain Acyl-CoA Dehydrogenase. The synthesis of 2-azaacyl-CoA analogues is described in Materials and Methods. Both the thiocarbamates and dithiocarbamates (such as 2-azaoctanoyl-CoA and 2-azadithiooctanoyl-CoA: Chart 1, compounds 6 and 7, respectively) are readily prepared using the appropriate isocyanate or isothiocyanate derivatives (Crosby & Niemann, 1954). The compounds were purified by HPLC and characterized by NMR and UV spectroscopy (see Materials and Methods). All the 2-aza- derivatives proved sufficiently stable for the titrations performed here (see Materials and Methods).

Figure 1 shows the marked spectral changes induced upon the addition of 2-azaoctanoyl-CoA (compound 6, Chart 1) to the medium chain enzyme with the formation of a distinct

shoulder at 486 nm and an approximately 6 nm red-shift in the overall absorbance envelope. No significant long wavelength band is apparent in any of the titrations with 2-aza- derivatives. This is in marked contrast to a number of derivatives at the 3-position, which yield intense charge transfer bands between 560 and 820 nm (Engel & Massey, 1971; Powell et al., 1987; Lau et al., 1988). The spectral changes shown in Figure 1 are completed as fast as measurement can be made in a conventional spectrophotometer. In the stopped flow spectrophotometer, the increase in absorbance accompanying binding (Figure 1) is half-complete in 14 ms when the dehydrogenase is mixed with 2-azaoctanoyl-CoA (at 1 °C to final concentrations of 10 and 43 μM, respectively; not shown). The spectral changes shown in Figure 1 remain stable for the time required to complete a titration. Nevertheless over a period of many hours a slow apparent reduction of the flavin chromophore is observed. These slow, secondary changes do not affect the conclusions of this paper and will not be considered further here.

The inset in Figure 1 shows the absorbance changes at 492 nm from a separate titration using 0.85 μM enzyme fitted to a curve corresponding to a dissociation constant of 51 nM and a stoichiometry of 1.0 ligand/active site. Because of this tight binding to the oxidized enzyme, 2-azaoctanoyl-CoA can compete effectively with octanoyl-CoA (apparent K_d ca. 20 nM; Thorpe et al., 1981; data not shown). Thus when 2-azaoctanoyl-CoA is added to the reduced enzyme•*trans*-2-octenoyl-CoA complex, reappearance of the oxidized enzyme occurs on reversal of equilibria K_1 and K_2 (eq 1; not shown).

Spectrophotometric titrations with 2-azaacyl-CoA analogues comparable to thioesters of C2 to C15 chain length[2] are essentially identical in form to that shown in Figure 1. Further, the magnitude of the difference spectrum extrapolated to saturating ligand does not vary over 20% for chain lengths of C4 to C15 (not shown). The binding energy, obtained from the dissociation constant for each analogue, is plotted as a function of chain length in Figure 2A. In marked contrast to the linear chain length dependence manifest with alkyl-SCoA thioethers (open circles, Figure 2A; Powell et al., 1987), 2-azathioesters show an optimum which matches the expected specificity profile of the pig kidney medium chain acyl-CoA dehydrogenase (Lau et al., 1988; see Discussion). In addition, there are significant differences in the incremental binding energy per methylene group between the two analogues. Over the C4−C8 range the presence of the thioester carbonyl oxygen effects an increase in slope corresponding to about 800 cal versus 390 cal/CH_2 group for the thioethers (Figure 2A). These data cast doubt on our previous suggestion that alkyl-CoA thioethers are appropriate substrate analogues of the dehydrogenase (see later).

As expected from the spectral changes shown in Figure 1, 2-azaoctanoyl-CoA is not a detectable substrate of the acyl-CoA dehydrogenase in the standard assay (not shown). 2-Azabutyryl-CoA was found to be a competitive inhibitor of the medium chain acyl-CoA dehydrogenase in the standard ferricenium assay (see Materials and Methods) with a K_i of

[2] For simplicity the length of an acyl chain containing a heteroatom, e.g., 2-aza- or 4-thia- analogues, is designated counting the heteroatom as a methylene group. Thus, 4-thiatetradecanoyl-CoA is abbreviated 4-thia-C14.

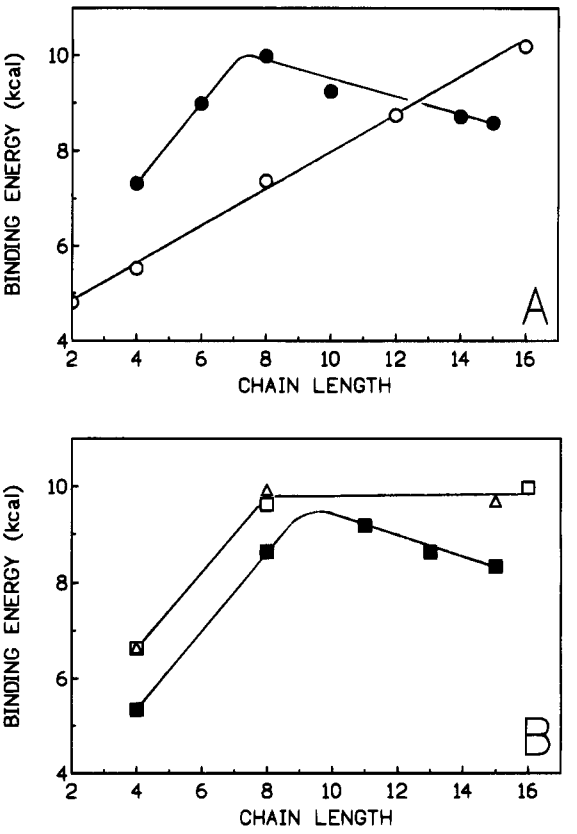


FIGURE 2: Chain length dependence of the apparent free energy of binding acyl-CoA analogues to the medium chain acyl-CoA dehydrogenase. (Panel A) Free energies, calculated from dissociation constants obtained spectrophotometrically at pH 7.6, 25 °C (see Materials and Methods), are shown for 2-aza- derivatives of the chain lengths shown (●). The corresponding values determined under the same conditions for a series of alkyl-SCoA thioethers analogues are shown for comparison (○; Powell et al., 1987). (Panel B) Binding free energies for *trans*-2-enoyl- (□), 3-ketoacyl- (△), and 3-thiaacyl-CoA derivatives (■) over a similar range of chain length.

4 μM, in good agreement with the corresponding value of 4.4 μM determined spectrophotometrically.

Chain Length Discrimination with Other Thioester Analogues. Investigation of three additional types of analogues (Figure 2B; using *trans*-2-enoyl-CoA, 3-ketoacyl-CoA, and 3-thiaacyl-CoA; Chart 1, compounds 2, 4, and 5) confirms the anomalous chain length response of the thioethers seen in Figure 2A. Like the 2-aza- derivatives, these thioesters are not substrates of the acyl-CoA dehydrogenase, although both 3-keto- and 3-thia- derivatives undergo α-proton abstraction within the active site to generate bound enolates (Engel & Massey, 1971; Massey & Ghisla, 1974; McKean et al., 1979; Powell et al., 1987; Lau et al., 1988; Nishina et al., 1993). Figure 2B shows that these additional thioester categories show either optimal binding in the medium chain length range (the 3-thia- analogues) or an essentially flat response with longer chain lengths (3-ketoacyl- and *trans*-2-enoyl-CoA derivatives).

Binding of 2-Azadithioacyl-CoA Derivatives to the Acyl-CoA Dehydrogenase. The data cited above indicate that the presence of a carbonyl group in four widely differing types of thioester ligands changes the chain length response shown by alkyl-SCoA thioethers. A further indication of the importance of the thioester oxygen atom is provided by 2-azadithioester analogues in which the larger sulfur atom replaces the thioester carbonyl oxygen (compound 7, Chart

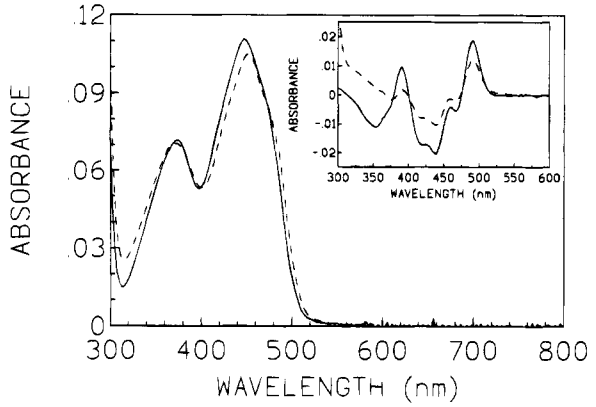


FIGURE 3: Spectral effects of 2-azadithiooctanoyl-CoA with the medium chain acyl-CoA dehydrogenase. The dehydrogenase (7.55 μM in 0.8 mL of phosphate buffer, pH 7.6, 25 °C; —) was mixed with 76 μM 2-azadithiooctanoyl-CoA (– – –). The inset shows the difference spectra (E·L − E) for 2-azaoctanoyl-CoA (—) and 2-azadithiooctanoyl-CoA (– – –) normalized to an enzyme concentration of 7.55 μM.

1). The spectral changes generated by 2-azadithiooctanoyl-CoA are shown in Figure 3. Note that the spectral shifts are less pronounced than those generated by the thioester analogue. The dithio- derivative binds some 80-fold weaker to the acyl-CoA dehydrogenase (data not shown; K_d 4 μM compared to 51 nM) whereas the difference is only about 10-fold for the butyryl-CoA derivatives (47 versus 4.4 μM). In the case of the 2-azaphenylacetyl-CoA (Chart 1, compound 8), the corresponding values are only about 3-fold different (18 versus 6.3 μM; not shown).

The spectral changes for 2-azaoctanoyl-CoA and its dithioester counterpart are compared in the difference spectrum shown as an inset to Figure 3. Above 380 nm, the spectral changes for both ligands are qualitatively very similar although the dithio- analogue perturbs the flavin spectrum to an extent of only about 60% that of the 2-azaoctanoyl-CoA analogue. The additional absorbance centered around 340 nm with 2-azadithiooctanoyl-CoA may reflect the weakly absorbing bands observed in this region of the spectrum with dithiocarbamates in solvents of low polarity (Djerassi et al., 1962; Toniolo, 1973).

Dithiooctanoyl-CoA as a Substrate of the Medium Chain Acyl-CoA Dehydrogenase. Replacement of a thioester oxygen atom by a sulfur thus weakens the binding of a nonsubstrate analogue by up to 80-fold. It was therefore of interest to compare catalytic turnover for the normal substrate octanoyl-CoA with the corresponding dithiooctanoyl-CoA (Chart 1, compound 9). Dithiooctanoyl-CoA is a relatively sluggish substrate with a maximal turnover number of 15/min compared to 1400/min in the ferricenium assay at pH 7.6 (Lehman & Thorpe, 1990). Oxidation of dithiooctanoyl-CoA is accompanied by the disappearance of the substrate peak at 308 nm and the appearance of a new absorbance at 342 nm expected for the corresponding *trans*-2-enoyl-CoA derivatives. These changes in the UV region of the spectrum interfere with quantitation of the disappearance of the ferricenium ion at 300 nm (Lehman & Thorpe, 1990). Accordingly, the decrease of ferricenium ion was followed using the much less sensitive wavelength 618 nm (Lehman & Thorpe, 1990; $\epsilon = 0.41$ mM^{-1} cm^{-1}). A low micromolar K_m for dithiooctanoyl-CoA, together with a marked curvature of the assays at low substrate concentrations, made accurate K_m determinations impractical with the current assay system.

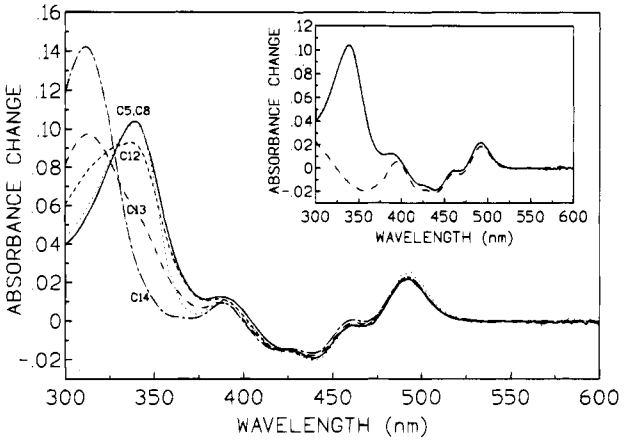


FIGURE 4: Difference spectra generated on the addition of 4-thia-*trans*-2-enoyl-CoA analogues to the medium chain dehydrogenase. Titrations of the medium chain acyl-CoA dehydrogenase (7 μM in 50 mM phosphate, pH 7.6, 25 °C) were performed and difference spectra (E·L − E) selected from ca. 70% saturation were extrapolated to completion. The main panel shows extrapolated difference spectra using 4-thia-*trans*-2-pentenoyl- to 4-thia-*trans*-2-tetradecenoyl-CoA as indicated on the curves. The inset is a comparison of extrapolated difference spectra between 4-thia-*trans*-2-octenoyl-CoA (—) and *trans*-2-octenoyl-CoA (– – –).

Since the K_m value for the normal substrate octanoyl-CoA appears even lower in this assay system (R. C. Trievel and C. Thorpe, unpublished observations), it is clear that replacement of the thioester by a dithioester group is tolerated very poorly during catalysis by the medium chain acyl-CoA dehydrogenase.

Polarization of the Thioester Carbonyl Group is Chain Length Dependent. 4-Thia-*trans*-2-octenoyl-CoA (compound 11, Chart 1) binds tightly to the medium chain acyl-CoA dehydrogenase with a pronounced red-shift of the enoyl chromophore (from 312 to 340 nm; Lau et al., 1989). This bathochromic shift was attributed to polarization of the carbonyl moiety on binding (Lau et al., 1989), and the crystal structure of the medium chain acyl-CoA dehydrogenase provides a molecular basis for this effect (see Discussion; Kim et al., 1993, 1994). This section shows that the degree of polarization of the enoyl chromophore is strongly dependent on acyl chain length.

Titrations of the medium chain dehydrogenase were performed with 4-thia-*trans*-2-enoyl-CoA derivatives from C5 to C16 at pH 7.6 (see Materials and Methods). Spectra at about 70% saturation were extrapolated to completion to avoid any interference from the 312 nm absorbance peak of the free enoyl-CoA ligand (see Materials and Methods). In all cases the binding of the 4-thia-*trans*-2-enoyl-CoA derivatives was sufficiently tight to ensure insignificant levels of free ligand at 70% saturation. Difference spectra [(E.FAD$_{ox}$·4-thiaenoyl-CoA) − (E.FAD$_{ox}$)] are presented in Figure 4. Above 390 nm, all chain lengths yield difference spectra which resemble that produced on binding *trans*-2-octenoyl-CoA to the medium chain enzyme (inset Figure 4; Lau et al., 1989). The enoyl chromophore of this normal product does not extend into the visible region of the spectrum, and so the difference spectrum for *trans*-2-octenoyl-CoA over the 300−550 nm range represents perturbation of just the bound flavin chromophore. Thus the deviation of the difference spectra below 390 nm from that expected for the flavin chromophore alone (solid line; inset) reflects the added contribution of the bound chromophoric ligand. The C5 and C8 analogues give a prominent additional peak at 340 nm

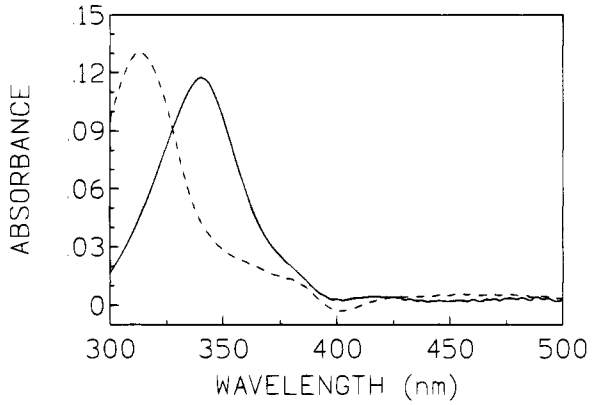


FIGURE 5: Estimation of the spectrum of polarized and unpolarized 4-thia-*trans*-enoyl-CoA chromophores. A difference spectrum for the normal product *trans*-2-octenoyl-CoA was subtracted from that induced on binding 4-thia-*trans*-2-octenoyl-CoA (—) and 4-thia-*trans*-2-tetradecenoyl-CoA (— — —) to approximate the spectrum of the bound 4-thiaenoyl chromophore. Data were taken from Figure 4.

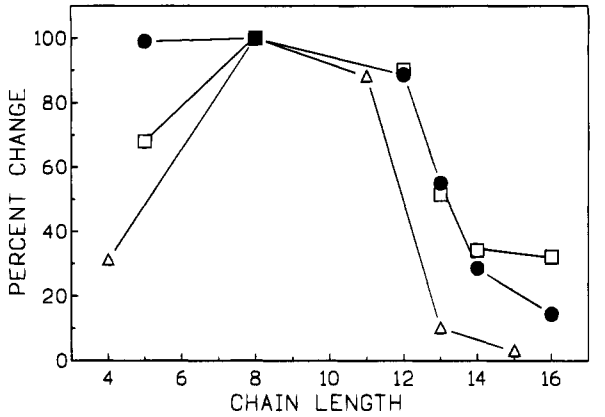


FIGURE 6: Chain length dependence of three equilibrium parameters in the medium chain acyl-CoA dehydrogenase. The polarization of 4-thia-*trans*-2-enoyl-CoA ligands (●) is expressed as a percentage of the absorbance at 340 nm observed with the C8 analogue in Figure 4. Similarly, the percent reduction of the dehydrogenase (□) was determined on the addition of excess 4-thiaacyl-CoA ligands of the indicated chain length. Reduction was evaluated at an isosbestic point for the interconversion of free oxidized and ligated forms of the oxidized enzyme (472 nm) rather than the maximum for the free enzyme (446 nm). This wavelength more cleanly defines reduction. However, use of 446 nm gives a qualitatively similar chain length response (not shown). The intensity of the charge transfer band observed with saturating levels of 3-thiaacyl-CoA analogues is compared with that produced by 3-thiaoctanoyl-CoA [△; data from Lau et al. (1988)].

in the difference spectrum whereas the flavin contribution alone would be expected to be slightly negative (see inset). In contrast, the C14 analogue (and the C16 analogue; not shown) gives a strong positive maximum at 312 nm, close to the wavelength of the free ligand. Note, however, that free ligand contributes insignificantly to these spectra (see earlier) and all chain lengths induce red-shifts similar to the flavin spectrum evident from the peak at 490 nm.

An estimate of the spectrum of 4-thia-*trans*-2-enoyl-C8 and C14 thioesters bound to the medium chain dehydrogenase at pH 7.6 is shown in Figure 5. It was obtained making the reasonable assumption (see Discussion) that the similarities in the flavin difference spectra between 4-thiaenoyl-CoA and normal enoyl-CoA products noted earlier extend below 390 nm where they are obscured by the strong absorbance of the 4-thiaenoyl chromophore. Note that 4-thia-C12 and C13 show intermediate behavior with contributions at both 312 and 340 nm (Figure 4). This behavior can be simulated by linear combinations of the polarized and unpolarized spectra (not shown). Further evidence for an equilibrium between two forms of the enoyl-CoA chromophore on the enzyme is provided by pH effects (see later).

The Chain Length Dependence of the Polarization of the Enoyl Chromophore Correlates with the Extent of Flavin Reduction by 4-Thiaacyl-CoA Substrates. The spectral changes seen above reflect the binding of 4-thiaenoyl-CoA product analogues to the oxidized enzyme. Figure 6 shows that chain length discrimination is also observed at the level of the more catalytically significant internal redox equilibrium for the reductive half-reaction:

$$E{\cdot}FAD_{ox}{\cdot}\text{4-thiaacyl-CoA} \overset{K_2}{\rightleftharpoons} E{\cdot}FAD_{2e}{\cdot}\text{4-thiaenoyl-CoA}$$

Saturating levels of 4-thia- substrates (e.g., compound 10; Chart 1) were added to the oxidized medium chain acyl-CoA dehydrogenase and the extent of reduction plotted as a function of chain length in Figure 6 (square symbols). Note that there is a marked decrease in the degree of reduction between C8 and C16 analogues with the transition half-complete between C12 and C13. Similar behavior has been observed with normal substrates of the medium chain acyl-CoA dehydrogenases (Beinert, 1963; Hall et al., 1979; Thorpe et al., 1979; Ikeda et al., 1985). Figure 6 also shows that the extent of polarization of the enoyl chromophore in the abortive $E.FAD_{ox}$·4-thiaenoyl-CoA complexes follows a similar chain length dependence (circles). Thus the polarization of the carbonyl group observed with these product analogues appears to have general relevance for the reductive half reaction of the medium chain enzyme. Figure 6 also depicts the intensity of the charge transfer bands observed with 3-thiaacyl-CoA analogues (Lau et al., 1988). This last parameter seems to have the most exacting chain length dependence (Figure 6, triangles) but the general trends are the same for all three.

Polarization of Thioester Is pH Dependent. Figure 4 shows that the difference spectrum induced on binding 4-thia-*trans*-2-tridecenoyl-CoA at pH 7.6 contains about 40% of the component which absorbs at 340 nm. Figure 7 (main panel) shows that the extent of polarization is strongly pH dependent, with lower pH values favoring the 340 nm form. Polarization is lost at higher pH values with an increase in the 312 nm peak. The isosbestic point at 328 nm is consistent with an equilibrium involving two states of the bound ligand (see Discussion). The inset to Figure 7 shows that the apparent pK for this interconversion is 7.5 (circles). The other data represent titrations with 4-thia-*trans*-2-enoyl-CoA analogues from C-8 to C-14 over a pH range of 6−9. Apparent pK values range from 6.6 for C14 to greater than 9 for C8 (the dotted line is a fit to a pK of 9.3).

DISCUSSION

The most obvious role for the thioester carbonyl group of acyl-CoA substrates is to acidify the α-protons such that removal of the *pro-R*-α-proton by the acyl-CoA dehydrogenase is sufficiently rapid to sustain catalysis. Thus the pK of the α-protons of uncomplexed acyl-CoA derivatives are about 21 (Amyes & Richard, 1992), whereas the corresponding protons in alkyl-SCoA thioethers have pK values of around 40. Further marked acidification of the

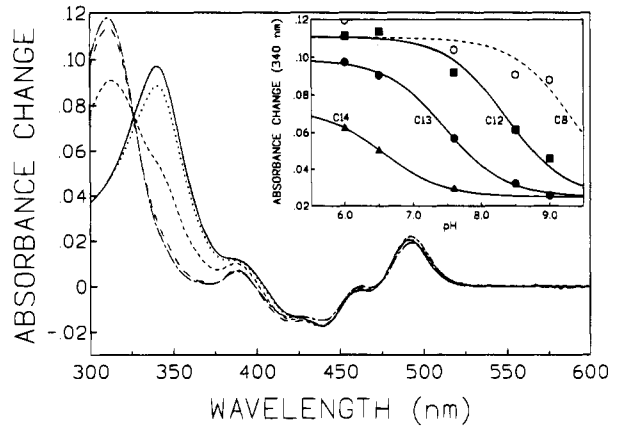


FIGURE 7: pH Dependence of the polarization observed in 4-thia-*trans*-2-tridecenoyl-CoA complexes with the oxidized enzyme. Extrapolated difference spectra for 4-thia-*trans*-2-tridecenoyl-CoA titrations were obtained as in Figure 5 at pH 6.0 (—), 6.5 (• • •), and 7.6 (— — —) using 50 mM phosphate buffer and pH 8.5 (— • —) and 9.0 (— — —) in 50 mM Tris-HCl buffer. The spectrum at pH 9.0 was extrapolated from data at 35% saturation. The pH dependence of the 340 nm absorbance in difference spectra obtained with C8 to C14 enoyl analogues was determined from pH 6.0 to 9.0 and is shown in the inset. The curves are computer generated nonlinear least-square fits reflecting the removal of a single proton with apparent pK values of 9.3, 8.4, 7.5, and 6.6 using an absorbance limit at high pH of 0.025.

pro-R-α-proton accompanies binding of acyl-CoA thioesters to the dehydrogenases with a strong preferential binding of the deprotonated enolate to the enzyme (Lau et al., 1988; Ghisla et al., 1994; Thorpe & Kim, 1995). The crystal structure of the medium chain acyl-CoA dehydrogenase shows that the substrate carbonyl oxygen atom makes two hydrogen-bonding interactions within the enzyme: with the peptide N-H of GLU376 and with the 2′-OH group of the ribityl side chain of the FAD prosthetic group (Kim et al., 1993, 1994). Removal of this latter interaction by reconstitution of the medium chain enzyme with 2′-deoxy-FAD leads to an essentially inactive enzyme (Ghisla et al., 1994). Thus these hydrogen-bonding interactions appear crucial for normal catalysis in the acyl-CoA dehydrogenases. They presumably help stabilize the partial negative charge developed on the carbonyl oxygen atom in the transition state for substrate dehydrogenation (Gerlt & Gassman, 1993; Engel, 1992; Ghisla et al., 1994; Thorpe & Kim, 1995).

Hydrogen-bond formation has been suggested as a significant contributor to the polarization and red-shift of the carbonyl group in keto steroid isomerase (Austin et al., 1992; Hawkinson & Pollack, 1993). In addition, the red-shifts of 20−90 nm observed on binding a variety of cinnamoyl-CoA thioesters to enoyl-CoA hydratase (D'Ordine et al., 1994) are clearly relevant to the present studies. Raman and NMR spectroscopy on enoyl-CoA hydratase complexes suggest that polarization is largely confined to the acryloyl-CoA moiety of cinnamoyl-CoA and results in the development of a partial positive charge at the β-carbon (D'Ordine et al., 1994). Comparable resonance Raman experiments with *trans*-2-octenoyl-CoA complexes of the reduced medium chain acyl-CoA dehydrogenase also suggest strong polarization of the thioester carbonyl group with the development of a partial positive charge at the C-3 position (Nishina et al., 1992, 1993). Polarization of enoyl-CoA product is also believed to modulate the redox properties of acyl-CoA dehydrogenase product complexes (Johnson & Stankovich, 1993; Becker et al., 1994).

In addition to the crucial role of hydrogen bonds in polarization of enoyl-CoA derivatives in the acyl-CoA dehydrogenase suggested by the work described above, stacking of the enoyl moiety over the electron-deficient flavin nucleus may also contribute to the modulation of the properties of the bound enoyl-CoA analogues (Nishina et al., 1993). Thus any movement of the thioester carbonyl group out of its binding pocket in the acyl-CoA dehydrogenase would disrupt hydrogen-bonding interactions and also perturb the orientation of the enoyl moiety with respect to the flavin (Kim et al., 1993, 1994). Both effects are likely to disrupt polarization of the enoyl-CoA derivatives within the active center of the acyl-CoA dehydrogenase.

Figure 6 shows that three distinct equilibrium properties of the enzyme show a very similar chain length dependence. Polarization of the enoyl-CoA moiety within the oxidized enzyme at pH 7.6 does not involves a covalent transformation of the bound ligand and is half-maximal at a chain length of about C13. The internal equilibrium K_2 (eq 1), involving dehydrogenation of the substrate and transfer of a hydride equivalent to the enzyme, also shows a similar chain length dependence. Modulation of the redox potential of bound enoyl-CoA product will depend on the polarization of the enoyl moiety (Johnson & Stankovich, 1993; Becker et al., 1994) and therefore affect K_2. Finally, α-proton abstraction, uncoupled from subsequent β-hydride transfer (Lau et al., 1988), is also optimal with medium length chains and declines sharply with increasing chain length. Lowering the pK of the bound acyl-CoA thioesters to permit enolate formation requires polarization of the carbonyl group as described above. While there are differences in the exact chain length dependence of the parameters shown in Figure 6, they are all clearly dependent on the polarization of the carbonyl group and they all show a decline centered at about C12 or C13. How, then, are these effects modulated by acyl chain length?

The crystal structure of the medium chain dehydrogenase showed that no discernible changes to tertiary or quaternary structure occur on binding thioester ligands from C6 to C12; however, there is movement of the acyl chain within the binding pocket (Kim et al., 1993, 1994). Consistent with this crystallographic work, we have searched for evidence of significant conformational changes by circular dichroism (300−185 nm), by UV-difference spectroscopy, and by protein fluorescence but found no convincing evidence using a variety of thioester and thioether ligands (e.g., 2-aza-octanoyl-CoA, 3-thiaoctanoyl-CoA, 3-ketooctanoyl-CoA, and octyl-SCoA; not shown). It should be noted, however, that this does not mean that structural adjustments to the Michaelis complex are not catalytically unimportant. Thus the studies of Srivastava and co-workers (Johnson et al., 1992; Johnson & Srivastava, 1993) indicate that a relatively slow isomerization of enzyme·indoleacryloyl-CoA complexes does occur. Further, a structural isomerization appears to limit the rate of reduction of the medium chain enzyme by the preferred substrate octanoyl-CoA (Thorpe & Kim, 1995).

The structure of the medium chain dehydrogenase also suggests that the hydrocarbon chain of thioester and thioether CoA derivatives must occupy the same pocket buried deep within the protein matrix (Kim et al., 1994). Both types of ligands must progressively displace the chain of water molecules arranged along this cavity (Kim et al., 1993, 1994), and this desolvation is presumably partly responsible for the similar red-shift observed with a range of alkyl-SCoA

thioethers and thioester ligands (see later). Given a common binding site, how can the apparent incremental binding energy per methylene group (the ascending slopes of Figure 2A,B) be 390 cal for thioethers but about 800 cal for the thioesters? We suggest that the steeper slope shown by thioesters in Figure 2A,B reflects an increasing contribution that the carbonyl oxygen group makes to the observed binding energy as the chain is lengthened. With the shortest chains, the thioester carbonyl group makes a negligible contribution to the apparent binding energy (K_d for acetyl-CoA is 450 versus 300 μM for ethyl-CoA; Powell et al., 1988). Possibly short hydrocarbon chains are inadequately constrained within the active site of the enzyme and allow the carbonyl oxygen atom to make frequent departures from its binding pocket. In addition, the inadequate desolvation of the active center produced by short analogues (Kim et al., 1993, 1994; Powell et al., 1987) would be expected to weaken hydrogen-bonding interactions between the thioester carbonyl group and the enzyme (Cleland, 1992). In contrast, medium chain lengths maximize the interaction between the carbonyl oxygen and the enzyme leading to the optima seen in the binding energy plots shown in Figure 2. Chains longer than C12 approach the bottom of the binding pocket (Kim et al., 1994), and the propagation of unfavorable steric interactions up the chain may decrease the frequency with which an effective alignment of the C1−C3 region of substrate with the active site can be maintained (Kim et al., 1993, 1994; Wang, 1992; Thorpe & Kim, 1995). In particular, oversized chains will spend less time in the strongly hydrogen-bonded polarized state than those of optimal length (see below). Kim et al. (1994) have observed that the addition of tetradecanoyl-CoA, but not shorter substrates, causes cracking of crystals of the medium chain dehydrogenase.

In contrast to thioester ligands, thioethers have no carbonyl interactions to gain or lose as the chain length is extended and hence no discontinuity in plots of binding energy are evident in Figure 2A. Lack of a carbonyl group would enhance the conformational mobility of the hydrocarbon chains and might allow longer chains to be accommodated without the constraining interactions of a carbonyl group.

The observation that dithioesters are poor substrates and ligands of the enzyme suggests that the larger =S moiety is accommodated with some difficulty by the medium chain dehydrogenase. Corroboration comes from molecular modeling studies of complexes of the medium chain acyl-CoA dehydrogenase with octanoyl-CoA and dithiooctanoyl-CoA (see Materials and Methods). Comparison of these structures showed that the 2′-OH- of the ribityl side chain of FAD would have to be rotated away from the carbonyl oxygen of the substrate to accommodate the larger sulfur atom. While some enzymes can tolerate such substitution with little effect (Storer & Carey, 1985), replacement of C=O by C=S generally significantly slows catalysis [e.g., Wlassics et al. (1988) and Anderson et al. (1990)]. Thus, the comparatively weak binding of 2-azadithiooctanoyl-CoA, the low turnover with dithiooctanoyl-CoA, and the molecular modeling results are consistent with a tight interaction between the substrate carbonyl oxygen and its binding pocket in the medium chain dehydrogenase. The substitution of a dithioester for the normal thioester would then have the most impact when the binding contribution of the carbonyl oxygen is maximized. This is what is observed: good thioester ligands such as 2-azaoctanoyl-CoA are drastically affected by replacement of the oxygen with a sulfur atom (80-fold), whereas binding of the 2-azaphenylacetyl-CoA derivative is only weakened 3-fold (see Results).

The correlations observed in Figure 6 suggests that all of the properties plotted are influenced by the polarization of the thioester carbonyl group (see above). These chain length correlations can be explained most simply by a two-state model reflecting the residence time spent in a singular polarized conformation. In contrast, a multiplicity of partially polarized states would be expected to lead to a continuum of wavelengths for the polarized 4-thia-*trans*-2-enoyl-chromophore as well as the charge transfer bands observed with both the oxidized and reduced enzyme (Lau et al., 1988, 1989).

A two-state model also adequately describes the interaction of the medium chain dehydrogenase with indoleacryloyl-CoA (Johnson et al., 1992; see below). However the red-shift of 30 nm observed with 4-thia-*trans*-2-enoyl-CoA thioesters (Lau et al., 1988; this work) is in marked contrast to a shift of only 8 nm reported by Johnson et al. (1992) for indoleacryloyl-CoA. The evidence presented below indicates that indoleacryloyl-CoA is polarized much more dramatically than previously believed. First, there is extensive overlap between the spectrum of indoleacryloyl-CoA (Johnson et al., 1992) and the flavin absorbance envelope (especially around 370 nm; Thorpe et al., 1979). Johnson et al. (1992) showed that the addition of indoleacryloyl-CoA to the medium chain dehydrogenase yields maxima at 375 and 425 nm and ascribed the latter peak to a blue-shifted and intensified flavin contribution. This proposed perturbation is unprecedented in enoyl-CoA complexes of the oxidized acyl-CoA dehydrogenases. Thus, for example, binding normal enoyl-CoA products (Powell et al., 1987; Schopfer et al., 1988), 4-thiaenoyl analogues (Lau et al., 1989; this work), cinnamoyl-CoA thioesters (Murfin, 1974), 3-(2-furyl)acryloyl-CoA (McFarland et al., 1982), and 3-(2-thienyl)acryloyl-CoA (Trievel and Thorpe, unpublished) causes the flavin spectrum to be red-shifted. Such shifts always yield a positive maximum at 490 nm in (E.L − E) difference spectra (e.g., as in that for *trans*-2-octenoyl-CoA; Figure 4). Similar difference spectra are also observed using 2-azaoctanoyl-CoA (Figure 1) and octyl-SCoA (Powell et al., 1987). Thus a red-shift with a peak at about 490 nm is a general consequence of binding a range of acyl-CoA analogues to the medium chain acyl-CoA dehydrogenase. In fact, the difference spectrum obtained with indoleacryloyl-CoA clearly show a small positive peak at 490 nm, although this ligand was suggested to induce a blue-shift in the flavin chromophore (Johnson et al., 1992). The 425 nm absorbance maximum therefore cannot be due to a blue-shifted flavin but must arise from the polarized indoleacryloyl-CoA chromophore. The magnitude of the red-shift (about 60 nm: from 367 to 425 nm) is in line with the highly polarizable indoleacryloyl moiety and compares with shifts of 20−90 nm observed for a series of cinnamoyl-CoA analogues with the enoyl-CoA hydratase (D'Ordine et al., 1994). Thus we ascribe the remaining 375 nm peak to the proportion of bound, essentially unpolarized, indoleacryloyl-CoA. The observation that both unpolarized and polarized peaks coexist on the enzyme is entirely consistent with the results of the present paper (see below).

This revised peak assignment provides a ready explanation for the pH dependence of the spectrum of complexes of the enzyme with indoleacryloyl-CoA (Johnson et al., 1992,

1993). Thus the decrease of the 425 nm peak which is observed as the pH is raised (apparent p*K* 7.45; Johnson et al., 1993) represents the loss of the polarized form of bound indoleacryloyl-CoA. As expected from the work reported here, this decrease is accompanied by a corresponding increase of the unpolarized species at 375 nm (Johnson et al., 1992). These intriguing results with both indoleacryloyl- and 4-thiaenoyl-CoA thioesters prompt two questions. First, what is the origin of these pH effects? Second, why is the apparent p*K* strongly influenced by the structure of the enoyl-CoA analogue used? The catalytic base, Glu376, which abstracts the *pro-R*-α-proton from bound substrates (see earlier), is an obvious candidate for the titrating group, especially since it lies close to the C-2 position of bound substrate. Future work will probe the role of Glu376 in thioester polarization in the medium chain acyl-CoA dehydrogenase and will examine whether the p*K* effects seen in Figure 7 are of catalytic significance.

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