The Reductive Half-Reaction in Acyl-CoA Dehydrogenase from Pig Kidney: Studies with Thiaoctanoyl-CoA and Oxaoctanoyl-CoA Analogues†

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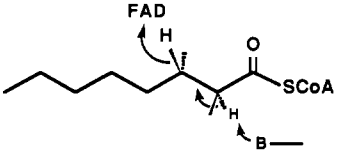
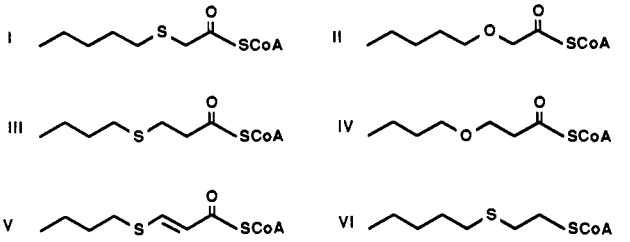
ABSTRACT: Thia- and oxaoctanoyl-CoA derivatives (substituted at the C-3 and C-4 positions) have been synthesized to probe the reductive half-reaction in the medium-chain acyl-CoA dehydrogenase from pig kidney. 3-Thiaoctanoyl-CoA binds to this flavoenzyme, forming an intense, stable, long-wavelength band (at 804 nm; extinction coefficient = 8.7 mM^{-1} cm^{-1} at pH 7.6). The intensity of this band increases about 20% from pH 6.0 to pH 8.8. This long-wavelength species probably represents a charge-transfer complex between bound acyl enolate as the donor and oxidized flavin adenine dinucleotide as the acceptor. Thus, the enzyme catalyzes α-proton exchange, and no long-wavelength bands are seen with 3-thiaoctyl-CoA (where the carbonyl moiety is replaced by a methylene group). 3-Oxaoctanoyl-CoA binds comparatively weakly to the dehydrogenase, with a long-wavelength band at 780 nm which is both less intense and less stable than the corresponding thia analogue. These data suggest that the enzyme can accomplish α-proton abstraction from certain weakly acidic acyl-CoA derivatives, without concerted transfer of a hydride equivalent to the flavin. 4-Thiaoctanoyl-CoA is dehydrogenated in the standard assay 1.5-fold faster than octanoyl-CoA. Titrations of the medium-chain dehydrogenase with the 4-thia derivative resemble those obtained with octanoyl-CoA, except for the contribution of the strongly absorbing 4-thia-*trans*-2-octenoyl-CoA product. The corresponding 4-oxa analogue is a much poorer substrate (10% of the rate shown by octanoyl-CoA) but again effects substantially complete reduction of the flavin chromophore in the dehydrogenase. These studies illustrate the utility of heteroatom-substituted acyl-CoA derivatives in the study of the acyl-CoA dehydrogenases.

Mammalian acyl-CoA[1] dehydrogenases are mitochondrial flavoproteins that catalyze the introduction of a trans double bond between C-2 and C-3 of their acyl-CoA thioester substrates (Beinert, 1963). The reductive half-reaction in these enzymes is probably initiated by removal of the *pro-R* α-hydrogen as a proton (Cornforth, 1959) by an active site base residue (Frerman et al., 1980; Gomes et al., 1981; Fendrich & Abeles, 1982; Ghisla et al., 1984). The *pro-R* β-hydrogen is transferred, probably as a hydride, to the N-5 position of the isoalloxazine ring (Ghisla et al., 1984). Murfin (1974) and Ghisla and co-workers (Pohl et al., 1986) have reported primary deuterium isotope effects at both α- and β-positions with approximately multiplicative effects upon dideuteriation. Similarly, large effects were observed with perdeuteriobutyryl-CoA (Reinsch et al., 1980). Thus α-proton abstraction and β-hydride transfer are thought to be concerted (Murfin, 1974; Ghisla et al., 1984; Pohl et al., 1986) as depicted:



Although this reaction is concerted with normal substrates, we were interested in the possibility of uncoupling proton abstraction from hydride transfer using analogues that lack a β-methylene group. The 3-thia- and 3-oxaoctanoyl-CoA derivatives synthesized in this study (Chart I; compounds I and II) show that proton abstraction and hydride transfer are not obligatorily coupled. This work investigates the nature of the intense long-wavelength bands encountered on binding these substrate analogues to the medium-chain acyl-CoA dehydrogenase from pig kidney. Further, the corresponding 4-substituted thioesters (Chart I; compounds III and IV) have been studied as alternate substrates of the enzyme.

Chart I: Octanoyl-CoA Analogues Used in This Study[a]



[a] Compound I, 3-thiaoctanoyl-CoA [(pentylthio)acetyl-CoA]; compound II, 3-oxaoctanoyl-CoA [(pentyloxy)acetyl-CoA]; compound III, 4-thiaoctanoyl-CoA [3-(butylthio)propionyl-CoA]; compound IV, 4-oxaoctanoyl-CoA [3-(butoxy)propionyl-CoA]; compound V, 4-thia-*trans*-2-octenoyl-CoA [3-(butylthio)acryloyl-CoA]; compound VI, 3-thiaoctyl-CoA [2-(pentylthio)ethyl-CoA].

In general, this work demonstrates the utility of heteroatom-substituted acyl-CoA derivatives as probes for the reductive half-reaction of the acyl-CoA dehydrogenases. These new derivatives may prove useful in the study of other acyl-CoA-requiring enzymes.

Materials and Methods

Materials. Medium-chain acyl-CoA dehydrogenase was isolated from pig kidney as described previously (Gorelick et

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[1] Abbreviations: CoA, coenzyme A; FAD, flavin adenine dinucleotide; HPLC, high-performance liquid chromatography; EDTA, ethylenediaminetetraacetic acid; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

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al., 1985), with incorporation of an octyl-Sepharose column as the final step (Lau et al., 1986). Acyl-CoA oxidase was purified from *Candida tropicalis* as in Jiang and Thorpe (1983). CoASH (lithium salt) and octanoyl-CoA were from B-L Biochemicals.

Preparation of 3-Thiacarboxylic Acids. These acids were made essentially according to the procedure of Uyeda and Reid (1920) for 3-thiaheptanoic acid, substituting an equivalent amount of *n*-pentyl, *n*-octyl-, *n*-decyl-, and *n*-dodecyl mercaptan for the *n*-butyl mercaptan. The steam distillation step was omitted. After acidification, 3-thiaoctanoic acid was purified by distillation under reduced pressure (bp 144–149 °C, 10 mmHg). The higher homologues were purified by recrystallization from heptane. All structures, including those described below, were confirmed by infrared and NMR spectra.

Preparation of 3-Oxaoctanoic Acid. This acid was synthesized essentially as described by Rule et al. (1928).

4-Thiaoctanoic Acid. Nine grams of butyl mercaptan was stirred with 0.1 g of sodium methoxide, and then 10 g of ethyl acrylate was added slowly with cooling to keep the temperature below 80 °C. The mixture was then stirred at room temperature for 1 h and poured into 70 mL of 10% NaOH. The ester was saponified for 1.5 h at 100 °C. After cooling, the mixture was acidified and the resulting oil distilled under reduced pressure (bp 152 °C, 10 mmHg).

4-Oxaoctanoic Acid. The ethyl ester was prepared from 30 mL of *n*-butyl alcohol and 20 g of ethyl acrylate (Rehberg et al., 1946). The crude ester was stirred overnight at room temperature with 12% NaOH. After acidification, the acid was extracted with methylene chloride and dried over $MgSO_4$. Solvent was removed and the acid distilled under reduced pressure (bp 140–142 °C, 10 mmHg).

Preparation and Purification of Thioesters. All thioesters were prepared by the mixed anhydride method (Bernert & Sprecher, 1977) and purified by HPLC as described earlier (Powell et al., 1987) except for 3-thiapentadecanoyl-CoA. The latter was purified by hydrophobic interaction chromatography on octyl-Sepharose as described for hexadecyl-CoA (Powell et al., 1987). CoA derivatives were stored as desalted lyophilized powders and were quantitated by using an extinction coefficient of 16 mM^{-1} cm^{-1} at 260 nm unless otherwise noted. All CoA derivatives were characterized by proton NMR in D_2O using a Bruker 250-mHz spectrometer with an Aspect 3000 computer system. Solutions of these thioesters were stable for several weeks at −20 °C.

Synthesis of 3-Thiaoctyl-CoA. This analogue was prepared by alkylation of CoASH with the alkyl chloride derived from 3-thiaoctan-1-ol. Sodium hydroxide (4.5 g) was dissolved in 40 mL of water followed by 10.2 g of 1-mercaptopentane. 2-Chloroethanol (8 g) was added slowly while the temperature was maintained at about 45 °C. After addition was complete, the biphasic mixture was stirred overnight at room temperature. The oily layer was washed with brine and distilled (bp 116–118 °C, 10 mmHg). Infrared and NMR spectra were consistent with that expected for the alcohol. Thionyl chloride (1 g) was added dropwise to 1 g of the alcohol, stirred for 15 min, and then heated to 100 °C for 15 min. The mixture was cooled to room temperature, and 4 mL of water was added, stirred for 30 min, and then extracted with 5 mL of diethyl ether. The ethereal solution was passed over a short column of anhydrous magnesium sulfate, and then the ether was removed to give a pale yellow oil. An infrared spectrum showed the absence of −OH absorbance. The oil (13 μL) was used to alkylate 40 mg of CoASH as described earlier (Ciardelli et al., 1981; Powell et al., 1987). The thioether was purified and stored as described above.

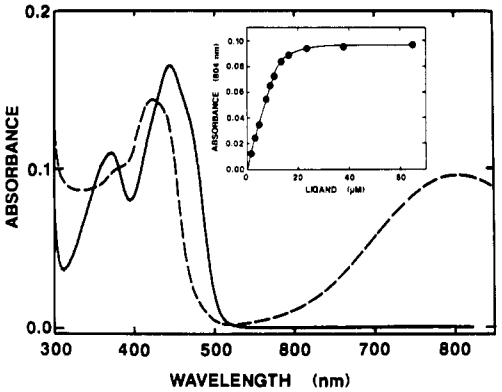


FIGURE 1: Effects of 3-thiaoctanoyl-CoA on medium-chain acyl-CoA dehydrogenase from pig kidney. The oxidized dehydrogenase (11 μM; —) in 0.86 mL of 50 mM phosphate buffer, pH 7.6, 25 °C, was mixed with 23 μM 3-thiaoctanoyl-CoA (---). The inset shows a spectrophotometric titration performed under the same conditions following absorbance changes at 804 nm. The points are best fit by the curve shown (see Materials and Methods; K_d = 0.47 μM with a stoichiometry of 1.1 per flavin).

Spectrophotometric Titrations. Unless otherwise stated, titrations were performed in 50 mM phosphate buffer, pH 7.6, 25 °C, containing 0.3 mM EDTA. Anaerobic experiments were performed as in Mizzer and Thorpe (1981). A Cary 219 spectrophotometer interfaced to a microcomputer was used for data acquisition and manipulation (Powell et al., 1987). Measurement of dissociation constants and binding stoichiometries was as described earlier (Powell et al., 1987). Concentrations of the dehydrogenase were determined by using an extinction coefficient of 15.4 mM^{-1} cm^{-1} at 446 nm (Thorpe et al., 1979).

Enzyme Assays. Assays were performed following the transfer of reducing equivalent to 2,6-dichlorophenolindophenol using 1.4 mM phenazine methosulfate (Thorpe et al., 1979). Standard conditions used 30 μM thioester substrate at pH 7.6, 25 °C.

RESULTS

3-Thiaoctanoyl-CoA. Figure 1 shows the unusually intense long-wavelength band (extinction coefficient of 8.7 mM^{-1} cm^{-1} at 804 nm) generated on addition of 3-thiaoctanoyl-CoA to the oxidized form of the medium-chain acyl-CoA dehydrogenase from pig kidney. The spectral changes are complete before measurement could be made and are stable for at least 3 h under these conditions. Formation of this green enzyme species is accompanied by a blue shift in the major flavin transition with a decrease in the intensity of the 370-nm peak (Figure 1). These spectral features resemble those of a charge-transfer interaction between oxidized flavin as acceptor and an electron-rich donor ligand (Massey & Ghisla, 1974; Abramovitz & Massey, 1976): the acyl-CoA dehydrogenases form a number of such complexes, for example, with CoA persulfide (Williamson et al., 1982a), with 3-ketoacyl-CoA derivatives (Engel & Massey, 1971; Benecky et al., 1979; Thorpe & Massey, 1983), and with *trans*-3-alkenoyl-CoA derivatives (Powell et al., 1987). The nature of the charge-transfer donor in Figure 1 will be discussed later. The points in the inset show absorbance changes at 804 nm during the titration and are fit to a line with a K_d of 0.47 μM and a stoichiometry of 1.1 molecules of ligand per flavin (see Materials and Methods).

Similar spectral changes to those seen at pH 7.6 (Figure 1) are also observed at pH 6.0 and at pH 8.8 (50 mM phos-

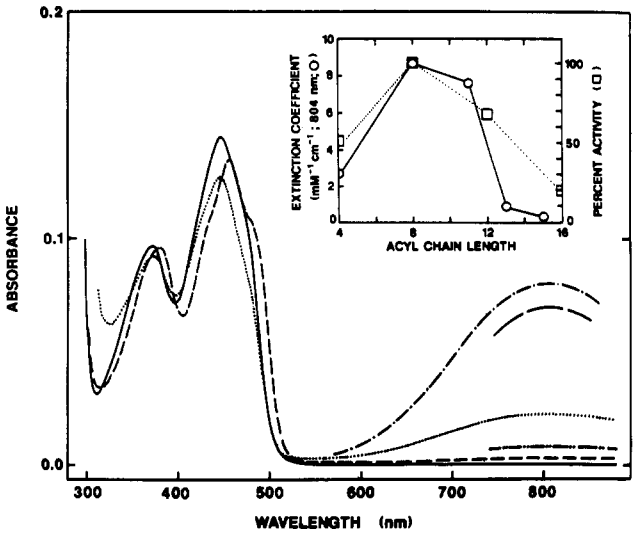


FIGURE 2: Comparison of the maximal spectral changes induced on binding the 3-thia analogues of butyryl-, octanoyl-, undecanoyl-, tridecanoyl-, and pentadecanoyl-CoA to the medium-chain acyl-CoA dehydrogenase. The solid line corresponds to 9.4 μM enzyme in 50 mM phosphate buffer, pH 7.6, 25 °C. The spectral change for the shortest analogue, 3-thiabutyryl-CoA (···) (K_d = 124 μM), was extrapolated to completion. The spectra for the remaining cases were recorded in the presence of saturating concentrations of ligand: 3-thiaoctanoyl-CoA (–·–), 3-thiaundecanoyl-CoA (––), 3-thiatridecanoyl-CoA (–··–), and 3-thiapentadecanoyl-CoA (–··–). The inset is a plot of the intensity of the long-wavelength bands as a function of chain length (circles). Enzyme activities (squares), extrapolated to saturating levels of butyryl-, octanoyl-, dodecanoyl-, and palmitoyl-CoA (see Materials and Methods), are expressed as a percentage of the rate with octanoyl-CoA.

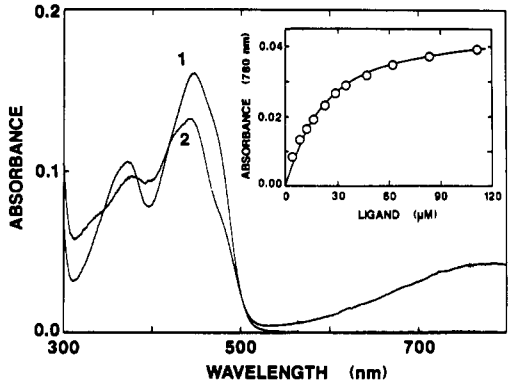


FIGURE 3: Spectral changes observed on the addition of 3-oxaoctanoyl-CoA to the medium-chain acyl-CoA dehydrogenase. The oxidized dehydrogenase (curve 1; 10.3 μM in 50 mM phosphate buffer, pH 7.6, 25 °C) was mixed with 139 μM 3-oxaoctanoyl-CoA and the spectrum recorded immediately (curve 2). The inset shows a titration monitored at 780 nm using 10.3 μM enzyme and is fit to a curve corresponding to a K_d of 14 μM.

phate and Tris-HCl, respectively) with no significant change in the position of the long-wavelength band. The intensity of this band increases slightly over this range however (corresponding to 7.1, 8.7, and 9.1 mM^{-1} cm^{-1} at pH values 6.0, 7.6, and 8.8, respectively). Except where indicated below, spectrophotometric titrations were routinely conducted at pH 7.6.

3-Thiaoctanoyl-CoA can be readily displaced from the enzyme upon the addition of an excess of the tightly binding substrate octanoyl-CoA (apparent K_d = about 20 nM; Thorpe et al., 1981) with disappearance of the 804-nm band (90% decrease on the addition of a 2.2-fold excess of substrate over 3-thiaoctanoyl-CoA; data not shown). Reduction of the flavin is accompanied by the appearance of the characteristic band at 570 nm ascribed to a reduced flavin–enoyl-CoA charge-transfer complex (Engel & Massey, 1971; Massey & Ghisla, 1974). Further, 3-thiaoctanoyl-CoA is an ineffective inhibitor of the dehydrogenation of octanoyl-CoA in the standard assay system (see Materials and Methods): when both substrate and analogue are present at 30 μM concentration, the initial rate decreased by only 10%. As expected from Figure 1, 3-thiaoctanoyl-CoA does not appear to reduce the bound prosthetic group of the dehydrogenase and is not itself a substrate in the standard assay system (data not shown).

Spectral Effects of 3-Thiaacyl-CoA Analogues of Differing Chain Lengths. Pig kidney medium-chain acyl-CoA dehydrogenase shows optimal activity toward acyl-CoA of medium chain length with considerably less activity toward butyryl- and palmitoyl-CoA (Thorpe et al., 1979; inset, Figure 2). It was therefore of interest to examine the response of the dehydrogenase to analogues that were either shorter or longer than 3-thiaoctanoyl-CoA. The spectral changes at saturation for 3-thia chains containing 4, 8, 11, 13, and 15 members are shown in Figure 2. Increasing chain length influences the intensity, but not the position, of the long-wavelength band. In the case of the longest analogue tested, 3-thiapentadecanoyl-CoA, the spectrum is dominated by a pronounced red shift and resolution of the main flavin envelope typical of the binding of an alkyl chain to the dehydrogenase (Figure 2; Powell et al., 1987). A very small long-wavelength band is observed, however, amounting to about 3% of that seen with 3-thiaoctanoyl-CoA. The intensity of this band is not significantly increased at pH 8.8 (data not shown). As expected (Powell et al., 1987), the shortest analogue, 3-thiabutyryl-CoA, binds relatively weakly to the medium-chain dehydrogenase (K_d = 124 μM; data not shown). The extrapolated intensity at 804 nm (Figure 2) is 31% of that seen with 3-thiaoctanoyl-CoA.

The inset to Figure 2 plots the intensity of the long-wavelength bands observed with 3-thiaacyl-CoA derivatives as a function of chain length. Interestingly, a qualitatively similar response is seen for the chain-length dependence of the maximal activity of the medium-chain dehydrogenase with normal substrates (butyryl- to palmitoyl-CoA; e.g., see inset, Figure 2).

3-Oxaoctanoyl-CoA. An obvious candidate for the charge-transfer donor in these 3-thiaacyl-CoA complexes is the thioether sulfur atom. Precedent for charge-transfer complexes between sulfur as the donor and oxidized flavin as the acceptor exists in the green species formed upon the addition of CoA persulfide to the acyl-CoA dehydrogenases (Williamson et al., 1982a,b) and in the two-electron-reduced form of glutathione reductase and related enzymes (Williams, 1976). If this ascription is correct, substitution of the sulfur atom for an oxygen atom, as in compound II (Chart I), would be expected to lead to a large (about 300-nm) blue shift in the long-wavelength band. This is because ethers have ionization potentials about 1.2 eV larger than those of thioethers (Rosenstock et al., 1977). In fact the 3-oxa analogue yields a long-wavelength band at 780 nm when added to the medium-chain enzyme (Figure 3), only about 25 nm blue shifted from the thia derivative. This strongly suggests that the heteroatom is not the charge-transfer donor in these complexes (see below).

The new band in Figure 3 is less intense than observed with the sulfur derivative (extrapolated extinction coefficient of 4.3 mM^{-1} cm^{-1}). Further, it is unstable, decaying with a half-time of about 6 h (after the addition of 340 μM 3-oxaoctanoyl-CoA to 27 μM dehydrogenase at 25 °C) with the reappearance of a spectrum resembling uncomplexed enzyme. This effect does not reflect instability of 3-oxaoctanoyl-CoA in free solution

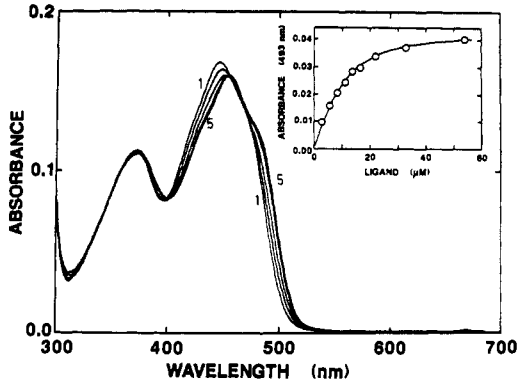


FIGURE 4: Binding of 3-thiaoctyl-CoA to the pig kidney dehydrogenase. The enzyme (10.8 μM in 0.865 mL of 50 mM phosphate buffer, pH 7.6, 25 °C; curve 1) was titrated with 2.3, 8.3, 22, and 54 μM 3-thiaoctyl-CoA (curves 2, 3, 4, and 5, respectively). Intermediate spectra are omitted for clarity. The inset shows absorbance changes at 493 nm (O) with the line corresponding to a K_d of 3.6 μM and a stoichiometry of 1 per flavin.

(data not shown) but is a characteristic of the complexed ligand. A similar series of spectral changes is seen under anaerobic conditions (data not shown). The long-wavelength band seen in Figure 3 can be readily displaced by the addition of excess octanoyl-CoA, as observed with 3-thiaoctanoyl-CoA. In contrast, the species formed after its decay (see above) is not reducible by substrate and will be the subject of a future investigation.

The dissociation constant for binding the 3-oxa analogue to the dehydrogenase was estimated as 14 μM by titrations monitored at 780 nm (see legend, Figure 2). Binding is some 25-fold weaker than for the corresponding 3-thia derivative. It seems unlikely that this difference derives from steric or electronic effects alone, but it might reflect a less favorable association of the more polar ether with the hydrocarbon binding cleft of the medium-chain enzyme (Powell et al., 1987).

As expected, 3-oxaoctanoyl-CoA is neither a substrate nor a significant inhibitor of the dehydrogenase (98% activity is retained when 30 μM 3-oxaoctanoyl-CoA is included in the standard assay system; see Materials and Methods).

3-Thiaoctyl-CoA. Further evidence against the heteroatom at position 3 serving as the charge-transfer donor in Figures 1–3 is provided by 3-thiaoctyl-CoA (compound VI, Chart I). This derivative retains a heteroatom at position 3, but the carbonyl group previously at C-1 is now a methylene group. This dithioether analogue binds to the medium-chain enzyme with no significant long-wavelength band up to 900 nm (Figure 4). In fact, the spectral changes shown in Figure 4 are very similar to those produced by octyl-CoA, where the 3-thia substituent is replaced by a methylene group (Frerman et al., 1980; Powell et al., 1987). Both derivatives bind with comparable K_d values (4.0 and 3.6 μM for octyl- and 3-thiaoctyl-CoA, respectively), suggesting, in the absence of compensating factors, that the 3-sulfur substituent is a valid replacement for a methylene group.

Thus it appears that the charge-transfer bands formed on binding compounds I and II to the medium-chain dehydrogenase are not due to the heteroatom per se but are associated with the carbonyl oxygen in some way (see Discussion).

4-Thiaoctanoyl-CoA. An anaerobic titration of the medium-chain acyl-CoA dehydrogenase with 4-thiaoctanoyl-CoA (compound III, Chart I) is shown in Figure 5. The titration resembles that of octanoyl-CoA (inset, Figure 5), a preferred substrate of the enzyme. In both cases, there is extensive

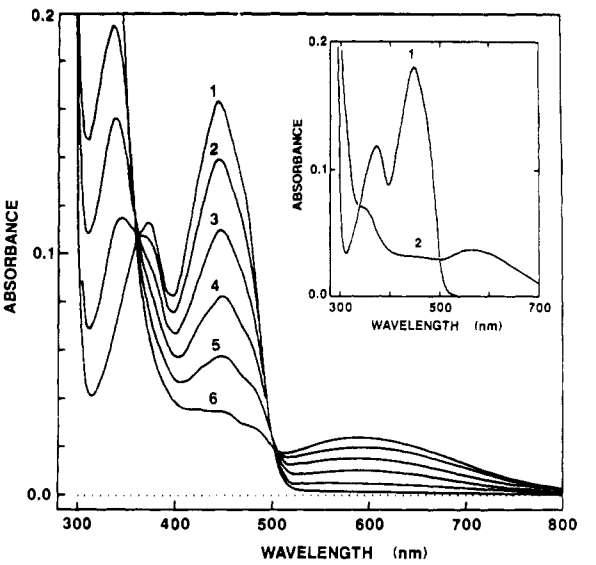


FIGURE 5: Anaerobic titration of medium-chain acyl-CoA dehydrogenase with 4-thiaoctanoyl-CoA. The oxidized dehydrogenase (10.4 μM enzyme in 1.28 mL of 50 mM phosphate buffer, pH 7.6, 25 °C) was deoxygenated (curve 1) and titrated with 3.1, 6.1, 9.2, 12.2, and 21 μM 4-thiaoctanoyl-CoA (curves 2, 3, 4, 5, and 6, respectively). The inset shows a spectrum of the dehydrogenase (curve 1) reduced with excess octanoyl-CoA (curve 2).

bleaching of the flavin chromophore and the appearance of a long-wavelength band (maximal at 570 nm with octanoyl-CoA and at about 585 nm with the 4-thia analogue). In addition, however, a prominent peak at 340 nm develops during reduction of the flavin with 4-thiaoctanoyl-CoA which is not apparent with the normal substrate (see inset, Figure 5). This new feature is due to the bound 4-thia-*trans*-2-octenoyl-CoA product (see below). The spectral changes resulting after each addition of titrant are complete before measurement could be made and are stable over several hours under anaerobic conditions. The reduced enzyme is however less resistant toward oxygen than the extremely air stable complexes formed when octanoyl-CoA is the reductant (Beinert & Page, 1957). Thus, the decay of the long-wavelength band generated on the addition of 33 μM 4-thiaoctanoyl-CoA to 11 μM dehydrogenase in aerobic phosphate buffer (pH 7.6, at 25 °C) is half complete in 100 min. Under comparable conditions, no appreciable decay is seen over 2 h when octanoyl-CoA is the reductant.

Compound III is a surprisingly good substrate of the dehydrogenase. In the standard assay (see Materials and Methods; whereby reducing equivalents are transferred from substrate to enzyme and then passed from phenazine methosulfate to the terminal acceptor 2,6-dichlorophenolindophenol) the 4-thia analogue is oxidized 1.5-fold faster than octanoyl-CoA.

The putative product of the dehydrogenation of 4-thiaoctanoyl-CoA was prepared by enzymatic oxidation of this substrate with the peroxisomal acyl-CoA oxidase from *C. tropicalis* (Lau, Brantley, and Thorpe, unpublished observations). The unsaturated 4-thia derivative (compound V, Chart I) exhibits a strong new band at 312 nm (extinction coefficient of 21 mM^{-1} cm^{-1}). This band is shifted to 340 nm on binding to the oxidized medium-chain dehydrogenase (data not shown), suggesting that the peak at this position in Figure 5 is due to 4-thia-*trans*-2-octenoyl-CoA binding to the reduced enzyme (Lau, Brantley, and Thorpe, unpublished observations). Characterization of the properties of this interesting enoyl-CoA derivative will appear in a separate paper.

4-Oxaoctanoyl-CoA. In marked contrast to the thia analogue, 4-oxaoctanoyl-CoA is a poor substrate of the medium-

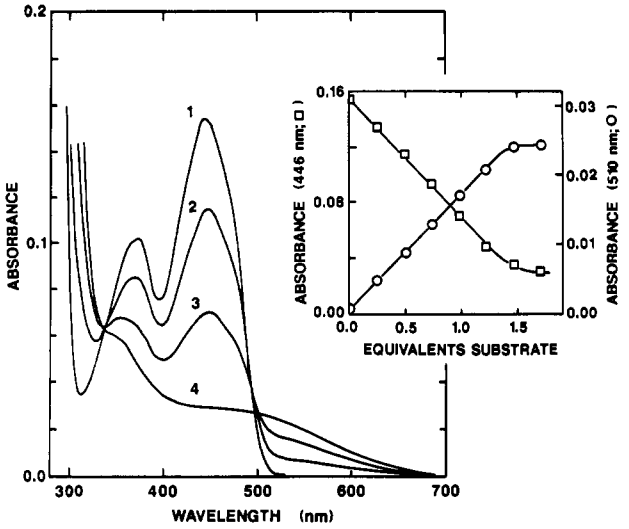
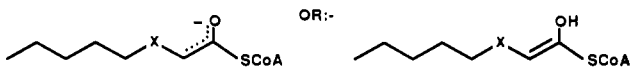


FIGURE 6: Anaerobic titration of the kidney dehydrogenase with 4-oxaoctanoyl-CoA. The dehydrogenase was deoxygenated (curve 1; 10 μM enzyme in 50 mM phosphate, pH 7.6, 25 °C) and titrated with 4.9, 9.8, and 17 μM 4-oxaoctanoyl-CoA (curves 2, 3, and 4, respectively). Intermediate spectra are not shown for clarity. The inset records the changes in absorbance at 446 and 510 nm throughout the titration.

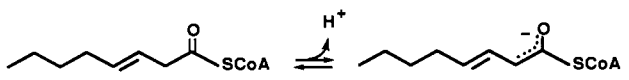
chain dehydrogenase, being dehydrogenated at about 10% of the rate of octanoyl-CoA in the standard assay. A spectrophotometric titration of the dehydrogenase by 4-oxaoctanoyl-CoA is shown in Figure 6. Reduction is accompanied by a long-wavelength band that extends into the flavin absorption envelope. Enzyme reduced with 4-oxaoctanoyl-CoA is reoxidized slightly more rapidly by molecular oxygen (half complete in 65 min when 11 μM enzyme is reduced with 3 equiv of 4-oxaoctanoyl-CoA in air-saturated buffer) than in the corresponding 4-thiaoctanoyl-CoA case.

Discussion

An important question raised by this work is the nature of the charge-transfer donor in the long-wavelength bands seen in Figures 1–3. Evidence has been presented that appears to rule out the 3-heteroatom as the donor, and which suggests the involvement of the thioester carbonyl group (see above). Clearly, the thioester per se would not be expected to be the donor but would yield either the enolate or the enol on α-proton abstraction (see below):



Consistent with this ascription, one α-proton is rapidly exchanged in D_2O upon incubation of 8 mM 3-thiaoctanoyl-CoA with 5 μM medium-chain dehydrogenase ($t_{1/2}$ = 12 min; in 50 mM phosphate buffer, pD 6.0; data not shown; Ghisla et al., 1984). Of the two species shown above, the enolate (left) would represent a much better charge-transfer donor (Ellison et al., 1982). Similarly, α-proton abstraction yielding a delocalized enolate has been invoked to explain the intense 820-nm band observed on the addition of *trans*-3-octenoyl-CoA to the medium-chain enzyme (Powell et al., 1987):



Indeed, the spectra of the 3-thiaoctanoyl-CoA and the *trans*-3-octenoyl-CoA complexes of the dehydrogenase are rather similar. Further, the *trans*-3-octenoyl derivative is also subject to rapid α-proton exchange (Powell et al., 1987), again presumably involving the *pro-R* proton by analogy with normal substrates (Biellmann & Hirth, 1970; Murfin, 1974; Ghisla et al., 1984; Ikeda et al., 1985).

Spectroscopic evidence for enolate complexes of the comparatively acidic 3-ketoacyl-CoA derivatives (pK 8–9; Auer & Frerman, 1980; Gilbert et al., 1981) with acyl-CoA dehydrogenases has been obtained (McKean et al., 1979; Benecky et al., 1979; Williamson et al., 1982b; Powell et al., 1987). In addition to a prominent increase in absorbance at 300 nm due to the bound enolate, a new charge-transfer band at about 570 nm is observed in these complexes.

These considerations suggest that the long-wavelength bands observed upon addition of 3-thia and 3-oxa analogues to the oxidized dehydrogenase represent charge-transfer complexes between their enolates and oxidized flavin. What then are the likely pK values for the α-protons of these analogues in free solution, and how might these enolates be stabilized within the active center of the dehydrogenase?

The α-protons in acetyl-CoA are believed to have a very similar pK value to those of acetone in water (i.e., about 20; Wessely & Lynen, 1953; Gilbert, 1981; Chiang et al., 1984), reflecting the basic similarity of the carbonyl group in thioesters and ketones (El-Assar et al., 1982). The corresponding α-protons in longer chain thioesters such as octanoyl-CoA are likely to be of similar acidity (Bordwell et al., 1977a). Thioether sulfur atoms have a marked tendency to increase the kinetic and thermodynamic acidity of adjacent C–H bonds (Cram, 1965; Price & Oae, 1962), possibly via conjugation between carbanion and the sulfur d orbitals. This acidification is less pronounced when oxygen is substituted for sulfur (Bordwell et al., 1976). Thus, CH_3O– and CH_3S– substituents at the α-position lower the pK of the parent phenyl ketone by about 2 and 5 pH units, respectively (Bordwell et al., 1976). Thus we may expect the α-protons of 3-thiaoctanoyl-, 3-oxaoctanoyl-CoA, and octanoyl-CoA to have approximate pK values of 15, 18, and 20, respectively. *trans*-3-Octenoyl-CoA would be expected to have a corresponding pK of about 19 (Fedor & Gray, 1968). Thus all these analogues remain only weakly acidic in free solution: their carbanions would require considerable thermodynamic stabilization to populate the active site of the dehydrogenase significantly.

The general issue of the formation and stabilization of weakly acidic enolate carbanions has been considered by a number of workers (Thibblin & Jencks, 1979, Jencks, 1975; Rose, 1975; Gilbert, 1981). One strategy for promoting ionization in the acyl-CoA dehydrogenases is to increase the strength of the base that functions in α-proton abstraction. This residue may well be a glutamate carboxylate (Fendrich & Abeles, 1982; Powell and Thorpe, unpublished observations), and desolvation upon substrate binding would be expected to raise its pK markedly. Second, there could be electrostatic and structural complementarity with the planar enolate, e.g., by placement of a suitable positive charge or dipole adjacent to the enolate oxygen, and possibly by stacking over the planar electron-deficient isoalloxazine ring. There is presumably no free energy minimum corresponding to the carbanion along the reaction coordinate for the dehydrogenation of normal substrates: the reaction proceeds with concerted elimination of the hydride to give the *trans*-2-alkenoyl product (Murfin, 1974; Ghisla et al., 1984; Ghisla, 1984; Pohl et al., 1986). This discharge of reducing equivalents into the flavin is prevented with the 3-hetero-substituted thioesters, and the enolate accumulates. In these cases at least, α-proton abstraction can occur without being obligatorily coupled to

hydride transfer (Jencks, 1981).

The factors that drive the enolization of these analogues within the active site may become clearer when a high-resolution structure of the medium-chain acyl-CoA dehydrogenase becomes available (Kim et al., 1984). It is likely that a significant fraction of the intrinsic binding energy of these thioesters is utilized in promoting this unfavorable ionization (Jencks, 1975). Possibly, the 3-heteroatom-substituted and *trans*-3-alkenoyl thioesters are preceived by the dehydrogenase as hybrids between substrate and product. Their α-positions are tetrahedral and substratelike, whereas their β-positions are more or less planar, resembling the *trans*-2-alkenoyl product. Enolization of the α-position would render both loci approximately planar and leave a negative charge on the resulting ligand. Preferential binding of anionic CoA derivatives to the acyl-CoA dehydrogenases has already been noted (Powell et al., 1987).

Finally, the rapid dehydrogenation of 4-thiaoctanoyl-CoA, taken with the correlation between the catalytic specificity of the dehydrogenase toward normal substrates and the chain-length dependence of the intensity of the long-wavelength band in the 3-thia series, argues strongly for the validity of a thioether sulfur atom as a replacement for a methylene group in acyl-CoA derivatives. Ether linkages appear less satisfactory in the acyl-CoA dehydrogenase case, presumably because of their increased polarity and decreased polarizability. Clearly, the interaction between heteroatom-substituted acyl-CoA derivatives and other CoA-dependent enzymes deserves more study.

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Kinetics of Inactivation of Creatine Kinase during Modification of Its Thiol Groups†

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ABSTRACT: Kinetics of inactivation and modification of the reactive thiol groups of creatine kinase by 5,5′-dithiobis(2-nitrobenzoic acid) or iodoacetamide have been compared, the former by following the substrate reaction in presence of the inactivator [Wang, Z.-X., & Tsou, C.-L. (1987) *J. Theor. Biol. 127*, 253]. The microscopic constants for the reaction of the inactivators with the free enzyme and with the enzyme–substrate complexes were determined. From the results obtained it appears that with respect to ATP both inactivators are noncompetitive whereas for creatine iodoacetamide is competitive but DTNB is not. The formation of the ternary complex protects against the inactivation by both DTNB and iodoacetamide. The inactivation kinetics is monophasic with both inactivators, but under similar conditions, the modification reactions in the presence of the transition-state analogue of creatine–ADP–Mg^{2+}–nitrate show biphasic kinetics as also reported by Price and Hunter [Price, N. C., & Hunter, M. G. (1976) *Biochim. Biophys. Acta 445*, 364]. If the reactive ternary complex and the enzyme complexed with the transition-state analogue react in the same way with these reagents, the modification of one fast-reacting thiol group for each enzyme molecule leads to complete inactivation, indicating that the enzyme has to be in the dimeric state to be active.

Some years ago, a systematic study on the kinetics of irreversible modification of enzyme activity was presented [Tsou, 1965a,b; see Tian and Tsou (1982)]. From the equations derived for the substrate reaction in the presence of the modifier, the rate constant for the irreversible inhibition of enzyme activity can be obtained in one single experiment. Recently, this method has been employed for the determination of the rate constants of irreversible modification of a number of enzymes in different laboratories (Tian & Tsou, 1982; Bieth, 1984; Harper & Powers, 1984; Harper et al., 1985; Mason et al., 1985; Liu & Tsou, 1986). In the above studies, the kinetics of irreversible inhibition of enzymes reacting with a single substrate was considered, and the kinetic treatment has since been extended to enzyme reactions involving two substrates (Wang & Tsou, 1987).

Creatine kinase (EC 2.7.3.2) is a dimeric enzyme composed of identical subunits with eight Cys residues (Putney et al., 1984). Of the eight thiol groups of the native enzyme, only two are capable of reacting with a number of modification reagents, and the modified cysteine residues are situated at the same position in the primary sequence of each subunit (Zhou & Tsou, 1987). They are believed to be essential for the activity of the enzyme on the basis of extensive modification studies with a large number of reagents including *N*-ethylmaleimide (Ennor & Rosenberg, 1954), iodoacetic acid (Watts & Rabin, 1962), iodoacetamide and derivatives (Price, 1979), 2,4-dinitrofluorobenzene (O'Sullivan & Chen, 1966), (dimethylamino)naphthalenesulfonyl chloride (Brown & Cunningham, 1970), DTNB[1] (Price & Hunter, 1976), and iodomethane (Reddy & Watts, 1979). It has also been reported that substrates protect these thiol groups from modification (Watts & Rabin, 1962; Price & Hunter, 1976), and a mechanism of action of this enzyme with an SH group in each subunit taking an active part was proposed by Watts and Rabin (1962). It was also reported that the reaction between these two thiol groups with some reagents shows biphasic kinetics in the presence of the transition-state analogue creatine–ADP–Mg^{2+}–NO_3^-. On the basis of some intriguing experiments in which the two reactive thiol groups behave differently, Degani and Degani (1979) suggested that the two subunits of this enzyme are asymmetrically arranged and the

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[1] Abbreviations: DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); Cr, creatine (used only in equations).

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