Inactivation of General Acyl-CoA Dehydrogenase from Pig Kidney by 2-Alkynoyl Coenzyme A Derivatives: Initial Aspects†

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ABSTRACT: Pig kidney general acyl-CoA dehydrogenase is rapidly, stoichiometrically, and irreversibly inactivated by the acetylenic thio ester 2-octynoyl coenzyme A (2-octynoyl-CoA). The inhibitor binds initially to the dehydrogenase with a 10-nm red shift and increased resolution of the flavin chromophore, followed by the generation of a charge-transfer complex between some form of the bound inhibitor and oxidized flavin (λ_{max} 800 nm; ϵ_{app} = 4.5 mM^{-1} cm^{-1}; k_1 = 1.07 min^{-1}, at pH 7.6, 25 °C). The rate of formation of the long wavelength band is increased markedly with increasing pH (pK_{app} = 7.9). This intermediate then decays with release of about 0.6 mol of CoASH at pH 7.6, yielding a final form with a spectrum typical of bound oxidized flavin. Both irreversible inactivation and covalent modification of the protein occur prior to the decay of the long wavelength species. The modified dehydrogenase is not reduced on prolonged anaerobic incubation with the substrate octanoyl-CoA. The inactive enzyme is unusually resistant to dithionite reduction but may be readily photoreduced via the blue semiquinone to the dihydroflavin form. This reduced enzyme is rapidly reoxidized by electron-transferring flavoprotein, the physiological electron acceptor of the dehydrogenase. General acyl-CoA dehydrogenase is also inactivated by 2-pentynoyl- and 2-pentadecynoyl-CoA with formation of an 800-nm band of lower intensity and by propiolyl-CoA, phenylpropiolyl-CoA, and 2-octynoylpantetheine without the appearance of detectable intermediate species. These data are compared with the behavior of acyl-CoA dehydrogenases toward mechanism-based inactivators carrying an acetylene function at C-3, e.g., 3-butynoyl-CoA.

The flavoprotein general acyl-CoA dehydrogenase catalyzes the α-β desaturation of acyl coenzyme A (acyl-CoA) thio esters with transfer of reducing equivalents to electron-transferring flavoprotein (ETF)[1] and thence to the respiratory chain (Beinert, 1963). The reductive half-reaction

$$E\cdot FAD\cdot R\text{-}CH_2CH_2CO\text{-}SCoA \rightleftharpoons E\cdot FADH^-\cdot R\text{-}CH{=}CHCO\text{-}SCoA$$

is probably initiated by abstraction of an α-proton (Cornforth, 1959; Fendrich & Abeles, 1982; Frerman et al., 1980; Thorpe et al., 1981; Wenz et al., 1981, 1982) followed by transfer of a β-hydrogen to the 5-position of the isoalloxazine ring as a hydride (Ghisla et al., 1984).

Several suicide substrates for the acyl-CoA dehydrogenases have been described that are presumably activated by an initial proton abstraction. Two of these, methylenecyclopropylacetyl-CoA and 3,4-pentadienoyl-CoA, form reduced flavin adducts with loss of enzyme activity (Wenz et al., 1981, 1982). Another class, 3-alkynoyl thio esters, attacks the protein moiety probably after base-catalyzed isomerization to the corresponding 2,3-allenic species (Frerman et al., 1980; Gomes et al., 1981; Fendrich & Abeles, 1982). Fendrich and Abeles have identified a glutamate residue as the target of this modification and suggest that this residue may be the active center base that services normal substrate.

This work describes the covalent inactivation of pig kidney general acyl-CoA dehydrogenase by 2-alkynoyl-CoA derivatives:

$$R\text{-}C{\equiv}C\text{-}CO\text{-}SCoA$$

These acetylenic analogues are already Michael acceptors and would not be expected to require further activation by their target enzymes. Nevertheless, the interaction of 2-alkynoyl-CoA derivatives with general acyl-CoA dehydrogenase exhibits several interesting and unanticipated features which are described in this paper. It should be noted that 2-butynoyl-CoA has been shown to slowly inactivate pig heart thiolase (Holland et al., 1973), and Robinson et al. (1963) have found that propiolyl- and 2-butynoyl-CoA inactivate the fatty acid synthetase system. More recently, 2-hexadecynoate has been shown to inhibit fatty acid elongation (Wood & Lee, 1981).

Experimental Procedures

Materials

D-Pantetheine was obtained from Sigma. Ethyl chloroformate, triethylamine, propiolic acid, and phenylpropiolic acid were from Aldrich. 2-Pentynoic acid and 1-tetradecyne were from Farchan Labs Inc. 2-Octynoic acid was purchased from Pfaltz & Bauer. CoASH, lithium salt, and octanoyl-CoA were from P-L Biochemicals and [^{3}H(G)]CoA, 550 mCi/mmol, was purchased from New England Nuclear. 8-Cl-FAD and 5-deaza-FAD were generous gifts from Dr. Vincent Massey. General acyl-CoA dehydrogenase was purified from pig kidney as described previously (Gorelick et al., 1982), except that the Cibacron Blue–Sepharose column step was replaced by a Sephacryl S-200 column (Thorpe et al., 1979). Pig kidney electron-transferring flavoprotein was a gift from Robert

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[1] Abbreviations: DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); ETF, electron-transferring flavoprotein; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane.

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Gorelick (Gorelick et al., 1982).

Methods

General. Unless otherwise stated, all buffers contained 0.3 mM EDTA. UV and visible spectra were recorded on Cary 219 or Perkin-Elmer 552 or 559 spectrophotometers. Fluorescence spectra were recorded on a Perkin-Elmer 650-10S instrument. NMR spectra were recorded on Perkin-Elmer R-12 60-MHz and Bruker Aspect 3000 250-MHz spectrometers. Concentrations of acyl-CoA dehydrogenase and electron-transferring flavoprotein were calculated by using the following extinction coefficients: 15.4 mM^{-1} cm^{-1} at 446 nm (Thorpe et al., 1979), and 13.3 mM^{-1} cm^{-1} at 436 nm (Gorelick et al., 1982), respectively. Enzyme assays were performed as described in these references. Anaerobic experiments were conducted as in Mizzer & Thorpe (1981). Small-scale ultrafiltration was performed by using Centricon microconcentrators (M_r 10000 cutoff; Amicon Corp.) following the manufacturer's instructions. Radiolabeled samples were counted with a Beckman LS-110C scintillation counter and Amersham ACS cocktail. Counting efficiencies for tritium were approximately 20%.

Synthesis of 2-Pentadecynoic Acid. *n*-Butyllithium (7 mL of a 2.5 M solution in hexane) was added dropwise to 3 g of 1-tetradecyne in 50 mL of anhydrous stirred tetrahydrofuran. After 10 min, the solution was mixed with a slurry of powdered dry ice in 50 mL of anhydrous ether. The mixture was allowed to warm to room temperature, and the lithium salt of 2-pentadecynoic acid was extracted with three 50-mL aliquots of water. The extracts were combined, acidified to pH 2 with HCl, and extracted with methylene chloride. The acid had the expected ^1H NMR spectrum (yield = 79%).

Preparation and Purification of 2-Alkynoyl-CoA Derivatives. 2-Octynoyl-CoA was prepared by the mixed anhydride procedure (Bernert & Sprecher, 1977) starting with up to 100 mg of CoASH and purified by chromatography on DEAE-cellulose using an increasing gradient of LiCl in 1 mM HCl (Lau et al., 1977). Fractions were combined according to their UV spectra (see below) and concentrated by lyophilization. The material was then desalted on a Bio-Gel P-2 column and freeze-dried to give a white powder (in yields of 15–40%). The thio ester showed one spot on thin-layer chromatography on cellulose or silica gel plates (butanol/acetic acid/water, 5/2/3 and 6/2/2 v/v; R_f values of 0.67 and 0.50, respectively) using a short wavelength UV light, iodine vapor, or nitroprusside spray (Stadtman, 1957).

2-Octynoyl-CoA at 20 mg/mL in D_2O showed the expected ^1H NMR spectrum. Notably, the terminal methylene group of pantetheine (C-9; Fung et al., 1976) shifts to 3.06 ppm (triplet; two protons) on thioesterification, and the following additional resonances due to the 2-octynoyl moiety are observed: 2.55 ppm (triplet; two protons), C-4 methylene group; 1.62 ppm (multiplet; two protons), C-5; 1.41 ppm (multiplet; four protons), C-6 and C-7 methylene groups; and 0.96 ppm (triplet; three protons), C-8 methyl group.

2-Pentynoyl-, 2-pentadecynoyl-, propiolyl-, and phenylpropiolyl-CoA were prepared as described above, except that 2-pentadecynoyl-CoA was purified by hydrophobic interaction chromatography. The long-chain analogue was dissolved in 2 M LiCl and applied to an octyl-Sepharose column (Merrill et a., 1983; 0.6 × 8 cm) equilibrated with 1 M LiCl. The column was eluted with a decreasing salt gradient (LiCl/water). CoASH and its disulfide elute in 1 M LiCl, whereas the inhibitor is eluted at very low ionic strength.

UV Spectrum and Extinction Coefficient of 2-Octynoyl-CoA. The UV spectrum of 2-octynoyl-CoA shows a peak at 260 nm, a trough at 235 nm, and shoulders at 230 and 210 nm (235/260 nm = 0.45). The thio ester exhibits no significant absorbance at wavelengths greater than 320 nm. An impurity that elutes immediately after 2-octynoyl-CoA on the DE-52 column has an additional prominent peak at 314 nm (absorbance ratio: 314/260 nm = 0.56). This species is thio ester negative when the nitroprusside test is used and does not inactivate general acyl-CoA dehydrogenase. The extinction coefficient of 2-octynoyl-CoA was determined by phosphate analysis using the acid ammonium molybdate/ascorbate method (Ames, 1965) with CoA, and octanoyl-CoA as standards. The value obtained, 20.9 ± 1.1 mM^{-1} cm^{-1} (four determinations) at 260 nm, should be compared to 23 mM^{-1} cm^{-1} for crotonyl-CoA and to the value of 16 mM^{-1} cm^{-1} for octanoyl-CoA (Stadtman, 1957). Alkaline hydrolysis followed by determination of the CoASH released with DTNB cannot be used with 2-alkynoyl derivatives, because the liberated coenzyme A thiolate reacts with the acetylenic function of residual unhydrolyzed thio ester (Stadtman, 1957).

Preparation of 2-Octynoylpantetheine. Pantetheine was generated by borohydride reduction of the disulfide (Gomes et al., 1981). The solution was brought to pH 8 with HCl and thioesterified as described earlier. 2-Octynoylpantetheine was purified by chromatography on silica gel in 96% ethyl acetate/4% methanol. The product showed a single spot on thin-layer chromatography visualized with iodine vapor.

Analysis of Absorbance Traces. Absorbance traces were fit to two sequential first-order reactions

$$A \xrightarrow{k_1} B \xrightarrow{k_2} C$$

(see the text; Frost & Pearson, 1961) in which only B absorbs at 800 nm. The mole fraction of intermediate B is given by

$$\text{mole fraction} = \frac{e^{-\tau} - e^{-K\tau}}{1 - K}$$

where $\tau = k_1 t$ and $K = k_2/k_1$. Mole fractions were calculated by microcomputer as a function of time by using estimated k_1 and k_2 values, plotted on a Houston Instruments HiPlot digital plotter, and compared to absorbance data normalized so that both theoretical and experimental curves had the same peak amplitude. Best fits were decided in most cases by inspection. Alternatively, a nonlinear least-squares fitting program was used (MLAB; Knott, 1979).

Resolution and Reconstitution of 2-Octynoyl-CoA-Treated General Acyl-CoA Dehydrogenase. The dehydrogenase (50 μL of 200 μM in 50 mM phosphate buffer (pH 7.6, 25 °C) was treated for the required time with 2 equiv of 2-octynoyl-CoA, and 50 μL of this solution was quenched into 500 μL of the acid ammonium sulfate/KBr/charcoal suspension used to prepare apoprotein (Mayer & Thorpe, 1981). The apoprotein precipitate was then centrifuged and redissolved with 1 mL of 100 mM Tris buffer, pH 8.9, at 4 °C as described previously (Mayer & Thorpe, 1981). FAD was then added to 50 μM, and the samples were dialyzed overnight vs. 100 mM phosphate buffer, pH 7.6. As a control, samples of enzyme were incubated with additions of water, rather than inhibitor, and taken through the above procedure.

RESULTS

General Aspects of the Interaction of General Acyl-CoA Dehydrogenase with 2-Octynoyl-CoA. Figure 1 shows the spectral changes encountered on the addition of 2-octynoyl-CoA to pig kidney general acyl-CoA dehydrogenase at pH 7.6, 25 °C. Initially there is a rise in long wavelength absorbance (centered at about 800 nm) with a concomitant decrease in intensity and skewing of the main flavin absorbance peak. This

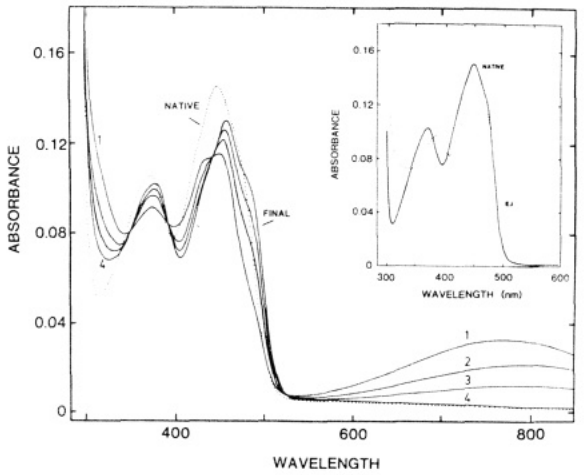


FIGURE 1: Spectral changes observed on the addition of 2-octynoyl-CoA to pig kidney general acyl-CoA dehydrogenase. A solution of enzyme (9.7 μM; dotted line) in 50 mM phosphate buffer, pH 7.6, 25 °C, was treated with 28 μM 2-octynoyl-CoA: curves 1–4 represent spectra recorded at 2 nm/s 1, 13, 29, and 75 min after mixing. The long wavelength region of curve 1 is distorted because the 800-nm species forms relatively slowly (see the text). The inset shows the spectrum recorded at 16 nm/s immediately after the addition of 25 μM inhibitor to 10 μM dehydrogenase at 4 °C in 50 mM phosphate buffer, pH 7.6.

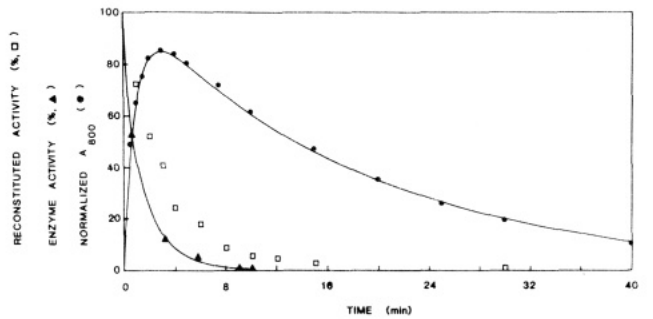


FIGURE 2: Interaction of 2-octynoyl-CoA with general acyl-CoA dehydrogenase. The solid circles represent absorbance data at 800 nm recorded on mixing 4.5 μM dehydrogenase flavin with 13.6 μM 2-octynoyl-CoA in 50 mM phosphate, pH 7.6, 25 °C. The line through the absorbance data points is calculated for two sequential exponential reactions with rate constants of 1.07 and 0.055 min^{-1}, respectively, and represents the concentration of the intermediate species in percent mole fraction (see the text). The absorbance points (●) are normalized to the theoretical curve by matching values at the peak. Samples were withdrawn for activity measurements from an identical incubation and diluted into the standard enzyme assay system (▲) (see Methods). The extent of covalent modification of the enzyme (□) was assessed by preparing apoprotein from enzyme samples incubated for the indicated times and reconstituting with excess FAD (see Methods).

intermediate clearly resembles an oxidized flavin–donor charge-transfer complex (Massey & Ghisla, 1974; see later). This species then decays with loss of the long wavelength band yielding an oxidized flavin spectrum (Figure 1; curve 4), which is both red shifted and more resolved than that of the native enzyme. When experiments of this type are repeated at 4 °C with scanning at 960 nm/min, an additional intermediate is clearly seen immediately after mixing. Compared to the native enzyme, this species shows prominent red shifts of both absorbance peaks, a lowered absorbance at 446 nm, and the appearance of a shoulder at 480 nm (see inset to Figure 1). These spectral changes are consistent with binding of an appropriate CoA derivative to the acyl-CoA dehydrogenases (Steyn-Parvé & Beinert, 1958; Thorpe et al., 1981; Frerman et al., 1980; Thorpe & Massey, 1983).

Enzyme assayed after completion of the spectral changes shown in Figure 1 is inactive with either phenazine methosulfate or electron-transferring flavoprotein to mediate the transfer of reducing equivalents from octanoyl-CoA to 2,6-dichlorophenolindophenol (see Methods). Activity is not regained upon prolonged dialysis in 50 mM phosphate, pH 7.6, neither are there sizable associated spectral changes. Inactivation does not reflect modification of the flavin moiety, since it can be released unchanged on denaturation of the protein (see later).

Since the experiments shown in Figure 1 reveal three distinct species (i.e., the initial enzyme–inhibitor complex, the long wavelength species and the final form), it was of interest to establish at what stage inactivation of the enzyme occurred. Accordingly, the course of an incubation was followed at 800 nm (see Figure 2), and aliquots were removed periodically and assayed. These assays were as linear as those of the native enzyme, and significant reactivation of the treated enzyme did not occur in the presence of a 500-fold excess of the tightly binding substrate, octanoyl-CoA. Further, no additional inactivation occurs after dilution into the assay mixture. Octanoyl-CoA strongly protects acyl-CoA dehydrogenase against inactivation by 2-octynoyl-CoA. Thus, by use of 5.4 μM enzyme in 50 mM phosphate buffer, pH 7.6, with 16 μM 2-octynoyl-CoA, 15% activity remained after 5 min at 25 °C, whereas 94% activity was retained including 50 μM octanoyl-CoA in the incubation. A control sample incubated in the absence of inhibitor lost no significant activity over this time period.

It should be noted that the line drawn through the 800-nm absorbance values in Figure 2 was calculated for two sequential first-order reactions (Frost & Pearson, 1961)

$$A \xrightarrow{k_1} B \xrightarrow{k_2} C$$

in which $k_1 = 1.07$ min^{-1} and $k_2 = 0.055$ min^{-1}. These values were selected by matching real and theoretical data visually. However, very similar values (1.17 ± 0.06 and 0.055 ± 0.002 min^{-1}, respectively) were obtained by using a nonlinear least-squares fitting procedure (see Methods).

Figure 2 also shows the kinetics of covalent modification of the protein. Since [1-^{14}C]-2-octynoyl-CoA was not available for this work, modification of the protein was assessed by quenching samples at the times indicated into the acid ammonium sulfate/activated charcoal suspension which is used to prepare apoprotein (Mayer & Thorpe, 1981; see Methods). The resulting apoproteins were redissolved in Tris buffer, reconstituted with FAD, and assayed (see Methods). Incubation of the enzyme with 2-octynoyl-CoA for long periods results in apoprotein that is still able to bind as much FAD as a control sample but yields a spectrum very similar to that of curve 4 of Figure 1. This reconstituted holoenzyme is inactive (Figure 2), confirming that the protein moiety is the target of this inhibitor. Samples taken at intermediate times exhibit spectra and activities, after reconstitution, which reflect mixtures of native and modified enzymes. Although this method of assessing incorporation is indirect, irreversible covalent attachment to the protein clearly occurs prior to the decay of the long wavelength absorbing species (Figure 2).

The stoichiometry of the interaction of the inhibitor with general acyl-CoA dehydrogenase could be determined spectrophotometrically at 442 nm, which is an isosbestic point for the interconversion of the long wavelength and final species (Figure 1). Absorbances were recorded 10 min after each addition and show an end point corresponding to 1.1 mol of inhibitor per FAD (Figure 3). This stoichiometry is supported by kinetic experiments, following the absorbance at 800 nm with time as in Figure 2. Traces obtained with 1.3, 3, and 8

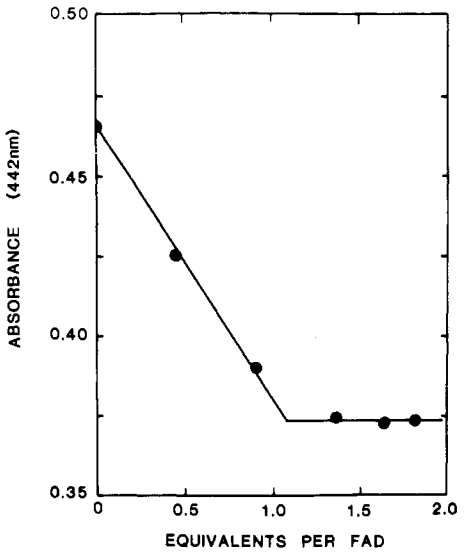


FIGURE 3: Titration of general acyl-CoA dehydrogenase with 2-octynoyl-CoA. The indicated levels of 2-octynoyl-CoA were added to 30 μM of enzyme flavin in 50 mM phosphate buffer, pH 7.6, 25 °C, and the absorbance at 442 nm recorded 10 min after each addition.

equiv of 2-octynoyl-CoA and 10 μM enzyme at pH 7.6, 25 °C, are superimposable (data not shown).

These experiments outline several aspects of the interaction of 2-octynoyl-CoA with general acyl-CoA dehydrogenase which will be amplified later in this paper.

Properties of the Dehydrogenase Modified with 2-Octynoyl-CoA. Native general acyl-CoA dehydrogenase is rapidly and extensively reduced by 1 equiv of octanoyl-CoA with bleaching of 450-nm absorbance and the appearance of a prominent charge-transfer band at 570 nm (Thorpe et al., 1979). In contrast, the treated enzyme undergoes no significant spectral changes on incubation with 10 equiv of substrate over 24 h in phosphate buffer, pH 7.6, 25 °C. The modified enzyme is also apparently unable to bind acetoacetyl-CoA. The oxidized flavin–enolate charge-transfer complex observed with the native enzyme (K_d = 4.3 μM; McKean et al., 1979) is not formed, and no spectral changes were seen with 3.3 μM modified enzyme and 100 μM acetoacetyl-CoA in phosphate buffer, pH 7.6 (data not shown).

Since 2-octynoyl-CoA treatment abolishes the reductive half-reaction in the dehydrogenase, it was of interest to assess whether the derivative could still reduce its physiological electron acceptor, electron-transferring flavoprotein. It was therefore necessary to prepare the dihydroflavin form of the enzyme derivative. This may be readily accomplished, with the native enzyme, by dithionite titration or by catalytic photoreduction (Thorpe et al., 1979) under anaerobic conditions. Interestingly, the inhibited enzyme is extremely slowly reduced by dithionite, with only partial reduction of the flavin over 12 h in the presence of a 6.6-fold excess. No semiquinone species accumulated during this reduction. In contrast, photochemical reduction of the enzyme derivative (see Methods) proceeded smoothly, with the formation of low levels of the blue radical form (apparent ϵ = 1.4 mM^{-1} cm^{-1} at 570 nm). Note that in the native enzyme, bound substrates or products, but not competitive inhibitors, profoundly stabilize the red anionic flavosemiquinone (Mizzer & Thorpe, 1981). After photoreduction, the reduced enzyme derivative was mixed anaerobically with 1 equiv of oxidized ETF from a side arm. The resulting spectrum showed the characteristic red anionic ETF semiquinone (Gorelick et al., 1982), and no further changes were observed over 30 min. This experiment shows

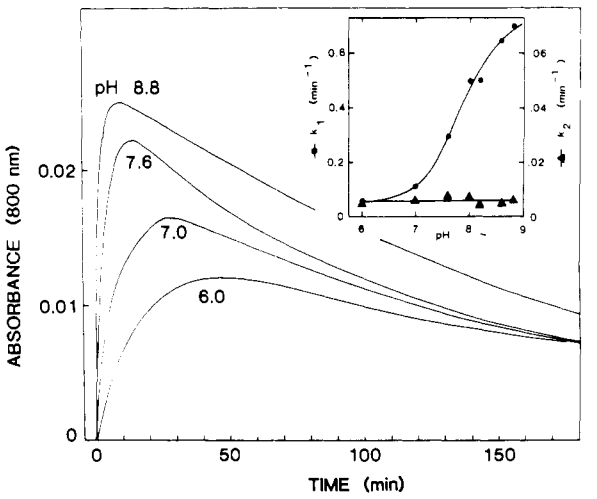


FIGURE 4: pH dependence of the appearance and decay of the 800 nm absorbing species. Absorbance traces were collected at pH values of 6.0, 7.0, 7.6, and 8.0 in 50 mM phosphate buffer, pH 8.2, in 50 mM *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonate buffer, and 8.6 and 8.8 in 50 mM 2-(*N*-cyclohexylamino)ethanesulfonate buffer at 10 °C. The curves were analyzed for two sequential first-order reactions (as described in the text), and the values of k_1 (●) and k_2 (▲) are plotted vs. pH in the inset. Intermediate traces have been omitted for clarity.

that, although the modified enzyme is no longer reducible by octanoyl-CoA, it is still able to transfer electrons to ETF.

pH and Temperature Dependence of the Appearance and Decay of the 800-nm Species. Figure 4 shows absorbance traces collected at pH values between 6.0 and 8.8 with the pH dependence of k_1 and k_2 plotted in the inset. The rate constant for the appearance of the 800 nm absorbing species increases strongly with increasing pH, with an apparent pK of 7.9. In contrast, k_2 is pH independent; hence, the maximal absorbance seen in Figure 4 increase, and the corresponding peak times decrease, with increasing pH (Figure 4). When the peak absorbances are corrected for the mole fractions expected at the peaks (determined by the ratio of k_2/k_1; see Methods), the extinction coefficient of the long wavelength species is found to decrease slightly with decreasing pH (at 8.8, = 5.3 mM^{-1} cm^{-1}; at pH 6.0, 3.2 mM^{-1} cm^{-1}, 10 °C). In contrast to these data, the rate of inactivation of general acyl-CoA dehydrogenase by 3-butynoyl-CoA decreases with increasing pH (apparent pK = 7.0; Frerman et al., 1980).

Lowering the temperature increases the separation between fast and slow phases. Thus, at pH 7.6, the value of k_1 is 3.5-fold slower at 10 °C than at 25 °C, whereas k_2 is correspondingly 7.3-fold slower.

Effects of Other Alkynoyl-CoA Derivatives. Pig kidney general acyl-CoA dehydrogenase has a rather broad substrate specificity, exhibiting a ratio of activities toward butyryl-, octanoyl-, and palmitoyl-CoA of 0.1:1.0:0.2, respectively, in the phenazine methosulfate assay system (Thorpe et al., 1979). It was thus possible that 2-alkynoyl inhibitors of different chain lengths might allow the long wavelength intermediate to accumulate by, e.g., a selective enhancement of the formation of this species. Table I summarizes data obtained with 2-pentynoyl-, 2-octynoyl-, and 2-pentadecynoyl-CoA. Both the C-5 and C-15 compounds are effective inactivators of the dehydrogenase, resulting in complete and irreversible inactivation (see k_i values in Table I). Both form long wavelength intermediates at 800 nm, but the observed maximal intensity of these bands is considerably less than that seen with octynoyl-CoA under the same conditions. This difference is clearly due to a decreased extinction coefficient of the 800-nm species

Table I: Rate Constants from Inactivation and 800-nm Absorbance Data during Interaction of General Acyl-CoA Dehydrogenase with Alkynoyl Thio Ester Derivatives[a]

compound	k_1 (min^{-1})	k_2 (min^{-1})	k_i (min^{-1})	ϵ_{800}, app (mM^{-1} cm^{-1})
2-pentynoyl-CoA	1.4	0.045	0.46	1.8
2-octynoyl-CoA	1.07	0.055	0.6	4.5
2-pentadecynoyl-CoA	0.45	0.116	0.16	2.2
propiolyl-CoA			0.06	0
phenylpropiolyl-CoA			0.46	0
2-octynoylpanthetheine			0.004	0

[a] Determined at 25 °C in 50 mM phosphate buffer, pH 7.6, using 5 μM enzyme and 13.5 μM of the CoA inhibitors. 2-Octynoylpantetheine was used at a much higher concentration of 235 μM with 2.3 μM dehydrogenase. k_1, k_2, k_i, and $\epsilon_{800,app}$ were calculated as described in the text.

in the pentynoyl- and pentadecynoyl-CoA cases. Thus, the ratio of k_2/k_1 for pentynoyl-CoA dictates a corresponding peak mole fraction of 0.89 for the long wavelength intermediate, whereas the observed absorbance is considerably smaller than that observed for octynoyl-CoA (mole fraction at the peak = 0.85 under the same conditions). These trends are in keeping with the spectral behavior of the dehydrogenase upon substrate titrations (Thorpe et al., 1979; Hall et al., 1979).

The data in Table I illustrate that for 2-pentynoyl-, octynoyl-, and pentadecynoyl-CoA the appearance of the long wavelength species (k_1) is faster than the rate of inactivation. This observation has been confirmed repeatedly. The discrepancy is smallest for octynoyl-CoA (1.8-fold and is not immediately apparent on inspection of Figure 2) but is about 3-fold with 2-pentadecynoyl-CoA. These data suggest that there are two 800-nm species with very similar visible spectra. One yields active enzyme when diluted into the standard assay system; the other does not. This aspect has not been investigated further.

Propiolyl-CoA and phenylpropiolyl-CoA also inactivate general acyl-CoA dehydrogenase but without formation of long wavelength bands (Table I). Murfin has utilized dihydrocinnamoyl-CoA as a substrate for general acyl-CoA dehydrogenase, and like octanoyl-CoA, this derivative yields a reduced flavin–enoyl-CoA charge-transfer complex (Murfin, 1974).

2-Octynoylpantetheine. Inactivation of general acyl-CoA dehydrogenase by 2-octynoylpantetheine requires much higher concentrations of thio ester to be effective. Thus, with a 100-fold excess (2.3 μM enzyme and 235 μM inhibitor in 50 mM phosphate, pH 7.6, 25 °C, containing 5% methanol), inactivation is half-complete in 164 min. A control incubation, using the same solvent system minus inhibitor, lost insignificant activity over 30 h. No long wavelength band accompanies inactivation, instead the spectrum of the native enzyme is converted to a resolved red-shifted species similar to that shown by the final form in Figure 1.

Flavin Analogues. The addition of 32 μM octynoyl-CoA to 9 μM 8-Cl-FAD substituted dehydrogenase (Thorpe & Massey, 1983) in 50 mM phosphate buffer, pH 7.6, 25 °C, leads to spectral changes very similar to those shown in Figure 1. Formation of the long wavelength band (λ_{max} 820 nm; maximal apparent extinction coefficient = 4.8 mM^{-1} cm^{-1}) is complete in about 2 min and subsequently decays in a first-order reaction (k_2 = 0.02 min^{-1}). The red shift in the long wavelength band upon replacement of FAD [E_0' = −208 mV (Draper & Ingraham, 1968)] with 8-Cl-FAD [E_0' = −152 mV (Moore et al., 1978)] is consistent with the formation of a charge-transfer complex in which flavin is the acceptor (Massey & Ghisla, 1974; Thorpe & Massey, 1983).

In contrast, addition of 22 μM octynoyl-CoA to 6.2 μM 5-deaza-FAD dehydrogenase (Thorpe & Massey, 1983) only perturbs the spectrum of the oxidized flavin, with a 5-nm red shift and a 6% decline in absorbance of the main absorbance peak. These changes are apparently complete before measurement could be made and are very similar to those obtained after the addition of octanoyl-CoA to the 5-deaza-FAD-substituted enzyme (Thorpe & Massey, 1983). The failure of octanoyl-CoA to reduce the 5-deaza-FAD enzyme has been ascribed to the unfavorable redox potential of 5-deaza-FAD (Thorpe & Massey, 1983; E_0' = −311 mV; Walsh et al., 1978). In the present case, no long wavelength band is formed even after 12-h incubation of the substituted enzyme with 2-octynoyl-CoA.

Evidence That 2-Octynoyl-CoA Treatment Involves Loss of the CoA Moiety. The spectrum of a dialyzed sample of 2-octynoyl-CoA-treated enzyme, when compared to an equimolar amount of a control sample incubated in buffer alone, did not show significant differences in the 300–230-nm region. The incorporation of 1 mol of CoA/mol of subunit would be expected to increase the extinction coefficient of the modified enzyme by about 1.4-fold. Dialyzed samples were denatured with 6 M guanidine hydrochloride and reexamined. Again no difference ascribable to the presence of CoA–adenine could be detected. The CoA moiety could conceivably have been lost during overnight dialysis. However, reaction mixtures rapidly denatured with 5% trichloroacetic acid or with 80% methanol or by boiling for 10 min also showed no significant spectral differences from controls after redissolving the precipitated apoproteins in 6 M guanidine hydrochloride (see Methods).

This apparent loss of CoA was confirmed in several ways. Ultrafiltrates from enzyme treated with 1.2 equiv of 2-octynoyl-CoA until the spectral changes shown in Figure 1 were complete contained 0.46 equiv of thiol assessed by using the reaction with DTNB. Control experiments with enzyme or 2-octynoyl-CoA alone did not show significant free thiol. A second approach was using inhibitor prepared with tritiated CoA (see Methods). Enzyme exhaustively treated with 2-octynoyl-[^{3}H(G)]CoA and then ultrafiltered and washed twice with buffer retained only 0.28 mol of bound CoA/FAD. No activity was recovered, and neither was significant flavin lost during this treatment, as expected from results presented earlier. The time course for the release of CoASH from the dehydrogenase was addressed (Figure 5) by mixing enzyme, inhibitor, and DTNB and following the generation of the thionitrobenzoate species at 412 nm (Degani & Patchornik, 1971). This experiment is feasible since the native dehydrogenase (curve 2), or its complex with octanoyl-CoA (curve 3), reacts sluggishly with DTNB (Thorpe et al., 1979; Figure 5). Approximately 0.62 equiv of thiolate are generated in a reaction that is half-complete in 18 min at 25 °C, pH 7.6, in agreement with the 18 min required for half-disappearance of the long wavelength species (Figure 2). In the absence of enzyme, very little increase in absorbance is observed (Figure 5). When the same experiment is performed with 1.5 equiv of 2-pentynoyl-CoA, significantly less thiol is liberated (0.16 equiv after 120 min; data not shown).

Discussion

The interaction of 2-octynoyl-CoA with oxidized general acyl-CoA dehydrogenase is of surprising complexity, e.g., with the observation of the 800-nm intermediate (Figure 1) which appears somewhat faster than inactivation of the enzyme (Figure 2) and with the finding that CoASH is released during

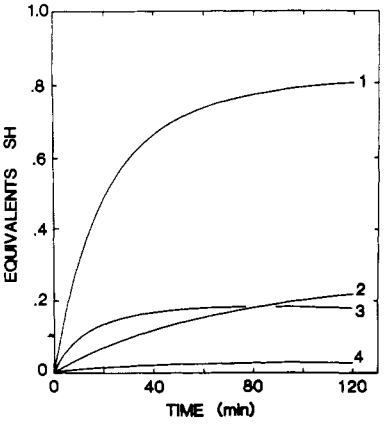
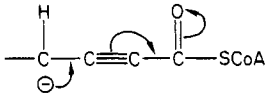


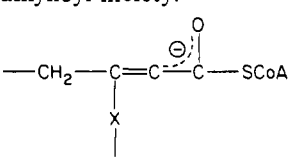
FIGURE 5: Release of CoASH on incubation of general acyl-CoA dehydrogenase with 2-octynoyl-CoA. The enzyme (21 μM) was mixed with 100 μM DTNB followed by 27 μM 2-octynoyl-CoA (curve 1), 29 μM octanoyl-CoA (curve 3), or no addition (curve 2). Curve 4 is a control with DTNB and 2-octynoyl-CoA but minus enzyme. Absorbance traces were followed at 412 nm and converted to equivalents of thiol by using a molar extinction coefficient for the thionitrobenzoate of 13.6 mM^{-1} cm^{-1} (Degani & Patchornik, 1971).

decay of this species (Figure 5). Although the 800 nm absorbing species clearly resembles a charge-transfer complex, in which oxidized flavin is the acceptor, the identity of the donor ligand remains to be established. Several possibilities should be considered. It seems unlikely that the donor represents the unreacted 2-alkynoyl moiety, since this species is somewhat electron deficient. Further, the initial complex, E·I, does not show long wavelength absorbance (Figure 1, inset), and the time and pH dependence of the formation of this band are more consistent with a covalent modification of the acetylene. A second possibility is that the 800-nm species is a resonance stabilized carbanion derived from abstraction of the relatively acidic C-4 proton:



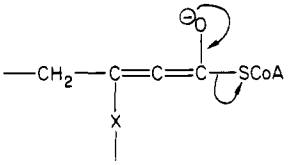
Studies with glutaryl-CoA dehydrogenase (Gomes et al., 1981) and general acyl-CoA dehydrogenase (Wenz et al., 1982) have shown that these enzymes can catalyze a C-2 to C-4 proton shift. In the case of glutaryl-CoA dehydrogenase this transfer has been shown to proceed without loss of tritium to solvent (Gomes et al., 1981). Consistent with the possibility of α-proton abstraction, propiolyl-CoA and phenylpropiolyl-CoA do not form detectable long wavelength bands, although they inactivate the dehydrogenase (Table I). However, 2-octynoylpantetheine, unlike its CoA thio ester counterpart, does not yield significant 800-nm absorbance, and thus, the 800-nm species could intervene in all three cases but remain undetected for kinetic reasons. Clearly, however, accumulation of the long wavelength species is not a prerequisite for inactivation by these acetylenic thio esters (Table I).

Another explanation for the 800-nm species is that it results from Michael attack of a protein nucleophile on the electron-deficient 2-alkynoyl moiety:



An attractive candidate for the nucleophile would be the glutamate residue identified by Fendrich & Abeles (1982) as the target of 3-butynoyl-CoA treatment of butyryl-CoA dehydrogenase. This carboxylate may be the active site base that abstracts the *pro-R* α-proton from normal substrates (Fendrich & Abeles, 1982). The pH dependence observed in the formation of the long wavelength species (apparent pK = 7.9: Figure 4) is not inconsistent with attack of such a nucleophile, since considerable increases in pK are expected for carboxylic acids in media of low dielectric (Gilbert, 1981). It should be noted, however, that the low pH-limiting value for k_1, though small, is not zero (see inset to Figure 4). This situation is similar to that seen by Murfin (1974) in the reduction of general acyl-CoA dehydrogenase by dihydrocinnamoyl-CoA derivatives (apparent pK = 7.6).

Thus, both species depicted above appear plausible candidates for the charge-transfer donor, and there are chemical precedents for their decomposition with the elimination of CoA, for example



Thus, α-proton abstraction from acetoacetyl-CoA (pK = 9.0; Gilbert et al., 1981) is followed by unimolecular rate-limiting elimination of CoASH and the formation of a ketene intermediate (Douglas & Yaggi, 1977). Elimination–addition reactions involving a ketenoid transition state have also been discussed by Pratt & Bruice (1970). Since release of CoASH parallels the disappearance of the 800-nm intermediate in 2-octynoyl-CoA-treated enzyme (Figure 5), phenylpropynoyl-CoA was tested. The latter rapidly inactivates general acyl-CoA dehydrogenase without detectable accumulation of long wavelength species (Table I). No thiol release was detected (data not shown; conditions as in Figure 5). Further, analogous experiments with 2-pentynoyl-CoA yield 0.16 mol of CoASH released (vs. 0.62 mol of thiol with 2-octynoyl-CoA) in accord with the lower yield of 800-nm intermediate (see Table I). Thus, there is a correlation between the presence of long wavelength species and the release of CoA consistent with the scheme depicted above. However, it should be stressed that both events follow inactivation of the enzyme. Whether the elimination of CoA results in a secondary attachment of the inhibitor to the protein remains to be established.

This report has described initial aspects of the effects of 2-alkynoyl-CoA derivatives on general acyl-CoA dehydrogenase. It is interesting to note that no long wavelength species has been reported for the 3-alkynoyl thio ester inhibitors (Frerman et al., 1980; Gomes et al., 1981; Fendrich & Abeles, 1982), and presumably no loss of CoA occurs in these cases. Isolation of labeled peptides and identification of the target nucleophile(s) clearly require the synthesis of [^{14}C]-2-octynoyl-CoA. In addition, preparation of 2-octynoyl-CoA dideuterated at C-4 will permit examination of the possibility of an isotope effect on the formation of the long wavelength species (see above). Since acyl-CoA oxidase from *Candida* tropicalis (Jiang & Thorpe, 1983) is also inactivated irreversibly by 2-octynoyl-CoA (Z.-Y. Jiang and C. Thorpe, unpublished observations), it will be interesting to compare the sequences of labeled peptides from these two enzymes that share the same reductive half-reaction.

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