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2-Octynoyl Coenzyme A Is a Mechanism-Based Inhibitor of Pig Kidney Medium-Chain Acyl Coenzyme A Dehydrogenase: Isolation of the Target Peptide†

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ABSTRACT: Pig kidney medium-chain acyl-CoA dehydrogenase (EC 1.3.99.3) is irreversibly and stoichiometrically inactivated by [1-^{14}C]-2-octynoyl coenzyme A. The linkage is stable at pH 2-6, but labile under basic conditions. The inhibitor labels a unique tryptic peptide, Ile-Tyr-Gln-Ile-Tyr-Glu-Gly-Thr-Ala-Gln-Ile-Gln-Arg, close to the C-terminus of the protein. The peptide is labeled at Glu-401 with the acyl moiety of the inhibitor but does not contain detectable coenzyme A. Both the inactivation of the dehydrogenase and the appearance of an absorption band at 800 nm show large primary deuterium isotope effects using 4,4′-dideuterio-2-octynoyl-CoA (7.3 and 6.3, respectively). Thus, 2-octynoyl-CoA is a mechanism-based inactivator of the dehydrogenase and is activated by rate-limiting γ-proton abstraction. Glutamate-401 may be the base that abstracts the *pro-R* α-proton during the dehydrogenation of normal substrates.

The short-, medium-, and long-chain acyl-CoA[1] dehydrogenases are immunologically distinct (Ikeda et al., 1985a) flavoproteins that catalyze the first oxidative step of β-oxidation (Beinert, 1963). These enzymes introduce a trans double bond between C-2 and C-3 of their acyl-CoA substrates (Beinert, 1963) and are subsequently reoxidized by electron-transferring flavoprotein (ETF; Crane & Beinert, 1956):

$$dH_{ox}\cdot RCH_2CH_2COSCoA \rightleftharpoons dH_{red}\cdot RCH{=}CHCOSCoA$$

$$dH_{red}\cdot RCH{=}CHCOSCoA + 2ETF_{ox} \rightleftharpoons dH_{ox}\cdot RCH{=}CHCOSCoA + 2ETF_{red}$$

The most thoroughly studied of these flavoproteins is the mammalian medium-chain acyl-CoA dehydrogenase. Recently the gene sequences of both the human and rat liver enzymes have been reported (Kelly et al., 1987; Matsubara et al., 1987, respectively). A deficiency of this enzyme in humans leads to sometimes fatal organic acidurias, and this

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[1] Abbreviations: CoA(SH), coenzyme A; HPLC, high-performance liquid chromatography; ETF, electron-transferring flavoprotein; TFA, trifluoroacetic acid; TPCK, L-1-(tosylamino)-2-phenylethyl chloromethyl ketone; PTH, phenylthiohydantoin; TCA, trichloroacetic acid; dH, dehydrogenase; DTT, dithiothreitol; Tris, tris(hydroxymethyl)aminomethane.

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inborn error is more common than previously believed (Stanley et al., 1983; Rhead et al., 1983; Divry et al., 1983). Despite the importance of these enzymes, active-site sequences have yet to be identified, although a crystal structure determination of the medium-chain dehydrogenase is in progress (Kim et al., 1984).

3-Alkynoyl-CoA or -pantetheine derivatives have been reported to be mechanism-based inactivators of the acyl-CoA dehydrogenases, undergoing base-catalyzed isomerization to the 2,3-allene prior to covalent labeling of the protein (Frerman et al., 1980; Gomes et al., 1981; Fendrich & Abeles, 1982). Fendrich and Abeles (1982), working with a bacterial short-chain acyl-CoA dehyrogenase, suggested that the susceptible amino acid was a glutamate residue since they recovered 5-OH-2-NH_2-valerate after acid hydrolysis of borohydride-treated modified enzyme.

This work describes the isolation of an active-site peptide from pig kidney medium-chain acyl-CoA dehydrogenase labeled with 2-octynoyl-CoA.

We have previously shown that this acetylenic thioester inactivates the dehydrogenase rapidly, irreversibly, and stoichiometrically with loss of CoASH (Freund et al., 1985). This paper demonstrates, somewhat surprisingly, that 2-octynoyl-CoA is a mechanism-based inactivator of the dehydrogenase. The 2-acetylenic function of the inhibitor is not merely a Michael acceptor but is activated by γ-proton abstraction prior to enzyme inactivation and loss of CoASH. The susceptible nucleophile is again a glutamate residue, suggesting that this carboxylate may be the base that abstracts the α-proton during the dehydrogenation of normal substrates (Biellmann & Hirth, 1970; Murfin, 1974; Ghisla et al., 1984; Ikeda et al., 1985b). Finally, examination of the sequence of yeast acyl-CoA oxidase (Okazaki et al., 1986) reveals a similar peptide close to the C-terminus of this peroxisomal protein.

Materials and Methods

Materials. Pig kidney medium-chain acyl-CoA dehydrogenase was purified as described previously (Lau et al., 1986). CoASH (lithium salt) and octanoyl-CoA were from P-L Biochemicals. Propiolic acid, 1-heptyne, methyl valerate, lithium aluminum deuteride, methyllithium, and 2-octynoic acid were from Aldrich. TPCK-treated trypsin, pepsin, and *N*-ethylmaleimide were from Sigma. $Ba^{14}CO_3$ was purchased from New England Nuclear.

General Methods. All buffers used for the preparation of modified and native enzyme contained 0.3 mM EDTA. UV–vis spectra were recorded on Perkin-Elmer 552 or Cary 219 spectrophotometers. Concentrations of native enzyme were calculated by using an extinction coefficient of 15.4 mM^{-1} cm^{-1} at 446 nm (Thorpe et al., 1979). Enzyme assays were as described previously (Thorpe et al., 1979). NMR spectra were run by using a Bruker 250-MHz spectrometer with an Aspect 3000 data system. Radiolabeled samples were counted on a Beckman LS-110C scintillation counter using Amersham ACS cocktail. Centricon microconcentrators (M_r 10 000; Amicon Corp.) were used for small-scale ultrafiltrations according to the manufacturer's instructions. HPLC was performed by using a Perkin-Elmer Series 400 solvent delivery system.

Synthesis of [1-^{14}C]-2-Octynoic Acid. Radiolabeled 2-octynoic acid was prepared by carboxylation of the lithium acetylide of 1-heptyne using $^{14}CO_2$. Briefly, the acetylide was formed in one flask of a two-flask closed system that had been flushed with dry nitrogen and then partially evacuated: 2 mmol of *n*-butyllithium was added with stirring to 3 mmol of 1-heptyne in 5 mL of freshly distilled tetrahydrofuran at −70 °C. Carbon dioxide was generated in the other flask by the addition of 1.2 mL of concentrated sulfuric acid to 75 mg of barium carbonate (^{14}C; 2.5 mCi) diluted with 125 mg of unlabeled material. After 1 h, the reaction was quenced by the addition of 5 mL of water to the stirred solution. The apparatus was flushed with a slow stream of nitrogen, trapping any unreacted CO_2 by bubbling through 0.1 M NaOH. Tetrahydrofuran was removed, and the crude product was extracted with four aliquots of ether (3 mL) to remove unreacted heptyne. The product was then acidified and extracted into ether. The combined extracts were dried over anhydrous sodium sulfate and evaporated. The resulting pale yellow oil gave the expected ^{1}H NMR spectrum in deuteriochloroform: 11.05 ppm (singlet, one proton), carboxyl proton; 2.55 ppm (triplet, two protons), C-4 methylene protons; 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 protons. It showed one spot by thin-layer chromatography on silica using (12:3:5) butanol/acetic acid/water (Stadtman, 1957) and comigrated with a 2-octynoic acid standard (R_f = 0.65).

Synthesis of 4,4'-Dideuterio-2-octynoic Acid. Lithium aluminum deuteride (1 g) was added to 75 mL of diethyl ether over nitrogen at 0 °C. Methyl valerate (5.4 g) was added in small portions to the solution and the mixture allowed to stir overnight at room temperature. Water (about 1 mL) was then added cautiously followed by 50 mL of 6 M HCl. The aqueous layer was removed, extracted with ether, and discarded. The combined ether layers were dried over anhydrous potassium carbonate and magnesium sulfate and filtered. The deuteriated alcohol in 75 mL of ether was refluxed with 6 g of phosphorus tribromide for 1 h and then stirred overnight at room temperature. The ether was shaken with water and then with a saturated solution of sodium bicarbonate. The ether layer was dried and evaporated, and the 1-bromo-1,1'-deuteriopentane distilled. Methylithium (29 mL of a 1.4 M solution) was added to 1.4 g of propiolic acid dissolved in 10 mL of freshly distilled dry tetrahydrofuran over nitrogen at −20 °C. Enough hexamethylphosphoramide was added to keep the dianion in solution. Bromopentane (3 g) was added in portions with enough hexamethylphosphoramide to allow magnetic stirring at −20 °C for 1 h. The mixture was stirred for a further 1.5 h at 0 °C, quenched by the addition of 2 M HCl, and extracted with benzene. After drying over magnesium sulfate, the solvent was evaporated, yielding 2 mL of 4,4'-dideuterio-octynoic acid. ^{1}H NMR showed greater than 95% deuteriation of C-4.

Preparation and Purification of 2-Octynoyl-CoA. 2-Octynoyl-CoA was prepared by the mixed anhydride procedure (Bernert & Sprecher, 1977) as described previously (Freund et al., 1985) using unlabeled, radiolabeled, or deuteriated 2-octynoic acid. Thioesters were purified by chromatography on DEAE-cellulose (Lau et al., 1977) or by HPLC (Powell et al., 1987) and stored as lyophilized powders. Deuteriated and ^{14}C-labeled 2-octynoyl-CoA, when mixed with a sample of the unlabeled material, eluted as a single sharp peak on HPLC (at 16.3 min, using the gradient described previously). 2-Octynoyl-CoA was quantitated by using an extinction coefficient of 20.9 mM^{-1} cm^{-1} at 260 nm (Freund et al., 1985). The specific activity of the radiolabeled thioester was 5200 dpm/nmol. Each thioester showed the expected ^{1}H NMR

spectrum. The ^1H NMR spectrum of 4,4'-dideuterio-2-octynoyl-CoA in D_2O showed a greater than 95% loss of the multiplet at 1.62 ppm due to the methylene protons at C-4.

Preparation of Modified Enzyme. Medium-chain acyl-CoA dehydrogenase was inactivated with a 1.3–3-fold excess of radiolabeled 2-octynoyl-CoA in 50 mM potassium phosphate buffer, pH 7.6, 25 °C. Excess inhibitor was removed by ultrafiltration.

Denaturation of Modified Enzyme and Stability of Covalent Label. Aliquots of the modified enzyme (40 600 dpm, in plastic centrifuge tubes) were diluted to a final volume of 1 mL in 50 mM phosphate buffer, pH 7.6, and placed in a boiling water bath for 5 min. The suspension was centrifuged and the precipitate washed once with this buffer. Both supernatant and washings consistently accounted for a combined total of 20–27% of the total counts, possibly reflecting some heterogeneity of the modification reaction. The radioactivity remaining in the precipitate was thereafter normalized to 100% (see later). The precipitates were then redissolved in buffer containing 6 M guanidine hydrochloride adjusted to the pH values shown in Table I and incubated at 25 °C for 12 h. The solutions were then ultrafiltered, and the denatured protein was washed twice with 1 mL of fresh incubation solution. Filtrates and retained fractions were counted.

Trichloroacetic acid precipitations were performed in dim light at 0 °C by using 450 μL of 1.2 μM enzyme in 50 mM phosphate buffer and 50 μL of 50% TCA for a final concentration of 5% TCA. The precipitate was washed with two 220-μL aliquots of 5% TCA and dissolved in 100 μL of 6 M guanidine hydrochloride. Both protein and supernatants fractions were counted.

Modified enzyme was precipitated with methanol by adding 0.2 mL of 5.5 μM enzyme to 0.8 mL of methanol at 0 °C and incubating on ice for 10 h. The suspension was centrifuged and the precipitate redissolved in 6 M guanidine hydrochloride. Protein and supernatant fractions were counted.

Denaturation in TCA or methanol consistently released 20–25% of the label.

Stability of Modified Enzyme in Guanidine Hydrochloride. Labeled holoenzyme (1.5 mL, 13 μM) was incubated in 6 M guanidine hydrochloride, pH 8, 25 °C. At several time points the solution was filtered (see above) and the retentate diluted to 1.5 mL with the same solution. The filtrate and retentate were measured spectrophotometrically to determine the amount of flavin released. Radiolabeled enzyme (1 mL of 4.3 μM, 22 400 dpm) was incubated in the same solution. Aliquots (300 μL) were removed and gel filtered (G-25 Sephadex, 20 × 1 cm). Fractions were counted, and the amount of radiolabel associated with enzyme was determined.

Alkylation of Denatured Enzyme. Modified enzyme (11 mg) was denatured by boiling, as described above, and the washed precipitate dissolved in 1 mL of a mixture of 6 M guanidine hydrochloride and 50 mM phosphate adjusted to pH 6.0 with KOH. The solution was incubated with a 2-fold excess of *N*-ethylmaleimide over total thiols (Thorpe et al., 1979) for 1 h. Alkylation at pH 6 can be more readily accomplished with *N*-ethylmaleimide rather than with iodoacetamide. The denatured protein was then dialyzed against two changes of 2 M urea containing 30 mM phosphate buffer adjusted to pH 6.

Trypsin Digestion. The modified protein (with or without a ^{14}C label) in dialysis buffer (see above), 37 °C, was digested with 2% w/w aliquots of TPCK-treated trypsin at 0, 3, 11, and 20 h and the mixture ultrafiltered after 24 h to separate peptides from trypsin, undigested protein, and core material. The retentate (representing 55% of the total radioactivity before digestion) was suspended in 0.5 mL of 5% formic acid, and 1.2% w/w aliquots of pepsin (1 mg/mL in 0.5 M NaCl) were added at 0 and 1 h. After 2 h, the clear solution was adjusted to pH 6 and ultrafiltered. Peptic "core" peptides and the tryptic peptides were purified by HPLC (see below).

Pepsin/Trypsin Digestion. The denatured alkylated protein was prepared as before but dialyzed against 5% formic acid containing 50 mM phosphate to provide buffering capacity at higher pH values. The white suspension was treated for 10 min at 37 °C with 1.2% w/w pepsin, and then the pH was raised to 6.0 by the addition of 1 M KOH. Digestion was continued after the addition of aliquots of 2% w/w TPCK-treated trypsin at 0 and 4 h. After 8 h, the pH of the cloudy solution was adjusted to 2 with HCl and the solution treated with a further aliquot of pepsin for 15 min. The clear solution was ultrafiltered, leaving only 2% of radioactivity in the retentate. Peptides were separated by HPLC.

Purification of Peptides. Solutions of peptides were taken nearly to dryness by lyophilization and applied to octadecylsilica reverse-phase columns (Fisher Resolvex 4.6 mm × 25 cm or Zorbax semipreparative column 9.4 mm × 25 cm). Typical analytical elution profiles comprised a 5-min wash with 0.1% TFA followed by a linear gradient to 60% acetonitrile containing 0.1% TFA over 3 h. One-minute fractions were collected for counting. Preparative runs were tailored for the region(s) of interest. Radioactive peptides were collected and lyophilized. Portions of the purified peptides were rechromatographed to check for purity and eluted as single peaks.

Amino Acid Analysis and Sequencing Peptides. Amino acid analyses of peptides were performed on the Pico·Tag amino acid analysis system from Waters (using the WISP 712 coupled to the Waters 721 system controller, the Waters 740 data module, and control station interfaced to the 510 HPLC), following manufacturer's instructions. Automated sequencing was performed on an Applied Biosystems gas-phase protein sequencer, Model 470A/120A/900A, according to manufacturer's instructions. Typically, 1–3 nmol of peptide was used for each analysis.

Kinetics of Inactivation of Dehydrogenase with 2-Octynoyl-CoA. A 1.3-fold excess of 2-octynoyl-CoA (protio- or 4,4'-dideuterio-) was added to a 2.75 μM solution of the dehydrogenase in 50 mM phosphate buffer, pH 7.6, 25 °C. The inactivation was sampled by withdrawing 5-μL aliquots and diluting them directly into assay cocktail containing 30 μM octanoyl-CoA (Thorpe et al., 1979). The assay was started by adding phenazine methosulfate as described previously.

RESULTS

Isolation and Stability of Enzyme Adduct with [1-^{14}C]-2-Octynoyl-CoA. Since inactivation of the medium-chain acyl-CoA dehydrogenase is accompanied by the loss of CoA (Freund et al., 1985), inhibitor radiolabeled in the acyl chain was synthesized (see Materials and Methods). The dehydrogenase was inactivated with a 3-fold excess of [1-^{14}C]-2-octynoyl-CoA (see Materials and Methods) following the reaction spectrophotometrically at 800 nm (Freund et al., 1985). Upon completion, the enzyme was separated from excess inhibitor by ultrafiltration (see Materials and Methods). The extent of incorporation of radiolabel (1.01 mol/mol of enzyme flavin) is in good agreement with the value of 1.1 determined spectrophotometrically (Freund et al., 1985).

Table I shows an experiment to assess the stability of the modified enzyme under various denaturing conditions. The modified enzyme was denatured by boiling and the precipitate dissolved in 6 M guanidine hydrochloride adjusted to the pH

Table I: Stability of the Enzyme Inhibitor Adduct

incubation solution	pH	% radioactivity released[a]
50 mM phosphate	2	1
50 mM phosphate	5	2
50 mM phosphate	6	3
50 mM phosphate	8	21
50 mM phosphate + DTT	8	67
50 mM phosphate	11	93
0.1 M Tris	8	85

[a] Incubation of heat-denatured enzyme for 12 h at 25 °C (see Materials and Methods). DTT, where included, was 5 mM.

values shown. Less than 3% of the label is released after 12 h of incubation at pH 2–6 in phosphate. Higher pH values, the substitution of Tris buffer, or the inclusion of 5 mM dithiothreitol promotes the release of radiolabel (Table I). Accordingly, the isolation of labeled peptides was performed at pH values of 6 and below.

It should be noted that the inactive labeled holoenzyme was denatured by boiling in Table I because it proved surprisingly stable in 6 M guanidine hydrochloride. Thus, at pH 8, 25 °C, both the release of flavin (monitored spectrophotometrically; see Materials and Methods) and the release of radiolabel were half-complete in about 1 day. This contrasts sharply with the few seconds required for the native flavoprotein (data not shown).

Isolation of Peptides. A sample of freshly prepared modified enzyme was denatured by boiling and alkylated in 6 M guanidine hydrochloride, pH 6.0, with a 2-fold molar excess of *N*-ethylmaleimide over total thiols (see Materials and Methods). The protein was dialyzed versus 2 M urea containing 30 mM phosphate buffer adjusted to pH 6 and digested with TPCK–treated trypsin in this buffer (see Materials and Methods). The solution was ultrafiltered to remove undigested protein and core material (amounting to about 55% by radioactivity).

Figure 1 shows an HPLC chromatogram of the supernatant monitored both by absorbance at 220 nm and by radioactivity. The peak at 75 min contained 70% of the counts injected, with no other major radioactive peaks in the chromatogram (Figure 1). Material was combined from several injections, lyophilized, and rechromatographed to check purity. Rechromatographed samples eluted as a single sharp peak. The corresponding peak on HPLC from digests of enzyme inactivated with nonradiolabeled 2-octynoyl-CoA was isolated for comparison.

The amino acid sequence obtained for both radiolabeled and nonradiolabeled peptide is shown in Table II, line 1, and the chromatograms of the PTH amino acids released in cycles 5–7 of the gas-phase sequencer are shown in Figure 2. The residue in cycle 6 emerges later than any of the standard amino acids (Figure 2) and carries the radioactive label (Table II). Comparison of the observed sequence with the complete sequence of the human (Kelly et al., 1987) and rat (Matsubara et al., 1987) liver medium-chain acyl-CoA dehydrogenases

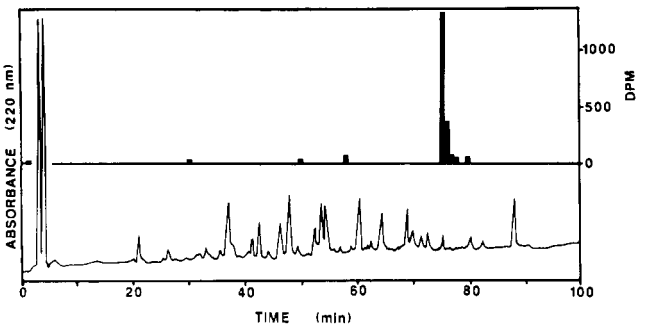


FIGURE 1: Isolation of the ^{14}C-labeled tryptic peptide from medium-chain acyl-CoA dehydrogenase treated with 2-octynoyl-CoA. The peptides were applied to a C_{18} reverse-phase HPLC column and eluted at 3 mL/min with the following program: 0–5 min, 0.1% TFA; 5–125 min, a linear gradient from 0.1% TFA to 60% acetonitrile containing 0.1% TFA. The upper portion shows the distribution of radioactivity followed by counting 100 μL from each 1-min fraction. The peak at 75–76 min was collected and sequenced.

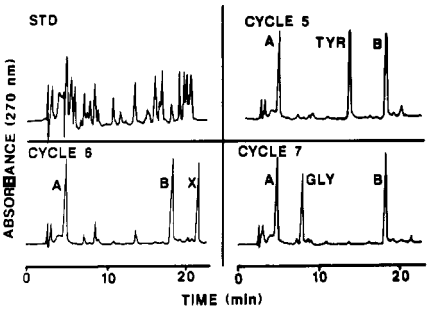


FIGURE 2: PTH amino acid sequencing of modified peptide and the elution profile for a standard (STD) and cycles 5–7 of Edman degradation of the purified tryptic peptide (see Materials and Methods). Peaks A and B, present in all cycles, represent breakdown products of phenyl isothiocyanate; (A) is *N*,*N*-dimethyl-*N*′-phenylthiourea, and (B) is *N*,*N*′-diphenylthiourea. The last amino acid in the standard mixture (LEU) elutes at 21.29 min, while (×) in cycle 6 elutes at 22.64 min.

(Table II) shows identity with the rat sequence 396–408 and close homology to the human sequence (one amino acid substitution). These peptides are close to the C-terminus of the protein. The pig kidney peptide is also identical with a tryptic peptide obtained from the pig liver enzyme (Kelly et al., 1987). In all these published sequences, a glutamate residue occupies position 6, indicating that 2-octynoyl-CoA modifies this carboxylate in the pig kidney medium-chain acyl-CoA dehydrogenase. Derivatization with the eight-carbon chain renders the modified glutamate highly hydrophobic, consistent with the elution of the modified residue on reverse-phase chromatography (Figure 2). Finally, amino acid analysis of the labeled peptide (see Materials and Methods) is consistent with the sequence shown in Table II with four residues of Glu per mole of peptide (data not shown). Fendrich and Abeles (1982) have also suggested that a glutamate residue is the target of inactivation of a bacterial short-chain acyl-CoA dehydrogenase by 3-pentynoylpantetheine (see Discussion).

Table II: Comparison of Peptide Sequences from Medium-Chain Acyl-CoA Dehydrogenase

	peptide source	amino acid sequence												
(1)	tryptic	Ile	Tyr	Gln	Ile	Tyr	X	Gly	Thr	Ala	Gln	Ile	Gln	Arg
	dpm	15	118	126	140	196	986	340	52	35	36	33	25	48
(2)	core (peptic)				Ile	Tyr	X	Gly	Thr	Ala	Gln			
(3)	peptic/tryptic 1				Ile	Tyr	X	Gly	Thr	Ala	Gln			
(4)	peptic/tryptic 2				Ile	Tyr	X	Gly	Thr	Ala	Gln	Ile	Gln	
(5)	human liver[a]	Ile	Tyr	Gln	Ile	Tyr	Glu	Gly	Thr	Ser	Gln	Ile	Gln	Arg
(6)	rat liver[b]	Ile	Tyr	Gln	Ile	Tyr	Glu	Gly	Thr	Ala	Gln	Ile	Gln	Arg

[a] Kelly et al. (1987). [b] Matsubara et al. (1987). The counts emerging at each cycle are shown for the tryptic digest (line 1).

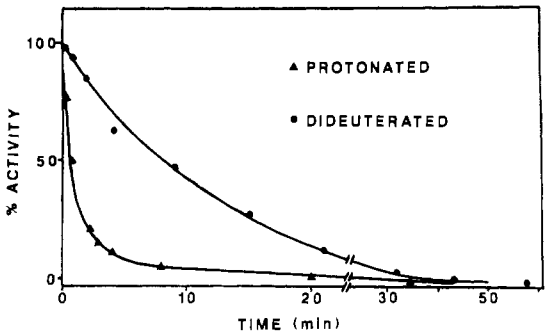


FIGURE 3: Inactivation of medium-chain acyl-CoA dehydrogenase with 4,4′-dideuterio-2-octynoyl-CoA versus the protonated analogue. A 1.3-fold excess of thioester was added to a 2.75 μM solution of enzyme in 50 mM phosphate buffer, pH 7.6, 25 °C. Aliquots (5 μL) were removed, and the reaction was stopped by dilution into assay cocktail. Activity was determined by adding phenazine methosulfate as described under Materials and Methods.

Despite the inclusion of 2 M urea, approximately 55% of the radioactivity was retained as undigested protein or core material after tryptic digestion at pH 6 (see earlier). Digestions performed without urea under otherwise identical conditions resulted in 83% of the radioactivity remaining with the undigested or core material. The 55% undigested material was largely solubilized with pepsin (see Materials and Methods), and the peptides were purified by HPLC as before. The major radioactive peak (81% of that applied) was sequenced and was found to be a shorter version of the tryptic sequence (Table II, line 2). Finally, in an effort to eliminate the problem of undigested material, a sample of the original alkylated protein was treated briefly with pepsin (10 min at pH 2) followed by trypsin digestion at pH 6 (see Materials and Methods). Negligible amounts of radioactivity were retained in core (less than 2%). The sequences of the two major radioactive peptides isolated on HPLC representing 51% and 32% of the counts applied to the column are shown in lines 3 and 4, respectively, in Table II.

Thus, inactivation of medium-chain acyl-CoA dehydrogenase by 2-octynoyl-CoA apparently leads to stoichiometric and unique modification of glutamate-401 in the enzyme. The isolated peptide did not contain detectable levels of β-alanine on amino acid analysis (data not shown), consistent with the loss of CoA observed during inactivation (Freund et al., 1985).

Interaction of the Medium-Chain Dehydrogenase with 4,4′-Dideuterio-2-octynoyl-CoA. 2-Octynoyl-CoA deuteriated at the γ-position was synthesized to examine whether the reagent might be a mechanism-based inhibitor or simply an affinity label of the dehydrogenase (see Materials and Methods and Discussion). Figure 3 shows that the dehydrogenase is indeed inactivated about 7.3-fold more slowly by the deuteriated material. A large primary kinetic isotope effect is also manifest in the spectral changes that accompany inactivation of the enzyme (Figure 4). The interaction of the enzyme with protio-2-octynoyl-CoA was previously shown to involve a transient appearance of a long-wavelength band at 800 nm followed by its slower decay to a spectrum resembling oxidized enzyme (Freund et al., 1985). The inset to Figure 4 compares the rate of appearance and decay of the 800-nm band for protio and deuterio inhibitor under otherwise identical conditions. The appearance of the band is markedly slowed (about 6.3-fold) upon deuteriation, while its decay is unaffected.

The possibility that a reactive intermediate accumulates in solution prior to inactivation of the enzyme was tested by the inclusion of 5 mM dithiothreitol as a potential scavenger. No

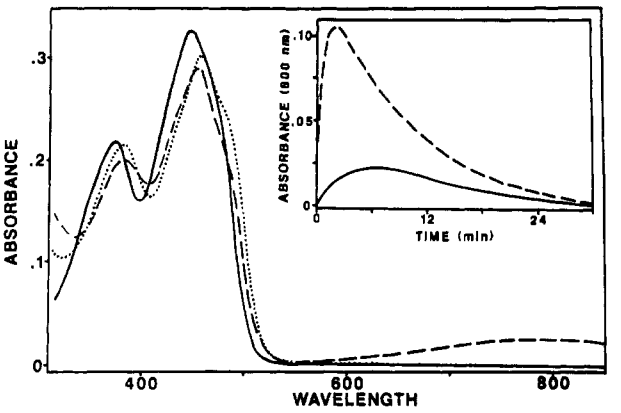


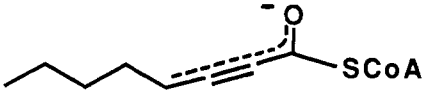
FIGURE 4: Spectral changes observed on the addition of 4,4′-dideuterio-2-octynoyl-CoA to pig kidney medium-chain acyl-CoA dehydrogenase. The oxidized dehydrogenase (22.5 μM; —) in 50 mM phosphate buffer, pH 7.6, 25 °C, was treated with 45 μM 4,4′-dideuterio-2-octynoyl-CoA: (- - -) maximal formation of the 800-nm band (11.0 min after mixing); (···) final spectrum (after 165 min). The inset shows a comparison of the absorption changes at 800 nm when protio (- - -) or 4,4′-dideuterio (—) 2-octynoyl-CoA was used.

effect was observed on the spectral changes or the kinetics of inactivation using a 3- or 5-fold molar excess of 2-octynoyl-CoA, respectively (data not shown).

Discussion

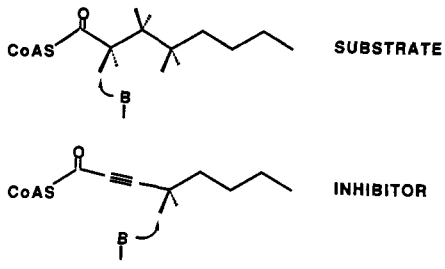
Previous studies with 3-alkynoyl thioesters are consistent with base-catalyzed isomerization to the corresponding 2,3-allene within the active center of the acyl-CoA dehydrogenases (Frerman et al., 1980; Gomes et al., 1981; Fendrich & Abeles, 1982):

In contrast, 2-octynoyl-CoA would not be expected a priori to be a mechanism-based inhibitor of the dehydrogenase. It is already a potent Michael acceptor and, lacking α-protons, could not be activated as above. However, large primary kinetic isotope effects (of 6–7) were observed by using 4,4′-dideuterio-2-octynoyl-CoA both on the appearance of the 800-nm band and on the rate of inactivation of the enzyme. Thus, somewhat surprisingly, 2-octynoyl-CoA is a mechanism-based inactivator of the medium-chain dehydrogenase undergoing rate-limiting removal of one of the C-4 hydrogens. Proton abstraction at this relatively acidic position would generate a resonance-stabilized enolate



that could serve as the charge-transfer donor (Massey & Ghisla, 1974; Thorpe & Massey, 1983; Freund et al., 1985) in the 800-nm absorbing intermediate. Indeed, a number of bands of similar (780–820 nm) wavelength have been ascribed to enolate-oxidized flavin charge-transfer complexes, e.g., those involving *trans*-3-octenoyl-CoA (Powell et al., 1987) and 3-thia- and 3-oxaoctanoyl-CoA (Lau et al., 1988; Thorpe, 1988). The dehydrogenase exhibits a marked propensity to stabilize these weakly acidic enolates when transfer of a β-hydride is prevented (Thorpe, 1988). In the case of 2-octynoyl-CoA, formation of the 800-nm charge-transfer band implies a close proximity with the flavin prosthetic group, and there appears little doubt that the ultimate target of this inhibitor is an active-site residue.

The arguments discussed below suggest that the active-site base servicing normal substrates of the acyl-CoA dehydrogenase is also involved in activation of 2-octynoyl-CoA. The ability of a single active-site base to interact with both α- and γ-positions during allylic rearrangements has been inferred for a number of enzymes [for example, Talalay and Benson (1972), Hashimoto et al. (1973), Hanson and Rose (1975), Schwab and Klassen (1984), and Schwab and Lin (1985)]. Butyryl-CoA dehydrogenase from *Megasphaera elsdenii* catalyzes such a rearrangement: the interconversion of vinylacetyl-CoA and crotonyl-CoA (Fendrich & Abeles, 1982). Although the involvement of a single base in this reaction has not been proved, it appears likely (Hanson & Rose, 1975). Further, in a mechanistically related enzyme, glutaryl-CoA dehydrogenase, a 2–4-proton shift has been observed without substantial exchange with solvent protons (Gomes et al., 1981). Thus, a single monoprotic base appears to be involved (Gomes et al., 1981). Both α- and γ-positions could be accessible to a base B, and the γ-protons in 2-octynoyl-CoA occupy positions analogous to those of a normal substrate.



Inactivation of *M. elsdenii* butyryl-CoA dehydrogenase by 3-pentynoyl-CoA leads to derivatization of a glutamate residue identified by amino acid analysis after reduction with borohydride (Fendrich & Abeles, 1982). Fendrich and Abeles (1982) suggest that this residue is in fact the base that abstracts an α-proton during catalysis. The present work, using pig kidney medium-chain acyl-CoA dehdyrogenase, has directly identified a glutamate residue as the target of a mechanistically different suicide substrate. We suggest that glutamate-401 plays the same role in the mammalian enzyme. One advantage of a carboxylate is that desolvation upon binding amphipathic acyl-CoA substrates would be expected to raise the pK of this residue markedly, contributing to the stabilization of the enolate within the active center (Jencks, 1975; Rose, 1975; Thibblin & Jencks, 1979; Gilbert, 1981).

It will be interesting to examine other members of the acyl-CoA dehydrogenase family to see whether they are also inactivated by 2-alkylnoyl-CoA derivatives and, where appropriate, to sequence their target peptides. It is of interest that sequences somewhat similar to those in Table II appear close to the carboxyl terminus of acyl-CoA oxidase from both *Candida tropicalis* (Okazaki et al., 1986) and rat liver (Miyazawa et al., 1987):

C. tropicalis

Asp-Gly-Asp-Ile-Tyr-Glu-Asn-Tyr-Phe-Asp-Leu-Val-Lys

rat liver

Asp-Gly-Asn-Val-Tyr-Glu-Asn-Leu-Phe-Glu-Trp-Ala-Lys

Comparison of the dehydrogenase and oxidase sequences aligned by using the presumed catalytically essential glutamate residue shows about 60% similarity using the Dayhoff MDM-78 matrix method (Dayhoff et al., 1983). The yeast enzyme is inactivated by 2-octynoyl-CoA, and it will now be interesting to investigate this reaction further.

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Registry No. EC 1.3.99.3, 9027-65-0; Glu, 56-86-0; D_2, 7782-39-0; [1-^{14}C]-2-octynoic acid, 116374-62-0; 1-heptyne lithium acetylide, 42017-07-2; 1-heptyne, 628-71-7; 4,4′-dideuterio-2-octynoic acid, 116374-63-1; methyl valerate, 624-24-8; 1,1′-dideuteriopentanol, 35658-10-7; 1-bromo-1,1′-dideuteriopentane, 77734-75-9; 2-octynoyl-CoA, 96448-59-8.

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On the Intermediacy of Carboxyphosphate in Biotin-Dependent Carboxylations†

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ABSTRACT: In the ATP-dependent carboxylation of biotin that is catalyzed by most biotin-dependent carboxylases, a fundamental mechanistic question is whether the ATP activates bicarbonate (via the formation of carboxyphosphate as an intermediate) or whether the ATP activates biotin (via the formation of *O*-phosphobiotin). We have resorted to three mechanistic tests using the biotin carboxylase subunit of acetyl-CoA carboxylase from *Escherichia coli*: positional isotope exchange, intermediate trapping, and ^{18}O tracer experiments on the ATPase activity. First, no catalysis of positional isotope exchange in adenosine 5′-([α,β-$^{18}O,\beta,\beta$-$^{18}O_2$]triphosphate) was observed when either biotin or bicarbonate was absent, nor was any exchange seen in the presence of both *N*-1-methylbiotin and bicarbonate. Second, the putative carboxyphosphate intermediate could not be trapped as its trimethyl ester, under conditions of incubation and analysis where the authentic triester was shown to be adequately stable. In the third test, however, we showed that the ATPase activity of biotin carboxylase that is seen in the absence of biotin, an activity that is known to parallel the normal carboxylase reaction when biotin is present, occurs with the transfer of an ^{18}O label directly from [^{18}O]bicarbonate into the product P_i. This result suggests that the bicarbonate-dependent biotin-independent ATPase reaction catalyzed by biotin carboxylase goes via carboxyphosphate and that the carboxylation of biotin itself may proceed analogously.

The carboxylation reactions that are catalyzed by biotin-dependent enzymes all involve 1-carboxybiotin (*N*-1-carboxybiotin), the intermediacy of which has been demonstrated both by direct trapping (Lynen et al., 1959, 1961; Knappe et al., 1961) and by experiments that have shown the chemical and kinetic competence of the synthetic material (Guchhait et al., 1974b). The *N*-1-carboxybiotin intermediate is normally produced from biotin and bicarbonate in an ATP-dependent reaction that is common to all biotin-dependent carboxylases (except transcarboxylase, where the carboxyl group donor is a β-keto or a β-thioester acid derivative). The mechanism by which the carboxylation of the N-1 ureido nitrogen of biotin is driven by ATP has remained a vexatious issue, despite considerable experimental scrutiny (Kaziro et al., 1962; Moss & Lane, 1971; Wood & Barden, 1977; Kluger et al., 1979; Attwood & Keech, 1984; Hansen & Knowles, 1985). There have been three favored pathways proposed that seek in different ways to overcome the two mechanistic problems: that biotin is a poor nucleophile and that bicarbonate is a poor electrophile (see Figure 1). Each of these three pathways accommodates the important finding that one of the three bicarbonate oxygens ends up in product phosphate in every turnover of a biotin-dependent carboxylase (Kaziro et al., 1962). In mechanism 1 (stepwise), bicarbonate is phosphorylated by ATP to form the highly reactive mixed-anhydride carboxyphosphate, which is then attacked by the N-1 nitrogen of enzyme-bound biotin (either directly or after collapse to enzyme-bound CO_2; Sauers et al., 1975) to yield carboxybiotin. This pathway has the attraction of involving a precedented species, carboxyphosphate (Powers & Meister, 1976, 1978; Wimmer et al., 1979), though this is balanced by the evident lack of nucleophilicity of the ureido nitrogen of biotin (Caplow, 1965; Caplow & Yager, 1967; Bruice & Hegarty, 1970). In mechanism 2 (concerted), ATP is used to activate biotin rather than bicarbonate by phosphorylation on the ureido *oxygen* to give the reactive species *O*-phosphobiotin. In a subsequent (chemically unprecedented)

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