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Mechanism of Action of Glutaryl-CoA and Butyryl-CoA Dehydrogenases. Purification of Glutaryl-CoA Dehydrogenase†

Bruce Gomes,‡ Gabriele Fendrich, and Robert H. Abeles*

ABSTRACT: Glutaryl-CoA dehydrogenase, a flavoprotein, catalyzes the reaction $^{-}OOCCH_2CH_2—CH_2COSR$ (FAD → $FADH_2$) → $CH_3CH{=}CHCOSR + CO_2$ (SR = CoA or pantetheine). With the isolated enzyme, a dye serves as the final electron acceptor. The enzyme from *Pseudomonas fluorescens* (ATCC 11250) has been purified to homogeneity. It was established with appropriate isotopic substitutions that the proton which is added to the γ position of the product, subsequent to decarboxylation, is not derived from the solvent but is derived from the α position of the substrate. Under conditions where no net conversion of substrate occurs, i.e., in the absence of electron acceptor, the enzyme catalyzes the exchange of the β hydrogen of the substrate with solvent protons. Butyryl-CoA dehydrogenase (*M. elsedenii*), which catalyzes an analogous reaction, catalyzes the exchange of both the α and β hydrogens with solvent protons in the absence of electron acceptor. Glutaryl-CoA dehydrogenase and butyryl-CoA dehydrogenase are irreversibly inactivated by the substrate analogues 3-butynoylpantetheine and 3-pentynoylpantetheine. These inactivators do not form an adduct with the flavin and probably react with a nucleophile at the active site. Upon inactivation, the spectrum of the enzyme-bound flavin is essentially unchanged, and the flavin can be reduced by $Na_2S_2O_4$. We suggest that inactivation involves intermediate allene formation. We proposed that these results support an oxidation mechanism for glutaryl-CoA dehydrogenase and butyryl-CoA dehydrogenase which is initiated by proton abstraction. With glutaryl-CoA dehydrogenase, the base, which abstracts the substrate α proton, is shielded from the solvent and is then used to protonate the carbanion (CH_2⁻⁻CH⁻⁻CHCOSCoA) formed after oxidation and decarboxylation.

Several different experimental approaches have led to the proposal that the initial step in reactions catalyzed by some flavoproteins is the abstraction of the substrate α hydrogen as a proton (Bright & Porter, 1975). The resulting carbanion then transfers electrons to the flavin by a currently unknown mechanism. Enzymes for which evidence for a carbanion mechanism has been obtained are the following: D- and L-amino acid oxidase (Walsh et al., 1971, 1973; Massey et al., 1976; Voet et al., 1972; Porter et al., 1972), L-lactic acid oxidase (Ghisla et al., 1976; Schonbrunn et al., 1976), and D-lactic acid dehydrogenase (Massey et al., 1979). The extensive model studies carried out by Bruice and his collaborators establish that in nonenzymatic reactions oxidations involving flavins can involve carbanionic intermediates when substrates are involved which form stable carbanions (Bruice, 1980). It should be noted that the above-mentioned enzymes act on substrates which are carboxylic acids; i.e., the hydrogen which is removed is α to a carboxyl group, and hence a mechanism for carbanion stabilization exists.

Several flavin dehydrogenases catalyze oxidations which lead to the formation of carbon–carbon double bonds. Examples of enzymes in this category are acyl-CoA dehydrogenases, glutaryl-CoA dehydrogenase, and succinic dehydrogenase. One of the hydrogens removed in these reactions is α to a carboxyl group or to a thioester group, and therefore the possible involvement of a carbanionic intermediate should be considered. The chemical mechanism of these reactions has not been extensively studied. It has been established that succinic dehydrogenase can catalyze the elmination of HF from difluorosuccinate (Tober et al., 1970). This observation is consistent with a carbanion mechanism. The reaction catalyzed by glutaryl-CoA dehydrogenase (eq 1) (Numa et

$$^{-}OOCCH_2CH_2—COSCoA \xrightarrow{Fl} {}_{Fl_{red}} CH_3CH{=}CHCOSCoA + CO_2 \quad (1)$$

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‡ Present address: Laboratory of Neurochemistry, National Institute of Mental Health, Bethesda, MD 20205.

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al., 1964) attracted our attention since it differs from other dehydrogenations involving flavoproteins in that it involves a decarboxylation. A reasonable reaction sequence for the reaction catalyzed by glutaryl-CoA is shown in eq 2.

$$^{-}OOCCH_2CH_2{-}CH_2COSCoA \xrightarrow{Fl_{ox}\ \rightleftharpoons\ Fl_{red}} {}^{-}OOCCH_2CH{=}CHCOSCoA \xrightarrow{-CO_2} [CH_2{\cdots}CH{\cdots}CHCOSCoA]^{-} \xrightarrow{H^+} CH_3CH{=}COSCoA \quad (2)$$

According to this mechanism, a dehydrogenation takes place initially. The resulting unsaturated product then decarboxylates. Finally, a proton is added to the allylic anion formed through the decarboxylation to form the γ-methyl group of crotonyl-CoA. Regardless of the details of the mechanism of the conversion of glutaryl-CoA to crotonyl-CoA, the γ-carbonyl group of glutaryl-CoA must be replaced by a proton. The enzyme, therefore, clearly has the ability to protonate the terminal carbon of the reaction product. If evidence can be obtained that the proton added to the γ position is abstracted from the α position of the substrate in the oxidation, it would provide evidence for the carbanion mechanism. These considerations led us to undertake a study of the mechanism of action of glutaryl-CoA dehydrogenase. In the course of this work, it became of interest to compare some properties of glutaryl-CoA dehydrogenase wtih those of butyryl-CoA dehydrogenase, an enzyme which also catalyzes the formation of a carbon–carbon double bond.

Materials and Methods

Assays

Assay for Glutaryl-CoA Dehydrogenase. The standard assay solution was 75 μM 2,6-dichlorophenol–indophenol (DCIP), 0.6 mM phenazine methosulfate (PMS), 100 mM potassium phosphate buffer, pH 7.0, and substrate (50 μM glutaryl-CoA or 10 mM glutarylpantetheine). The reaction was started by addition of the enzyme solution. The decrease in the optical density at 600 nm was followed. The extinction coefficient of DCIP in 100 mM phosphate buffer, pH 7.0, was determined by anaerobic titration with ascorbate to be 21 450 M^{-1} cm^{-1}. The assays were performed at 25 °C. The assay is linear for at least 15 min when the substrate is saturating. However, at low enzyme or low substrate concentrations, curvature is evident even in the initial rates. In addition, at these low rates, small blank rates (without substrate) are significant. Both of these problems can be eliminated almost completely by preincubation of the enzyme with a concentrated DCIP solution (5 μL of 5% mg/mL DCIP per 20 μL of enzyme solution) and by including 1 mg/mL bovine serum albumin in the assay mixture. A unit of activity is defined as μmol/min.

An alternate assay was to follow decarboxylation by monitoring $^{14}CO_2$ release. The same assay conditions as above were used, except the reaction was carried out in a tube stoppered with a serum cap; [1,5-$^{14}C_2$]glutaryl-CoA or [1,5-^{14}C]-glutarylpantetheine were used as the substrates. At various time points after the initiation of the reaction (by addition of the enzyme), an aliquot was removed with a syringe and added through a serum stopper to a vial containing 0.5 mL of 0.6 M H_2SO_4. This vial was equipped with a suspended cup containing 0.2 mL of 0.2 M NaOH an a magnetic stirrer. After 15 min, 100 μL of 1 M $NaHCO_3$ was added, and the solution was stirred an additional 15 min. The NaOH solution was removed and counted in a scintillation counter, using ACS counting fluid (Amersham).

Assay of Butyryl-CoA Dehydrogenase. The assay mixture contained 0.2 M sodium phosphate, pH 8.0, 1 mg of BSA, 75 μM DCIP, 0.6 mM PMS, and 100 μM butyryl-CoA or 500 μM butyrylpantetheine in a total volume of 1.0 mL at 25 °C. The decrease in absorbance at 600 nm is measured (DCIP ϵ_{600} = 22 × 10^3 M^{-1} cm^{-1}). After the blank rate is determined, the assay is started by addition of enzyme.

Assay of the Electron Transferring Flavoprotein. The electron-transferring flavoprotein (ETF) was assayed by enhancement of the activity of glutaryl-CoA dehydrogenase.

The assay conditions were the same as in the assay of the dehydrogenase except that PMS was omitted. A saturating amount of glutaryl-CoA, 50 μM (or 30 mM glutarylpantetheine), was added to the DCIP solution. Then approximately 2 mg of glutaryl-CoA dehydrogenase was added. The ΔOD_{600} was monitored. Then an aliquot of the ETF solution to be tested was added. The ΔOD_{600} was again observed. The difference between the two readings was the activity of the ETF solution. At these high concentrations of glutaryl-CoA dehydrogenase, it was difficult to saturate with respect to ETF, and, therefore, the ETF assay seemed linear over a fairly wide range of ETF concentrations.

Identification of FAD. The flavin cofactor resolved from the enzyme was identified by paper electrophoresis and TLC. Electrophoresis was carried out at pH 1.9 (450 mL of H_2O, 10 mL of 98% HCOOH, and 40 mL of glacial CH_3COOH) and 3000 V (75 V/cm) for 2 h. FMN migrated 32 cm from the origin and FAD 24.5 cm. TLC plates were developed in 1-butanol/acetic acid/water (12:3:5): R_f(FMN) 0.19, R_f(FAD) 0.09.

NMR Experiments. NMR spectra were taken on a Bruker WH90 spectrometer. All deuterium spectra were taken on a 270-MHz spectrometer (Redfield et al., 1975).

Synthetic Procedures

Synthesis of Pantetheine. Pantetheine was made by reduction of pantethine (Sigma, Calbiochem, or Vega Biochemicals Corp.) by using the following procedure. In an Erlenmeyer flask, equipped with a magnetic stirrer and a pH electrode and cooled in an ice bath, was placed 1 g (1.9 mmol) of pantetheine in 30 mL of water. N_2 was bubbled through the solution. Sodium borohydride solid (1 g or 27 mmol) was added slowly over 30 min. The pH of the solution was maintained between 8 and 8.5 by addition of 1 M HCl. After addition of all of the borohydride, the solution was stirred until gas evolution slowed significantly. The pH was then lowered to 2 in order to destory any excess borohydride. The pH was then adjusted to 7.5 for use in subsequent experiments. These pantetheine solutions were used immediately.

Glutarylpantetheine. Glutarylpantetheine was prepared by using a modification of the procedure of Simon & Shemin (1953). A 30-mL aqueous solution of pantetheine (1.19 mmol) at pH 7.5 was stirred in an ice bath. The reaction flask was equipped with a pH electrode. Solid glutaric anhydride (Aldrich) was added over 15 min, and the pH was maintained at 7.5 by addition of solid $NaHCO_3$. The solution was tested periodically with the nitroprusside reagent to detect free thiol. When no further thiol was detected, the addition of glutaric anhydride was terminated. This usually required approximately 0.8 g (6 mmol). The solution was then acidified with Dowex 50 (H^+ form) to pH 2.5. This was followed by three extractions with 2 volumes of ether to remove any glutaric acid. The solution was shell frozen and lyophilized.

Purification of glutarylpantetheine was accomplished by high-performance liquid chromatography on a C_{18} reverse-phase preparative column (Waters) by using 12% methanol

in water adjusted to pH 2.5. The flow rate was 3 mL/min, and the retention time was 30 min (variable dependent on injection size). After collection of the effluent from the column, the solution was lyophilized. The residue contained no free thiol by the nitroprusside (Stadtman, 1957) test until after base hydrolysis. The yield of this reaction was about 40% with reference to pantetheine. Glutaryl thioesters were stored at pH 3.

The NMR spectrum of the residue resembled the sum of the spectra of glutaric acid and pantetheine. The NMR spectrum consists of a doublet at δ 0.8 (J = 36 Hz), triplets at 2.3 (J = 6.2 Hz), 2.6, and 2.98 (J = 7.6 Hz), a multiplet at 3.3, and a broad singlet at 3.9.

Glutaryl-CoA. Glutaryl-CoA was obtained from Sigma or was prepared analogously to glutarylpantetheine, and 50 mg (65 μmol) of CoA lithium salt (Sigma) was used. Purification of glutaryl-CoA was via the descending paper chromatography system of Numa et al. (1964). The nitroprusside positive band after base hydrolysis was eluted with 10^{-3} M HCl, and the eluant was lyophilized.

[1,5-$^{14}C_2$]Glutaryl-CoA. [1,5-$^{14}C_2$]Glutaryl-CoA was made by a modification of a published procedure (Willadsen & Eggerer, 1975). The [1,5-$^{14}C_2$]glutaryl-CoA was purified by the same method as glutaryl-CoA. The thioester was in a single spot when chromatographed on paper (Numa et al., 1964). When the chromatogram was viewed with a strip scanner, virtually all (>99%) of the radioactivity was in the single thioester positive spot.

Crotonylpantetheine. Crotonylpantetheine was synthesized by using the method employed for [1,5-$^{14}C_2$]glutaryl-CoA except that a 2-fold excess of the mixed anhydride of ethyl chloroformate and crotonic acid (Aldrich) over the thiol was used. Purification of crotonylpantetheine was accomplished by high-performance LC on a C_{18} reverse-phase column with 22% aqueous methanol. At a flow rate of 3 mL/min, the retention time was approximately 30 min. The effluent was lyophilized. The NMR of the crotonylpantetheine was the summation of the spectra of crotonic acid and pantetheine. The NMR spectrum consists of a doublet at δ 0.8 (J = 4 Hz), a doublet of doublets at 1.8 (J = 9 and 2.5 Hz), triplets at 2.4 and 3.0 (J = 7 Hz), a multiplet at 3.4, a broad singlet at 3.9, a doublet of multiplets at 6.3 (large J = 16 Hz), and a multiplet centered at 6.9.

Synthesis of 3-Pentynoylpantetheine. 3-Pentynoylpantetheine was synthesized according to eq 3.

$$\mathrm{CH_3CH_2C{:}CH} \xrightarrow[\mathrm{NH_3}]{\mathrm{NaNH_2}} \mathrm{CH_3CH_2C{:}C^-\ Na^+} \xrightarrow[\mathrm{CO_2}]{\mathrm{THF}}$$
$$\mathrm{CH_3CH_2C{:}CCO_2H} \xrightarrow[\text{(or } \mathrm{NaNH_2/NH_3})]{^-\mathrm{OH}}$$
$$\mathrm{CH_3C{:}CCH_2CO_2H} \xrightarrow[\text{THF, acetone}]{\text{DCC, pantetheine}}$$
$$\mathrm{CH_3C{:}CCH_2C(=O)S\text{–pantetheine}} \quad (3)$$

Synthesis of 2-pentynoic acid was accomplished by the method used to synthesize tetrolic acid (Kauer & Brown, 1973). Under a N_2 atmosphere, 150 mL of anhydrous ammonia was condensed into a three-neck flask equipped with a dry ice condenser and magnetic stirrer. A freshly crushed crystal of $Fe(NO_3)_3 \cdot 9H_2O$ was added along with a small piece of Na (~100 mg). The solution was stirred until all the blue color was replaced with a gray suspension ($NaNH_2$). Sodium metal (2.3 g or 0.1 mol) was added in small pieces over 30 min. The solution was stirred until all the sodium reacted (30 min). The solution was dark gray black. Ethylacetylene (1-butyne, Pfaltz & Bauer, Inc.) was gently bubbled in with vigorous stirring. After 10 min, the solution had turned a light gray, signaling the formation of sodium ethylacetylide. The gas flow of ethylacetylene was stopped, and a nitrogen stream was maintained overnight to remove all NH_3 and exclude moisture.

To the residue was added 100 mL of ether and 100 mL of THF (both freshly distilled from Na/benzophenone). A CO_2 stream (Matheson) was bubbled through the solution. After 4 h, the solution had turned brown. The solvent was removed with a rotary evaporator. To the residual light tan powder residue was added approximately 25 mL of water. The solution was extracted twice with a half-volume of ether and then acidified (pH 2) with concentrated HCl, saturated with NaCl, and continuously extracted overnight with ether. The ether (~200 mL) was dried over Na_2SO_4 and removed with a rotary evaporator. An oil resulted, which was crystallized from toluene (mp 49–50 °C; lit. mg 50.0 °C). The NMR spectrum consists of a triplet (δ 1.18) and a quartet (δ 2.36, J = 7 Hz). The yield was approximately 45%.

Rearrangement of 2-pentynoic acid to 3-pentynoic acid was accomplished in either or two ways: rearrangement was done with $NaNH_2/NH_3$ (Craig & Moyle, 1963) (procedure III) or with NaOH (Jones et al., 1954).

For the small-scale synthesis used in the NaOH rearrangement, the procedure was modified in that the product was recovered by continuous ether extraction. The product recrystallized from toluene gave white needles (mp 101–103.5 °C; lit. mg 102–104 °C) whose NMR spectrum consists of a triplet (δ 1.8) and quartet (δ 3.28, J = 2 Hz). The yield is approximately 80%.

Thioesterification was by the procedure used for 3-butynoylpantetheine (see below). After that procedure, the compound could be further purified on a C_{18} reverse phase using 30% aqueous methanol. The retention time is about 30 min (depends strongly on concentration) at 2 mL/min. The FT NMR of the resultant compound was a broad singlet of δ 3.8, a complex multiplet at 3.2 (clearly containing the quartet of the acid moiety), two broad multiplets at 2.3 and 2.6, a skewed triplet at 1.5, and a skewed doublet at 0.8.

Synthesis of 3-Butynoylpantetheine. 3-Butynol was oxidized to 3-butynoic acid (Heibron et al., 1949). The NMR spectrum of the acid consists of a doublet (δ 3.4) and a triplet (δ 2.3, J = 3 Hz), in a ratio of 2:1; yield 26%.

Thioesterification was accomplished by the following procedure: 1 g of pantetheine (1.0 mmol) was reduced to pantetheine as described above. The solution was adjusted to pH 5. The water was removed with a rotary evaporator. The residue was suspended in 25 mL of acetone (dried over $CaSO_4$ and distilled just prior to use). In another flask was placed 1.2 g of dicyclohexylcarbodiimide (5.8 mmol, Eastman) and 25 mL of THF (freshly distilled from Na/benzophenone). To the THF solution was added 0.5 g of 3-butynoic acid (5.8 mmol); quickly the THF and acetone solutions were mixed. The solution was then stirred overnight in a sealed flask at 4 °C.

The solution was filtered on a sintered glass filter to remove dicyclohexylurea and sodium borate. The solvent was removed with a rotary evaporator to yield an oil. The oil was triturated with ether and dried in vacuo. Purification was accomplished with a C_{18} reverse-phase high-performance liquid chromatography column using 25% aqueous methanol. The material gave a single nitroprusside positive peak with a retention time of approximately 30 min (2 mL/min flow rate).

Synthesis of the 5-Methyl Ester of Glutaryl-CoA. The half-ester of glutaric acid was synthesized by a method similar to a published procedure (Eliel & Burgstahler, 1949). Ten

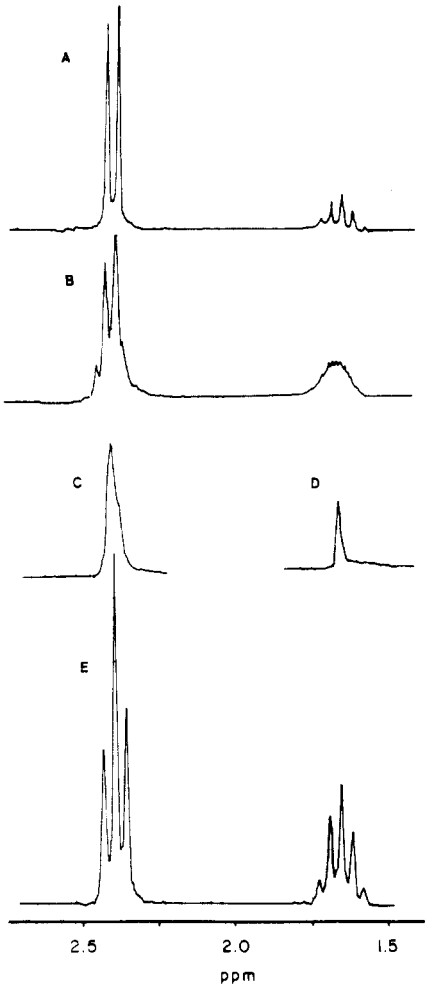


FIGURE 1: 270-MHz ^1H FT NMR spectra of deuterated glutaric acid standards: (A) β-monodeuterioglutaric acid (deuteron decoupled); (B) α,β-dideuterioglutaric acid (deuteron decoupled); (C) α,β-dideuterioglutaric acid (deuteron decoupled and proton decoupled at C-3 position); (D) α,β-dideuterioglutaric acid (deuteron decoupled and proton decoupled at C-2 and C-4 positions); (E) glutaric acid. The solvent in all cases was D_2O.

grams of glutaric anhydride (76 mmol, Aldrich), 100 mL of methanol (dried over $CaSO_4$), and 1 drop of H_2SO_4 were refluxed for 3 h. Most of the methanol was removed with a rotary evaporator. The remaining solution (~15 mL) was dissolved in approximately 50 mL of water. The pH (2) was adjusted to 7 with NaOH. The solution was extracted with 2 volumes of ether. The water solution was adjusted to pH 2.5, and the solution was extracted several times with ether (total volume ~500 mL). The ether was dried over $CaSO_4$ and removed with a rotary evaporator to yield monomethylglutaric acid. The NMR showed a singlet (δ 3.6), a triplet of doublets (δ 2.6, J = 8 and 2 Hz), and a quartet (δ 2.2, J = 8 Hz) in a ratio of approximaately 3:4:2. Yield is essentially quantitative.

The thioesterification was done by the procedure used for [1,5-$^{14}C_2$]glutaryl-CoA. The compound was chromatographed on Eastman cellulose plates in 0.1 M sodium acetate (pH 4.5)/95% ethanol (1:1) to show that there was no glutaryl-CoA contaminating the 5-methylglutaryl-CoA. The R_f of glutaryl-CoA is 0.59. 5-Methylglutaryl-CoA ran as a single spot with an R_f of 0.74. There was no glutaryl-CoA contamination detected by UV absorbance or by staining with 1 N NaOH and nitroprusside reagent.

Synthesis of α,β-Dideuterioglutaric Acid. α,β-Dideuterioglutaric acid was made by catalytic hydrogenation with

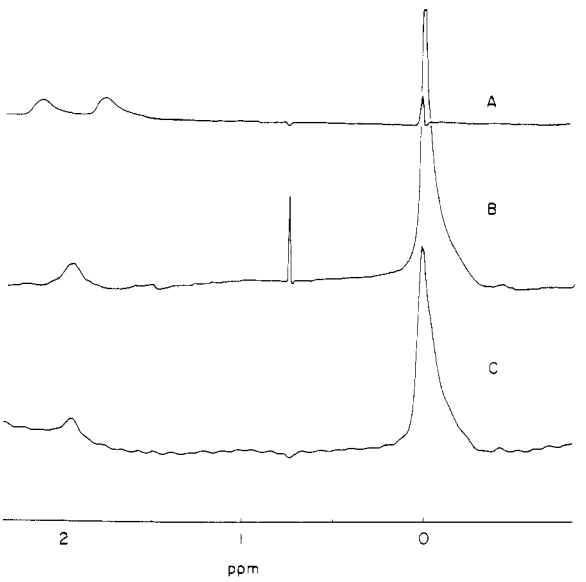


FIGURE 2: ^2H FT NMR spectra of glutaric acid: (A) α,β-dideuterioglutaric acid (672 transients); (B) β-monodeuterioglutaric acid (480 transients); (C) glutaric acid isolated from enzymatic reaction carried out in deuterated water (>5000 transients). The peak on the right of all three spectra is the D_2O resonance. The peak in the center of each spectrum is a machine artifact. All spectra were carried out in H_2O.

deuterium gas and the corresponding α,β-unsaturated pentanedioic acid (glutaconic acid). Glutaconic acid (1 g, 7.7 mmol, Aldrich) and palladium on charcoal (250 mg; 10% catalyst, Alfa) were stirred in 75 mL of ethyl acetate for 5 h with a constant stream of detuerium gas (Matheson) bubbling through the solution. The catalyst was removed by filtration (care should be taken not to allow the catalyst to dry out completely or it will ignite). The ethyl acetate was removed by rotary evaporation. A deuterium-decoupled ^1H NMR spectrum showed two multiplets (see Figure 1) at δ 1.3 and 2.4 are in a ratio of 1:3. A ^2H NMR spectrum is shown in Figure 2 which consists of two resonances of equal intensity. The yield was quantitative.

Synthesis of 3-Iodoglutarate. 3-Iodoglutarate was synthesized by a procedure similar to one published elsewhere (Stone & Schechter, 1963). In a round-bottomed flask equipped with a condenser was placed 100 g of 85% phosphoric acid (0.87 mol) and 45 g of phosphorous pentoxide (0.33 mol). These were cooled on ice, and then 30 g of potassium iodide (0.18 mol) was added. Not all the KI went into solution. Glutaconic acid (10 g, 77 mmol) was added, and the reaction was boiled gently for 3 h. The resultant black solution was cooled and diluted with an equal volume of water. The solution was extracted twice with 1 volume of ether. The combined, red-brown extract was dried over magnesium sulfate and decolorized by stirring over sodium thiosulfate. The ether was removed by rotary evaporation, and a pale yellow powder resulted. The crude 3-iodoglutaric acid was recrystallized twice from toluene to give white needles (mp 130–131.5 °C; 46% yield). Anal. Calcd: C, 23.28; H, 2.7; O, 24.80; I, 49.19. Found: C, 24.42; H, 2.75; O, 24.69; I, 48.24; oxygen value was by difference.

Synthesis of β-Deuterioglutaric Acid. Hydrogenolysis of iodoglutarate was similar to a published procedure (Carlsson & Ingold, 1968). A mixture of 250 mg (0.97 mmol) of D,L-3-iodoglutaric acid, 10 mg of α,α'-azobis(proprionitrile) (61 μmol, Aldrich), and 3 mL of tributyltin deuteride (9.3 mmol, Alfa) was heated at reflux in 5 mL of tetrahydrofuran (dried just before use by reflux and distillation from $LiAlH_4$). The

THF was removed with a rotaroy evaporator. Approximately 20 mL of water was added, and the solution was made basic with 1 N NaOH. The solution was extracted with 2 volumes of ether and then acidified with concentrated HCl and extracted several times with ether (total volume ~100 mL). The ether extract from the acidic solution was dried over sodium sulfate and brought to dryness with a rotary evaporator. A deuterium-decoupled ^1H NMR spectrum gave a doublet and quintuplet in the ratio of 4:1 (δ 1.3 and 2.4, J = 9 Hz). The NMR spectrum is shown in Figure 1A. The ^2H NMR is shown in Figure 2B, which consists of a singlet.

Exchange Experiments

Tritium Incorporation into Crotonylpantetheine. In a final volume of 1.1 mL of tritiated water (1.20×10^5 cpm/μmol of H), 10 μmol of potassium phosphate buffer, pH 7.0, glutaryl-CoA dehydrogenase solution (0.35 unit), ETF solution (0.11 units), and 6.1 μmol of glutarylpantetheine were incubated at 20 °C. A 7.03 mM DCIP solution (in 100 mM potassium phosphate buffer, pH 7.0) was added in 100-μL aliquots as the dye was consumed. The extent of product formation was assayed by the amount of dye reduced. After 20 min, the reaction mixture was shell frozen and lyophilized. The residue was resuspended in 1 mL of water and lyophilized. Resuspension and lyophilization were repeated 3 more times. The residue and 10.5 μmol of carrier crotonylpantetheine in 1 mL of water were applied to a 1 × 31 cm Sephadex G-25 column. Fractions which tested positive to a thioester test (nitroprusside after base hydrolysis) were pooled and dried with a rotary evaporator. The residue was taken up in a minimum amount of water and purified by high-performance LC on a C_{18} reverse-phase column (Waters) with 25; aqueous methanol. (The retention time at 2 mL/min is ~30 min.) The peak, detected by a differential refractometer, was pooled and dried by a rotary evaporator. The thioester concentration was determined by base hydrolysis of an aliquot (0.1 N NaOH, 30 min, 37 °C), followed by DTNB titration. In a control experiment, crotonylpantetheine was maintained in 100 mM potassium phosphate buffer for 2 days. No deuterium incorporation into the γ position could be detected by NMR.

Exchange of Tritium from Water into Unreacted Substrate. The conditions for the reaction were 301 nmol of glutarylpantetheine, 30 μmol of potassium phosphate, pH 7.0, in 340 μL of tritiated water (3.9×10^6 cpm/μmol of H) (New England Nuclear), 8.7 nmol of 2,6-dichlorophenol–indophenol solution, 195 nmol of phenazine methosulfate, 42.2 nmol of FAD, and 0.7 IU of glutaryl-CoA dehydrogenase. The reaction was allowed to proceed for 5 min at room temperature, at which time the dye was consumed. The solution was shell frozen and the solvent removed by lyophilization. Glutaric acid (12.3 mg, 92.8 μmol) was added as a carrier, followed by 1 mL of 0.5 N NaOH. The solution was incubated at 37 °C for 10 min and then frozen and lyophilized. To the residue was added 1 mL of 0.5 N H_2SO_4, and the solution was frozen and lyophilized again. Finally, 2 mL of H_2O was added, and the solution was frozen and lyophilized. The residue was taken up in a minimum volume of 0.5 N H_2SO_4, added to 1 g of silicic acid, and applied to the top of a 1 × 30 cm column containing 3 g of silicic acid [the silicic acid and solvents for the chromatography were prepared in accordance with the published procedure (Varner, 1957)]. The column was eluted with approximately 15 mL of 5% butanol in chloroform, followed by 15% butanol in chloroform. The fractions were collected. For determination of the content of glutaric acid, each fraction was titrated with 0.1 N NaOH. All fractions were then diluted to the same volume (10 mL), and 2-mL aliquots were counted for radioactivity in 18 mL of ACS scintillation fluid (Amersham). The same conditions were used to determine the incorporation in the absence of electron acceptors, except that the dyes were omitted.

Deuterium Exchange from Water into Unreacted Substrate. In 5.3 mL of (94%) D_2O was placed 0.065 IU of glutaryl-CoA dehydrogenase (dialyzed against D_2O), 250 μmol of potassium phosphate, pH 7.0, and 21 μmol of glutarylpantetheine. The reaction was incubated for 90 min at 23–25 °C. The solution was then shell frozen and lyophilized. To the residue was added 5 mL of 0.5 N NaOH, and the mixture was incubated at 37 °C for 10 min. Denatured protein was removed by centrifugation, and the solution was lyophilized. [3-^{3}H]Glutaric acid (0.17 μmol, 7.76×10^5 cpm/μmol) was added to aid in the detection of glutaric acid in the subsequent purification. The residue was chromatographed on silicic acid. The conditions for the chromatography were the same as those employed in the experiment described for tritium incorporation from solvent into glutarylpantetheine. The glutaric acid was located with the radioactive marker. The pooled fractions were dried with a rotary evaporator, and a ^2H NMR spectrum was recorded.

CO_2 Release in the Absence of Electron Acceptor. A small vial was equipped with a serum cap and a suspended cup containing 0.2 mL of Protosol (National Diagnostics). A small magnetic stirring bar was put in the vial. Reaction conditions were 1.5 mL of 0.2 M potassium phosphate buffer, pH 7.5, at 27 °C. To the vial was added 50 μL of [1,5-$^{14}C_2$]-glutaryl-CoA solution (360 nmol, 1.06×10^6 cpm/μmol) and 0.5 mL of enzyme solution (14.83 mg, 211 nmol, 0.24 IU/mg). After 5 s or 60 min, 0.5 mL of 1 N H_2SO_4 was added, and the stirring was continued for 15 min. The Protosol was then counted.

For determination of the efficiency of the CO_2 trap, a reaction mixture was set up in the same manner as above without substrate. Ten microliters of a $NaH^{14}CO_3$ solution (6.6×10^5 cpm) was added through the serum cap. After 20 min, the same workup procedure as above was employed. A total of 5.0×10^5 cpm was found, giving an efficiency of 75.95%.

A control was done in which the enzyme was omitted and the reaction stopped after 60 min.

Exchange Study with Butyryl-CoA Dehydrogenase. Butyryl-CoA dehydrogenase[1] (1.6×10^{-2} IU), 3 mg of butyryl-CoA (3.6 μmol), and 100 μmol of sodium phosphate, pH 8, in 300 μL of tritiated water (2.75×10^5 cpm/μmol of H) were incubated for 1 h at room temperature. The solution was shell frozen and lyophilized. Two milliliters of 0.5 M NaOH was added, and the solution was incubated at 37 °C for 30 min to hydrolyze the thioester, followed by lyophilization of the butyric acid carrier (15 μL, 0.17 mmol); 1 mL of water was added, and the mixture was lyophilized. The residue was acidified and loaded on a silicic acid column as described elsewhere. The column was washed with 15 mL of 1% 1-butanol/$CHCl_3$ and eluted with 3% 1-butanol/$CHCl_3$. The peak was titrated with 0.1 N NaOH, pooled, and dried with a rotary evaporator. Butyric acid (150 μL, 1.7 mmol) was added, and the specific radioactivity was determined. Butyric acid was then degraded to propionic acid by a Shmitt degradation (Greenberg & Rothstein, 1957), followed by $KMnO_4$ oxidation. The acid was transferred to a three-neck flask equipped with a condensor and containing 1.7 mL of 100% H_2SO_4. This was cooled on ice. Sodium azide (150 mg, 2.2

[1] The butyryl-CoA dehydrogenase (*M. elsedenii* ATCC 25940) used in these experiments was a generous gift of Professor V. Massey (Engel & Massey, 1971).

mmol) was added, followed by slow warming to 60–70 °C. This temperature was maintained for 30 min. The solution was cooled on ice and adjusted to pH 12. N_2 was blown through the solution as the temperature was raised to 100 °C. The effluent N_2 stream containing propylamine was bubbled into 4 mL of 0.5 N H_2SO_4 in a flask fitted with a condenser. The temperature was maintained at 100 °C for 15 min. To the acid trap was added 5 mL of 5% $KMnO_4$. The solution was cooled on ice and adjusted to pH 12. The tightly capped flask was incubated at 37 °C for 15 min. At the end of the time, all the $KMnO_4$ had been consumed. A total of 4 mL more of the $KMnO_4$ solution was added, and the incubation was resumed for an additional 35 min. MnO_2 was removed by filtration, and the solution was acidified. At the end of the oxidation, the solution was extracted several times with ether (total volume 500 mL). To the ether extract was added 1.5 mL of 1 N NaOH, and the biphasic system was brought to dryness with a rotary evaporator. The residue was purified by silicic acid chromatography. The column was washed with 1% 1-butanol/$CHCl_3$ and eluted with 2.5% 1-butanol/$CHCl_3$. The fractions were titrated with 0.1 N NaOH, to determine their acid content and then dried with a rotary evaporator. The radioactivity of the remaining salts of the propionic acid in each tube was determined.

To test that an artifact was not generated by exchange of the β protons of the propylamine during its dissociation, propylamine was oxidized under the conditions above except that the reaction was done in tritiated water (approximaately 5.60×10^5 cpm/μmol of H). A <0.02% exchange into propionic acid was observed.

Results

Properties of the Enzyme. The enzyme obtained by the purification procedure described in the Appendix gives a single band on polyacrylamide (Gabriel, 1971) and $NaDodSO_4$ gel electrophoresis (Weber et al., 1972). The absorption spectrum of the enzyme is typical of a flavoprotein. Acid precipitation of the protein releases a flavin which was identified as FAD by paper electrophoresis and TLC. The identification of the cofactor as FAD was further verified by reconstitution experiments. Addition of FAD to the apoenzyme (see Materials and Methods) restored 10–30% of the original activity. No activity was restored by addition of FMN. On the basis of flavin content, a minimal molecular weight of 63 000 was calculated. From $NaDodSO_4$ gel electrophoresis, a subunit molecular weight of 70 000 was determined, which is in agreement with the molecular weight based on flavin content.

Glutaryl-CoA and glutarylpantetheine are substrates for the enzyme. For both substrates, V_{max} is 0.24 μmol min^{-1} mg^{-1} under standard assay conditions. K_m values are 3.4 μM and 2 mM, respectively. A glutaryl-CoA analogue in which the δ-carboxyl group is converted to a methyl ester is also oxidized, as evidenced by dye reduction. Presumably, no decarboxylation occurs, although the product of this reaction has not been identified. V_{max} for the oxidation of this compound is the same as that for glutaryl-CoA.

Tritium Incorporation from Water into Crotonylpantetheine and Glutarylpantetheine. During the normal enzymatic reaction, the γ carbon of glutaryl-CoA must acquire a proton to become a methyl group (see eq 1). Whether this proton is derived from the solvent or from one of the two hydrogens abstracted from C-2 and C-3 of the substrate was established by the conversion of glutarylpantetheine to crotonylpantetheine; this was carried out in ^3H_2O (specific activity 1.2×10^5 cpm/μmol of H). The specific activity of the enzymically formed crotonylpantetheine was 5.0×10^2 cpm/μmol, i.e., approximately 0.5% that of the water. Thus, 1 out of 200 of the hydrogens at the C-4 position is dervied from the solvent. Barring an unusual large isotope effect, it can be concluded that the hydrogen which replaces the caroboxyl group of glutaryl-CoA is not derived from the solvent but must be derived from either the C-2 or C-3 position of the substrate.

In another experiment, glutarylpantetheine was converted to crotonylpantetheine in ^3H_2O (specific activity 3.5×10^6 cpm/μmol of H). Th reaction was allowed to proceed until approximately one-third of the substrate was consumed. The unreacted glutarylpantetheine was then hydrolyzed to glutaric acid which was purified by silicic acid chromatography. The specific activity of the glutaric acid was 1.0×10^5 cpm/μmol. A similar experiment was carried out in which the dyes were omitted; i.e., no net turnover occurs. The specific activity of the isolated glutaric acid was 2.7×10^5 cpm/μmol, i.e., 2.7 times higher than in the presence of electron acceptor dyes. It can be concluded that glutaryl-CoA dehydrogenase catalyzes the exchange of either the α or β hydrogen of the substrate with solvent protons.

Deuterium Exchange from Water into Glutarylpantetheine. For determination of which position of substrate is subject to exchange, glutarylpantetheine was added to glutaryl-CoA dehydrogenase in the absence of an electron acceptor (hence, without turnover) in deuterated water. The reisolated substrate was hydrolyzed and purified as detailed under Materials and Methods. The resultant deuterated glutaric acid was subject to deuterium NMR. The 270-MHz ^1H FT NMR spectrum is shown in Figure 2C. For comparison, the spectra of chemically synthesized α,β-dideuterioglutaric acid and β-monodeuterioglutaric acid are given in Figure 2, parts A and B, respectively. The spectra of β-monodeuterioglutaric acid and the glutaric acid extracted from the reaction mixture closely match. Therefore, the enzyme catalyzes the exchange of only the β proton of the substrate.

CO_2 Release in the Absence of Electron Acceptor. An experiment was carried out to determine whether decarboxylation in the absence of an electron acceptor. Under these conditions, the amount of CO_2 produced would, at most, be stoichiometric with the enzyme. Hence, relatively large amounts of enzyme were used. Glutaryl-CoA dehydrogenase (211 nmol) and [1,5-^{14}C]glutaryl-CoA (specific activity 1.06×10^6 cpm/μmol) were allowed to react for 5 s without the addition of an electron acceptor. The $^{14}CO_2$ which was evolved was collected and counted as described under Materials and Methods. A single turnover of enzyme would yield 1.1×10^5 cpm. A total of 302 cpm was found; i.e., 0.58 nmol of CO_2 was produced. This represents 0.27% of a single turnover. A turnover takes 3.6 s. A similar experiment was carried out in which the reaction proceeded for 60 min, at which point 67.3 nmol of CO_2 was released. During the 60-min incubation, the enzyme had lost considerable activity. Therefore, the values derived for this time point should only be considered as qualitative. It can be concluded that in the absence of an electron acceptor decarboxylation is extremely slow relative to the catalytic process. It is possible that O_2 can serve as a poor electron acceptor and that the decarboxylation which occurred is partially or entirely due to reaction with O_2.

Exchange Studies with Butyryl-CoA Dehydrogenase. We wished to test whether another flavin enzyme might exhibit the same exchange behavior as glutaryl-CoA dehydrogenase, i.e., whether it specifically catalyzes the exchange of the β hydrogen. Therefore, butyryl-CoA dehydrogenase (0.016 IU) was added to butyryl-CoA (3.6 μmol) in the absence of an electron acceptor (conditions under which there is no net re-

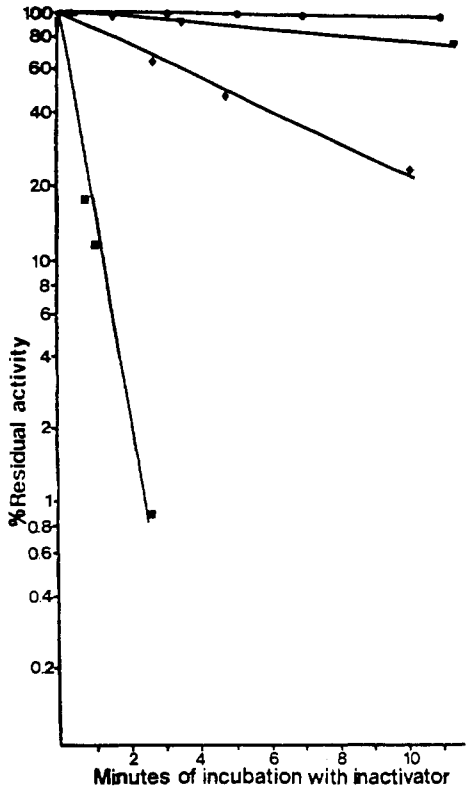


FIGURE 3: Kinetics of inactivation of glutaryl-CoA dehydrogenase by 3-pentynoylpantetheine. Inactivation was carried out in 100 mM potassium phosphate buffer, pH 7.0, 0.2 mg of BSA, 1.7×10^{-3} IU glutaryl-CoA dehydrogenase, and 3-pentynoylpantetheine as indicated at a final volume of 105 μL, 25 °C. At various time points, 10-μL aliquots were assayed in a 1-mL standard assay mixture. The following concentrations of 3-pentynoylpantetheine were used: (■) 425 μM, (♦) 85 μM, and (▼) 425 μM plus 2.7 mM glutaryl-CoA; (●) enzyme incubated without 3-pentynoylpantetheine.

action) in tritiated water (final specific activity 6.6×10^6 cpm/μmol of H). After 60 min, butyryl-CoA was hydrolyzed, and the resultant butyric acid was purified by silicic acid chromatography. The purified butyric acid after carrier addition was then subjected to a Schmitt degradation. The reactions involved in this degradation are shown in eq 4.

$$CH_3CH_2CH_2\text{—}COOH \xrightarrow[-CO_2]{} CH_3CH_2CH_2\text{—}NH_3 \xrightarrow{[O]} CH_3CH_2COOH \quad (4)$$

The specific activity of the butyric acid prior to the Schmitt degradation was 7.6×10^5 cpm/mmol. The specific activity of the propionic acid dervied from butyric acid was 4.2×10^5 cpm/mmol. The amounts of ^3H incorporated into the α and β position of butryl-CoA are therefore nearly equal. These results show that butyryl-CoA dehydrogenase does not show the same absolute conservation of the α hydrogen as glutaryl-CoA dehydrogenase. However, the results obtained so far do not exclude the possibility that the rates of exchange of the α and β protons are not identical. This question is now under further investigation.

Inactivation of Butyryl-CoA Dehydrogenase and Glutaryl-CoA Dehydrogenase by Acetylenic Substrate Analogues. Acetylenic substrate analogues have been used as suicide inactivators in enzymatic reactions involving carbanionic intermediates. We therefore tested the effects of 3-butynoylpantetheine and 3-pentynoylpantetheine on glutaryl-CoA dehydrogenase and butyryl-CoA dehydrogenase. Both enzymes were irreversibly inactivated by these acetylenic substrate analogues. The kinetics of the inactivation of glutaryl-CoA dehydrogenase by 3-pentynoylpantetheine is shown

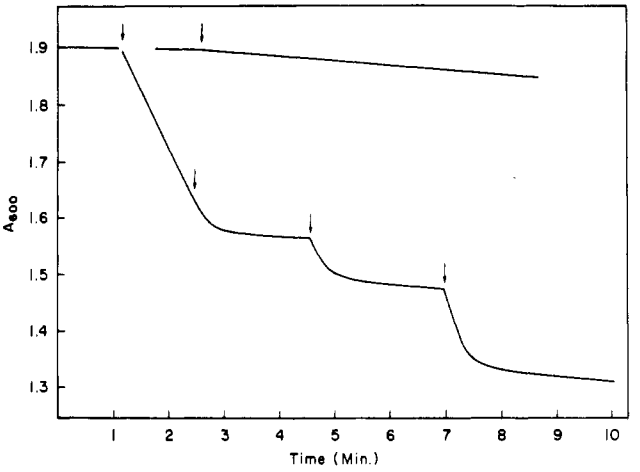


FIGURE 4: Inactivation of butyryl-CoA dehydrogenase by 3-pentynoylpantetheine. The reaction contained 0.2 M potassium phosphate buffer, pH 7.0, 1 mg of BSA, 80 μM DCPIP, 600 μM, PMS, and 620 μM butyrylpantetheine in a total volume of 1.0 mL at 25 °C. The reaction was started by addition of 0.1 nmol of butyryl-CoA dehydrogenase (yellow form), and 90 s later, 3-pentynoylpantetheine (5 μM) was added. Additional enzyme was added at 4.5 min (0.1 nmol) and at 7 min (0.2 nmol). (Upper line) Blank rate in the absence of enzyme. Additions indicated by arrows.

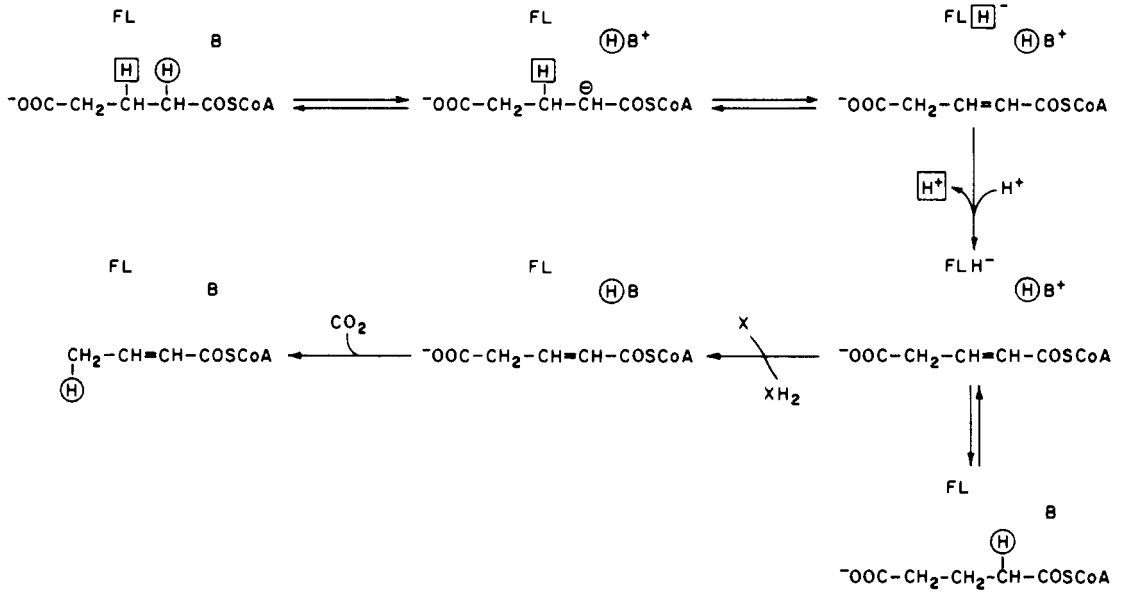
in Figure 3. The reaction shows first-order kinetics. The rate of inactivation is reduced by the presence of substrate. This inactivation, therefore, meets the criteria for a suicide inactivator. The rate of inactivation of butyryl-CoA dehydrogenase by 3-pentynoylpantetheine was too fast to measure. Results obtained when inactivator is added to the enzyme in the presence of the substrate are shown in Figure 4. Essentially complete inactivation occurs.

A number of examples of the inactivation of flavoproteins by acetylenic substrate analogues have been described (Walsh et al., 1973; Massey et al., 1979). In all cases, the inactivator reacts with flavin, as evidenced by changes in the spectrum of the flavin. We therefore examined the spectral changes which occur when glutaryl-CoA dehydrogenase and butyryl-CoA dehydrogenase are inactivated by 3-pentynoylpantetheine. It was found that the inactivation did not produce significant changes in the flavin spectrum. Furthermore, addition of $Na_2S_2O_4$ to the inactivated enzyme resulted in the reduction of the enzyme-bound flavin. Typical results for butyryl-CoA dehydrogenase are shown in Figure 5. These results show that the inactivation of butyryl-CoA dehydrogenase and glutaryl-CoA dehydrogenase probably does not occur through covalent modification of the flavin.

Discussion

In the conversion of glutarylpantetheine to crotonylpantetheine, a proton replaces the ω-carboxyl group of glutarylpantetheine. We investigated this reaction to establish the source of this proton. Essentially no solvent ^3H is incorporated when glutarylpantetheine is converted to crotonylpantetheine in ^3H_2O; therefore, the proton must be derived from the α or β position of the substrate. We have, however, also shown that the β position of the substrate is subject to exchange with solvent protons while no exchange into the α position could be detected by NMR. Hence, the α position of the substrate must be the source of the proton which replaces the ω-carboxyl group of glutarylpantetheine. In Scheme I, we show a reaction sequence which can account for the experimental results. According to this scheme, a base at the active site abstracts the substrate α hydrogen. This hydrogen is shielded from exchange with solvent protons. The β hy-

Scheme I: Mechanism of Action of Glutaryl-CoA Dehydrogenase[a]



[a] FL = enzyme-bound FAD; X = electron acceptor.

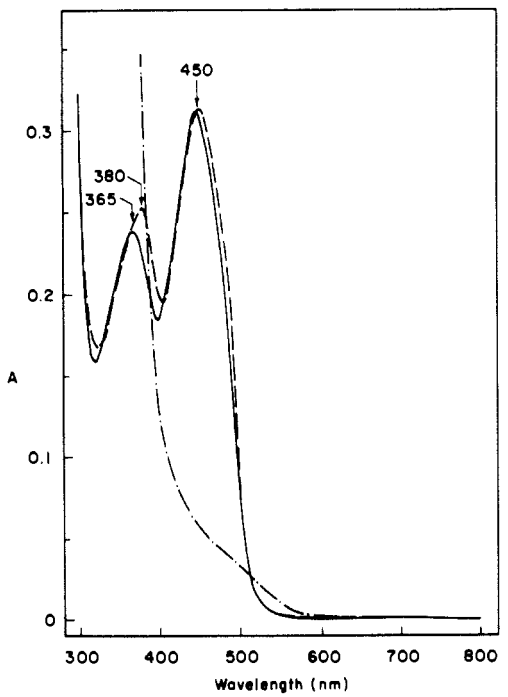


FIGURE 5: Spectrum of butyryl-CoA dehydrogenase after inactivation by 3-pentynoylpantetheine. (—) Butyryl-CoA dehydrogenase (yellow form) (9 nmol) in 370 μL of 0.1 M potassium phosphate buffer, pH 7.0, at 25 °C. (- - -) 1 min and 40 min after addition of 0.5 μL of 20 mM 3-pentynoylpantetheine; (···) after addition of a few grains of solid $Na_2S_2O_4$. An aliquot was assayed after 20 min and found to be inactive.

drogen is transferred to the flavin, where it is subject to exchange with solvent protons. In the absence of electron acceptors, when no decarboxylation occurs, the enzyme catalyzes the exchange of the substrate β proton with solvent protons. In the presence of an electron acceptor, the flavin is reoxidized and decarboxylation occurs. Decarboxylation leads to the formation of an allylic anion which is then protonated by the active-site base. The base must be a monoprotic base since the proton is transferred essentially without dilution.

The results which we have obtained are clearly consistent with a carbanion mechanism but do not provide absolute proof for the mechanism. An alternative explanation of the results is possible: oxidation of the substrate and formation of reduced flavin occur by some mechanism which results in transfer of the α and β hydrogen to the flavin, possibly as hydrogen atoms. One of the two hydrogens of the reduced flavin, i.e., the hydrogen dervied from the β position of the substrate, is exposed to solvent protons and subject to exchange with solvent protons. The other hydrogen, dervied from the α position of the substrate, is sequestered from the solvent. Upon oxidation of the flavin, this hydrogen is transferred to a base at the active site and from there to the γ position of the reaction product. According to this interpretation, the base at the active site is not involved in the oxidative process but only in the decarboxylation. We believe, however, that the alternative mechanism is more difficult to reconcile with the results of the inactivation experiments which will be discussed below than the mechanism represented in Scheme I.

Glutaryl- and butyryl-CoA dehydrogenases are inactivated by acetylenic substrate analogues. Inactivation does not alter the flavin spectrum significantly. Furthermore, the flavin of the inactivated enzyme can be reduced with dithionite. It is, therefore, likely that the inactivator does not interact with the flavin but with a functional group at the active site. This inactivation, therefore, differs from other cases where flavoproteins have been inactivated with acetylenic substrate analogues, where a covalent adduct between inactivator and flavin is formed. The acetylenic inactivators used with butyryl- and glutaryl-CoA dehydrogenases differ from previously used acetylenic inactivators in another important respect. In all other cases, the structure of the inactivator was such that it could undergo oxidation prior to inactivation. The interaction of flavin and inactivator could occur between reduced flavin and oxidized inactivator. If oxidation of the inactivator occurs prior to reaction with the flavin, no information concerning the chemical mechanism of the oxidative process could be gained from these inactivators. The acetylenic inactivators (butynoyl- and pentynoylpantetheine) used with glutaryl- and butyryl-CoA dehydrogenases cannot be oxidized without breaking carbon–carbon bonds. The inactivation therefore must occur prior to complete electron transfer from inactivator to flavin and therefore reflects a process which has occurred prior to oxidation.

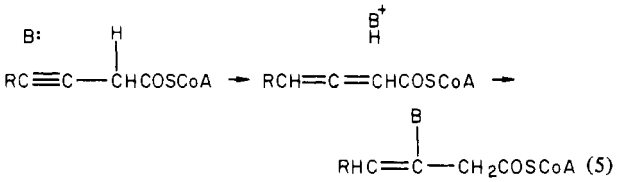
The mechanism of inactivation of glutaryl-CoA dehydrogenase and butyryl-CoA dehydrogenase is not known. It is likely, however, in view of the results obtained with other

Table I: Purification of Glutaryl-CoA Dehydrogenase[a]

procedure (H_2O extraction)	vol (mL)	unit (mL)	total units	[protein] (mg/mL)	unit/mg $\times 10^3$	$OD_{260/280}$
before centrifugation	2000	0.02	42	59	0.36	1.2
after centrifugation	1500	0.023	34	13	1.8	1.6
streptomycin	1600	0.023	37	13	1.8	1.4
35% $(NH_4)_2SO_4$	1850	0.023	43	14	1.6	1.7
55% $(NH_4)_2SO_4$	60	0.15	9.1	51	3.0	0.7
affinity column	18	0.063	1.1	30	2.1	0.7

[a] Assays for this purification were done as in the standard assay except PMS was omitted. This results in low specific activities in the later stages of purification since ETF is removed. Final activity in the standard assay (with PMS) was 0.24 IU/mg.

acetylenic inactivators (Bloch, 1971) (Washtien & Abeles, 1977), that it involves allene formation followed by addition of a nucleophile (eq 5).



If inactivation occurs by the mechanism of eq 5, it is initiated by α-proton abstraction and thus establishes that the enzyme can abstract a substrate α proton. In the case of glutaryl-CoA dehydrogenase, it might be argued that the enzyme's ability to abstract a proton is related only to the decarboxylation reaction. However, this argument does not apply to butyryl-CoA dehydrogenase since that enzyme does not catalyze a decarboxylation. Therefore, it is very likely that the ability to abstract a proton, as reflected by the inactivation process, is also utilized in the oxidative reaction.

In summary, we believe that the results presented here provide evidence that the reactions catalyzed by butyryl-CoA dehydrogenase and glutaryl-CoA dehydrogenase proceed through a carbanion mechanism. Model studies have established that analogous nonenzymatic oxidations can proceed by a carbanion mechanism (Bruice, 1980). It probably is not possible to provide absolute proof for a carbanion mechanism for an enzymatic reaction. The mechanism becomes acceptable when sufficient supporting experiments are presented and none contradict it. This now appears to be the case with a number of flavoproteins.

Appendix

Purification of Glutaryl-CoA Dehydrogenase. Growth of Bacteria. The enzyme source was *Pseudomonas fluorescens* ATCC 11250. Growth conditions and acetone extraction were modifications of the procedure of Numa et al. (1964). Bacteria were grown either in 20-L carboys containing 10 L of growth media or in a 100-L fermentor.

The growth medium for the innocula and the final growth vessels was 0.2% bactopeptone (Difco), 0.1% yeast extract (Nutritional Biochemicals), 0.5% NH_4Cl, 0.15% K_2HPO_4, 0.05% KH_2PO_4, and 0.15% lysine monohydrochloride (ICN). $MgSO_4 \cdot 7H_2O$ was sterilized separately and added to a final concentration of 0.02%. Cultures were grown at 26 °C with vigorous aeration for 18 h. A continuous feed centrifuge was used for harvesting. Yields of cell paste were 3–4.5 g/L.

Purification of Glutaryl-CoA Dehydrogenase. Acetone Extraction. In batches of approximately 20 g, the bacterial paste was added to 3 volumes of acetone previously chilled to −20 °C. The suspension was homogenized in a Waring blender for 1 min. The homogenate was filtered on a refrigerated Buchner funnel (−10 °C). The solid was subjected to two more cycles of acetone, homogenization, and filtration. The acetone extracted cells were put in a desiccator and dried under vacuum at 4 °C. Yields were approximately 2 g of acetone powder from 10 g of bacterial paste. All subsequent steps were performed at 4 °C.

Water Extraction. A total of 100 g of the acetone powder was ground to a fine consistency with a mortar and pestle. This powder was suspended in distilled water to a final volume of 2 L, and 20 mg of FAD was added. An overhead stirrer was used to blend the solution for 1 h. The tan mixture was centrifuged for 2 h at 12200*g*. The loose pellet was discarded (see Table I).

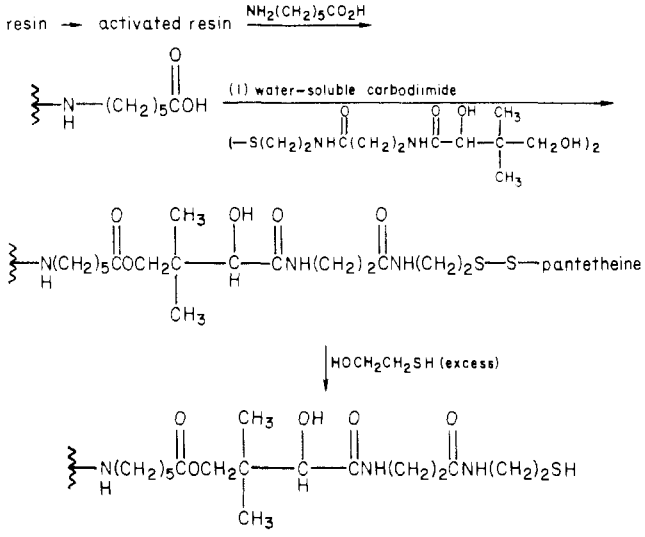
Streptomycin Sulfate Fractionation. To the supernatant (1.5 L) was added dropwise 200 mL of 10% (w/v) streptomycin sulfate (Sigma) over 30 min. An overhead stirrer was again employed. Care should be taken to occassionally clean the fibrous precipitate from the stirrer, as the accumulated mass occludes a large amount of solution which would otherwise be discarded. After addition of the streptomycin, the solution was stirred for an additional hour. The suspension is centrifuged for 1 h at 12200*g*. The pellet is discarded.

Ammonium Sulfate Fractionation. To the supernatant (1.6 L) was added 320 g of $(NH_4)_2SO_4$ slowly with stirring. The addition took approximately 35 min. The addition of $(NH_4)_2SO_4$ brings the concentration to 35% saturation (calculated for 0 °C). The solution was stirred an additional hour after completion of addition of $(NH_4)_2SO_4$ and then centrifuged for 30 min at 12200*g*. The pellet was discarded. To the supernatant (1.85 L) was added 222 g of $(NH_4)_2SO_4$, in the same manner as before. This brought the concentration to 55% saturation (calculated for 0 °C). The suspension was centrifuged for 30 min at 12200*g*. The pellet was slurried in 40 mL of 20 mM potassium phosphate buffer, pH 7.0, which contained 5 mg/mL FAD. The slurry was dialyzed against the same buffer (2 × 2.5 L) overnight. A small amount of precipitate was removed by centrifugation (5900*g* for 10 min).

Pantetheine Affinity Column. A 2 × 19 cm Sepharose 4B 6-aminocarpproylpantetheine column was washed with β-mercaptoethanol (as described in the synthesis of the affinity matrix) and then equilibrated with the dialysis buffer. The protein was loaded on the top of the column. The column was washed with the same buffer until the OD_{280} decreased to the background level (this required approximately 3 L). (The flow-through is an excellent source of crude ETF.) The top 2 cm of the column, containing a gray-brown material, was removed and discarded. The column was then eluted with 0.2 M NaCl. The elution of the enzyme can be easily followed by its bright green color or by measuring the OD_{435}. The protein concentration of the eluted enzyme is sufficiently high so that no further concentration is required. The eluted enzyme can be frozen for storage and is stable for at least 2 months.

Resolution and Reconstitution of Glutaryl-CoA Dehydrogenase. Glutaryl-CoA dehydrogenase can be resolved from its flavin cofactor by acidic ammonium sulfate treatment.

Scheme II



At 0 °C, 0.5 mL of enzyme solution (6 mg/mL) was added to 100 mg of ammonium sulfate and 35 μL of 1 N H_2SO_4 containing 0.22 mg/mL $(NH_2)_2SO_4$. The solution was quickly centrifuged (~1 min). The pellet was resuspended in 0.5 mL of 130 mM H_2SO_4 containing 200 mg/mL $(NH_4)_2SO_4$. This suspension was rapidly centrifuged. The resuspension and centrifugation were repeated twice more. After the final centrifugation, the pellet containing the apoenzyme was resuspended in 0.2 M potassium phosphate buffer, pH 7.0. This solution must be used immediately, as the apoenzyme phosphate buffer, pH 7.0. This solution must be used immediately, as the apoenzyme is very unstable (within 2 or 3 min, no activity can be reconstituted). The apoenzyme has no detectable activity in the normal assay.

For reconstitution of the enzyme, the final pellet from acidic ammonium sulfate treatment is resuspended in 0.2 M potassium phosphate buffer, pH 7.0, containing 0.3 mM FAD. In those cases where FMN was tested as a possible cofactor, the resuspension was the same, but FMN was substituted for FAD. In either case, a final centrifugation to remove denatured protein was required.

Synthesis of Sepharose 4B 6-Aminocaproylpantetheine—The Affinity Column. The affinity matrix was synthesized according to Scheme II. Approximately 20 g of wet Sepharose 4B in 100 mL of water in a beaker equipped with a magnetic stirrer and pH electrode was cooled in an ice bath and approximately 7 g of cyanogen bromide slurried in 50 mL of water was added. The pH was kept constant at 11 for 0.5 h by addition of 5 N NaOH. The resin was then quickly filtered on a Buchner funnel and, while still in the funnel, was washed with 150 mL of cold water, 1 L of cold 0.5 M $NaHCO_3$, and finally 0.5 L of cold water. The activated resin was transferred to a beaker and 5 g of 6-aminocaproic acid (Aldrich) in 40 mL of water was added. The pH was adjusted to 8, and the solution was stirred overnight at 4 °C.

The Sepharose aminocaproate resin was filtered and washed with water. The resin was suspended in approximately 60 mL of water in a beaker equipped with a magnetic stirrer and pH electrode and cooled in an ice bath. One gram of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (Sigma) was added, quickly followed by 30 mL of cold water containing 2 g of pantetheine which had previously been adjusted to pH 5. The pH was maintained at 5 for 0.5 h, after which time it did not vary. The suspension was stirred overnight at 4 °C. The resin was filtered and washed extensively with water. The disulfide of the bound pantetheine was reduced just prior to use of the column by passing 1 mL of β-mercaptoethanol through the affinity column. This was followed by washing with water and then buffer until no more thiol could be detected in the effluent by the nitroprusside test. The resin was stored at −20 °C in 50% glycerol.

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