Mechanism of Action of Butyryl-CoA Dehydrogenase: Reactions with Acetylenic, Olefinic, and Fluorinated Substrate Analogues†

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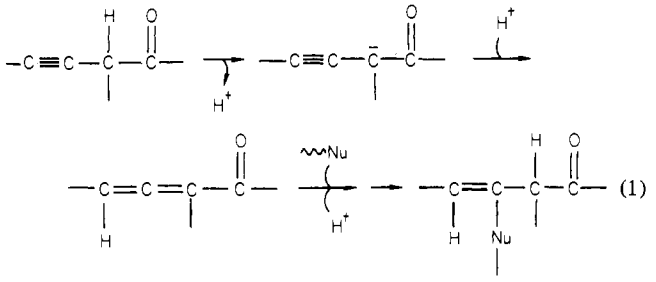
ABSTRACT: The acetylenic thio ester (3-pentynoyl)pantetheine irreversibly inactivates butyryl-CoA dehydrogenase from *Megasphaera elsdenii*. The inactivator becomes covalently attached to the protein (0.61 ± 0.1 mol of ^{14}C-labeled inactivator/mol of enzyme flavin). No modification of the flavin cofactor is seen. The covalent enzyme–inactivator adduct is labile toward base and neutral hydroxylamine. These treatments release 85 ± 5% of the incorporated ^{14}C label from the protein. Base-catalyzed hydrolysis of the adduct releases 3-oxopentanoic acid (0.6 mol/mol of incorporated inactivator). Treatment with hydroxylamine leads to formation of a hydroxamic acid on the protein (0.64 ± 0.09 mol/mol of incorporated inactivator). The covalent adduct can be reduced with sodium borohydride with release of 1,3-pentanediol. Hydrolysis of the protein with 6 N HCl after sodium borohydride reduction yields 2-amino-5-hydroxyvaleric acid and proline. We conclude that the inactivator has reacted with the γ-carboxyl group of a glutamate residue at the enzyme active site. The inactivation proceeds through enzyme-catalyzed rearrangement of the acetylene to an allene, followed by nucleophilic addition of the carboxyl group to the allene. (3-Chloro-3-butenoyl)pantetheine irreversibly inactivates the enzyme in a fashion similar to the acetylenic thio ester and also modifies a glutamate residue. Butyryl-CoA dehydrogenase catalyzes the isomerization of (3-butenoyl)pantetheine to (2-butenoyl)pantetheine. The enzyme catalyzes the elimination of HF from 3-fluoropropionyl-CoA and (3,3-difluorobutyryl)pantetheine. We suggest, that these results together support an oxidation mechanism for butyryl-CoA dehydrogenase which is initiated by α-proton abstration.

Acetylenic substrate analogues have frequently been used as inactivators for enzymes which are believed to catalyze reactions involving carbanionic intermediates (Abeles & Maycock, 1976; Rando, 1977; Walsh, 1977). In many cases, the inactivation mechanism may involve enzyme-catalyzed carbanion formation, followed by rearrangement of the carbanion to an allene, and finally nucleophilic addition of a group at the active site of the enzyme to the allene (eq 1).



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It has been proposed that the flavoenzyme-mediated dehydrogenation of acyl-CoA esters is initiated by abstraction of the α hydrogen as a proton (Cornforth, 1959). The carbanion, stabilized by the adjacent thio ester group, then undergoes electron transfer to the enzyme flavin by a currently unknown mechanism. Chemical model studies have established that flavin-catalyzed oxidation reactions can involve carbanionic intermediates, when the substrate provides sufficient carbanion stabilization (Bruice, 1980).

Acetylenic thio esters inactivate general acyl-CoA dehydrogenase from pig liver (Frerman et al., 1980) as well as the bacterial enzymes glutaryl-CoA dehydrogenase and butyryl-CoA dehydrogenase (Gomes et al., 1981). We have now investigated the mechanism of inactivation of butyryl-CoA dehydrogenase by (3-pentynoyl)pantetheine. Specifically, we wished to establish whether the inactivation proceeds through intermediate formation of an allene. Additionally, since the inactivator does not react with the flavin, a study of the inactivation could lead to the identification of a functional group at the active site. We also investigated reactions in which butyryl-CoA dehydrogenase catalyzes α,β elimination as well as an allylic isomerization. These reactions probably proceed by carbanion mechanism and thus provide further indication that the enzyme is capable of catalyzing the formation of substrate-derived carbanions.

Materials and Methods

Enzyme Preparation. Low iron cultures of *Megasphaera elsdenii* (ATCC 25940) were grown, harvested, and dried as described by Mayhew & Massey (1969), except that commercial sodium lactate (60% syrup) was used. Butyryl-CoA dehydrogenase was isolated from dried cells by a modification of the procedure of Engel & Massey (1971a). After the acid precipitation step, the protein solution was dialyzed against two additional changes of 0.2 M potassium phosphate, pH 7, and then applied to the DEAE-cellulose column. The final step of chromatography on Sephadex G-200 was omitted. The protein was at least 90% pure by $NaDodSO_4$–polyacrylamide gel electrophoresis. The absorbance ratios obtained from different enzyme preparations were $A_{710}:A_{430}$ = 0.3–0.35, $A_{360}:A_{430}$ = 0.8–0.83, and $A_{266}:A_{430}$ = 8.0–8.4. Enzymatic activity was assayed in 0.1 M potassium phosphate buffer, pH 7, 70 μM DCPIP,[1] 0.6 mM PMS, and 1 mM butyryl-pantetheine by using ϵ_{600} = 21450 M^{-1} cm^{-1} for DCPIP

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[1] Abbreviations: DCPIP, 2,6-dichlorophenolindophenol; PMS, phenazine methosulfate; Gdn·HCl, guanidine hydrochloride; Cl_3CCOOH, trichloroacetic acid; $NaDodSO_4$, sodium dodecyl sulfate; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; HPLC, high-pressure liquid chromatography; TLC, thin-layer chromatography.

0006-2960/82/0421-6685$01.25/0

(Gomes et al., 1981). All enzyme concentrations refer to bound FAD by using $\epsilon_{430} = 9600\ M^{-1}\ cm^{-1}$ (Engel & Massey, 1971a).[2]

Synthetic Procedures. Pantetheine was prepared by $NaBH_4$ reduction of pantethine (Vega Biochemicals Corp. or Calbiochem) (Gomes et al., 1981). Pantetheine thio esters were synthesized by two methods: (A) from the acid with dicyclohexylcarbodiimide in tetrahydrofuran/acetone (Gomes et al., 1981); (B) from the acid anhydride by the procedure described for glutarylpantetheine (Gomes et al., 1981). Pantetheine thio esters were purified by HPLC system D. They were characterized by NMR. 2-Pentynoic acid was prepared by carboxylation of lithium 1-butynide (Brandsma, 1971), mp 50 °C (reported mp 50 °C; Zoss & Hennion, 1941). 3-Pentynoic acid was prepared by chromic acid oxidation of 3-pentyn-1-ol (ICN) (Jones et al., 1954) and by rearrangement of 2-pentynoic acid sodium salt in 6 N NaOH at 60 °C, 90 min [mp 100 °C; reported mp 101–104 °C (Jones et al., 1954)]. The NMR spectra of both acids were identical with the published spectra (Bushby & Whitham, 1969). 2-Amino-5-hydroxyvaleric acid was kindly donated by Dr. W. P. Jencks.

2-[1-^{14}C]Pentynoic acid was synthesized by a modification of the procedure of Brandsma (1971). To 0.5 mL (~6 mmol) of 1-butyne (Pfaltz & Bauer) in 2 mL of dry tetrahydrofuran was added 2.4 mmol of 1-butyllithium in ether at −70 °C. The lithium 1-butynide solution was carboxylated in a closed apparatus first with $^{14}CO_2$ generated from 0.94 mmol of $Ba^{14}CO_3$ (5.39 mCi/mmol; ICN) followed by nonisotopic CO_2 generated from 1 mmol of $NaHCO_3$. The acid was isolated by ether extraction and purified by sublimation (0.3 mm, 45 °C bath temperature): yield ~130 mg (1.3 mmol). At least 90% of the radioactive compound comigrated with authentic 2-pentynoic acid in HPLC systems A and B. A specific activity of 4.6×10^6 cpm/μmol was determined in HPLC system A. Rearrangement to 3-[1-^{14}C]pentynoic acid was accomplished in 6 N NaOH at 60 °C, 90 min. The acid was isolated by ether extraction and recrystallized from hexane: yield 20 mg (0.2 mmol). At least 90% of the radioactive material comigrated with authentic 3-pentynoic acid in HPLC system A and on TLC [silica, chloroform/acetic acid (9:1)]. (3-[1-^{14}C]-Pentynoyl)pantetheine was prepared by method A. At least 90% of the radioactive material cochromatographed with nonisotopic (3-pentynoyl)pantetheine in HPLC system D and on TLC [silica, chloroform/methanol (9:1)]. A specific activity of $(4.1 \pm 0.3) \times 10^6$ cpm/μmol was determined by HPLC and TLC.

The Li salt of 3-oxopentanoic acid was synthesized by hydrolysis of ethyl propionylacetate (Pfaltz & Bauer) with LiOH as described for the preparation of lithium acetoacetate (Hall, 1963). The Li salt was ≥95% pure by HPLC system A and by NMR: NMR (D_2O) δ 3.34 (s, 2 H, $-CH_2COO$), 2.56 (q, 2 H, J = 7 Hz, CH_3CH_2CO), and 0.9 (t, 3 H, J = 6.5 Hz, CH_3CH_2-). Concentrations of standard solutions of the ketoacid were determined by titration and enzymatically with D-3-hydroxybutyrate dehydrogenase (Mellanby & Williamson, 1974).

3-Hydroxypentanoic acid was prepared by reduction of lithium 3-oxopentanoate with a 3-fold molar excess of $NaBH_4$ in 10^{-4} M NaOH at room temperature. The reaction was stopped after 7 h and brought to dryness, and the residue was repeatedly treated with methanol to decompose borate esters. The residue was then taken up in water and extracted with ether to yield 3-hydroxypentanoic acid as a colorless syrup: NMR ($CDCl_3$) δ 6.47 (br s, 2.3 H, exchangeable with D_2O), 4.07–3.77 (m, 1 H, $-CHOH$), 2.53–2.37 (m, 2 H, $-CH(OH)CH_2COOH$), 1.66–1.30 [m, 2 H, $-CH_2CH(OH)$], and 0.9 (t, 3 H, J = 6.5 Hz, CH_3-). The concentration of 3-hydroxypentanoic acid solutions was determined by titration and enzymatically with D-3-hydroxybutyrate dehydrogenase (Williamson & Mellanby, 1974).

1,3-Pentanediol was prepared by reduction of ethyl propionylacetate (Pfaltz & Bauer) with a 5-fold molar excess of $NaBH_4$ in refluxing methanol for 2 h. After removal of the solvent the residue was taken up in water, neutralized with concentrated HCl, and continuously extracted with ether. A mixture of free diol and borate esters was obtained, the latter could be decomposed by repeated treatment with methanol/HCl. The residue was then applied to a Dowex 1-OH^- column and eluted with water. Elution of the diol was followed by a spot test with vanadium oxinate reagent (Zweig & Sherma, 1972). After the solution was evaporated and dryed in vacuo, 1,3-pentanediol was obtained as a clear, viscous liquid. A small amount of impurity (≤5% of the total material) could be removed by HPLC system C: NMR ($CDCl_3$) δ 4.0–3.63 [m, 3 H, $-CH_2OH$ and $-CH(OH)-$], 3.13 (s, 2 H, −OH, exchangeable with D_2O), 1.84–1.34 (m, 4 H, CH_3CH_2- and $-CH_2CH_2OH$), and 0.92 (t, 3 H, J = 6.5 Hz, CH_3CH_2-). Concentrations of pentanediol solutions were determined from weight and enzymatically with horse liver alcohol dehydrogenase (Bernt & Gutman, 1974). The enzymatic assay was modified by using 0.2 M semicarbazide to trap the produced aldehyde and by increasing the NAD concentration to 10 mM. Oxidation of the primary alcohol group of commercial 1,3-butanediol (Aldrich, 99% purity) was to ≥90% complete under the assay conditions used.

3-Chloro-3-butenenitrile was prepared from 2,3-dichloro-1-propene (Aldrich) and NaCN (Kurtz et al., 1960; Vessière, 1959). The crude product was a 3:1 mixture of the nitrile and 3-chloro-3-buten-1-ol, which could not be cleanly separated by distillation: bp 35 °C (10 mm) for the fraction containing ≥95% nitrile (reported bp 39.5–40.5 °C (11 mm); Vessière, 1959); NMR ($CDCl_3$) δ 5.55 (d, 1 H, J = 1.5 Hz, cis H—C=C—Cl), 5.45 (d, 1 H, J = 2 Hz, trans H—C=C—Cl), and 3.4 (s, 2 H, $C{=}CCH_2CN$). The nitrile was hydrolyzed with concentrated HCl (10 equiv) at 80 °C, 2 h. The reaction mixture was then diluted with 1 volume of water and the acid isolated by ether extraction. Purification was achieved by sublimation (0.04 mm/30 °C bath temperature) or by silicic acid chromatography (Varner, 1957). The acid was eluted with chloroform/1-butanol (98:2) and isolated as the sodium salt: NMR free acid (acetone-d_6) δ 5.52 (d, 1 H, J = 1.5 Hz, cis H—C=C—Cl), 5.41 (d, 1 H, J = 1.5 Hz, trans H—C=C—Cl), and 3.48 (d, 2 H, J = 1 Hz, C=C—CH_2—COOH); free acid (D_2O) δ 5.47 (s, 2 H, $H_2C{=}C-$), 4.8 (s, 1.6 H, H_2O and −COOH), and 3.5 (s, 2 H, $C{=}CCH_2COOH$). The collapse of the two vinyl proton signals to a singlet in D_2O appears to be a solvent effect. The collapse is reversed, when the solvent is changed back to acetone-d_6. IR (free acid) (neat, between NaCl disks) 1718 (ν C=O), 1640 (ν $H_2C{=}C<$), 1430–1410 (δ $H_2C{=}C<$), 903 (δ $H_2C{=}C<$), and 660 (ν C—Cl) cm^{-1}. Anal. Calcd for (Na salt) $C_4H_4ClO_2Na$ (M_r 142.517): Cl, 24.876. Found: Cl, 24.7.

The pantetheine ester was prepared by method A: NMR (D_2O) δ 5.47 (s, 2 H, $H_2C{=}C$) and 3.73 (s, 2 H, $-CH_2COSR$); the other signals at 3.96, 3.4, 3.1, 2.44, and 0.88 ppm belonged to the pantetheine moiety. The integrated

[2] A revised extinction coefficient ($\epsilon_{430} = 10\,400\ M^{-1}\ cm^{-1}$) has been published recently (Engel, 1981).

intensities of the acid and the pantetheine protons were of the correct ratio.

3,3-Difluorobutyric acid was a generous gift from Dr. JoAnne Stubbe: bp 30 °C (1 mm); NMR ($CDCl_3$) δ 3.47 (t, 2 H, J_{CHCF} = 14 Hz, CF_2CH_2COOH) and 1.82 (t, 3 H, J_{CHCF} = 18.5 Hz, CH_3CF_2–). The pantetheine ester was prepared by method A: NMR (D_2O) δ 0.86 (d, 6 H, J = 3.5 Hz, pantetheine), 1.68 (t, 3 H, J_{CHCF} = 19 Hz, CH_3CF_2–), 2.43 (t, 2 H, J = 6.5 Hz, pantetheine), 2.92–3.2 (m, 2.5 H, 2 H pantetheine + 0.5 H CF_2CH_2COSP.), 3.2–3.58 (m, 7.5 H, 6 H pantetheine + 1.5 H CF_2CH_2COSP.), and 3.97 (s, 1 H, pantetheine).

3-Fluoropropionic acid was synthesized by chromic acid oxidation of 3-fluoro-1-propanol (Columbia Organic Chemicals Co., Inc.) by a modification of the procedure of Pattison et al. (1956). The oxidation was performed in the presence of Florisil (Fisher Scientific Co.; 10 g/0.1 mol of fluoropropanol). The ether extract which contained considerable amounts of chromium salts was passed through a Florisil column (3.5 × 5.2 cm) and the column washed with 150 mL of ether. After the extract was dried over Na_2SO_4, the ether was distilled off through a 13-cm Vigreux column under normal pressure. The residue was fractionally distilled to yield the acid: bp 55-60 °C (1.6 mm) [lit. bp 51–52 °C (2 mm); Gryszkiewicz-Trochimowski, 1947]; NMR ($CDCl_3$) δ 4.78 (dt, 2 H, J_{CHF} = 47.5 Hz, J_{CHCH} = 6 Hz, FCH_2–) and 2.82 (dt, 2 H, J_{CHCF} = 25.5 Hz, J_{CHCH} = 6.5 Hz, CH_2FCH_2–). 3-Fluoropropionyl chloride was prepared with thionyl chloride as described previously (Stubbe & Abeles, 1977). The crude reaction mixture contained 90% of the desired product and 10% starting material by NMR and was used directly for the synthesis of 3-fluoropropionyl-CoA. The published procedure (Stubbe & Abeles, 1977) was modified by using 1 M $KHCO_3$ to buffer the excess HCl introduced by addition of the crude acid chloride. Fluoropropionyl-CoA was identified by NMR.

(3-Butenoyl)pantetheine was prepared from 3-butenoic acid (Polysciences, Inc.) by method A: NMR (D_2O) δ 6.18–5.70 (m, 1 H, CH_2=CH—), 5.29 (dm, 2 H, large J = 14.4 Hz, H_2C=CH—), 4.0 (s, 1 H, pantetheine), 3.67–3.25 (m, 8 H, 6 H pantetheine + 2 H –CH_2COSP.), 3.08 (t, 2 H, J = 6.5 Hz, pantetheine), 2.49 (t, 2 H, J = 6.5 Hz, pantetheine), and 0.89 (d, 6 H, J = 3 Hz, pantetheine). Butyrylpantetheine was prepared from butyric anhydride by method B.

Analytical Procedures. Radioactivity on chromatograms was determined by liquid scintillation counting. The paper or the TLC sheets were cut in 0.5–1.0-cm segments, placed into scintillation vials, and eluted with 0.1 N HCl (amino acids) or water (all other compounds). After addition of ACS counting fluid (Amersham) radioactivity was measured with a Beckman Model LS-100 C scintillation counter. The specific activity of radioactive compounds was determined by HPLC. Fractions (0.25–1.0 mL) were collected along the entire chromatogram to determine radioactivity by liquid scintillation counting. The peak area of the radioactive compound was compared to the peak areas obtained from injections of known amounts of the nonisotopic compound. The specific activity of individual fractions across the radioactive peak was estimated from their recorded refractive index. Alternatively, the peak was traced on paper, and the segments corresponding to each fraction were cut out and weighed.

Concentration of thio ester solutions were determined after hydrolysis in 0.5–1.0 N NaOH at 37 °C, 30 min, or after cleavage in 1 M neutral NH_2OH at 25 °C, 12 min, by reaction of the liberated thiol with Ellman's reagent (Ellman, 1959). Alternatively, the hydroxamic acid formed upon NH_2OH cleavage was assayed by the ferric chloride procedure (Jencks, 1958).

Fluoride was determined with a fluoride electrode (Model 96-09, Orion Research Inc.) in connection with an Orion 701 A pH meter in a continuous assay (method 1) or by aliquot assay (method 2). *Method 1*: The fluoride electrode was immersed directly into the reaction vial containing 0.1 M potassium phosphate, pH 7, 5×10^{-6} M NaF, and 100 μM 3-fluoropropionyl-CoA or 2 mM (3,3-difluorobutyryl)pantetheine in a total of 0.5 mL. The temperature was maintained at 24 ± 1 °C with a circulating water bath. When a constant background rate was observed (after 4–6 min), the reaction was started by addition of enzyme. The decrease in potential was recorded as a function of time and compared with a calibration curve by using known concentrations of NaF. *Method 2*: Twenty-microliter aliquots were added to 2 mL of 0.5 M sodium acetate, pH 5.0, 0.5 M NaCl, and 5×10^{-3} M ethylenediaminetetraacetate, 25 °C. Readings were taken when the fluoride electrode had reached a relatively stable potential (after 3–5 min, drift <0.5 mV/min). They were compared to a calibration curve by using known amounts of NaF.

The following HPLC systems were used in the course of this work: system A, Aminex HPX 87 ion-exclusion column (Bio-Rad, organic acid analysis column, 0.78 × 30 cm), 6 mM H_2SO_4, 0.6 mL/min; system B, C_{18} reverse phase column (0.39 i.d. × 30 cm; Waters Associates), water, pH 2.4, with H_3PO_4 or H_2SO_4, 3 mL/min; system C, C_{18} reverse phase column (0.39 i.d. × 30 cm or radial compression column RCM-100, i.d. 8 mm, both Waters Associates), distilled water, 1.5 mL/min; system D, C_{18} reverse phase column (0.39 i.d. × 30 cm; Waters Associates), 30–40% aqueous methanol, 1–1.5 mL/min; system E, C_{18} reverse phase column (0.39 i.d. × 30 cm; Waters Associates), 50 mM potassium phosphate (pH 5.7)/methanol (82:18), 1.5 mL/min.

A refractive index detector was used for all compounds except CoA–thio esters which were detected by UV absorbance (254 nm).

All NMR spectra were taken on a Bruker FT WH 90 spectrometer. Absorbance spectra were recorded with a Perkin-Elmer Model 559 spectrophotometer.

Preparation and Denaturation of the (3-[1-^{14}C]Pentynoyl)pantetheine–Protein Adduct. To a (1–3) × 10^{-4} M solution of the green enzyme in 0.1 M potassium phosphate, pH 7, was added (3-[1-^{14}C]pentynoyl)pantetheine to a final concentration of (2–6) × 10^{-4} M. The solution was incubated at room temperature and periodically assayed for loss of activity. When inactivation was ≥95% complete (after 30–50 min), the yellow protein solution (200–300 μL) was passed through a Sephadex G-25 column (0.7 × 30 cm) equilibrated with 0.1 M potassium phosphate, pH 7, at 4 °C. The absorbance spectrum of the eluted protein was recorded, and an aliquot was removed to determine the amount of incorporated radioactivity. The labeled protein was then immediately precipitated with Cl_3-CCOOH (5% final concentration) at 4 °C. The protein pellet was washed 3 times with 200 μL of ice-cold water and then redissolved at pH 5.5 in 7 M Gdn·HCl (Heico, Inc) or 8 M urea (recrystallized from 95% ethanol) for 1 h at 30 °C.

Titration of Butyryl-CoA Dehydrogenase with (3-Pentynoyl)pantetheine. The enzyme (25 μM) in 0.1 M potassium phosphate, pH 7, was incubated with 0, 12.5, 13.8, 16.2, 25.2, and 47 μM inactivator. After 40 min at 25 °C aliquots (10 μL) were added to 1 mL of standard assay to determine residual enzymatic activity.

Rate of Hydrolysis of the Enzyme–Inactivator Linkage. To a solution of ^{14}C-labeled protein (12 nmol, 2.9×10^4 cpm) in 0.4 mL of 8 M urea, pH 5, was added 1 M K_2HPO_4 in 8 M urea to adjust the pH to 8.43 (experiment 1) or 8.73 (experiment 2). The mixture was stirred at room temperature, and 50-μL aliquots were removed at intervals and passed through a Bio-Gel P-6 column (0.55×16 cm) equilibrated with 0.1 M potassium phosphate–8 M urea, pH 6.5. Fractions of 0.2 mL were collected into counting vials to determine radioactivity; recovery of the applied radioactivity was ≥90%. Half-lives were determined from semilogarithmic plots of percent protein-bound radioactivity vs. time. The fraction of radioactivity which remained protein bound after <10 half-lives (18% at pH 8.43, 15% at pH 8.73) was taken as the end point.

Identification of the Radioactive Compounds Released by Base Treatment. A solution of ^{14}C-labeled protein (60 nmol, 1.29×10^5 cpm) in 300 μL of 8 M urea–0.1 M Tris-HCl was adjusted to pH 11 by addition of 15 μL of 1 N NaOH. The mixture was stirred 1 h at room temperature. 3-Oxopentanoic acid (Li salt) (170 μmol) was then added and the protein solution applied to a Bio-Gel P-6 column (0.6×24.5 cm) equilibrated with 0.1 M Tris-HCl–7 M urea, pH 7.8; 2.24×10^4 cpm (20% of the originally incorporated radioactivity) eluted with the protein peak. Radioactive fractions eluting after the protein peak were pooled (3.25 mL total, 8.5×10^4 cpm), the pH was adjusted to 9.8, and the solution was treated with 8 mg (0.2 mmol) of $NaBH_4$ for 5.5 h at room temperature. After acidification to pH 2.4, the reaction mixture was continuously extracted (12 h) with 20 mL of ether, which removed 80% of the radioactivity (7×10^4 cpm) from the aqueous phase. The ether was dried and concentrated and the residue treated with methanol to remove borate. After the mixture was dried in vacuo, a colorless syrup remained, which contained 5.4×10^4 cpm or 62% of the total radioactivity originally released from the labeled protein. The specific activity of the isolated 3-hydroxypentanoic acid was determined by HPLC systems A and C.

Reaction of Hydroxylamine with [^{14}C]Butyryl-CoA Dehydrogenase. To a solution of ^{14}C-labeled protein (52 nmol, 1.3×10^5 cpm) in 200 μL of 7 M Gdn·HCl, pH 6.5, was added neutralized NH_2OH·HCl [recrystallized from ethanol/water (7:1)] to obtain a final concentration of 0.4 M NH_2OH. After the solution was stirred for 2 h at room temperature, the protein was separated from released small molecules and excess reagent by passage through a Sephadex G-25 column (0.8×33 cm) equilibrated with 7 M Gdn·HCl. Protein-containing fractions were pooled and assayed for hydroxamic acid formation. Protein-free fractions either were used entirely for determination of released radioactivity or were analyzed to identify the released compound(s). Noninactivated protein (52 nmol) was treated identically and served as the control for the hydroxamate assay.

Hydroxamic acids were estimated by a modification of the procedure of Yasphe et al. (1960). The assay was scaled down 10-fold to a final volume of 0.85 mL. *N*-(1-Naphthyl)-ethylenediamine dihydrochloride was used instead of α-naphthylamine, and the absorbance was read at 550 nm. Protein samples and the acetohydroxamic acid standard were used as solutions in 7 M Gdn·HCl. The color yield obtained from the inactivated hydroxylamine-treated protein was about twice that obtained from the control protein at identical protein concentrations in the assay. The released radioactive compounds were purified on a Dowex 1-OH^- column (1.4 mL bed volume) in the presence of 14 μmol of synthetic 3-oxopentanoic acid. After the column was washed with 8 mL of water, 80% of the applied radioactivity could be eluted with 1 N HCl. The acidic eluate was extracted with ether and analyzed by HPLC system A.

Reduction of Enzyme Inactivator Adduct with NaB^3H_4. A solution of ^{14}C-labeled protein (85 nmol, 2×10^5 cpm) in 400 μL of 8 M urea was cooled in ice. Tris-HCl (1 M) was added to pH 8.0, followed by 21 μmol of NaB^3H_4 (25 mCi/mmol; New England Nuclear). The mixture was stirred at 4 °C until the initial vigorous reaction subsided, then allowed to warm up to room temperature, and stirred an additional hour. The pH was maintained at 8.0–8.5 to addition of 1 N HCl. The reaction was stopped by acidification to pH 5–6 and then passed through a Bio-Gel P-6 column (0.7×35 cm) equilibrated with 8 M urea–0.1 M Tris-HCl, pH 7.8. Protein-containing fractions were pooled (1.9 mL total) and dialyzed against three changes of 800 mL of distilled water for 20 h, 4 °C. The protein, which precipitated inside the dialysis bag, was recovered by centrifugation and the pellet dried in vacuo. The dried protein was then hydrolyzed with 0.3 mL 6 N HCl at 100 °C for 21 h. The hydrolysate was repeatedly brought to dryness with a stream of nitrogen and resuspended in distilled water, finally dried in vacuo over NaOH. The residue was taken up in 150 μL of distilled water, and 1-μL aliquots were subjected to high-voltage paper electrophoresis at pH 8.9 [1% $(NH_4)_2CO_3$]. Enzyme inactivated with (3-chloro-3-butenoyl)pantetheine (85 nmol) and noninactivated enzyme (85 nmol) were treated identically.

The hydrolysate of the reduced (3-pentynoyl)pantetheine–protein adduct was purified on a small column of Dowex 1-OH^- (0.8 mL bed volume). The column was washed with distilled water (4–5 column volumes) until no more radioactivity appeared in the eluate. The bulk of amino acids (together with 40% of the applied radioactivity) was then eluted with 0.2 M HCOOH and lyophilized. The residue was analyzed by two-dimensional chromatography: first dimension, high-voltage paper electrophoresis, pH 8.9; second dimension, paper chromatography using the solvent system P1 = 2-methyl-2-propanol/methanol/water (6:5:2), descending, and P2 = 1-butanol/acetic acid/water (12:3:5), ascending.

Identification of the Radioactive Compounds Released by NaB^3H_4 Reduction. (3-[1-^{14}C]Pentynoyl)pantetheine–protein adduct was prepared and reduced with $NaBH_4$ as described above. Protein-free fractions from the Bio-Gel P-6 column were pooled (3.9 mL total, 2.9×10^4 cpm of ^{14}C). 1,3-Pentanediol (65 μmol) was added, and the neutral solution was continuously extracted with 50 mL of ether for 48 h. The ether was dried and evaporated and the residue purified by HPLC system C. The 1,3-pentanediol peak was collected and continuously extracted as before. The residue obtained after concentration of the dried ether extract was analyzed by two thin-layer chromatographic systems [silica plates; solvent 1, chloroform/methanol (92:8); solvent 2, 1-butanol/water (9:1)]. The specific activity of the purified diol was determined by HPLC systems A and C. Extraction of the pooled protein-free fractions at neutral pH (as described above) did not remove all ^{14}C radioactivity. Thus an additional continuous ether extraction was carried out after adjusting the pH to 2.5 and addition of 3-hydroxypentanoic acid (140 μmol) as carrier. The concentrated ether extract was treated with methanol to remove borate esters and boric acid. The residue was then applied to a column of Dowex 1-formate (0.5×5.0 cm), and the hydroxy acid eluted with 0.1 N HCl together with the bulk of radioactivity. The specific activity of the isolated 3-hydroxypentanoic acid was determined by HPLC system A.

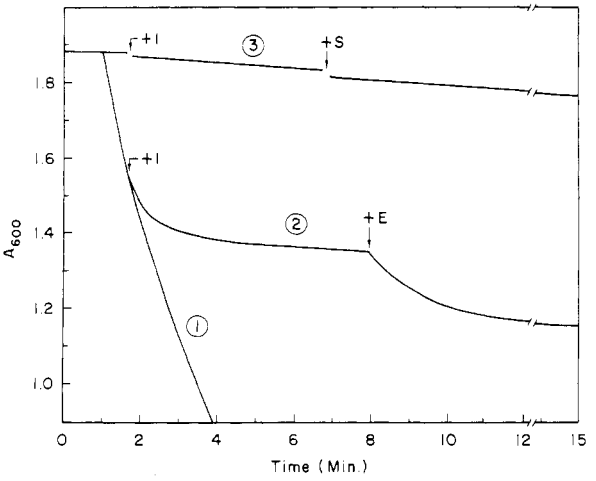


FIGURE 1: Time course of inactivation of green butyryl-CoA dehydrogenase by (3-pentynoyl)pantetheine. The reaction mixtures contained 0.1 M potassium phosphate, pH 7, 80 μM DCPIP, and 0.6 mM PMS in a total of 990 μL at 25 °C. Enzyme (0.13 μM) was added, and 1 min later reactions 1 and 2 were started by addition of butyrylpantetheine (1.2 mM final). After 38 s, (3-pentynoyl)-pantetheine (I) (3.7 μM final) was added to reaction 2. When dye reduction had leveled off (after 6.5 min) a second aliquot of enzyme (E) (0.13 μM) was added. Reaction 3 was started by addition of (3-pentynoyl)pantetheine (I) (3.7 μM final). Butyrylpantetheine (S) (1.2 mM final) was added after the enzyme was incubated with the inactivator for 5 min.

Identification of the Radioactive Compounds Released by NaB^3H_4 Reduction of the (3-Chloro-3-butenoyl)pantetheine–Protein Adduct. The (3-chloro-3-butenoyl)pantetheine–protein adduct was prepared and reduced with NaB^3H_4 as described for (3-[1-^{14}C]pentynoyl)pantetheine.

Protein-free fractions from the Bio-Gel P-6 column containing ^{3}H radioactivity were pooled (5.3 mL, 9×10^7 cpm of ^{3}H), and 147 μmol of 1,3-butanediol (Aldrich) was added. The radioactive material obtained after continuous ether extraction of the neutral solution for 48 h was purified on a Dowex 1-OH^- column (0.5 × 2.0 cm). The concentrated ether extract (225 μL) was applied and the column washed with distilled water. Elution of the diol was followed by spot tests with vanadium oxinate. The two main diol positive fractions containing 82% of the applied radioactivity (3.43×10^6 cpm) were combined (275 μL total) and further purified in HPLC system C. The butanediol peak was collected and reextracted with ether for 36 h. The residue obtained after concentration of the ether extract was subject to thin-layer chromatography on silica plates using chloroform/methanol (92:8) and 1-butanol/water (9:1) as solvents. Authentic 1,3-butanediol was cochromatographed with the radioactive material. The diol was visualized with vanadium oxinate spray.

Analysis of the Reaction Products Derived from 3-Fluoropropionyl-CoA. The enzymatic reaction contained 120 μM 3-fluoropropionyl-CoA in 525 μL of 0.1 M potassium phosphate, pH 7. A 25-μL aliquot was removed as zero point, added to 5 μL of precooled 1 N $HClO_4$ in a Eppendorf centrifuge tube, and centrifuged at 4 °C for 1 min. The supernatant fluid was removed, brought to pH 5.5 with 5 μL of 1 N KOH, and again centrifuged at 4 °C, 3 min, to remove $KClO_4$. To the supernatant fluid was added 50 μL of propionyl-CoA (4.8×10^{-5} M in water) as internal standard and the solution immediately applied to the HPLC column. Analysis was performed with HPLC system E. Enzymatic fluoride release was then started by addition of enzyme (0.036 μM) to the remaining 500 μL of fluoropropionyl-CoA solution. Fluoride release was monitored with the fluoride electrode and

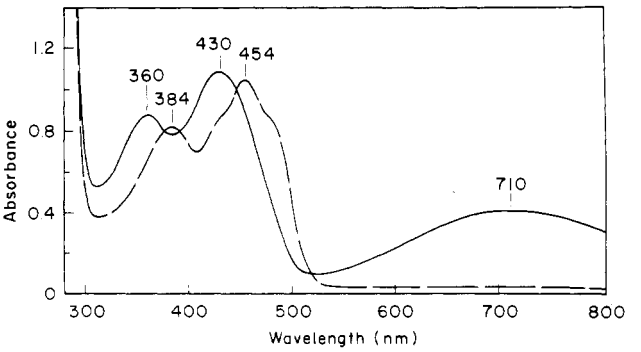


FIGURE 2: Absorbance spectrum of green butyryl-CoA dehydrogenase after inactivation by (3-pentynoyl)pantetheine. The enzyme (3×10^{-4} M) was incubated with 5.2×10^{-4} M (3-pentynoyl)pantetheine at 25 °C in 0.1 M potassium phosphate, pH 7, until catalytic activity was no longer detectable (25 min). The absorbance spectrum (---) was recorded after filtration of the inactivated protein through a Sephadex G-25 column equilibrated with the same buffer. Final enzyme concentration is estimated to be 8.67×10^{-5} M. For comparison (—) the spectrum of native green enzyme is shown (1.12×10^{-4} M in 0.1 M potassium phosphate, pH 7).

Table I: Substrate Protection of Butyryl-CoA Dehydrogenase against Inactivation by (3-Pentynoyl)pantetheine[a]

[butyrylpantetheine] (mM)	$t_{1/2}$ of inactivation[b] (s)
0.84	13
2.8	18
5.6	28.5
7.0	40.2

[a] The inactivation of butyryl-CoA dehydrogenase was followed by the change of absorbance at 600 nm (DCPIP reduction). Enzyme (0.3 μM final) was added to 1-mL standard assay mixtures containing various concentrations of butyrylpantetheine. The initial rate of DCPIP reduction was recorded for 0.5 min. The inactivation was then started by addition of (3-pentynoyl)-pantetheine (4 μM final). The rate of reaction decreased with time. Tangents were drawn on the chart recorder traces at 0.2-min intervals, and the residual activity was calculated as percent of the initial rate. A semilogarithmic plot of these values gave straight lines up to 70–75% complete inactivation. Half-lives were estimated from the part of the inactivation which showed first-order kinetics. [b] $t_{1/2}$ values ± 10%.

was to >90% complete after 15 min. A 25-μL aliquot was removed and prepared for HPLC analysis as described above.

Results

Inactivation by (3-Pentynoyl)pantetheine. We have previously reported that (3-pentynoyl)pantetheine causes a time-dependent inactivation of yellow butyryl-CoA dehydrogenase. In the experiments to be described here, the green form of the enzyme was used. The time course of inactivation is shown in Figure 1, and the spectral changes observed when (3-pentynoyl)pantetheine is added to the green form of the enzyme are shown in Figure 2. With the yellow enzyme, minor spectral changes occurred after addition of the inactivator (Gomes et al., 1981). In contrast, the spectrum of the green enzyme is significantly changed. The long wavelength absorbance centered around 710 nm is lost, and the absorbance maxima of the resulting yellow protein are red shifted to 380 and 454 nm. These changes seem to be due to alteration of the flavin environment, since extraction of the cofactor with methanol or Cl_3CCOOH yielded a flavin with absorbance properties identical with those of the flavin released from native green enzyme. Filtration of the inactivated enzyme (0.2 mL, 0.04 μmol) through a Sephadex column (G-25, 0.7

× 30 cm) did not restore catalytic activity.

Data summarized in Table I show that substrate protects against inactivation by (3-pentynoyl)pantetheine. An attempt was made to titrate the enzyme with inactivator. Enzyme (25 μM) was treated with varying amounts of inactivator (12–47 μM) as described under Materials and Methods. The amount of inactivator required to achieve at least 95% inactivation varied from 0.7 to 2 mol per mol of enzyme-bound flavin. The reason for this variability is unknown. A possible explanation for the requirement of inactivator in excess of the flavin present could be that the enzyme catalyzes conversion of the inactivator to an unreactive compound.

Isolation and Characterization of Enzyme–(3-Pentynoyl)pantetheine Adduct. The enzyme was inactivated with (3-[1-^{14}C]pentynoyl)pantetheine. When inactivation was complete (>95%), the enzyme was separated from excess inactivator by gel filtration. Radioactivity remained associated with the protein. This amount of radioactivity corresponds to 0.61 ± 0.1 nmol of inactivator incorporated per nmol of enzyme flavin. Precipitation of the labeled protein with Cl_3CCOOH or perchloric acid (5% final concentration, 4 °C) or with methanol (80 vol % final concentration, 4 °C) released less than 10% of the radioactivity from the enzyme. Under these conditions, at least 80% of the flavin was removed from native and inactivated enzyme. The labeled protein was also denatured in 8 M urea or 7 M Gdn·HCl at pH 6 and then passed through a gel filtration column, equilibrated with the same denaturants, without significant (<5%) loss of radioactivity. Thus a covalent linkage is formed between protein and inactivator.

The stability of the covalent linkage between enzyme and inactivator was determined. To the labeled protein was added urea to a final concentration of 8 M, and the pH was adjusted to 2.4. Aliquots were filtered through a Bio-Gel P-6 column, and the amount of radioactivity in the protein and small molecule fractions was determined. After 1.5 h (25 °C) maximally 10% of the protein-bound radioactivity was released. Similar results were obtained when the labeled protein was first precipitated with Cl_3CCOOH and then redissolved in 8 M urea at pH 2.5. When the protein was dissolved in 8 M urea with or without prior denaturation by Cl_3CCOOH and exposed to pH 11, 80–90% of the radioactivity was released from the protein after 5 min. The rate constant for the hydrolysis of the base-sensitive linkage was determined in 8 M urea at pH 8.4 and 8.7 (for experimental conditions see Materials and Methods). First-order kinetics were observed with half-lives of 90 and 52 min. Similar base lability has been reported for protein enol esters obtained by modification of protein carboxyl groups with isoxazolium salts (Bodlaender et al., 1969). These results suggested that the inactivator molecule is bound to the protein through an ester linkage, possibly an enol ester. Since, under basic conditions, 10–20% of the radioactive material associated with the protein was not released, it is possible that more than one adduct is formed.

An attempt was made to identify the radioactive molecule released when the enzyme–inactivator adduct was exposed to base. The molecule(s) released from the adduct was (were) separated from the protein by gel filtration. However, during subsequent workup, most of the radioactivity was lost. This suggested that a β-keto acid was released, which decarboxylated. $NaBH_4$ was added after base treatment to avoid decarboxylation. By this procedure the compound released from the enzyme–inhibitor adduct was identified as 3-hydroxypentanoic acid, which accounted for 75% of the material released from the protein. The remaining radioactivity was not identified. It is likely that some decarboxylation occurred prior to addition of carrier 3-oxopentanoic acid. This would result in loss of radioactivity. These results show that base hydrolysis of enzyme–inhibitor adduct releases 3-oxopentanoic acid.

To obtain further evidence for an ester linkage, we examined the effect of NH_2OH at neutral pH on the enzyme–inactivator complex. Initially, the experiment was done with protein denatured in 8 M urea without prior precipitation by Cl_3CCOOH. Under these conditions, a 15-h incubation with 1 M neutral NH_2OH was required to obtain extensive (>80%) release of the label. In a control experiment, protein incubated without NH_2OH for 15 h lost 50% of the label due to hydrolysis of the enzyme–inactivator linkage. However, when we repeated the experiment with Cl_3CCOOH-denatured enzyme redissolved in 7 M Gdn·HCl, 80% of the label was released by 0.4 M neutral NH_2OH after 2.5 h at 25 °C (experimental values ranged from 80 to 88%). Apparently more extensive denaturation is required for nucleophilic displacement of the label than for base-catalyzed hydrolysis, for which 8 M urea was a sufficient denaturant. The NH_2OH-treated protein was then assayed for hydroxamic acid formation (see Materials and Methods), and 0.75 ± 0.08 mol of protein hydroxamate was found per mol of ^{14}C inactivator released. The results strongly suggest that the inactivator esterifies a protein carboxyl group. Analysis of the radioactive material released in the presence of NH_2OH by HPLC and TLC indicated that a mixture of three to four compounds was present. One product was identified as 3-oxopentanoic acid. It comigrated with added synthetic carrier on Dowex 1-OH^- and cochromatographed with 3-oxopentanoic acid in HPLC system A. Its specific activity (80 cpm/μmol) could account for 20% of the released ^{14}C label. Attempts to characterize the other radioactive compounds were unsuccessful.

The results obtained with hydroxylamine suggested that inactivation by (3-[1-^{14}C]pentynoyl)pantetheine led to esterification of a protein carboxyl group. In order to identify the amino acid residue which had been modified, we reduced the inactivated protein with NaB^3H_4. Reduction of the proposed ester linkage should release the ^{14}C label and convert the modified carboxyl group to a carbinol. When labeled denatured protein was reduced with NaB^3H_4 and then subjected to gel exclusion chromatography, 40–50% of the ^{14}C label was released. Incorporation of ^{3}H into inactivated protein (6×10^4 cpm/nmol of enzyme) was 2.7-fold higher than into the noninactivated control protein (2.2×10^4 cpm/nmol). After acid hydrolysis the crude hydrolysates were analyzed by high-voltage paper electrophoresis at pH 8.9 (see Figure 3). Two peaks of ^{3}H radioactivity were obtained from the inactivated reduced protein, which were not present in the control. The major peak (20% of total incorporated ^{3}H) comigrated with 2-amino-5-hydroxyvaleric acid. The second peak (9% of total incorporated ^{3}H) was found in the region of proline. In addition, the homoserine region of the electrophoretogram contained 5% of the incorporated ^{3}H.

The crude hydrolysate was purified on Dowex 1-OH^- to test if the radioactivity which comigrated on electrophoresis with 2-amino-5-hydroxyvaleric acid, homoserine, and proline was indeed identical with that of the carrier amino acids. Amino acids were eluted with 0.2 M HCOOH together with 40% of the applied radioactivity. After electrophoresis at pH 8.9, the areas around 2-amino-5-hydroxyvaleric acid, homoserine, and proline were cut out, sewed on another paper, and subjected to paper chromatography in solvent systems P1 and P2; 55% of the radioactivity from the 2-amino-5-hydroxyvaleric acid spot comigrated with the carrier in system P1 and 80% in

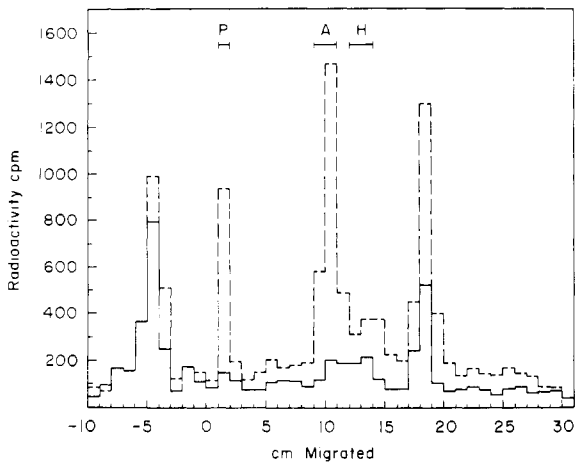


FIGURE 3: High-voltage paper electrophoresis of the acid hydrolysate of enzyme–inactivator adduct reduced with NaB^3H_4. The protein hydrolysates (1-μL aliquots) were cospotted with proline (P), 2-amino-5-hydroxyvaleric acid (A), and homoserine (H). After electrophoresis at pH 8.9 [1% $(NH_4)_2CO_3$, 56 V/cm, 65 min], amino acids were visualized with ninhydrin spray. The paper was cut in 1-cm segments and radioactivity determined. (—) Native enzyme (4750 cpm of ^3H total); (---) enzyme inactivated with (3-[1-^{14}C]pentynoyl)pantetheine (12879 cpm of ^3H total).

system P2. The contaminating radioactivity migrated with or close to the solvent front and is presumably due to unspecific ^3H incorporation from reduction of peptide bonds. No radioactivity from the homoserine region migrated with the carrier in both paper chromatography systems.

Of the radioactivity associated with the proline spot, 60% cochromatographed with the carrier upon chromatography in solvent P1. Thus proline or a structurally similar compound is formed upon $NaBH_4$ reduction of the inactivated enzyme.

Formation of 2-amino-5-hydroxyvaleric acid as well as proline indicates that the inactivator is bound to the γ-carboxyl group of a glutamate residue. The fact that the carboxyl group can be reduced by $NaBH_4$ is consistent with the presence of an enol ester.

Experiments were carried out to identify the small molecules released from the protein after $NaBH_4$ reduction. The molecules released were separated from the protein by gel filtration, and unlabeled 1,3-pentanediol was added as carrier. The solution was then continuously extracted at pH 7.8. Under these conditions 80% of the radioactive material(s) released from the enzyme by reaction with $NaBH_4$ was extracted. About 50% of the material extracted cochromatographed with 1,3-pentanediol in the following chromatographic systems: HPLC systems A and C and in two TLC systems [silica plates, $CHCl_3$/MeOH (92:8), 1-butanol/H_2O (9:1)]. The specific activity of the diol determined in both HPLC systems (210 ± 10 cpm of $^{14}C/\mu$mol, 2.2 × 10^4 cpm of $^3H/\mu$mol) can account for 50% of the ^{14}C label which was released through $NaBH_4$ reduction. After acidification of the aqueous phase which contained 5800 cpm of ^{14}C, a further ether extraction was carried out with 3-hydroxypentanoic acid as carrier. The extracted acid was purified by Dowex 1-formate. Analysis in HPLC system A established that 95% of the isolated ^{14}C radioactivity was identical with that of 3-hydroxypentanoic acid. The specific activity (10.5 cpm of $^{14}C/\mu$mol, 320 cpm of $^3H/\mu$mol) indicated that 5–6% of the borohydride-released ^{14}C label had been converted to 3-hydroxypentanoic acid. The identity of the remaining 45% of released ^{14}C radioactivity has not been identified. It is possible that they were borate esters of the pentanediol, which were lost during subsequent purification steps.

Inactivation of Butyryl-CoA Dehydrogenase by (3-Chloro-3-butenoyl)pantetheine. The mechanism we propose for the inactivation by (3-pentynoyl)pantetheine involves intermediate formation of the allenic thio ester as the true inactivating species. To test this proposal, we examined (3-chloro-3-butenoyl)pantetheine (CH_2=CHCl—COSPant.) as a potential inactivator of butyryl-CoA dehydrogenase. Enzyme-catalyzed abstraction of the α proton of this compound will lead to β elimination of chloride and generation of the allenic thio ester. The mechanism differs in so far that no protonation of an intermediate allenic anion is required. (3-Chloro-3-butenoyl)pantetheine is indeed a potent inactivator of the enzyme. When the dehydrogenase (4 μM) was incubated with 310 μM inactivator, complete loss of catalytic activity was observed after 40 s. The time course of inactivation under conditions described in the legend to Figure 1 is similar to the one shown for (3-pentynoyl)pantetheine. Inactivation is irreversible, since no enzymatic activity is restored after gel filtration. The spectral changes of the enzyme flavin are similar to those described for the inactivation by (3-pentynoyl)pantetheine. The green enzyme loses its long wavelength absorbance, and the main transitions at 365 and 430 nm are red shifted to 380 and 454 nm. The flavin of the inactivated enzyme is reducible with $Na_2S_2O_4$. Cl_3CCOOH denaturation releases a flavin with absorbance properties identical with flavin released from native enzyme. Experiments were done to determine the stoichiometry of inactivation. Incubations at various ratios of inactivator to enzyme flavin indicated that 3–5 mol of (3-chloro-3-butenoyl)pantetheine/mol of enzyme is required to achieve complete inactivation. It is possible that the inactivator is in part consumed in a reaction other than the inactivation process.

Experiments were carried out to establish the nature of the linkage between the protein and (3-chloro-3-butenoyl)pantetheine. We reduced the inactivated protein with NaB^3H_4 under conditions identical with those for the (3-pentynoyl)-pantetheine–enzyme adduct. The amount of tritium incorporated into the inactivated protein (4.9 × 10^6 cpm/nmol) was about 2.2-fold higher than into the noninactivated control (2.2 × 10^6 cpm/nmol). Analysis of the tritiated protein hydrolysate by high-voltage paper electrophoresis showed two peaks of radioactivity, which comigrated with 2-amino-5-hydroxyvaleric acid (21–29% of total incorporated ^3H) and proline (10–14% of total incorporated ^3H). Tritiated 1,3-butanediol was isolated by ether extraction in the presence of synthetic material as carrier. The radioactivity comigrated with authentic 1,3-butanediol in HPLC system C and in two TLC systems [silica plates, $CHCl_3$/MeOH (92:8), 1-butanol/H_2O (9:1)]. Thus inactivation by (3-chloro-3-butenoyl)pantetheine most probably leads to an adduct analogous to the one found for the acetylenic inactivator.

Isomerization of (3-Butenoyl)pantetheine. The mechanism of inactivation by (3-pentynoyl)pantetheine involves enzyme-catalyzed abstraction and addition of a proton from the α and γ positions of the substrate. To obtain evidence that the enzyme can interact with those positions of the substrate, we carried out experiments to determine whether butyryl-CoA dehydrogenase can catalyze the conversion of (3-butenoyl)-pantetheine [(vinylacetyl)pantetheine] to (2-butenoyl)pantetheine (crotonylpantetheine). When 0.1 μM enzyme was added to 88 μM (3-butenoyl)pantetheine in 0.1 M potassium phosphate, pH 7, at 25 °C, the absorbance at 260 nm increased until a constant value was reached after 18 min. The final spectrum was almost identical with the spectrum of crotonyl-*N*-acetylthioethanolamine (Lynen & Ochoa, 1953) for

which absorbance maxima at 224 nm (ϵ = 11 500 M^{-1} cm^{-1}) and 262 nm (ϵ = 6750 M^{-1} cm^{-1}) have been reported (Seubert & Lynen, 1953). Under the assumption that the extinction coefficients for the *N*-acetylthioethanolamine and the pantetheine thio ester are about equal, the final spectrum of the butyryl-CoA dehydrogenase catalyzed reaction amounts to 88 μM crotonylpantetheine formed. From the initial change in 260-nm absorbance, a rate of 238 μmol of crotonylpantetheine formed min^{-1} (μmol of enzyme)$^{-1}$ was estimated (using $\Delta\epsilon$ = 6300 M^{-1} cm^{-1}). Incubation of 190 μM (3-butenoyl)pantetheine in 0.1 M potassium phosphate, pH 7, at 25 °C in the absence of enzyme led to minor changes in 260-nm absorbance; after 50 min less than 1% of the thio ester appeared to have isomerized.

To obtain additional evidence for the formation of crotonylpantetheine, we took advantage of the fact that butyryl-CoA dehydrogenase has oxidase activity; i.e., it catalyzes the slow oxidation of substrates in the absence of electron acceptor dye (Engel & Massey, 1971b). Therefore, butyryl-CoA dehydrogenase should catalyze the conversion of butyrylpantetheine to crotonylpantetheine with O_2 as electron acceptor. Incubation of butyryl-CoA dehydrogenase (0.1 μM) with butyrylpantetheine (110 μM) under conditions described for (3-butenoyl)pantetheine causes similar, but much slower, spectral changes. These results therefore confirm that butyryl-CoA dehydrogenase catalyzes the isomerization of (3-butenoyl)pantetheine to crotonylpantetheine.

The reaction with (3-butenoyl)pantetheine proceeds without detectable oxygen consumption when monitored in an oxygen electrode. Incubation of 0.02 μM enzyme with 590 μM (3-butenoyl)pantetheine in the presence of PMS and DCPIP (as described for the standard assay) did not cause dye reduction. No loss of enzymatic activity was found when the enzyme (12 μM) was incubated with 1.6 mM (3-butenoyl)pantetheine in 0.1 M potassium phosphate, pH 7, for 40 min.

Fluoride Elimination from 3-Fluoro Thio Esters. We also examined the action of butyryl-CoA dehydrogenase on 3-fluoropropionyl-CoA and (3,3-difluorobutyryl)pantetheine. If the enzyme catalyzes abstraction of the α proton from the substrate, elimination of HF could occur from the fluorine-containing substrate analogues.

When butyryl-CoA dehydrogenase (0.008 μM) is incubated with 3-fluoropropionyl-CoA (100 μM), F^- is released. Initial rates of 1050 ± 200 μmol of F^- min^{-1} (μmol of enzyme)$^{-1}$ were measured with a fluoride electrode (see Materials and Methods). When enzyme inactivated with (3-pentynoyl)-pantetheine was added (0.36 μM final), the rate of fluoride release was 0.6% of the rate observed with active enzyme. Partial inactivation with (3-pentynoyl)pantetheine decreased the rate of butyrylpantetheine oxidation and the rate of fluoride release to a similar extent, suggesting that both reactions are catalyzed at the same site. The initial rates of fluoride release appeared to be at least 400 times faster than the turnover number for oxidation of propionyl-CoA [2 μmol min^{-1} (μmol of enzyme)$^{-1}$ determined under the conditions of the standard assay]. Addition of 0.01 μM enzyme to 100 μM fluoropropionyl-CoA led to increase in the absorbance at 260 nm, indicating that an α,β-unsaturated thio ester is formed. HPLC analysis of the reaction, as described under Materials and Methods, showed the disappearance of the fluoropropionyl-CoA peak and the appearance of one major new peak. The retention time of this new peak relative to the propionyl-CoA standard agreed with the retention time of the product obtained when acryloyl chloride is reacted with CoASH. Slow appearance of the same peak together with decrease in the fluoropropionyl-CoA peak was also observed in the nonenzymatic control incubation. These results show that butyryl-CoA dehydrogenase catalyzes the elimination of HF from 3-fluoropropionyl-CoA to form acrylyl-CoA.

3-Fluoropropionyl-CoA was also found to be a substrate for the enzyme in the PMS/DCPIP assay system. At comparable fluoropropionyl-CoA concentrations (100–200 μM), the observed rate of DCPIP reduction was less than 1% of the rate of fluoride release.

Butyryl-CoA dehydrogenase also catalyzes fluoride elimination from (3,3-difluorobutyryl)pantetheine, albeit at a much slower rate. At ≥2 mM (difluorobutyryl)pantetheine, initial rates of 20 ± 4 μmol of F^- min^{-1} (μmol of enzyme)$^{-1}$ were obtained after correcting for the nonenzymatic rate under these conditions (16.5 ± 2.5 μmol of F^- min^{-1}). This rate is about 2% of the rate obtained with 100 μM 3-fluoropropionyl-CoA [1050 ± 200 μmol of F^- min^{-1} (μmol of enzyme)$^{-1}$]. In the presence of 40 μM enzyme inactivated by (3-pentynoyl)pantetheine, the rate of fluoride elimination was identical with the nonenzymatic rate.

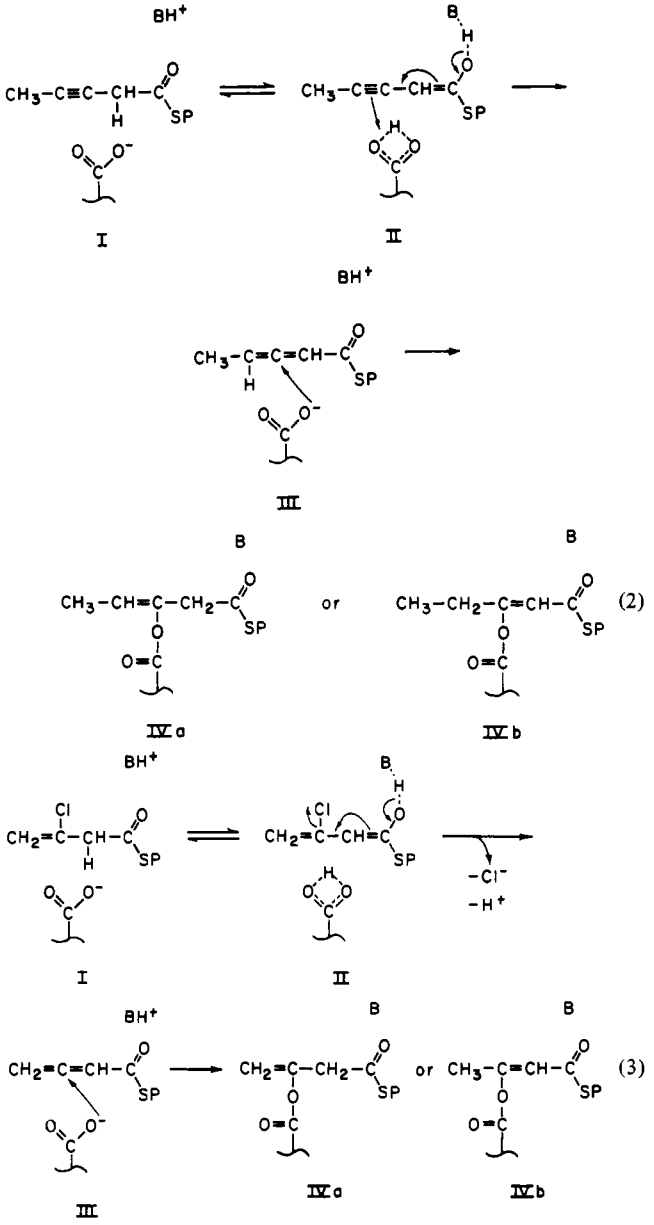
When the reaction was followed spectrophotometrically, an increase in absorbance at 260 nm was observed, suggesting that an α,β-unsaturated thio ester is formed as the reaction product. The rate of increase of absorbance at 260 nm is comparable to the rate of fluoride elimination. (Fluorocrotonyl)pantetheine was not actually identified but appeared to be highly unstable under our experimental conditions. It is converted to acetoacetylpantetheine which was identified enzymatically with β-hydroxyacyl-CoA dehydrogenase.

Discussion

Butyryl-CoA dehydrogenase from *Megasphaera elsdenii* is irreversibly inactivated by the acetylenic substrate analogue (3-pentynoyl)pantetheine. The inactivation is accompanied by covalent attachment of the inactivator to the enzyme with a stoichiometry of 0.61 ± 0.1 mol of ^{14}C-labeled inactivator/mol of enzyme flavin. The reason for this low stoichiometry is not understood. It is possible that the enzyme, as isolated, is partially inactive. The rate of inactivation is reduced in the presence of increasing concentrations of substrate (Table I), indicating that inactivation involves the active site. The kinetics of inactivation (Figure 1) show that addition of (3-pentynoyl)pantetheine to the enzyme causes time-dependent loss of activity with no lag period. A second addition of enzyme to the reaction mixture is inactivated with the same rate. These results demonstrate that inactivation does not involve an enzyme-generated reactive species which accumulates in solution. Thus (3-pentynoyl)pantetheine can be classified as a suicide inactivator (Abeles & Maycock, 1976).

The absorbance spectrum of the inactivated enzyme (Figure 2) suggests that the inactivator is bound at the active site. The fine structure of the 454-nm transition indicates a more hydrophobic flavin environment in the inactivated enzyme, presumably due to the alkyl chain of the inactivator. A similar spectrum was observed when various unsaturated acyl-CoA esters were added to the yellow form of the enzyme (Engel & Massey, 1971b). The green color of native butyryl-CoA dehydrogenase is attributed to a charge-transfer interaction between flavin and a CoA persulfide tightly bound at the active site (Williamson et al., 1982). Inactivation by (3-pentynoyl)pantetheine and (3-chloro-3-butenoyl)pantetheine disrupts this charge-transfer interaction as evidenced by loss of the green color. It is conceivable that both inactivators displace the CoA persulfide from the enzyme as observed for the "degreening" of the enzyme by acetoacetyl-CoA (Williamson

Scheme I: Proposed Mechanism of Inactivation by (3-Pentynoyl)pantetheine (eq 2) and (3-Chloro-3-butenoyl)pantetheine (eq 3)



et al., 1982). Acetoacetylpantetheine also causes a decrease in 710-nm absorbance, suggesting that the pantetheine moiety can substitute for the CoA moiety in this displacement (G. Fendrich, unpublished observations).

(3-Pentynoyl)pantetheine does not form an adduct with the flavin but becomes covalently attached to the apoprotein. We propose the reaction sequence shown in Scheme I (eq 2) for the inactivation. According to this mechanism, a base at the active site of the enzyme, pictured as a carboxyl group, abstracts a proton from the α carbon of the inactivator. The resulting enol then rearranges to an allenic carbanion. Formation of the allene is completed by addition of a proton to the γ carbon of the allenic ion, possibly mediated by the same carboxyl group. A glutamate residue at the active site then adds to the allene in a Michael-type addition. Model studies have established the facile addition of nucleophiles to allenes conjugated to a carbonyl function (Morisaki & Bloch, 1972; Covey et al., 1979). Two structures are shown for the final adduct. It is not possible to definitely assign either structure to the adduct. However, we prefer structure IVa. Structure IVb is expected to give an absorbance increase at 260 nm due to conjugation of the double bond with the thio ester group. Comparison of the UV spectra of native and inactivated holoenzyme and of the apoproteins obtained by Cl_3CCOOH denaturation did not show the expected absorbance difference. The model studies mentioned above showed that both conjugated and unconjugated adducts are formed. With acetic acid as the nucleophile, an unconjugated adduct was obtained (Covey et al., 1979).

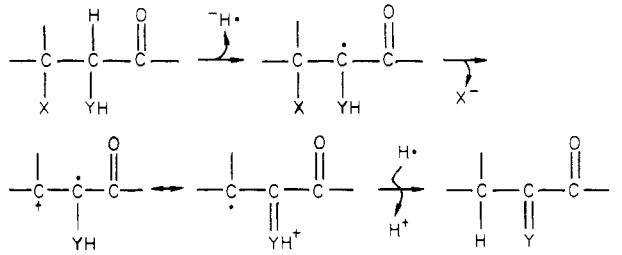
The nucleophile through which the inactivator is bonded to the protein is the γ-carboxyl group of a glutamic acid. This is supported by the lability of the adduct toward base, hydroxylamine, and borohydride reduction. Release of 3-oxopentanoic acid upon base-catalyzed hydrolysis is consistent with the addition of a nucleophile to the β carbon of the inactivator. Formation of a hydroxamic acid on the protein after treatment with hydroxylamine identified this nucleophile as a carboxyl group. Finally, reduction by $NaBH_4$ showed that this carboxyl group belongs to a glutamic acid. The reducibility of the adduct provides evidence for an activated ester linkage. Ordinary esters are reduced by borohydride in aqueous media only with great difficulties, whereas a glutamoyl enol ester was converted to the carbinol in 50% yield (Hall & Perfetti, 1974). The presence of proline (or a proline-like compound) in the protein hydrolysate can be explained by incomplete reduction of the glutamoyl enol ester to the γ-aldehyde. During subsequent acid hydrolysis the aldehyde can react with the liberated α-amino group to form Δ^1-pyrroline-5-carboxylic acid. Conversion to proline can occur upon prolonged heating in 6 N HCl (Vogel & Davis, 1952). The borohydride reduction also indicates that the inactivator is mainly present as intact thio ester in the covalent adduct. A neutral compound, 1,3-pentanediol, was the main reduction product which is derived from the reduction of a 3-keto thio ester. Reaction with hydroxylamine released 80–88% and exposure to base released 80–90% of the enzyme-bound radioactivity derived from the inactivator. Therefore the acetylenic inactivator reacted primarily with the γ-carboxyl group of a glutamate residue at the active site. This leaves the possibility that maximally 20% of the inactivator reacted with another functional group(s).

Butyryl-CoA dehydrogenase is also inactivated by (3-chloro-3-butenoyl)pantetheine. We have presented evidence that this compound reacts with a glutamate carboxyl group and forms an adduct analogous to that formed with (3-pentynoyl)pantetheine. A mechanism for inactivation by 3-chloro-3-butenoyl)pantetheine is shown in Scheme I (eq 3). As with the acetylenic inactivator, we propose that a proton is abstracted from the α position. Cl^- is then eliminated from the resulting carbanionic intermediate to form an allene.

The kinetics of inactivation of the green and yellow form of the enzyme by (3-pentynoyl)pantetheine and (3-chloro-3-butenoyl)pantetheine show no significant differences. The interaction of these inactivators with the yellow form of the enzyme was not examined in detail due to the instability of the yellow enzyme (Engel, 1981).

The present work is the third example of inactivation by an acetylenic substrate analogue in which the structure of the covalent protein inactivator adduct has been identified. Inactivation of β-hydroxydecanoyl thio ester dehydrase by (3-decynoyl)-*N*-acetylthioethanolamine led to modification of an imidazole residue (Endo et al., 1970). Recently, the modified residue of 3-oxosteroid Δ^5-isomerase inactivated by a β,γ acetylenic keto steroid, has been identified as asparagine (Penning et al., 1981). In all three cases, the enzyme nu-

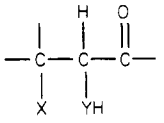
Scheme II



cleophile became attached to the inactivator at the carbon β to the carbonyl group.

A glutamate carboxyl group is labeled by (3-pentynoyl)-pantetheine and (3-chloro-3-butenoyl)pantetheine. This establishes that a carboxyl group is located at the active site and that this group is probably involved in the catalytic process. We suggest that the carboxyl group is the base which abstracts the α proton from the substrate and generates the substrate-derived carbanion from which electrons can be transferred to the flavin. It is noteworthy that for five enzymes which abstract protons from the substrate to form an enol intermediate, a carboxyl group has been identified at the active site. It has been argued that a carboxyl group may be the most suitable functional group for proton abstraction leading to enol formation (Gilbert, 1981). The mechanism shown in Scheme I (eq 2) requires that a functional group or groups are present at the active site which catalyze the abstraction and addition of a proton at the α and γ positions of the inactivator. Additional evidence for the presence of such a functional group(s) is provided by the conversion of (vinylacetyl)pantetheine to crotonylpantetheine catalyzed by butyryl-CoA dehydrogenase. This isomerization most probably proceeds by abstraction of the substrate α proton and addition of a proton to the γ position. A carboxyl group is particularly suitable for the catalysis of this isomerization.

Evidence for a carbanion intermediate is provided by the observation that a number of flavoproteins can catalyze elimination reactions when presented with a substrate with a good leaving group in the β position (Walsh et al., 1971, 1973). Although these elimination reactions can proceed through a carbanion mechanism, a radical process could also be involved. In the cases cited above the substrate has the following structure:



where X = Cl or any other good leaving group and YH = NH_2 or OH. For compounds of this structure elimination can occur through the mechanism shown in Scheme II.

Butyryl-CoA dehydrogenase catalyzes the elimination of HF from 3-fluoropropionyl-CoA and (3,3-difluorobutyryl)-pantetheine. This elimination probably cannot proceed by the radical mechanism shown in Scheme II since there is no electron-releasing group in the α position to stabilize the radical cation. This reaction therefore most probably proceeds through a carbanion mechanism.

In summary, we have described three reactions catalyzed by butyryl-CoA dehydrogenase which probably proceed through a carbanion mechanism: (1) the inactivation by (3-pentynoyl)pantetheine and covalent modification of an active site carboxyl group; (2) the rearrangement of (vinylacetyl)-pantetheine to crotonylpantetheine; (3) the elimination of HF from 3-fluoropropionyl-CoA and (3,3-difluorobutyryl)pantetheine. Since the enzyme has the ability to catalyze reactions involving carbanionic intermediates, it seems to us very likely that such intermediates are also involved in the normal catalytic reaction.

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Effect of Cryosolvent on Transient Kinetics of the Glutamate Dehydrogenase Reaction†

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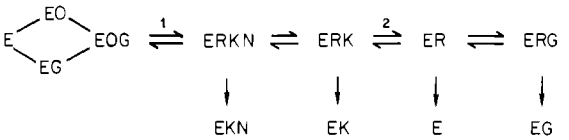
ABSTRACT: The transient and steady-state kinetics of the oxidative deamination of L-glutamate by glutamate dehydrogenase and NADP in both aqueous solution and 30% methanol are compared. Methanol causes an approximately 5-fold tightening of the enzyme–L-glutamate binary complex and an approximately 2-fold reduction of the interaction parameter for the ternary enzyme–NADP–L-glutamate complex. The most dramatic effect of methanol on the time course of the reaction is what appears to be a conversion of the enzyme at substoichiometric initial levels of reactant NADP to a form from which product α-ketoglutarate does not readily dissociate. This conversion appears only at NADP concentrations over one-third of the enzyme active site concentration.

Cryoenzymological studies of the glutamate dehydrogenase reaction (Johnson et al., 1981a,b) have revealed the existence of a stable less reactive form of the enzyme, identifiable by its distinctive spectral characteristics in the aromatic chromophore region. At normal temperatures in the absence of the cryosolvent (antifreeze) methanol, these spectral characteristics are observed only in the enzyme–NADPH–α-ketoglutarate ternary complex, but at cryogenic temperatures in solutions containing methanol the enzyme can be converted to a state for which the spectral characteristics are observed regardless of which enzyme–coenzyme complexes are formed. The purpose of the present experiments is to determine at which point in the reaction mechanism the existence of this new enzymatic form becomes mechanistically important and whether these effects are produced by the effects of temperature on free energy barriers, by methanol, or by both.

As it is currently understood from experiments in aqueous solution (diFranco, 1974; Colen et al., 1975, 1977, 1981; Brown et al., 1978; Fisher & Colen, 1978), the mechanism of the oxidative deamination of L-glutamate by glutamate dehydrogenase and NADP is given in Scheme I.

The catalytic hydride transfer step (step 1) converts oxidized coenzyme to complexes consisting of enzyme, reduced coenzyme, and α-ketoglutarate, which exhibit a blue-shifted reduced nicotinamide absorption peak, observed as a rapid transient production ("burst") of 336-nm absorbance. The release of α-ketoglutarate (step 2) is accompanied by a spectral shift of bound reduced nicotinamide absorbance to the red (346 nm) (Fisher et al., 1970; diFranco, 1971; diFranco & Iwatsubo, 1971, 1972). When free NADPH is produced (arrows pointing downward in Scheme I), its absorbance is, of course, unshifted (340 nm).

Scheme I[a]



[a] Abbreviations: E, enzyme, glutamate dehydrogenase; O, oxidized coenzyme, NADP; G, L-glutamate; N, ammonia; R, reduced coenzyme, NADPH; K, α-ketoglutarate.

Steps 1 and 2 may be studied directly by stopped-flow spectrophotometry and the production of free NADPH by studies of steady-state kinetics. Since step 2 is rate-limiting in steady-state studies under most experimental conditions, effects observed in transient-state studies will also be reflected in steady-state measurements (diFranco, 1974; Colen et al., 1975). In the work that follows, we compare the rapid initial burst of blue-shifted absorbance and the steady-state and transient modes of breakdown of these blue-shifted complexes at 5 °C both in aqueous solution and in 30% methanol.

Materials and Methods

Bovine liver glutamate dehydrogenase was obtained in ammonium sulfate suspension from Boehringer Mannheim. Before use, the enzyme was exhaustively dialyzed against 0.1 M potassium phosphate buffer at the appropriate pH and treated with activated charcoal to remove tightly bound organic material. We calculated enzyme concentrations using $A^{1\%}_{280}$ = 9.7 and M_r 56 100. The ratio A_{280}/A_{260} was always greater than 1.92, indicating the absence of significant amounts of nucleotide impurities.

NADP was obtained from Sigma and used without further purification. We calculated NADP concentrations using ϵ_{259} = 17 800 (Siegel et al., 1959). L-Glutamate acid was obtained from Calbiochem.

Solutions in 30% methanol were prepared with 0.1 M pH 7.6 phosphate buffer. On the basis of the data of Douzou et al. (1976), the pH of the solutions is estimated to be 8.2. For the sake of comparison, the experiments in aqueous solution

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