Mechanism-Based Inactivation of Thymine Hydroxylase, an α-Ketoglutarate-Dependent Dioxygenase, by 5-Ethynyluracil†

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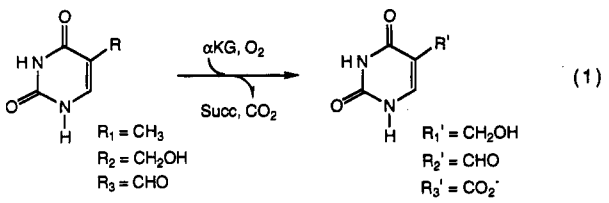
ABSTRACT: 5-Ethynyluracil was shown to be a mechanism-based inactivator of thymine 7-hydroxylase, with K_i = 22 μM and a k_2 = 2.6 min^{-1}. Inactivation resulted in covalent modification of the enzyme with a stoichiometry of ~1 adduct/enzyme molecule. The reaction of thymine 7-hydroxylase with 5-ethynyluracil also generated two products: 5-carboxyuracil and uracil–5-acetylglycine. The enzyme adduct was stable at pH 2, 8, and 10 and stable to treatment with hydroxylamine. Following trypsin digestion of labeled enzyme, two labeled peptides corresponding to 45% of the adduct were isolated and sequenced. The results demonstrated the presence of a single modified amino acid. Tandem mass spectrometry suggested that the modified amino acid is tyrosine, which is linked to the inhibitor in an unprecedented fashion.

Thymine hydroxylase (EC 1.14.11.6) is a non-heme Fe^{2+}, α-ketoglutarate-dependent dioxygenase, which catalyzes the three reactions shown in eq 1 (Holme et al., 1970, 1971; Liu et al., 1973). In the past 2 decades, a number of enzymes in



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this class have been partially characterized (Kivirikko et al., 1989; Gronke et al., 1990). Most possess the fascinating capability of catalyzing the hydroxylation of unactivated carbon–hydrogen bonds. Despite intense speculation, almost nothing is known about the detailed mechanisms employed by these enzymes. In contrast, a great deal is known about another class of enzymes which catalyze hydroxylations at unactivated carbon–hydrogen bonds: the heme Fe^{3+} dependent cytochrome P-450s (cyt P-450[1]). On the basis of elegant model chemistry, a consensus is emerging that the mechanism of these enzymes involves the formation of a high-valent iron–oxo species, which abstracts a hydrogen atom from the substrate. The resulting iron–hydroxyl species then undergoes recombination with the substrate radical to give product (McMurry & Groves, 1986; Ortiz de Montellano, 1986). A similar mechanism has been proposed for the α-ketoglutarate-dependent dioxygenases (Siegel, 1979; Hanauske-Abel & Gunzler, 1982).

cyt P-450 catalyzes a wide range of oxidation reactions in addition to hydroxylations, including epoxidation of alkenes, oxidation of sulfides to sulfoxides and sulfones, and demethylation of *N*-methylamines (Guengerich & MacDonald, 1984). In the preceding article in this issue, we show that thymine hydroxylase is capable of catalyzing a similar scope of reactions (Thornburg et al., 1993). Elegant studies by Ortiz de Montellano and co-workers have shown that terminal acetylenes, RC≡CH, are also substrates for cyt P-450; however, the enzymatic reaction with these compounds partitions between formation of a carboxylic acid product, $RCH_2CO_2^-$, and inactivation of the enzyme due to the formation of a heme adduct (Ortiz de Montellano & Komives, 1985). On the basis of these observations, we have investigated the interaction of thymine hydroxylase with 5-ethynyluracil (EU). Results are presented which demonstrate that this enzyme, like cyt P-450, catalyzes partitioning between RCH_2COX production and enzyme inactivation. The inactivation reaction has been investigated in detail, and the first active-site peptide of an α-ketoglutarate-dependent dioxygenase has been identified. A portion of these results has been published in preliminary form (Thronburg & Stubbe, 1989).

MATERIALS AND METHODS

General

All reagents used in the syntheses described below were obtained from Aldrich and were of the highest grade available. (Triphenylphosphine)palladium chloride was prepared by the method of Burmeister and Basolo (1964). Triethylamine was dried over calcium hydride and distilled immediately prior to use. 5-Acetyluracil was synthesized by a published procedure (Dewer & Shaw, 1961). 5-(Carboxymethyl)uracil was synthesized by the procedure of Fissekis and Sweet (1970). UV and NMR spectra were in agreement with published values.

Thymine, α-ketoglutarate, sodium ascorbate, hyamin hydroxide (1 M in methanol), 5-(hydroxymethyl)uracil, and PEI–cellulose TLC plates were purchased from Sigma. $FeSO_4 \cdot 7H_2O$ was obtained from Mallinkrodt. [2-^{14}C]Uracil (58 μCi/μmol) and [2-^{14}C]thymine (56 μCi/μmol) were supplied by ICN Radiochemicals and Moravek Biochemicals, respectively. [1-^{14}C]-α-Ketoglutarate (59 μCi/μmol) and

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[1] Abbreviations: cyt P-450, cytochrome P-450s; EU, 5-ethynyluracil; αKG, α-ketoglutarate; Succ, succinate; TLC, thin-layer chromatography; HPLC, high-pressure liquid chromatography; TFA, trifluoroacetic acid; DMSO, dimethyl sulfoxide; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; MeOH, methanol; PEI, poly(ethylene imine); SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel; TPCK–trypsin, trypsin treated with *N*-tosylphenylalanine chloromethyl ketone.

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[^{3}H]H_2O (1 Ci/g) were purchased from New England Nuclear. Silica TLC plates came from J. T. Baker. Ultrapure grade guanidine hydrochloride was supplied by ICN Biochemicals. Ultrafree MC filters are a product of Millipore. Iodoacetamide and DTT came from Fluka and United States Biochemical, respectively. TPCK–trypsin was supplied by Worthington Biochemicals. Sequencing-grade CH_3CN, water, and trifluoroacetic acid (TFA) were obtained from Pierce.

Protein concentrations were determined by the Bio-Rad microassay (Bradford, 1976) with BSA as the standard. Liquid scintillation counting was performed with a Packard TriCarb 1500 counter using Scint-A fluid (supplied by Packard). HPLC was carried out on a Beckman system (consisting of Model 110B pumps, a Model 421 controller, and a Model 163 variable-wavelength detector) to which was added a Hewlett-Packard HP3396A integrator. Analytical (4.6 × 250 mm) and semipreparative (7 × 250 mm) Econosil C_{18} and analytical Vydac peptide C_{18} HPLC columns were purchased from Alltech. A flow rate of 3 mL/min was used in all elutions from the semipreparative HPLC column. UV spectra were recorded on Cary 210, Beckman DU-50, and Hewlett-Packard 8452A spectrophotometers and were obtained in water unless stated otherwise. The following extinction coefficients were used to determine concentrations: thymine, 7.9 mM^{-1} cm^{-1} (Dunn & Hall, 1975); 5-(hydroxymethyl)uracil, 8.1 mM^{-1} cm^{-1} (Dunn & Hall, 1975); 5-ethynyluracil, 9.05 mM^{-1} cm^{-1} (Barr et al., 1976).

Synthesis of 5-Ethynyluracil

EU was initially synthesized by the method of Barr et al. (1976) or by the modified synthesis of Kundu and Schmitz (1982). Because neither procedure was suitable for the synthesis of ^{14}C-labeled material, a new synthetic route was developed on the basis of the methods of Robins and co-workers (Robins et al., 1982; Robins & Barr, 1983).

5-Iodouracil. Iodine monochloride (1.5 g, 9 mmol) was added to a stirred suspension of uracil (448 mg, 4.0 mmol) in 8 mL of methanol. The mixture was heated at reflux for 100 min, during which time the suspended solid never completely dissolved. However, TLC analysis (10% MeOH in CH_2Cl_2) indicated that the starting material (R_f = 0.33) had been completely converted to product (R_f = 0.47). The mixture was filtered, and the solid product was washed with ether and dried under vacuum over P_2O_5. A yield of 81% (774 mg, 3.25 mmol) was obtained: UV λ_{max} 292 nm; ^{1}H NMR (DMSO-d_6) δ 7.9 (d, J = 7 Hz, 1H, C6-H), 11.2 (d, J = 7 Hz, 1H, N1-H), 11.45 (s, 1H, N3-H); HPLC retention time, 24 min on a semipreparative C_{18} column eluted with water.

5-[2-(Trimethylsilyl)ethynyl]uracil. A suspension of 197 mg (0.83 mmol) of 5-iodouracil in 10 mL of dry triethylamine was purged with argon for 20 min. (Trimethylsilyl)acetylene (235 mL, 1.66 mmol) was added, followed by (triphenylphosphine)palladium chloride (4.0 mg, 5.7 μmol) and CuI (4.1 mg, 21 μmol). The reaction mixture was stirred at 50 °C under argon for 3 h. Solvent was then removed by evaporation. The residue was suspended in 20 mL of chloroform and extracted twice with 10-mL aliquots of 5% EDTA. The organic layer containing suspended product was evaporated to dryness. The resulting solid was dried under vacuum over P_2O_5 to give a 79% yield (136 mg, 0.65 mmol): UV λ_{max} 292 nm; ^{1}H NMR (DMSO-d_6) δ 0.2 (s, 9H, $Si(CH_3)_3$), 7.8 (s, 1H, C6-H), 11.3–11.5 (br, 2H, N1-H, N3-H) [for literature values, see Kundu and Schmitz (1982)].

5-Ethynyluracil. 5-[2-(Trimethylsilyl)ethynyl]uracil (67.9 mg, 0.33 mmol) was converted to EU by the procedure of Kundu and Schmitz (1982). An 85% crude yield (38 mg, 0.28 mmol) was obtained: UV λ_{max} 285 nm; ^{1}H NMR (DMSO-d_6) δ 4.05 (s, 1H, C≡CH), 7.82 (d, $J \approx 8$ Hz, 1 H, C6-H), 11.3 (br d, 1H, N1-H), 11.4 (s, 1H, N3-H); HPLC retention time, 13 min on a semipreparative C_{18} column eluted with water [for literature values, see Barr et al. (1976)].

[2-^{14}C]EU. The three-reaction sequence described above for the synthesis of EU was carried out on 54 μmol (300 μCi) of [2-^{14}C]uracil. Difficulties were encountered due to the small scale of the reactions. HPLC analysis of the final isolated solid indicated that it contained 1.3 μmol (2% yield) of [2-^{14}C]-EU and 3.0 μmol (5% yield) of [2-^{14}C]iodouracil. An additional 51 μmol (94% yield) of [2-^{14}C]iodouracil was recovered from the EDTA extracts of the second reaction. This material was carried through the second and third reactions once more. The resulting solid was analyzed by HPLC and found to contain 3.5 μmol (7% yield) of [2-^{14}C]-EU and 20.7 μmol (41% yield) of [2-^{14}C]iodouracil; 26.1 μmol (51%) of the latter compound was again recovered from the EDTA extracts. The [2-^{14}C]EU was isolated from the product mixture, in small batches, by HPLC on a semipreparative C_{18} column eluted with water. The specific activity of purified [2-^{14}C]EU was 4.6 μCi/μmol. The HPLC retention time and UV spectrum were identical to those for unlabeled EU.

[^{3}H]EU. The acetylenic hydrogen of 5-ethynyluracil was exchanged with [^{3}H]H_2O (1 Ci/g) by the method of Barr et al. (1979). The resulting [^{3}H]EU was purified by HPLC in small aliquots immediately before use to minimize exchange. The specific activity of [^{3}H]EU, determined at the start of each experiment, was typically 1.3–1.7 μCi/μmol. The UV spectrum and HPLC retention time were identical to those of unlabeled EU.

Due to the limited solubility of EU in aqueous buffer, stock solutions (~6 mM) were prepared in DMSO and subsequently diluted 50-fold into 50 mM HEPES (pH 7.5). Incubation of the DMSO/HEPES solutions at room temperature for 7 h resulted in decomposition of 32% of the EU. Significant decomposition was also observed when these solutions were stored at 4 °C for 5 days. The DMSO stock solutions, however, were stable when stored at –20 °C for several weeks. As a result, fresh solutions in HEPES buffer were prepared each day and were stored on ice until use. To minimize decomposition, the amount of [2-^{14}C]EU required for one experiment was typically isolated the day before use and stored as a solid at –20 °C; it was then dissolved in 50 mM HEPES (pH 7.5) containing 2% (v/v) DMSO and used within 15 min.

Synthesis of Uracil-5-Acetylglycine

Uracil-5-Acetylglycine Benzyl Ester. 5-(Carboxymethyl)-uracil (10.5 mg, 61 μmol), glycine benzyl ester hydrochloride (12.6 mg, 63 μmol), and 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide (11.8 mg, 62 μmol) were dissolved in 4 mL of anhydrous DMF under an atmosphere of argon. After cooling to 0 °C, 16 μL (115 μmol) of dry triethylamine was added. The reaction mixture was stirred for 5 h at 0 °C and then for another 44 h at room temperature. Solvent was removed *in vacuo*, and the residue was purified by flash chromatography on a column (10 × 150 cm) of silica gel eluted with CH_2Cl_2/MeOH/H_2O (90:10:1) (Still et al., 1978). Fractions containing UV-absorbing material were evaporated to dryness to give a white solid (7.9 mg, 23 μmol, 40% yield): ^{1}H NMR (DMSO-d_6) δ 3.09 (s, 2H, CH_2CONH), 3.87 (d, J = 5.7 Hz, 2H, $NHCH_2CO_2^-$), 5.12 (s, 2H, CO_2CH_2Ph), 7.33–7.36 (m, 6H, Ph-H, C6-H), 8.30 (t, J = 5.9 Hz, 1H, $CONHCH_2$), 11.1 (br s, 2H, N1-H, N3-H).

Uracil-5-Acetylglycine. Uracil-5-acetylglycine benzyl ester (0.91 mg, 2.72 μmol) was suspended in 50 μL of methanol, and a 3-μL aliquot of 2 N aqueous NaOH was added. After it was stirred at room temperature for 19 h, the mixture was neutralized with 2 μL of glacial acetic acid. Methanol was removed under a stream of argon. The resulting solid was dissolved in 500 μL of water and passed through a C_{18} Sep-pac (Waters) to remove benzyl alcohol. Subsequent purification by HPLC on a semipreparative C_{18} column equilibrated with 0.1% aqueous TFA (retention time, 5.7 min) afforded 2.1 μmol (78% yield) of uracil-5-acetylglycine: UV (pH 2) λ_{max} 264 nm; ^{1}H NMR (DMSO-d_6) δ 3.06 (s, 2H, CH_2-CONH), 3.71 (d, J = 5.7 Hz, 2H, $NHCH_2COO$), 7.33 (d, J = 5.4 Hz, C6-H), 8.15 (t, J = 5.3 Hz, $CONHCH_2$), 10.76 (d, J = 5.4 Hz, 1H, N1-H), 11.07 (s, 1H, N3-H); MS m/z 228 (M^+), 15 3, 125.

Enzyme Purification and Activity Assays

Thymine hydroxylase was purified from *Rhodotorula glutinis* by modifications to published methods (Warn-Cramer et al., 1983), as described in the preceding article in this issue (Thornburg et al., 1993). The resulting enzyme was typically 85% pure as determined by SDS–PAGE and had a specific activity of 20–30 μmol of CO_2/min/mg. To prevent loss of activity, all enzyme samples were stored in 20 mM potassium phosphate (pH 7.5) containing 100 mM glycine and 100 μM EDTA (hereafter referred to as buffer B; some samples contained glycerol as well). Assays for thymine hydroxylase activity were carried out at 30 °C as described (Thornburg et al., 1993, Thornburg & Stubbe, 1989). Time-dependent inactivation studies were carried out as previously described (Thornburg & Stubbe, 1989).

Enzyme Adduct Formation and Stoichiometry of Labeling

Thymine hydroxylase (248 μg, 26 units/mg) was inactivated with 97 μM [2-^{14}C]EU (9185 cpm/nmol) or [^{3}H-]EU (~1500 cpm/nmol) in 220 μL of 45 mM HEPES (pH 7.5), containing 0.45 mM α-ketoglutarate, 0.11 mM $FeSO_4$, 2.3 mM ascorbate, 11 mM glycine, 2.3 mM phosphate, and 0.2% DMSO. After 15 min at 30 °C, the sample was loaded onto a Sephadex G-25 column (0.8 × 28 cm) and eluted with buffer B; 0.5-mL fractions were collected. For each fraction, the absorbance at 280 nm was recorded, and a 100-μL aliquot was analyzed by liquid scintillation counting. Those fractions containing protein were pooled, and aliquots were analyzed for radioactivity, protein, and enzyme activity using the $^{14}CO_2$ assay. Control reactions lacking either α-ketoglutarate or EU were chromatographed and analyzed in an identical manner. The stoichiometry of labeling was calculated from the protein concentration assuming a molecular weight of 39 000 and 85% purity. The fractions from the G-25 column containing small molecules were also located by liquid scintillation counting, pooled, and concentrated for analysis by HPLC, as described below.

Test for Covalent ^{14}C Protein Adduct

Method 1. A sample of ^{14}C-labeled thymine hydroxylase (15 μg, 1.04 equiv of EU/equiv of enzyme) was subjected to SDS–PAGE in a 10% gel (Laemmli, 1970), and the protein bands were stained with Coomassie R-250. The gel was then cut into 10 strips such that each protein band was in a separate piece. Each piece was dried overnight, pulverized, and analyzed for radioactivity by liquid scintillation counting.

Method 2. A sample of the same ^{14}C protein (10 μg, 2060 cpm) was denatured by treatment with 300 μL of 8 M guanidine hydrochloride for 10 min at room temperature. The sample was then centrifuged (2000g for 60 min) through an Ultrafree-MC filter unit (30 000 M_r cutoff). Both the filter and the filtrate were analyzed for radioactivity.

Product Formation from Inactivation of Thymine Hydroxylase with EU

The small molecules obtained by G-25 chromatography in the experiments described above were chromatographed on a C_{18} semipreparative HPLC column eluted with water; 1-min fractions were collected. Analysis by liquid scintillation counting revealed ^{14}C label in the void volume and EU peaks (retention times of 2 and 13 min, respectively). The void volume fractions containing radioactivity were pooled and rechromatographed on a C_{18} semipreparative HPLC column eluted with 0.1% aqueous TFA. Fractions (1.5 mL) were collected and analyzed for radioactivity. Two peaks of UV-absorbing material, eluting at 5.5 and 7 min, were found to contain ^{14}C label. They were designated compounds I and II, respectively.

Characterization of Compound I. [^{14}C]-I (93 nmol) was isolated by HPLC and the solvent was removed *in vacuo*. The sample was analyzed by UV–visible spectroscopy in H_2O and by ^{1}H NMR spectroscopy in both DMSO-d_6 and DMSO-d_6 with a small amount of D_2O added. [^{14}C]-I (1540 cpm, 0.17 nmol) was mixed with 7.8 nmol of uracil-5-acetylglycine and injected onto an analytical C_{18} HPLC column that was then eluted with 0.1% aqueous TFA at 1.5 mL/min. Fractions (0.75 mL) were collected, and the amount of ^{14}C label present in each was determined by liquid scintillation counting. [^{14}C]-I (4.95 × 10^4 cpm, 5.4 nmol) was mixed with 0.3 μmol of uracil-5-acetylglycine in a final volume of 30 μL. Aliquots (2 μL) were spotted onto two silica TLC plates (2.5 × 20 cm); one plate was developed with CH_2Cl_2/MeOH/37% aqueous AcOH (3:2:1) and the other with iPrOH/CH_2Cl_2/H_2O/concentrated NH_4OH (52:20:20:10). Similarly, aliquots (2 μL) were spotted onto two PEI–cellulose TLC plates that had been prerun in water and air-dried prior to use; one of these was developed with 50 mM ammonium formate (pH 7.5) and the other with 0.2 M LiCl in water. Each TLC plate was dried, and the R_f of uracil-5-acetylglycine was recorded. The plates were then cut into 1-cm-wide strips. Each strip was placed in a scintillation vial with 1 mL of water and shaken gently overnight. An 8-mL aliquot of scintillation fluid was added to each, and the samples were analyzed for radioactivity.

Characterization of II. [^{14}C]-II (10 nmol) was isolated by HPLC, as described above, and the solvent was removed *in vacuo*. The UV spectrum was recorded in H_2O. A mixture of [^{14}C]-II and 5-(carboxymethyl)uracil was rechromatographed on an HPLC column eluted with aqueous TFA and analyzed by silica gel and PEI–cellulose TLC as described above for [^{14}C]-I.

Characterization of the Enzyme Adduct

UV Difference Spectrum. Thymine hydroxylase was inactivated with [2-^{14}C]EU and reisolated as described above. A control reaction lacking only inhibitor was run in parallel. A 1.0-mL sample containing 190 μg of protein and 3.68 nmol of ^{14}C label (0.92 equiv) in buffer B was placed in the sample cuvette. A solution with the same concentration of protein from the control reaction was placed in the reference cuvette. The difference spectrum was recorded. Two additional difference spectra were obtained for comparative purposes.

In the first, the sample cuvette contained 3.0 nmol of thymine and 200 μg of BSA in 1.0 mL of buffer B; the reference cuvette contained an equal concentration of BSA. In the second, 5-acetyluracil (3.0 nmol) replaced thymine in the sample cuvette.

Comparative Stability of [^{14}C]EU and [^{3}H]EU Adducts at pH 2 and pH 8. Thymine hydroxylase (0.83 mg, 27 units/mg) was inactivated with 0.19 mM [2-^{14}C]EU or 0.33 mM [^{3}H]-EU (~1500 cpm/nmol) as described above. After 15 min at 30 °C, each reaction mixture was divided in half and applied to two Sephadex G-25 columns; one was eluted with 100 mM Tris (pH 8.0) and the other with 0.1% aqueous TFA (pH 2). For each column, the protein-containing fractions were pooled and the stoichiometry of labeling was determined. Solid guanidine hydrochloride was added to each sample to give a final concentration of 6 M. A 200-μL aliquot was immediately removed from each sample and analyzed for covalently bound label by method 2. Samples were incubated at 30 °C, and additional aliquots were removed for analysis at various times over 3 days.

Stability of Protein Adduct(s) to Nucleophiles. A 200-μL aliquot of ^{14}C-labeled thymine hydroxylase (96 μg, 1.28 equiv of EU/equiv of enzyme) was denatured in 1.6 mL of 6 M guanidine hydrochloride for 10 min. An 800-μL aliquot of neutral hydroxylamine solution was added to give a final concentration of 0.6 M (Stadtman, 1957). The sample was incubated at 30 °C; 300-μL aliquots were withdrawn over a 3-day period and analyzed for covalently bound label by method 2.

Stability of Protein Adduct(s) to Base Hydrolysis. Thymidine hydroxylase (86 μg) with 1.04 equiv of ^{14}C label was incubated at 30 °C in 100 mM potassium carbonate (pH 10) containing 5 M guanidine hydrochloride. Aliquots (300 μL) were removed at various times over 4 days and analyzed for covalent label by method 2.

Trypsin Digest of ^{14}C-Labeled Thymine Hydroxylase. Thymine hydroxylase (960 μg, 20.9 nmol) with 1.28 equiv of bound ^{14}C label was isolated on a Sephadex G-25 column equilibrated with 50 mM ammonium formate (pH 7.5), lyophilized, and redissolved in 490 μL of 100 mM Tris (pH 8.0) containing 10 mM EDTA and 6 M guanidine hydrochloride. The solution was degassed for 5 min using a water aspirator and then reequilibrated with argon. DTT (0.5 μmol in 5 μL of water) was added to the sample and allowed to react for 30 min at room temperature. Iodoacetamide (2.5 mmol in 5 mL of water) was then added, and the reaction was left in the dark for 3 h. The sample was chromatographed on a Sephadex G-25 column (1.1 × 28 cm, 27 mL) equilibrated with 200 mM acetic acid. The resulting protein sample (629 μg, 1.44 × 10^5 cpm) was lyophilized and redissolved in 500 μL of 100 mM ammonium bicarbonate (pH 8.0). TPCK–trypsin (21 μg in 2 μL of 1 mM HCl) was added, and the reaction mixture was incubated at 37 °C for 18 h. The sample was divided into three equal aliquots, and each was lyophilized.

Isolation of ^{14}C-Labeled Peptides. Each lyophilized trypsin digest sample was dissolved in 200 μL of 0.1% aqueous TFA. One was used to optimize the conditions required to separate the peptides, and the other two samples were used to isolate sufficient amounts of material for further analysis. Separation of the peptides was effected on a Vydac C_{18} HPLC column (flow rate of 1.0 mL/min and UV detection at 220 nm). The following two solvent systems were used for gradient elution of the peptides: (1) A, 0.1% aqueous TFA and B, CH_3CN with 0.1% TFA; (2) A, 20 mM potassium phosphate (pH 7.2) and B, CH_3CN. Initial separation was accomplished using solvent system 1 and the following linear gradients: 0–20% B over 30 min; 20–30% B over 30 min; 30–50% B over 30 min. The predominant radiolabeled fraction was chromatographed using a shallower gradient (15% B for 15 min, 15–20% B over 20 min, and 20% B for 15 min) in the same solvent system. The fraction containing ^{14}C label was then chromatographed in solvent system 2 (10% B for 10 min, 10–15% B over 20 min, 15% B for 20 min). The two ^{14}C-labeled peptides isolated by this protocol (designated peptides A and B) were then rechromatographed separately in solvent system 1 (10% B for 10 min, 10–20% B for 20 min, 20% B for 20 min). The final recovery of ^{14}C label in peptide A was 850 pmol, while that in peptide B was 260 pmol.

Amino Acid Sequence of Labeled Peptides. Samples (100 pmol) of peptides A and B were submitted to the Harvard Microchemistry Facility for sequencing by automated Edman degradation.

Mass Spectrometry of Peptide A. A sample (625 pmol) of peptide A was submitted to the MIT Mass Spectrometry Facility for analysis. Laser desorption time-of-flight mass spectrometry (TOF-MS) was carried out as described by Juhasz et al. (1992). Fast atom bombardment (FAB-MS) and tandem mass spectrometries were performed as described by Papayannopoulos et al. (1989).

RESULTS

Time-Dependent Inactivation of Thymine Hydroxylase by EU. Incubation of thymine hydroxylase with EU was previously shown to result in time-dependent loss of enzyme activity in an apparent first-order process (Thornburg & Stubbe, 1989). No loss of activity was observed in the absence of EU, and the rate of inactivation was dependent on the concentration of EU. A double-reciprocal plot of rate constant (ρ) vs EU concentration (I) gave a straight line described by eq 2, with values for k_2 and K_I of 2.6 ± 0.8 min^{-1} and 22 ± 7 μM, respectively. This kinetic pattern is consistent with

$$1/\rho = (K_I/k_2)(1/I) + 1/k_2 \quad (2)$$

reversible, saturable binding of inhibitor to the enzyme followed by one or more irreversible steps leading to enzyme inactivation (Main, 1973). The apparent dissociation constant for EU, K_I, is approximately one-half the apparent K_m value for thymine (58 μM) determined under similar conditions (Thornburg et al., 1993). The rate constant for inactivation with saturating EU, k_2, is ~500-fold lower than the turnover number for the normal enzyme reaction with thymine (~1000 min^{-1}).

As previously reported, no loss of thymine hydroxylase activity occurs in the absence of molecular oxygen. When α-ketoglutarate is omitted from the inactivation reaction, slow loss of activity is observed after a lag of ~3 min. However, the same slow inactivation is also observed when both EU and α-ketoglutarate were omitted from the reaction. Therefore, inactivation of thymine hydroxylase by EU has the same substrate requirements as the normal enzyme reaction with thymine. Furthermore, the presence of 120 μM thymine completely protects the enzyme against inactivation. Taken together, the above results strongly suggest that EU is a mechanism-based inactivator of thymine hydroxylase.

Covalent Labeling of Thymine Hydroxylase. To determine whether EU forms a covalent adduct with the enzyme during inactivation, thymine hydroxylase was inactivated with ^{14}C- or ^{3}H-labeled EU and then chromatographed on a Sephadex G-25 column. In both cases, radiolabel was found to elute

with the protein peak. The enzyme activity after chromatography was ≤5% that of a control reaction which lacked EU. When α-ketoglutarate was omitted from the inactivation reaction, no radiolabel was associated with the protein after chromatography. The stoichiometry of ^{14}C labeling was 1.00 ± 0.16 equiv of inhibitor per enzyme equivalent (average of eight determinations).

To distinguish between tight-binding inhibition and covalent modification, ^{14}C-labeled thymine hydroxylase was subjected to gel electrophoresis under denaturing conditions. Analysis of gel segments by liquid scintillation counting demonstrated that radiolabel was associated with the major protein band (39 000), although only 15% of the initial label was recovered. Further evidence for covalent adduct formation was obtained when ^{14}C-labeled thymine hydroxylase was denatured with 6 M guanidine hydrochloride and separated from the small molecules by ultrafiltration. The reisolated protein contained 88% of the initial radiolabel. These results clearly demonstrate that inactivation of thymine hydroxylase by EU is accompanied by covalent binding of ~1 equiv of EU.

Product Formation from EU. To determine whether product formation accompanies enzyme inactivation in the thymine hydroxylase reaction with EU, the small molecules were isolated from inactivation reactions by Sephadex G-25 chromatography. Analysis of the small molecule fraction by HPLC on a reverse-phase column eluted with water revealed the presence of ^{14}C-labeled material in the void volume of the column, as well as unreacted [^{14}C]EU. The radioactivity eluting in the void volume was rechromatographed on a reverse-phase HPLC column equilibrated with 0.1% aqueous TFA (pH 2). Two ^{14}C-labeled compounds, designated I and II, were observed with retention times of 5.5 and 7.0 min, respectively. Neither compound was observed in a control reaction from which thymine hydroxylase was omitted. The distribution of ^{14}C label between enzyme adduct and the two products was determined in four separate experiments. The ratio of I to II was (3.5 ± 1.3):1, and the ratio of products (I and II) to labeled enzyme was (1.65 ± 0.65):1.

When [^{3}H]EU (1170 cpm/nmol) replaced [^{14}C]EU in the reaction, I and II were again isolated with specific activities of 630 and 790 cpm/nmol, respectively. Thus, the acetylenic hydrogen from EU is retained in both products. It is not possible to determine whether the lower specific activity of the products relative to the starting material is due to an isotope effect on the thymine hydroxylase catalyzed reaction, to an exchange from an intermediate in the enzyme-catalyzed reaction, or to a non-enzyme-catalyzed exchange from EU itself.

The migratory properties of I and II on reverse-phase HPLC (i.e., elution in the void volume at neutral pH, but retention at pH 2) suggested that they might be carboxylic acids. Activated cyt P-450 has been shown to react with acetylenic compounds to generate carboxylic acids via a ketene intermediate (Ortiz de Montellano & Komives, 1985). If an analogous reaction occurs with thymine hydroxylase, the resulting ketene could be trapped either by water to give 5-(carboxymethyl)uracil or by glycine (present in the assay buffer) to give uracil–5-acetylglycine. These two compounds were therefore synthesized for comparison with I and II.

The UV spectrum of I exhibits a λ_{max} of 264 nm, as does that of authentic uracil–5-acetylglycine. The NMR spectrum of I in DMSO, while containing major amounts of contaminating materials, exhibits resonances at 3.1, 3.7, 7.3, 8.2, 10.8, and 11.1 ppm; the latter three resonances are exchangeable in D_2O. These signals are identical to those of authentic uracil–5-acetylglycine. Furthermore, [^{14}C]-I was shown to comigrate with uracil–5-acetylglycine on a C_{18} HPLC column eluted with 0.1% aqueous TFA and in four different TLC systems (Table I). The UV spectrum of II, with a λ_{max} of 264 nm, is identical to that of 5-(carboxymethyl)uracil. The small amount of [^{14}C]-II that was isolated precluded obtaining an NMR spectrum of this compound. However, [^{14}C]-II was shown to comigrate with authentic 5-(carboxymethyl)uracil in four TLC systems (Table I) and on a C_{18} HPLC column eluted with 0.1% aqueous TFA. These results strongly suggest that thymine hydroxylase catalyzes the oxidation of EU to a ketene which, after dissociation from the active site, is trapped by nucleophiles in solution.

Table I: TLC Comigration Experiments

mixture	TLC plate	solvent system[a]	R_f	% ^{14}C in UV spot
[^{14}C]-I and **4**	silica	NH_4OH	0.47	98
	silica	AcOH	0.78	92
	PEI–cellulose	LiCl	0.55	93
	PEI–cellulose	pH 7.5	0.27	91
[^{14}C]-II and **5**	silica	NH_4OH	0.47	95
	silica	AcOH	0.85	90
	PEI–cellulose	LiCl	0.48	88
	PEI–cellulose	pH 7.5	0.21	88

[a] NH_4OH represents *i*PrOH/CH_2Cl_2/H_2O/concentrated NH_4OH (52:20:20:10); AcOH represents CH_2Cl_2/MeOH/37% aqueous AcOH (3:2:1); LiCl represents 0.2 M aqueous LiCl; pH 7.5, 50 mM ammonium formate, pH 7.5. **4** is uracil–5-acetylglycine and **5** is (carboxymethyl)-uracil.

Characterization of the Enzyme Adduct. Information about the structure of the enzyme adduct was obtained using a variety of approaches. The UV–vis difference spectrum between covalently modified and unmodified protein has a λ_{max} of 268 nm. Comparison with the spectra of thymine (λ_{max} = 268 nm) and 5-acetyluracil (λ_{max} = 303 nm) obtained under similar conditions indicates that the structure of the enzyme adduct resembles the former rather than the latter. Therefore, any proposed mechanism for inactivation must lead to the formation of an adduct which is a nonconjugated uracil derivative.

In an effort to gain further information about the chemical structure of the adduct, the stabilities of ^{14}C- and ^{3}H-labeled enzymes were determined under denaturing conditions at pH 8 and pH 2. At pH 8, 92% of the ^{14}C label remained bound to the protein after 67 h. Under the same conditions, 24% of the ^{3}H label was lost, suggesting that at least some of the tritium resides in an acidic position in the adduct. At pH 2, 97% of the ^{14}C label remained bound to the protein after 67 h. In contrast, 43% of the ^{3}H label was released from the protein within 90 min. An additional 7% of the ^{3}H label was lost during the subsequent 67-h incubation at pH 2. The observed loss of tritium under acidic conditions is somewhat surprising and will be discussed in more detail below.

To determine whether the enzyme adduct is labile to nucleophilic attack, ^{14}C-labeled thymine hydroxylase was treated with either neutral hydroxylamine or hydroxide (pH 10) under denaturing conditions. While 25% of the ^{14}C label was released by hydroxylamine within 24 h, no further loss of label was observed for up to 70 h. Incubation of ^{14}C-labeled enzyme at pH 10 resulted in the release of only 20% of the label after 90 h. These results demonstrate that there is a mixed population of structures on the enzyme and that 20–25% of the species present are labile to attack by both hydroxylamine and hydroxide ion. The remainder of the label appears to be chemically very stable. In conjugation with the

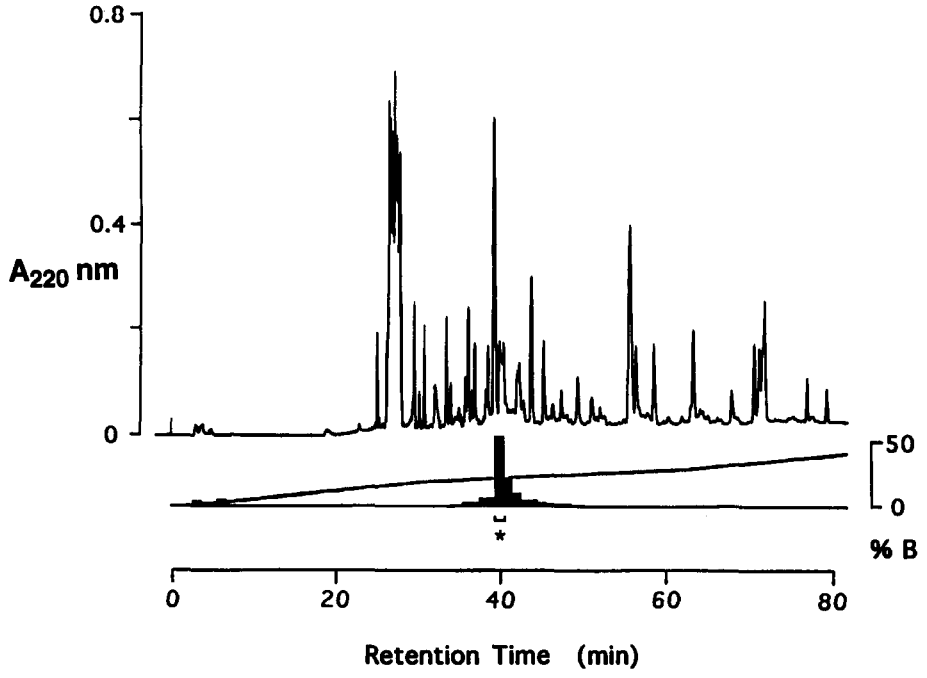


FIGURE 1: Initial HPLC separation of ^{14}C-labeled tryptic peptides. The analytical Vydac C_{18} peptide column was eluted with solvent system 1 at a flow rate of 1.0 mL/min; detection was by absorbance at 220 nm. The bar graph indicates the ^{14}C label in each fraction. Fraction 41 is marked with *.

studies at pH 2 and 8, these results suggested that isolation of ^{14}C-labeled peptide(s) would be possible.

Characterization of a Labeled Tryptic Peptide. ^{14}C-Labeled thymine hydroxylase was carbamidomethylated with iodoacetamide by standard procedures. The alkylated protein was then digested with TPCK–trypsin for 18 h. Initial HPLC separation of the resulting peptides revealed that 45% of the recovered ^{14}C label was present in fraction 41, with the remainder distributed in adjacent fractions (Figure 1). Rechromatography of the material in fraction 41 with a shallower gradient removed several unlabeled peptides. Subsequent chromatography in a second solvent system resolved the ^{14}C-labeled material into two peaks, designated A and B, with retention times of 39 and 43 min, respectively. The ratio of A to B was ~3:1. Final HPLC analysis in the initial solvent system demonstrated that each sample was a single, sharp peak that coincided with the radioactivity. The total recovery of radioactivity for each HPLC run ranged from 70 to 95%. The final yield of peptides A and B corresponded to 10% of the material injected onto the first HPLC column.

The amino acid sequence of peptide A, determined by Edman degradation, is N S I A F ? S N P S L R. The sequence of peptide B contains an additional unidentified amino acid at the N-terminus, but is otherwise identical to peptide A. This result demonstrates that at least 45% of the enzyme adduct is due to modification of the amino acid residue at position 6 of peptide A.

Because the sequence of peptide A is completely defined except for residue 6, the mass of this amino acid can be determined if the mass of the peptide is known. Laser desorption time-of-flight mass spectrometry gave an average mass of m/z 1519.8 for the $(M + H)^+$ ion of peptide A; the monoisotopic mass was found to be m/z 1519.7 by fast atom bombardment mass spectrometry. Subtraction of the calculated mass for the known amino acid sequence (m/z 1205.6) from the total mass of peptide A gives a residue mass of 314.2 for the modified amino acid.

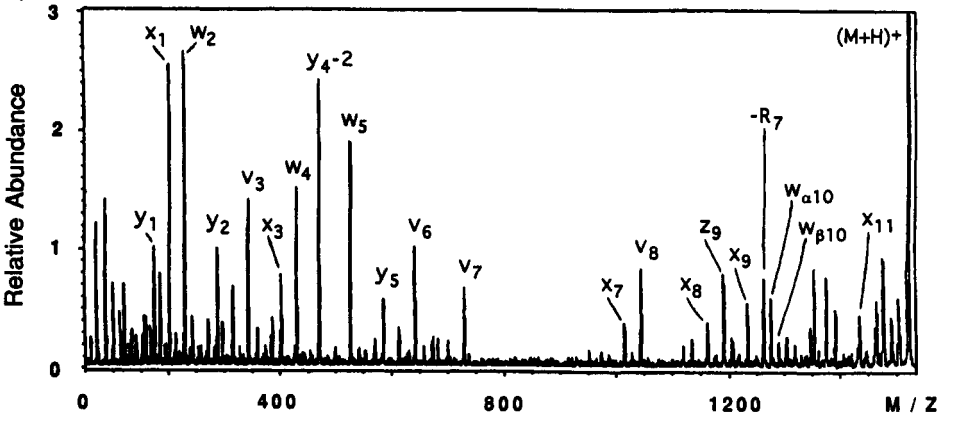
Tandem mass spectrometry was employed in the hope of obtaining more detailed information about the structure of the modified amino acid in peptide A (Biemann, 1988). The results are presented in Figure 2A and confirm the amino acid sequence determined by Edman degradation (Figure 2B). In addition, the lack of fragments between m/z 1051 and 729 strongly suggests that the unknown amino acid residue is large and quite stable. The fragment at 1261.6 results from loss of the side chain (mass 258.2) from the modified residue. The difference between 314.2 and 258.2 is entirely accounted for by the peptide backbone, demonstrating that the modification is localized on the side chain and confirming the residue mass of 314.2 for the modified amino acid.

DISCUSSION

Our previous studies have demonstrated that thymine hydroxylase possesses many of the same catalytic capabilities as cyt P-450 (Thornburg et al., 1993). The results of the current study show that these two enzymes also catalyze similar reactions with terminal acetylenes. Ortiz de Montellano and co-workers have made the interesting observation that incubation of phenylacetylene with cyt P-450 resulted in a partitioning between phenylacetic acid formation and N-alkylation of the heme cofactor in a ratio of 26:1. A detailed investigation of this reaction, as well as chemical model studies, led them to postulate the mechanism shown in Scheme I to account for their results (Ortiz de Montellano & Komives, 1985; Komives & Ortiz de Montellano, 1987). Phenylacetylene is proposed to bind to the cyt P-450 in two orientations. The partitioning between turnover and inactivation is determined by which carbon of the triple bond is positioned next to the active "iron–oxo" species. Addition of oxygen to the inner carbon results in enzyme inactivation to produce *N*-(2-phenyl-2-oxoethyl)heme. Addition to the outer carbon, concomitant with a 1,2-hydride shift, generates the ketene that leads to phenylacetic acid.

The reaction of thymine hydroxylase with EU also results in a partitioning between enzyme inactivation and production of products. Two products have been identified: 5-(carboxymethyl)uracil and uracil–5-acetylglycine. Tritium la-

A)

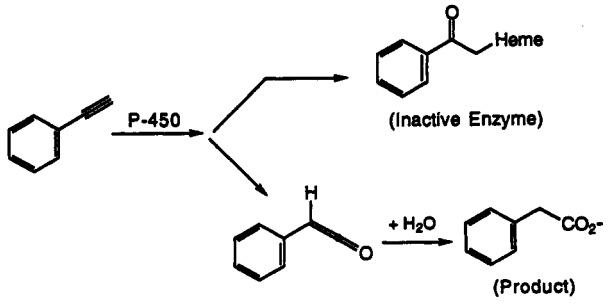


B)

	12	11	10	9	8	7	6	5	4	3	2	1
Seq:	N	S	I	A	F	?	S	N	P	S	L	R
x_n:	--	1433	--	1233	1161	1051	699	612	--	401	--	202
y_n:	--	--	--	--	--	--	--	586	473	--	288	175
z_n:	--	--	--	1190	1161	--	--	--	--	358	--	--
v_n:	--	--	--	--	1044	729	641	--	--	343	--	--
w_n:	--	--	1288 & 1274	--	--	--	--	527	429	--	229	--

FIGURE 2: Tandem mass spectrometry of peptide A. (A) Collision-induced dissociation spectrum of the $(M + H)^+$ ion of $m/z = 1519.8$. (B) The deduced sequence of peptide A and assignment of major ion fragments [see Biemann (1988)].

Scheme I



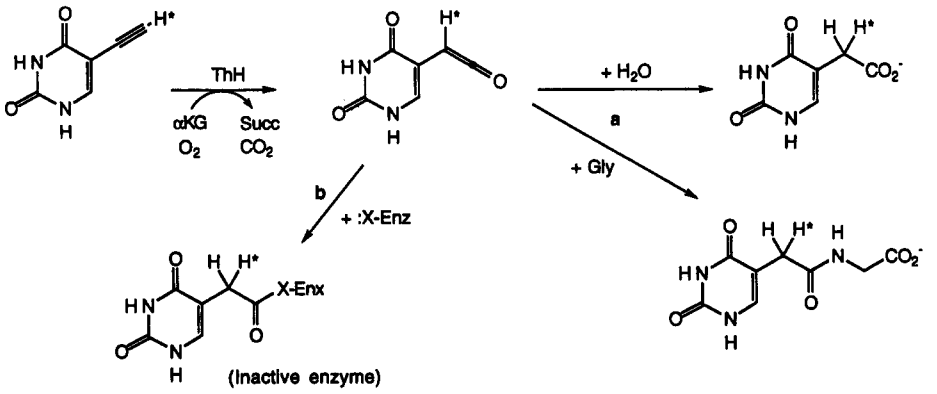
beling experiments indicate that the acetylenic hydrogen of EU is retained in both products. These results are consistent with a mechanism analogous to that proposed for cyt P-450, in which EU is first oxidized to a ketene intermediate with a concomitant 1,2-hydride shift; this species is then trapped by water or glycine in solution to give the observed products (Scheme II, path a). Unfortunately, the lability of the acetylenic hydrogen of EU precludes measuring isotope effects on product formation for comparison with the P-450 results.

The inactivation of thymine hydroxylase with EU occurs in one out of three turnovers. The normal substrates α-ketoglutarate and O_2 are required for this process. Complete inactivation of the enzyme is accompanied by stoichiometric covalent labeling, and the acetylenic hydrogen is retained in the adduct. If inactivation of thymine hydroxylase occurs via addition of oxygen to the inner carbon of the triple bond of EU, as is proposed for cyt P-450, then the resulting adduct would have a UV spectrum similar to that of 5-acetyluracil (λ_{max} = 303 nm). However, the observed spectrum for the enzyme adduct has a λ_{max} of 268 nm. Therefore, the mechanism for inactivation of thymine hydroxylase by EU is not the same as that for cyt P-450 inactivation by phenylacetylene.

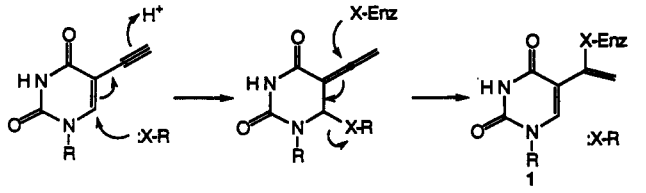
Extensive investigations of acetylenic mechanism-based inhibitors with a variety of enzymes suggest an alternate hypothesis for consideration. Studies on the mechanisms of inactivation of thymidylate synthase by 5-ethynyluridine (Barr et al., 1983) and of dihydroorotate dehydrogenase by EU (Porter et al., 1992) are consistent with the mechanism shown in Scheme III. In the case of thymidylate synthase, nucleophilic attack at the C-6 position of 5-ethynyluridine by an active site thiol generates an allene which, in the absence of the 5,10-methylenetetrahydrofolate cofactor, is attacked by buffer or β-mercaptoethanol. The resulting products have UV spectra whose λ_{max} values vary from 254 to 267 nm, depending on the nucleophile. In the case of dihydroorotate dehydrogenase, it is proposed that the reduced flavin cofactor transfers a hydride to the C-6 position of EU to form the allene, which is activated toward nucleophilic attack. In contrast to both of these systems, there is no mechanistic imperative for thymine hydroxylase to catalyze chemistry at the C-6 position of thymine. Therefore, a similar mechanism would require enzyme-catalyzed deprotonation of the N-1 position and protonation of the terminal carbon of the acetylene to generate an allene. Nucleophilic attack on the allene by an amino acid residue would result in covalent modification of the protein. The stability of the EU adduct, in conjunction with an extensive literature on the stability of compounds similar to **1** (Scheme III), suggests that all amino acid nucleophiles, except histidine, can be eliminated as reasonable candidates for the nucleophile responsible for the inactivation (Birch & Smith, 1956; Barr et al., 1983; Washtien & Abeles, 1977). To our knowledge, a histidine adduct has not been prepared; however, one would expect it to be more labile than the lysine adduct, which has already been eliminated from consideration. Therefore, it seems unlikely that thymine hydroxylase inactivation by EU proceeds by a mechanism involving an allene.

At present, the mechanism of inactivation by EU remains unknown. The favored hypothesis is that the putative ketene intermediate responsible for the formation of uracil–5-acetylglycine and 5-(carboxymethyl)uracil can also be trapped

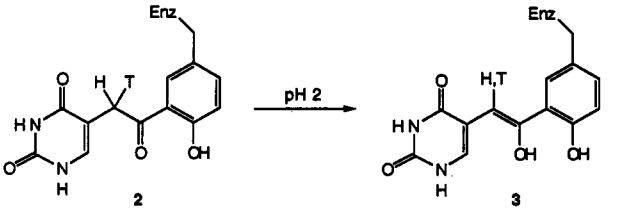
Scheme II



Scheme III



Scheme IV



by an enzyme nucleophile before leaving the active site (Scheme II, path b). This model predicts that the adduct would have a UV spectrum similar to that of thymine, as is the case. Moreover, the acetylenic hydrogen of EU would be retained in a somewhat acidic position α to the carbonyl in the postulated adduct. The observed slow loss of ^3H label, but not ^{14}C label, from the adduct at pH 8 supports this hypothesis. The adduct stability studies limit the possibilities for the attacking nucleophile in this model. If it was the side chain of the amino acid Y, C, H, E, or D, the resulting adduct would have been released from the enzyme during incubation with NH_2OH (Riordan & Vallee, 1967; Stadtman, 1957). Seventy-five percent of the ^{14}C label is stable to this treatment. The stability of the ^{14}C label at pH 10 also eliminates serine and threonine from consideration (Anderson et al., 1961). In both of these experiments, the portion of the label (approximately 20%) that was released may be due to nonspecifically labeled residues on the enzyme surface that have trapped the ketene after it dissociated into solution. Thus, lysine is the only enzyme nucleophile able to form a stable adduct in this postulated mechanism for inactivation.

The stability of the enzyme adduct allowed us to isolate two ^{14}C-labeled tryptic peptides, A and B. Sequencing of the two peptides by Edman degradation revealed that the same unidentified amino acid residue was modified in both. The fact that thymine hydroxylase is completely inactivated by alkylation with only 1 equiv of EU suggests that this residue is important for catalysis. The postulated mechanism for thymine hydroxylase does not require nucleophilic or acid/base catalysis by the enzyme (Siegal, 1979; Hanauske-Abel & Gunzler, 1982). Perhaps the modified amino acid is one of the iron ligands. Recall that phenylacetylene inactivation of cyt P-450 is due to alkylation of the N in the D ring pyrrole, one of the heme iron ligands.

The mass of peptide A was determined by two mass spectrometry techniques. Given the sequence for this peptide, we can calculate a residue mass of 314 for the modified amino acid. If this adduct did result from nucleophilic attack on a ketene, then the unmodified amino acid would have a residue mass of 162. Tyrosine, with a residue mass of 163, is the natural amino acid that comes closest to this value. However, the pH 10 and hydroxylamine stability data rule out the possibility of a tyrosine ester formed by trapping a ketene intermediate. The possibility of errors in the sequencing by Edman degradation was considered. However, the results of tandem mass spectrometry on peptide A are consistent with the sequence obtained by Edman degradation and confirm a mass of 314 for the modified amino acid. Furthermore, the mass spectral data establish that the modification is confined to the amino acid side chain. While no definitive assignment can be made, the tandem mass spectrometry data suggest that tyrosine is the modified amino acid in peptide A.

A postulated structure for the modified amino acid of peptide A is shown in Scheme IV. The adduct (**2**) could be obtained if a tyrosine residue attacked the ketene intermediate to form a reactive ester which subsequently underwent Frie's rearrangement. These reactions are catalyzed by Lewis acids and usually require reasonably strenuous conditions (March, 1977). In our case, the iron center of the enzyme would have to play an assertive role in this process. The same adduct could also be obtained if a tyrosine residue reacted with the ketene intermediate in a Friedel–Crafts (Lewis acid-catalyzed) acylation. This reaction would require that steric constraints by the enzyme prevent the oxygen of the tyrosine moiety from reacting with the ketene. Model acylation reactions with acid anhydrides or chlorides are also catalyzed by Lewis acids, including $FeCl_3$ (Pearson & Buhler, 1972). A ketene, especially if it is coordinated to a metal ion, is more reactive than either of these species. Model systems investigating the reactions of lithium and potassium enolates with phenylketenes indicate that O-acylation is kinetically favored in both cases, but the C-acylated products are thermodynamically more stable (Tidwell, 1990). Therefore, we currently favor the hypothesis that the modified amino acid results from the unprecedented acylation of a tyrosine moiety at its ortho position by a ketene derived from EU.

One piece of experimental data from the adduct stability studies is still puzzling. Fifty percent of the [^3H] label was lost rapidly from the adduct at pH 2, but much more slowly at pH 8. One possible explanation is that two types of adducts are formed with roughly equal probability, only one of which contains acid-labile tritium. A second possibility is that

exchange occurs through an intermediate, **3** (Scheme IV). However, formation of **3** might also be expected to be base-catalyzed (pH 8), and at present, no reasonable hypothesis exists to accommodate the less than stoichiometric washout of tritium, including our favored hypothesis in Scheme II (path b). It should also be noted that the postulated adduct **2** has a residue mass of 315, so that loss of an additional hydrogen is required to give the observed mass spectra. In addition, the possibility should not be ruled out that a posttranslationally modified amino acid, and not tyrosine, is responsible for the unusual stability of our adduct. Further degradative studies of peptide A clearly are required to determine unambiguously the structure of the modified amino acid. These studies should reveal the mechanism of inactivation.

SUMMARY

The reaction of thymine hydroxylase with EU has been shown to result in ketene-trapped products which retain the acetylenic hydrogen. This reaction is entirely analogous to that of cytochrome P-450 with phenylacetylene and, thus, provides an additional point of similarity between this Fe^{3+} heme enzyme and the α-ketoglutarate-dependent dioxygenases. EU has also been shown to inactivate thymine hydroxylase by a mechanism-based reaction. While the exact structure of the enzyme adduct has not yet been determined, it has been shown to differ from that obtained in the cytochrome P-450 reaction with phenylacetylene. Further characterization of the enzyme adduct may yield important information about the structure of the active site. In addition, the sequence information obtained should facilitate cloning of the gene for thymine hydroxylase. This in turn would allow the isolation of large quantities of the enzyme for physical characterization of the iron center.

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