A Non-Heme Iron Protein with Heme Tendencies: An Investigation of the Substrate Specificity of Thymine Hydroxylase†

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Received August 17, 1993; Revised Manuscript Received October 1, 1993®

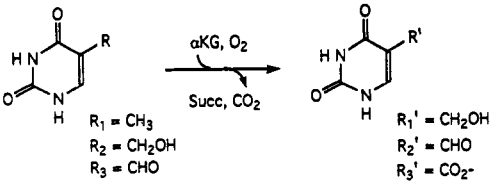
ABSTRACT: Thymine hydroxylase from *Rhodotorula glutinis* catalyzes the oxidation of thymine to its alcohol, aldehyde, and carboxylic acid in three successive reactions. Each step involves stoichiometric consumption of O_2 and α-ketoglutarate and formation of CO_2 and succinate. Given the promiscuity of this enzyme, it was hoped that it would serve as a prototype for understanding the mechanism of this class of enzymes, the non-heme Fe^{2+} dioxygenases. Kinetic parameters for thymine, O_2, Fe^{2+}, and α-ketoglutarate have been determined, and isotope effect analysis of (trideuteriomethyl)thymine with enzyme reveals $^D(V) = 2.08$ and $^D(V/K) = 1.11$ at saturating O_2. The kinetic parameters for (hydroxymethyl)uracil oxidation have been determined, and incubation of (5′-*R*)- and (5′-*S*)-[5′-^{2}H]-5-(hydroxymethyl)uracil with enzyme reveals stereospecific removal of the *pro-S* hydrogen. No apparent isotope effect is observed in this reaction. The substrate specificity of this enzyme has been examined in detail. The enzyme can catalyze epoxidation, oxidation of a thioether to a sulfoxide and a sulfone, hydroxylation of an unactivated carbon–hydrogen bond, and oxidation of a methylamine to formaldehyde, as revealed through studies with 5-vinyluracil, 5-(methylthio)uracil, 5,6-dihydrothymine, and 1-methylthymine, respectively. In each case, the products were identified by gas chromatography–mass spectrometry, and $^{18}O_2$-labeling studies revealed that one atom from O_2 is incorporated into each product. The enzyme has also been shown to catalyze an uncoupling of hydroxylation and decarboxylation in the presence of a substrate analog incapable of undergoing hydroxylation or a substrate that is difficult to oxidize. The substrate specificity studies and kinetic analysis reveal that this system is strikingly similar to the heme-dependent cytochrome P-450s.

In the past decade, it has become clear that the repertoire of reactions catalyzed by heme iron dependent proteins is mirrored by proteins containing either mononuclear or dinuclear non-heme iron centers (Ortiz de Montellano, 1986; Katopodis et al., 1988; Green & Dalton, 1989; Rataj et al., 1991; Stubbe, 1991; Liu et al., 1993). These non-heme iron proteins have escaped close scrutiny because their cofactor chemistries are not easy to monitor spectroscopically, unlike their more ostentatious heme counterparts.

Our laboratory is interested in several non-heme iron systems. Bleomycin is an antitumor antibiotic which, in the presence of Fe^{2+}, O_2, and a reductant, can catalytically hydroxylate the C-4′ carbon of a nucleotide in a defined DNA sequence (Hecht, 1986; Stubbe & Kozarich, 1987). The structure of the non-heme iron intermediate involved in hydroxylation of this unactivated C–H bond presently is not known. We are also interested in understanding the chemistry of nucleotide reduction catalyzed by ribonucleotide reductase (Fontecave et al., 1992; Stubbe, 1990). The enzyme from *Escherichia coli* possesses an unusual dinuclear non-heme iron center and a tyrosyl radical cofactor, which is essential for nucleotide reduction. The tyrosyl radical, located on one of the subunits of the protein, can be assembled from apoprotein, Fe^{2+}, O_2, and a reductant (Atkin et al., 1973). Recent studies in our laboratory have suggested that this non-heme iron chemistry involves an unprecedented diferric radical intermediate (Bollinger et al., 1991), in contrast to the well-characterized heme-dependent peroxidase systems which are capable of oxidizing phenols to phenoxide radicals and involve several different forms of ferryl intermediates (Sakurada et al., 1990; Job & Dunford, 1976).

These interests have led us to investigate an additional class of non-heme iron proteins that also acts on nucleosides and nucleic acid bases: the Fe^{2+}, α-ketoglutarate-dependent dioxygenases from *Rhodotorula glutinis*. Our initial interest focused on 2′-deoxyuridine hydroxylase, which catalyzes the reverse of the ribonucleotide reduction reaction (i.e., hydroxylation at C-2′ of the sugar to produce uridine), and on 1′-deoxyuridine hydroxylase, which catalyzes the conversion of 2′-deoxyuridine to uracil and deoxyribonolactone (Stubbe, 1985). However, the limited amounts of these proteins obtainable from *R. glutinus* and the availability of larger amounts of thymine hydroxylase from the same organism enticed us to shift our focus to this latter protein.

Thymine hydroxylase (EC 1.14.11.6) catalyzes the three oxidation reactions shown in eq 1 (Holme et al., 1970, 1971; Liu et al., 1973). These oxidations are not processive, with



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each subsequent oxidation requiring product release. This enzyme is a dioxygenase, as $^{18}O_2$-labeling studies demonstrated that one oxygen atom is incorporated into the pyrimidine product and the other is incorporated into succinate (Holme

† This research was supported by NIH Grant GM 32191.
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® Abstract published in *Advance ACS Abstracts,* November 15, 1993.

0006-2960/93/0432-14023$04.00/0

et al., 1971). The enzyme from *R. glutinis* was attractive for mechanistic investigations because Warn-Cramer et al. (1983) had previously reported its purification to homogeneity. In addition, its ability to catalyze three successive hydroxylation reactions suggested that its substrate specificity might be broad. The enzyme has also been partially purified and characterized from *Neurospora crassa*. Preliminary substrate specificity studies on this enzyme have provided evidence of its ability to accept a broad range of substrates (Bankel et al., 1977). In addition, the steady-state kinetic mechanism of the *N. crassa* hydroxylase has been studied in some detail by Holme (1975).

The present article reports preliminary kinetic characterization of the *R. glutinis* thymine hydroxylase and a detailed examination of its substrate specificity. The results provide evidence that this enzyme can catalyze allylic hydroxylations, epoxidation of an olefin, oxidation of a sulfide to a sulfoxide, N-demethylation of an amide, and hydroxylation of an unactivated carbon–hydrogen bond. In the following article in this issue, an acetylenic compound is also shown to be a substrate for this hydroxylase, partitioning between enzyme inactivation and conversion to CH_2CO_2H (Thornburg & Stubbe, 1993). Thus, this non-heme, mononuclear Fe^{2+} enzyme is shown to have catalytic capabilities similar to those of the well-characterized heme-dependent cytochrome P-450 (cyt P-450).

EXPERIMENTAL PROCEDURES

Materials

5-(Hydroxymethyl)uracil (HMU[1]), 5-fluorouracil, iodomethane, and ICD_3 (99+% D) were supplied by Aldrich Chemical Co. 5-Mercaptouracil was obtained from Chemical Dynamics. 5-Formyluracil, 5-vinyluracil, 5-acetyluracil, and (trideuteriomethyl)thymine (>99.8% deuterium incoporation) were synthesized by published procedures [Ressner et al. (1976), Jones et al. (1974), Dewar and Shaw (1961), and Shiue and Wolf (1980), respectively]. 5-(2-Hydroxyethyl)-uracil was a gift from D. Bergstrom, Purdue University. Thymine, 1-methylthymine, 5,6-dihydrothymine, 5-carboxyuracil, α-ketoglutarate, sodium ascorbate, protamine sulfate (grade X, from salmon), and hyamin hydroxide (1 M solution in methanol) were purchased from Sigma. Ferrous sulfate was purchased from Mallinkrodt. Protein assay dye reagent was obtained from Bio-Rad. Immobilon-P polyvinyl difluoride (PVDF) membranes (pore size 0.45 μm) were supplied by Millipore. [1-^{14}C]-α-Ketoglutarate (59 μCi/μmol) and [2-^{14}C]-thymine (56 μCi/μmol) were purchased from New England Nuclear and Moravek Biochemicals, respectively. *N*-Methyl-*N*-(trimethylsilyl)trifluoroacetamide (MSTFA) and CH_3CN were supplied by Pierce Chemical. $^{18}O_2$ (99.8%) was obtained from Amersham. *Rhodotorula glutinis* was obtained from American Type Culture Collection (ATCC No. 2527).

Synthesis of 5-(Methylthio)uracil. 5-(Methylthio)uracil was synthesized by the modification of a published procedure (Dinan et al., 1982). 5-Mercaptouracil (5.33 mg, 37 μmol; a mixture of thiol and disulfide) was suspended in 700 μL of 50% aqueous MeOH. Solid $NaBH_4$ was added in three aliquots (~2 mg each), until no further H_2 evolution was observed; the 5-mercaptouracil completely dissolved after addition of the third aliquot. The reaction vessel was flushed with argon and then cooled to 0 °C. Iodomethane (2.5 μl, 37 μmol) was added, and the reaction was stirred at 0 °C for 30 min, during which time a white solid formed. The mixture was then warmed to room temperature and stirred for another 30 min. The solid was isolated by centrifugation. The solid was washed with water (3 × 1 mL) and with 0.1 M HCl (1 × 200 μL), dissolved in MeOH (2 mL), evaporated to dryness, and dried under vacuum over P_2O_5 to give 2.1 mg (13.3 μmol, 36% crude yield): ^{1}H NMR (DMSO-d_6) δ 2.25 (s, 3H, SCH_3), 7.42 (d, 1H, C6-H), 11.06 (d, 1H, N1-H), 11.30 (s, 1H, N3-H); UV λ_{max} 234 and 278 nm (sh); GC–MS of TMS_2 deriv m/z 302 (M^+), 287 ($M^+ - CH_3$); TLC (1:9 MeOH/CH_2Cl_2) R_f = 0.48; HPLC retention time, 24 min on a semipreparative C_{18} column eluted with water at 3.0 mL/min.

Synthesis of 5-(Methylsulfinyl)uracil (Micetich et al., 1984). To a suspension of 5-(methylthio)uracil (10 mg, 0.06 μmol) in 1 mL of methanol was added a mixture of acetic acid (28.9 μL, 0.48 μmol) and hydrogen peroxide (14.4 μL, 0.12 μmol). The resulting mixture was maintained at room temperature for 1 week. The excess hydrogen peroxide was quenched by the addition of sodium sulfite. The suspension was then filtered and washed with methanol. The crude product was purified by flash chromatography with 10% methanol in methylene chloride: EI-MS $C_6H_6N_2SO_3$ (M^+) 174; ^{1}H NMR (DMSO-d_6) δ 2.77 (s, 3H, SCH_3), 7.55 (s, 1H, C6-H), 11.55–11.65 (br s, 2H, N1-H, N3-H).

Synthesis of 5-(Methylsulfonyl)uracil (Micetich et al., 1984). To a suspension of 5-(methylthio)uracil (10 mg, 0.06 μmol) in 1 mL of methanol was added a mixture of formic acid (95 μL, 2.4 μmol) and 30% hydrogen peroxide (72 μL, 0.72 μmol). The resulting solution was maintained at room temperature for 1 week. The suspension was then filtered and washed with methanol. The crude product was purified by flash chromatography with 10% methanol in methylene chloride: EI-MS $C_6H_6N_2SO_4$ (M^+) 190; ^{1}H NMR (DMSO-d_6) δ 3.15 (s, 3H, SCH_3), 7.98 (s, 1H, C6-H), 11.58–11.91 (br s, 2H, N1-H, N3-H).

Synthesis of Butyl Isoorotate (**2**). To a suspension of **1** (5 g, 32 mmol) in 250 mL of butanol was added 1.5 mL of H_2SO_4. The resulting solution was refluxed for 3 h, during which time the starting material slowly dissolved. The reaction was cooled and the precipitate was collected by filtration. The volume of the filtrate was reduced to 100 mL and then chilled in the refrigerator. Additional precipitate was collected and combined with the first proton to be used in the next reaction without further purification.

Synthesis of [5′,5′-$^2H_2$]-5-(Hydroxymethyl)uracil (**3**). To a suspension of lithium aluminum deuteride (2.0 g, 28.4 mmol) in 150 mL of dry THF was added **2** (3 g, 14.2 mmol in 100 mL of THF) in one portion. The resulting solution was stirred overnight at room temperature. The excess lithium aluminum deuteride was quenched carefully by adding water (5 mL). The mixture was concentrated to dryness. The gray solid was washed with hot water (~70 °C, 3 × 100 mL). The combined filtrates were evaporated to dryness under reduced pressure. The resulting residue was purified by flash chromatography with 20% methanol in methylene chloride: EI-MS $C_5H_4D_2N_2O_3$ 144; ^{1}H NMR (DMSO-d_6) δ 4.11 (s, 3.3%H, 5′-H), 7.25 (s, 1H, 6-H), 10.62–11.24 (br s, 2H, NH).

[1] Abbreviations: cyt P-450, cytochrome P-450s; DMSO, dimethyl sulfoxide; EDTA, ethylenediaminetetraacetic acid; HPLC, high-pressure liquid chromatography; MeOH, methanol; MSTFA, *N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide; PMSF, phenylmethanesulfonyl fluoride; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; TLC, thin-layer chromatography; TMS, trimethylsilyl; HMU, 5-(hydroxymethyl)uracil; PVDF, polyvinyl difluoride; EI, electron impact; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane.

Synthesis of [5′-^{2}H]-*5-Formyluracil* (**4**) (*Ressner et al., 1976*). To a suspension of **3** (300 mg, 2.1 mmol) was added 1 N aqueous ammonium cerium nitrate (2.3 g, 4.2 mmol) in one portion at room temperature. The completion of the reaction was indicated by a color change from brown to colorless (~15 min). The solution was then chilled in the refrigerator, and the precipitate was collected by filtration: EI-MS $C_5H_3DN_2O_3$ 141; ^{1}H NMR (DMSO-d_6) δ 8.15 (s, 1H, 6-H), 9.74 (s, 2.7%H, CHO), 11.53 (d, 1H, N1-H), 11.88 (s, 1H, N3-H).

Synthesis of (5′-*R*)-[5′-^{2}*H*]-*5-(Hydroxymethyl)uracil* (**6**) *and* (5′-*S*)-[5′-^{2}*H*]-*5-(Hydroxymethyl)uracil* (**5**) (*Midland et al., 1979*). To a suspension of **4** (50 mg, 0.36 mmol) in 2 mL of dry THF was added (*R*)-alpine borane (0.5 M in THF, 2.5 mL) dropwise. The resulting mixture was stirred at room temperature for 5 days. The excess alpine borane was quenched by the addition of acetaldehyde. The suspension was then filtered, and the residue was purified further by flash chromatography with 20% methanol in methylene chloride. When (*S*)-alpine borane was used as the reducing reagent, the same reaction procedure led to the formation of **6**: EI-MS $C_5H_5DN_2O_3$ 143; ^{1}H NMR (DMSO-d_6) δ 4.09 (d, 1H, 5′-H), 7.23 (d, 1H, 6-H), 10.72 (br s, 1H, N1-H), 11.05 (s, 1H, N_3-H).

General Methods

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 (Packard) fluid. HPLC was carried out on a Beckman system consisting of Model 110B pumps, a Model 421 controller, and a Model 163 variable-wavelength detector; a Hewlett-Packard integrator (Model HP3396A) was also used. Analytical (4.6 × 250 mm) and semipreparative (7 × 250 mm) Econosil C_8 and C_{18} and analytical Partisil C_{18} ODS-II HPLC columns were purchased from Alltech. UV spectra were recorded on Cary 210, Beckman DU-50, and Hewlett-Packard 8452A spectrophotometers. ^{1}H NMR spectra were obtained in DMSO-d_6 with trimethylsilane as internal standard, unless otherwise noted; Brüker 250-MHz and Varian 300-MHz NMR spectrometers were used.

UV and ^{1}H NMR spectra were obtained for each alternate substrate to confirm its identity. The purity of each was determined by HPLC analysis. If necessary, compounds were purified by semipreparative HPLC prior to use. Concentrations of substrate stock solutions were determined by UV absorbance using published extinction coefficients. Since 5,6-dihydrothymine has no UV absorbance above 240 nm, its concentration was based on the weight of the carefully dried solid used to prepare the stock solution.

Enzyme Purification

The procedure of Warn-Carmer et al. (1983) was modified extensively, as described below. Buffers were prepared at room temperature. All subsequent steps of the purification were carried out at 4 °C.

R. glutinis cultures were initiated in YM broth (pH 4.5) and then transferred into the carbon–base medium containing thymine, described by Warn-Cramer et al. (1983). Cultures for harvest were grown at 26 °C in a 60-L New Brunswick fermenter with stirring (120 rpm) and forced aeration. Cells were harvested in the late log phase using a DeLaval flow-through centrifuge and then frozen in liquid nitrogen.

R. glutinis cells (960 g) were thawed, suspended in 3 L of 50 mM Tris buffer (pH 8.0) containing 0.1 mM EDTA and saturating PMSF (buffer A), and pelleted by centrifugation (5000*g* for 20 min). The cells were resuspended in 600 mL of buffer A and ruptured by passage through a French press cell (14 000 psi). Cell debris was removed by centrifugation at 11000*g* for 45 min. The resulting supernatant was filtered through cheesecloth to give the crude extract. Protamine sulfate solution (10 mg/mL in buffer A) was added to give a final concentration of 0.23% (w/v). After stirring for 30 min, the sample was centrifuged at 15000*g* for 30 min. Solid ammonium sulfate (277 g/L) was added to the resulting supernatant to give a 45% saturated solution. After stirring for 30 min, the sample was centrifuged again at 15000*g* for 30 min. The supernatant was decanted, and additional solid ammonium sulfate (210 g/L) was added to bring it to 75% saturation. After stirring for 30 min, it was centrifuged at 15000*g* for 45 min.

The protein pellet was redissolved in 50 mL of buffer A. A small amount of insoluble denatured protein was removed by centrifugation at 12000*g* for 20 min. The supernatant was desalted on a Sephadex G-25 column (5.0 × 54 cm, 1060 mL) equilibrated with 20 mM potassium phosphate buffer (pH 7.5) containing 0.1 M glycine and 0.1 mM EDTA (buffer B). The protein sample (340 mL) was then loaded onto a Whatman DE-52 column (4.5 × 18 cm, 290 mL) equilibrated with buffer B. The column was eluted at a flow rate of 3.5 mL/min with 650 mL of this buffer, followed by a 2600-mL linear gradient of KCl (0–0.17 M) in buffer B. Fractions (~25 mL each) containing thymine hydroxylase activity (nos. 120–153) were pooled and concentrated in an Amicon pressure cell equipped with a PM-30 membrane. A portion of the sample from the DE-52 column (11 mL, 39 mg/mL) was centrifuged at 12000*g* for 20 min to remove any precipitated protein and was then loaded onto a Sephadex G-100 Superfine column (1.5 × 73 cm, 129 mL) equilibrated with buffer B. The column was eluted with same buffer at a flow rate of 1.7 mL/h; 1-mL fractions were collected. Fractions containing thymine hydroxylase activity (nos. 54–68) were pooled, concentrated to ~500 μL in an Amicon pressure cell equipped with a PM-30 membrane, and stored in aliquots at –20 °C.

The final purification step was performed on a Pharmacia FPLC system equipped with a MonoQ anion-exchange column. Because the enzyme obtained from this column was not stable to storage, it was prepared in small batches and used immediately. The MonoQ column (0.5 × 5 cm) was equilibrated at a flow rate of 1 mL/min using the following protocol: buffer B for 10 min; 350 mM KCl in buffer B for 15 min; and 15% buffer B for 35 min. An aliquot of thymine hydroxylase from the G-100 column, containing ≤4 mg of protein, was diluted to 400 μL with buffer B and injected onto the MonoQ column. It was eluted as follows (flow rate of 1 mL/min): 15% buffer B for 5 min; a linear gradient of 15–30% B over 15 min; 30% B for 15 min; and a linear gradient of 30–100% B over 15 min. Fractions (1 mL each) containing thymine hydroxylase (nos. 16–20) were pooled and concentrated to <50 μL as quickly as possible in a Centricon-30.

N-Terminal Sequence Analysis

Thymine hydroxylase (18 μg) isolated from the MonoQ column was subjected to SDS–PAGE in a 0.75 mm thick 10% gel using the buffer system of Laemmli (1970). Proteins were electroeluted from the gel onto an Immobilon-P PVDF membrane by the procedure of Matsudaira (1987). The membrane was then soaked in water for 5 min, stained with

0.06% Coomassie R-250 in 50% aqueous MeOH for 10 min, and destained with 50% aqueous MeOH for 10 min. The portion of membrane containing the major protein band (39 kDa) was cut out, washed with 10 1.5-mL aliquots of water, and air-dried. This sample was submitted to the Harvard Microchemistry Facility for sequencing by automated Edman degradation.

Enzyme Activity Assays

The standard assay mixture contained 10–25 μL of enzyme (typically 0.2–2 munits) in buffer B, 0.90 mM thymine, 0.45 mM α-ketoglutarate, 2.3 mM sodium ascorbate, 11 μM ferrous sulfate, and 50 mM HEPES (pH 7.5) in a total volume of 220 μL. Assays were carried out at 30 °C. Typical reaction times ranged from 1 to 5 min. One unit of thymine hydroxylase activity is defined as the amount of enzyme that catalyzes the formation of 1 μmol/min of product under these conditions.

$^{14}CO_2$ Assay. The assay mixture contained [1-^{14}C]-α-ketoglutarate (typically 0.05 μCi/μmol), in addition to the standard components given above. Each reaction was carried out in a glass vial sealed with a serum stopper, from which was suspended a cup containing a paper wick soaked with 20 μL of a 1 M solution of hyamin hydroxide in methanol. The reactions were initiated by the addition of thymine and quenched after the desired time with 100 μL of 20% (w/w) aqueous trichloroacetic acid. After incubation at 37°C for 1 h, each cup and wick were transferred into 4 mL of Scint-A for liquid scintillation counting. Control reactions lacking thymine were run in parallel, and this background value (typically 150–300 cpm) was subtracted from each data point.

HPLC Assay. These assays were carried out in test tubes (7 × 85 mm) open to the air. Reactions were initiated by the addition of either thymine or enzyme. After the desired reaction time, each sample was quenched with 40 μL of 10% (w/w) perchloric acid. To prevent reactivation of the enzyme upon neutralization, samples were treated with 50 μL of $CHCl_3$, vortexed, and centrifuged. Each sample was then neutralized with 10 μL of 6 N KOH in 1.3 M Tris and centrifuged to pellet the $KClO_4$. A 100-μL aliquot of the upper layer from each sample was injected onto an analytical C_{18} HPLC column and eluted with water at a rate of 1.5 mL/min; detection was by the absorbance at 260 nm. The retention times of HMU and thymine in this system are 4.6 and 11.6 min, respectively. The amount of HMU present in each sample was determined by comparing its peak height with a standard curve that had been prepared by chromatographing known amounts of authentic compound under the same conditions. Alternatively, the integrated area of the HMU peak was used for quantification instead of peak height. In some experiments, the enzyme reaction was quenched with 40 μL of 0.12 M periodic acid; aliquots were injected onto the HPLC column without further workup. For other applications, the HPLC assay was modified by including [2-^{14}C]-thymine (0.7 μCi/μmol) in the reaction mixture. Samples were quenched, neutralized, and chromatographed as described above. Fractions (1.5 mL) were collected from the HPLC column and analyzed by liquid scintillation counting in 12 mL of Scint-A. Control reactions lacking thymine hydroxylase were run in parallel; backgrounds of ~400 cpm were typical.

Modified Assays with Alternate Substrates. For most experiments, the standard assay conditions were used, except that thymine was replaced with one of the alternate pyrimidine substrates (0.9 mM final concentration) and the amount of thymine hydroxylase was adjusted to give reasonable rates. Reactions with 1-(methylthio)uracil contained 0.2% DMSO due to the low solubility of this uracil derivative in aqueous buffer. The $^{14}CO_2$ assay procedure was used without modification. The HPLC assay required modification for use with some of the alternate substrates. 5-Formyluracil assays were analyzed on an HPLC column eluted with ion-pairing buffer (5 mM tetrabutylammonium bromide in 40 mM sodium phosphate, pH 6.0). 5-(2-Hydroxyethyl)uracil and 5-vinyluracil assays were analyzed on an ODS-II C_{18} column eluted with water. 5,6-Dihydrothymine assays were analyzed on the standard C_{18} HPLC column with detection at 220 nm; the product was quantified by peak area relative to that of the initial substrate. Neither the $HClO_4/CHCl_3$/KOH–Tris nor the periodic acid quench method was satisfactory for assays with 5,6-dihydrothymine or 5-(methylthio)uracil; thus, samples were injected directly onto the HPLC column without quenching. For each alternate substrate, control reactions lacking either enzyme, O_2, or α-ketoglutarate were analyzed for pyrimidine product formation by HPLC.

Isolation of Products and GC–MS Analysis

Thymine hydroxylase reactions with each alternate substrate were carried out on large scale (440 or 1100 μL). The amount of enzyme used and the reaction times were chosen such that 50–100% of the substrate was consumed. The products were isolated from the reaction mixtures by HPLC on a semi-preparative C_{18} or C_8 column eluted with water at 3.0 mL/min. A sample of each isolated product (20–40 nmol) was placed in a silanized Wheaton vial, dried by repeated coevaporation with CH_3CN under a stream of argon, and then placed in a desiccator over P_2O_5 *in vacuo* for 6 h. Dry CH_3CN and MSTFA (10 μL each) were added to each sample. The vials were sealed with Teflon-lined septa and heated at 95 °C for 30–60 min. Aliquots (2 μL) were injected onto a Hewlett-Packard gas chromatograph–mass spectrometer (HP5989) operating in the EI mode. A 15-m fused silica capillary column coated with SPB-1 (DB-1 from J & W Scientific) was used. The injector temperature was 220 °C. The oven temperature was held at 70 °C for 30 s and then raised at 20 °C/min.

^{18}O-Labeling Studies

Reaction conditions were identical to those described above, except that the samples were degassed by four freeze-evacuate/thaw cycles and equilibrated with an atmosphere of $^{18}O_2$ prior to the addition of enzyme. Enzyme (0.2–2.5 units, depending on the substrate) was then added to start the reaction. After 5 min, the reaction mixtures were frozen in liquid nitrogen, evacuated, and filled with argon. Each sample was thawed, and the product was isolated by HPLC. Samples (20–40 nmol) of each were derivatized with MSTFA/CH_3CN and analyzed by GC–MS, as described above.

Kinetic Studies

Kinetic constants were determined for thymine, α-ketoglutarate, O_2, and Fe^{2+} by varying one substrate concentration while holding all others constant at the standard values. The HPLC assay ($HClO_4/CHCl_3$/KOH quench) was used with reaction times of 5 min. The assay was shown to be linear over this time range at the highest and lowest concentrations of each substrate. Data for each were fit to eq 2 by nonlinear least-squares analysis (Cleland, 1967). α-Ketoglutarate

$$v = VA/(K_m + A) \quad (2)$$

concentrations were determined by a glutamate dehydrogenase

end-point assay (Von Korff, 1969). The concentration of $FeSO_4$ was determined by the ferrozine assay of Stookey (1970). The oxygen concentration was varied by degassing assay mixtures on a vacuum manifold, adding the desired partial pressure of air or a nitrogen/air (4:1) gas mixture, and then adding argon to give a total pressure of 1 atm. The final concentration of dissolved oxygen was calculated using a value of 235 μM for air-saturated water at 30 °C and 760 mmHg (Hitchman, 1978).

Kinetic constants were likewise determined for each alternate substrate. Technical reasons required the use of the $^{14}CO_2$ assay with HMU, 5,6-dihydrothymine, and 5-fluorouracil; the HPLC assay and periodic acid quench were used with all other alternate substrates. Reaction times of 1 min were used in all cases. In each experiment, the activity of thymine hydroxylase was determined with thymine as substrate. Velocities were expressed as a fraction of this enzyme activity to facilitate comparison between experiments.

5-Acetyluracil, HMU, and 5-formyluracil were tested as inhibitors with respect to thymine. Initial velocities were measured at three concentrations of inhibitor with thymine varying from 30 to 300 μM. The HPLC assay with periodic acid quench and 1-min time points were used in all cases. With HMU and 5-formyluracil, the reactions contained [^{14}C]-thymine (800 cpm/nmol), and product formation was quantified by liquid scintillation counting. Data were fit by nonlinear least-squares analysis to the equations for competitive, noncompetitive, and uncompetitive inhibition; the criterion of lowest variance was used to determine which type of inhibition best fit the data (Cleland, 1979).

Isotope Effects. The direct comparison method was used to determine $^D(V/K)$ and ^DV isotope effects (Cleland, 1982; Northrop, 1977). Initial velocities were measured at 15 and 410 μM thymine and (trideuteriomethyl)thymine, with all other assay components held constant at standard concentrations. The HPLC assay, with periodic acid quench and 1-min reaction times, was used. The experiment was repeated in the presence of 34 μM dissolved O_2. In this case, samples were degassed by four freeze-evacuate/thaw cycles and then equilibrated with 1 atm of a 6:1 nitrogen/air mixture before the reaction was initiated.

Isotope Effects for Thymine Hydroxylase Catalyzed Oxidation of **5** *or* **6** *to 5-Formyluracil.* The methods are identical to those described above for (trideuteriomethyl)-thymine. In order to quantify the amount of product produced, a standard curve was generated using authentic 5-formyluracil and an ODS-II HPLC column eluted with water (flow rate, 1 mL/min; monitoring wavelength, 300 nm). The retention time of 5-formyluracil is 3.0 min under these conditions.

Stereochemistry

*Characterization of the 5-Formyuracil Produced when Thymine Hydroxylase Is Incubated with [5′-^{2}H]HMU (***5** *or* **6**). The reaction mixture contained, in a final volume of 6 mL, 0.9 mM **5** or **6**, thymine hydroxylase (1.0 unit), 50 mM HEPES (pH 7.5), 2.5 mM ascorbate, 0.45 mM ferrous sulfate, and 0.9 mM α-ketoglutarate. The reaction mixture was incubated at room temperature for 1 h and lyophilized, and the residue was chromatographed on an ODS-II HPLC column using 20 mM potassium phosphate buffer as eluate (pH 6.5; flow rate, 1 mL/min; monitoring wavelength, 300 nm). The fractions with retention times of 6.8 min were pooled and concentrated. The crude orange product was repurified using an Econosil C_8 column under identical conditions. The desired product, 5-formyluracil, eluted at 4.2 min. The appropriate fractions were pooled and lyophilized, and the 5-formyluracil was desalated using the same C_8 Econosil column washed for 10 min with water. The aldehyde was then eluted with 100% methanol. NMR spectra of the products from **5** to **6** were recorded. [^{1}H NMR (formyluracyl from **5**, ^2H_2O) δ 8.15 (s, 1H, 6-H), 9.48 (s, 1H, CHO); ^{1}H NMR (formyluracil from **6**, ^2H_2O) δ 8.15 (s, 1H, 6-H), 9.48 (s, 6%H, CHO)].

RESULTS AND DISCUSSION

Enzyme Purification. The protocol for purification of thymine hydroxylase is a modification of that reported by Warn-Cramer et al. (1983). They were able to prepare homogeneous protein by a 7-step procedure. Unfortunately, 85% of their enzyme activity was lost on the final preparative gel electrophoresis step. We have found that replacing this step with anion-exchange MonoQ chromatography using FPLC gives significantly better recovery of activity. In addition, we have reversed the order of the gel filtration and DEAE–cellulose columns and greatly decreased the size of both columns relative to the amount of protein loaded. From 1 kg of *R. glutinis* cells, we obtained 34 mg of protein with a specific activity of 27 μmol min^{-1} mg^{-1}. The protein was judged to be approximately 85% homogeneous on the basis of SDS gel electrophoresis. The procedure of Warn-Cramer et al. (1983) gave 6.3 mg of homogeneous protein per kilogram of cells (specific activity, 16 μmol min^{-1} mg^{-1}). This value, however, cannot be compared directly with the specific activity of our protein, because assay conditions different from those originally reported were required to overcome problems encountered, as discussed below.

N-Terminal Sequence Analysis. In preparation for cloning the hydroxylase gene, the N-terminal sequence of the protein was determined. Thymine hydroxylase obtained from the MonoQ column was separated from a contaminating 37-kDa protein by SDS–PAGE. The proteins were electroblotted onto a PDVF membrane by the procedure of Matsudairi (1987), and the 39-kDa protein was submitted for sequence analysis. The following two sequences were present in roughly equivalent amounts, where [L] is a probable assignment and (L) is a possible assignment:

(1) SSGIVPPINFEPFLSGTPEDK[L]ATA

(2) VSSGIVPPINFEPFLSGTPEDK[L]ATA

Evidence presented in the following article (Thornburg & Stubbe, 1993) confirms that this protein is in fact thymine hydroxylase.

Enzyme Assays. The conditions used by Warn-Cramer et al. (1983) to assay thymine hydroxylase activity caused a number of problems for us. First, the enzyme reaction was not linear with time. This problem was solved by decreasing the α-ketoglutarate concentration to 0.45 mM. At high concentrations, severe substrate inhibition is observed. Second, the phosphate buffer used by previous investigators formed an insoluble salt with the Fe^{2+}, resulting in low concentrations of free Fe^{2+}. HEPES buffer therefore was used instead. The ferrous sulfate concentration could then be reduced from 1 mM to 11 μM without significantly affecting the rate of reaction. Under these conditions, the presence of catalase (0.3 mg/mL, present in the original assay) inhibited the enzyme reaction by 60–70% and was therefore eliminated. With 0.5–2.5 m units of thymine hydroxylase, the new assay conditions resulted in linear product formation for 10 min and rates that were proportional to the enzyme concentration.

Table I: Kinetic Constants for Thymine Hydroxylase Reactions

variable substrate	K_m (μM)	V/K (μmol/min/mM/unit of enzyme)	V_{max} (μmol/min/units of enzyme)
Fe^{2+}	1.1 ± 0.1		1.13 ± 0.04
α-ketoglutarate	24 ± 2	51 ± 2	1.23 ± 0.04
O_2	36 ± 3	33 ± 2	1.17 ± 0.04
thymine	58 ± 7	21 ± 2	1.19 ± 0.06
5-(hydroxymethyl)uracil	118 ± 21	6.1 ± 0.8	0.72 ± 0.04
5-formyluracil	2340 ± 130	0.210 ± 0.005	0.49 ± 0.02
5-(2-hydroxyethyl)uracil	124 ± 13	2.6 ± 0.2	0.33 ± 0.02
1-methylthymine	1300 ± 65	0.175 ± 0.004	0.23 ± 0.03
5,6-dihydrothymine	87 ± 9.6	0.74 ± 0.06	0.064 ± 0.003
5-fluorouracil	322 ± 34	0.128 ± 0.009	0.041 ± 0.002

Kinetics of Thymine and (Trideuteriomethyl)thymine Oxidation. A detailed steady-state kinetic analysis of thymine oxidation has previously been carried out by Holme (1975) on the *N. crassa* enzyme. The K_m values reported for thymine and α-ketoglutarate were both 120 μM and that for O_2 was 60 μM; the effect of varying the Fe^{2+} concentration was not investigated. These and additional studies were interpreted to indicate a sequential ordered mechanism in which α-ketoglutarate binds first, followed by thymine and then O_2. Holme (1982) also showed with [*methyl*-^{3}H]thymine that there is an intramolecular isotope effect of 6.5 on thymine hydroxylation and no $^T(V/K)$.

As a first step in our investigation of thymine hydroxylase from *R. glutinis*, we have undertaken studies similar to those of Holme. The apparent kinetic constants for Fe^{2+} and the three substrates are presented in Table I. The V_{max} values indicate that the substrate concentrations in the standard assay are at near-saturating levels. From the specific activity of 27 μmol min^{-1} mg^{-1}, we estimate that the turnover number for the *R. glutinis* thymine hydroxylase is greater than 1000 min^{-1}. A more detailed kinetic analysis of our enzyme to determine the order of substrate binding has not been possible due to the insensitivity of the assay method and the nonlinearity of the progress curves with subsaturating substrate concentrations.

The isotope effects on the *R. glutinis* enzyme reaction with (trideuteriomethyl)thymine have been determined. They were initially measured under conditions where the dissolved O_2 concentration was 235 μM. The results are shown in Figure 1A and give values of 2.08 ± 0.12 for ^DV and of 1.11 ± 0.19 for $^D(V/K)$. ^DV reveals that carbon hydrogen-bond cleavage is at least partially rate-determining. The absence of an isotope effect on V/K demonstrates that an irreversible step (or steps) occurs prior to this bond-breaking process. If O_2 binds to the enzyme only after thymine, as reported for the *N. crassa* enzyme, then that step would be essentially irreversible under conditions in which O_2 is saturating. Therefore, in an effort to observe a $^D(V/K)$, the isotope effects were also determined under conditions where the dissolved O_2 concentration was ~36 μM. These results, shown in Figure 1B, reveal a ^DV of 1.22 ± 0.08 and a $^D(V/K)$ of 1.08 ± 0.33. The observation that ^DV is reduced demonstrates that O_2 binding is now partially rate-limiting. Since $^D(V/K)$ remains unchanged, we can conclude that some step other than O_2 binding is irreversible. These results are consistent with a mechanism (Siegal, 1970; Hanauske-Abel & Gunzler, 1982) in which decarboxylation of α-ketoglutarate and oxygen–oxygen bond cleavage of O_2 precede cleavage of the carbon–hydrogen bond of thymine (Scheme I). Both of these steps would be expected to be irreversible.

The observation of a $^T(V/K) > 1.0$ for any of the α-ketoglutarate dioxygenases would not be expected, given this proposed mechanism. However, Blanchard and Englard

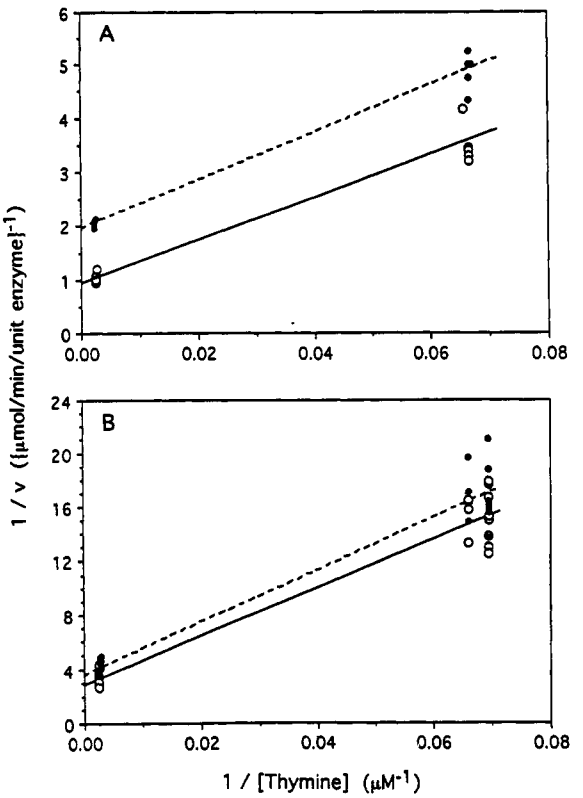
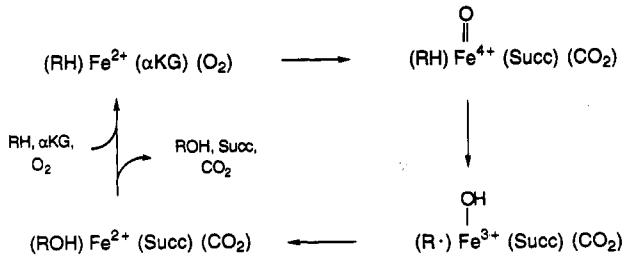


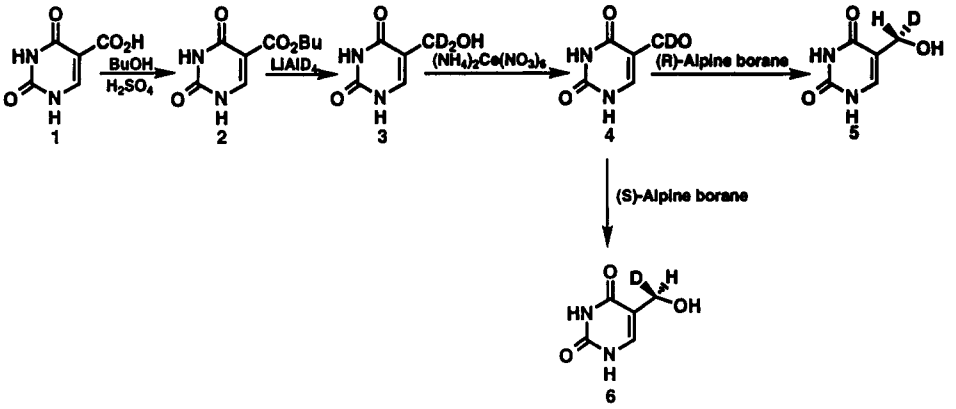
FIGURE 1: Isotope effect data for reactions carried out under an atmosphere of either (A) air or (B) 2.7% O_2: (–O–) thymine; (- -●- -) (trideuteriomethyl)thymine.

Scheme I



in 1983 reported a $^T(V/K)$ of ~15 for the reaction catalyzed by γ-butyrobetaine hydroxylase from calf liver. A reasonable explanation of this observed isotope effect was provided by the studies of Holme et al. (1984). Their studies demonstrated, with γ-butyrobetaine hydroxylase from human kidney, a $^D(V/K)$ of ~6 when production of the product carnitine was measured, but a $^D(V/K)$ of ~1 when production of the product CO_2 was measured. In addition, the ratio of CO_2 to carnitine increased from 1.15 when unlabled substrate was used in the assay to 7.5 in the presence of deuterated γ-butyrobetaine.

Scheme II



An uncoupling of the hydroxylation step from the decarboxylation step is apparent. For this enzyme, and presumably for the calf liver enzyme as well, the isotope effect on C–H bond cleavage results, therefore, from an artifact of the uncoupling reaction, rather than from the reversibility of the normal reaction sequence up to and including the cleavage of the C–H bond. A second example of uncoupling is provided by studies on clavaminate synthase. This enzyme catalyzes two successive α-ketoglutarate-dependent oxidations and, in the presence of a deuterium-labeled substrate, exhibits not only an increase in the uncoupled decarboxylation reaction but also formation of an alternate product (Salowe et al., 1991). These observations provide compelling evidence that α-ketoglutarate decarboxylation occurs prior to C–H bond cleavage in the mechanism of these enzymes.

To determine whether isotope-induced partitioning also occurs in the thymine hydroxylase reaction, the relative amounts of CO_2 and 5-(hydroxymethyl)uracil produced in the reaction with (trideuteriomethyl)thymine were measured. No significant difference was observed (data not shown), demonstrating that only the coupled reaction occurs with this substrate. We therefore can conclude that the ^{D}V obtained with (trideuteriomethyl)thymine is a direct reflection of the extent to which C–H bond cleavage is rate-limiting and not due to increased partitioning through the uncoupled reaction.

Kinetics and Stereochemistry of HMU Oxidation. Thymine hydroxylase from *N. crassa* has been shown to convert HMU into 5-formyluracil with concomitant formation of CO_2 and succinate (McCroskey et al., 1971; Liu et al., 1973). Holme (1975) demonstrated, with the *N. crassa* enzyme, that HMU is a competitive inhibitor with respect to thymine, suggesting that both reactions occur at the same active site. The enzyme from *R. glutinis* catalyzes this reaction as well (Warn-Cramer et al., 1983). We have examined the kinetics of the reaction with HMU; the results are summarized in Table I. HMU was also found to be a competitive inhibitor with respect to thymine; the observed K_i (156 ± 36 μM) is approximately equal to the K_m for this substrate (Table I). This result supports the proposal that both thymine and HMU oxidations are catalyzed by the same active site of thymine hydroxylase from *R. glutinis*.

We next addressed the questions of whether thymine hydroxylase could distinguish between the prochiral hydrogens of HMU and whether there was an isotope effect on this oxidation reaction. The dideuterio and *pro-R* and *pro-S* deuterated HMUs were synthesized (Scheme II); Experimental Procedures). Each substrate was enzymatically converted to product, which was subsequently isolated by HPLC and examined by NMR spectroscopy. The results shown in Figure 2 reveal that the *pro-S* hydrogen is uniquely

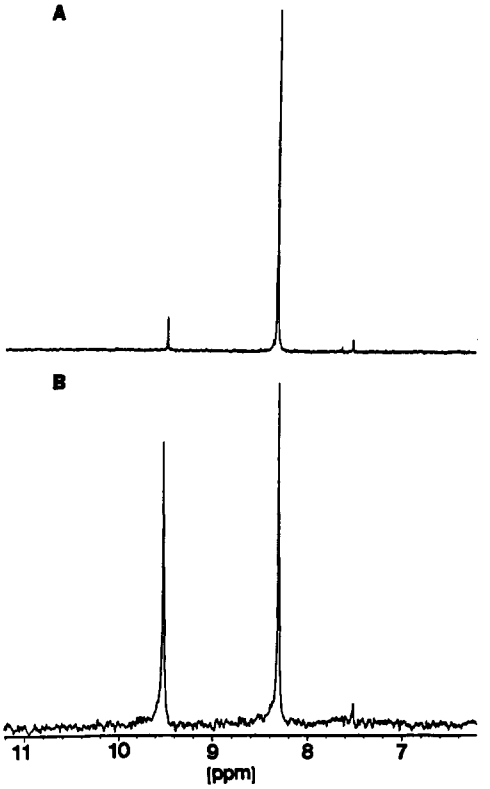


FIGURE 2: ^{1}H NMR spectra of the product produced on incubation of (5′-*R*)-[5′-^{2}H]-5-(hydroxymethyl)uracil (A) or (5′-*S*)-[5′-^{2}H]-5-(hydroxymethyl)uracil (B) with thymine hydroxylase.

removed during the enzyme-catalyzed oxidation. The small amount of protonated formyluracil detected in Figure 2A results from incomplete deuteriation of **4** (Scheme II) and stereospecific reduction of **4** to **5** with only ~95% enantiomeric excess. The isotope effects under V/K and V conditions were also determined. Within experimental error, both were found to be 1.0. Therefore, carbon–hydrogen bond cleavage is not rate-determining in this oxidation reaction.

Kinetics of 5-Formyluracil Oxidation. Thymine hydroxylase from *N. crassa* was shown to catalyze the oxidation of 5-formyluracil to 5-carboxyuracil (Holme et al., 1971; Liu et al., 1973). A major unresolved question is whether the aldehyde or the hydrate of the aldehyde is the actual substrate for the enzyme. Previous genetic and biochemical studies in *N. crassa* suggested that there is an NAD-dependent reaction capable of catalyzing the same oxidation and that this reaction is the physiologically significant one (Williams & Mitchell, 1969; Shaffer et al., 1975). Studies of Warn-Cramer et al. (1983) with the *R. glutinis* enzyme demonstrated that

Table II: GC–MS Data for Derivatized Products of Thymine Hydroxylase Reactions

alternate substrate	atmosphere	selected ions (m/z)	no. of TMSs	% ^{18}O
5-vinyluracil	air	445 ($M^+ - CH_3$), 357 ($M^+ - CH_2OTMS$)	4	
	$^{18}O_2$	447 ($M^+ - CH_3$), 357 ($M^+ - CH_2OTMS$)	4	97
5-(2-hydroxyethyl)uracil	$^{18}O_2$	447 ($M^+ - CH_3$), 359 ($M^+ - CH_2OTMS$)	4	88
1-methylthymine	air	300 (M^+), 285 ($M^+ - CH_3$)	2	
	$^{18}O_2$	302 (M^+), 287 ($M^+ - CH_3$)	2	95
5-(methylthio)uracil	air	390 (M^+), 375 ($M^+ - CH_3$)	3	
	$^{18}O_2$	392 (M^+), 377 ($M^+ - CH_3$)	3	97
5,6-dihydrothymine	air	358 (M^+), 343 ($M^+ - CH_3$)	3	
	$^{18}O_2$	360 (M^+), 345 ($M^+ - CH_3$)	3	95

α-ketoglutarate was decarboxylated in the presence of 5-formyluracil. However, the pyrimidine product was not identified. Using our HPLC protocol with an ion-pairing buffer, we have shown that 5-formyluracil is converted to a new product, which comigrates with authentic 5-carboxyuracil. The kinetic parameters for this reaction are summarized in Table I. The V/K value demonstrates that the enzyme specificity for 5-formylurcail is 1% that for thymine. 5-Formyluracil was also shown to be a competitive inhibitor with respect to thymine, with a K_i value of 1390 ± 380 μM. The large K_m and K_i values suggest that the hydrate, rather than the aldehyde, may be the actual substrate for the enzyme. The fact that 5-acetyluracil is a relatively poor competitive inhibitor against thymine ($K_i = 830 \pm 90$ μM) supports this hypothesis. In either case, the very large K_m for 5-formyluracil suggests that the thymine hydroxylase reaction with this substrate may not be physiologically significant in *R. glutinis*.

Uncoupling of the Decarboxylation and Hydroxylation Reactions. An interesting side reaction that has been detected with many oxygenases is an uncoupling reaction: the reduction of O_2 without the concomitant hydroxylation of substrate. For example, *p*-hydroxybenzoate hydroxylase, a flavin-dependent enzyme, catalyzes H_2O_2 formation in the presence of a dihydroxylated benzoate, a substrate analog incapable of undergoing a hydroxylation reaction (Walsh, 1979). cyt P-450 catalyzes the uncoupled oxidation of NADH with formation of H_2O_2, even in the presence of some substrates (Ortiz de Montellano, 1986; Harada et al., 1984). These side reactions have proven to be mechanistically informative. As discussed above, α-ketoglutarate dioxygenases also catalyze an uncoupled decarboxylation of α-ketoglutarate to CO_2 and succinate. Prolyl 4-hydroxylase and lysyl hydroxylase catalyze this reaction in the absence of their appropriate substrates at 1–4% the rate of the complete reaction; nonhydroxylatable substrate analogs increase these rates about 2-fold (Tuderman et al., 1977; Puistola et al., 1980; Myllyla et al., 1984). γ-Butyrobetaine hydroxylase catalyzes this uncoupled reaction in the presence of substrate analogs, deuterated substrate, and, under some conditions, with the normal substrate as well (Holme et al., 1982, 1984; Wehbie et al., 1988). Thymine hydroxylase from *N. crassa* catalyzes the uncoupled decarboxylation of α-ketoglutarate only in the presence of non-hydroxylatable substrate analogs, such as uracil or 5-fluorouracil, at a rate of 1–6% of the normal hydroxylation reaction with thymine (Hsu et al., 1981; Holme & Lindstedt, 1982).

We have found that thymine hydroxylase from *R. glutinis* likewise catalyzes the decarboxylation of α-ketoglutarate in the presence of 5-fluorouracil. The V_{max} for this reaction (Table I) is 3% of that for the normal reaction. No uncoupled reaction is observed in the absence of 5-fluorouracil under conditions where the lower limit of detection was 0.02% of the rate in the standard assay. This observation suggests that thymine binds to the enzyme prior to oxygen, as is the case for the *N. crassa* enzyme (Holme, 1975). Alternatively, a conformational change may take place, once all substrates are bound, which moves the α-ketoglutarate to within attacking distance of the oxygen. In either case, it is advantageous for the enzyme to avoid nonproductive consumption of α-ketoglutarate and generation of a (postulated) high-energy iron–oxo intermediate. Thymine hydroxylase clearly is able to control this unwanted reaction better than other α-ketoglutarate dioxygenases.

Examination of the Repertoire of Thymine Hydroxylase Catalyzed Oxidations

As outlined in the introduction, heme enzymes such as cyt P-450 (Guengerich & MacDonald, 1984) and non-heme iron dependent enzymes, such as methane monooxygenase (Green & Dalton, 1989) and ω-hydroxylase (Katopodis et al., 1984, 1988), catalyze a wide variety of reactions in addition to the hydroxylation of carbon–hydrogen bonds. These reaction types include allylic hydroxylations, epoxidation of olefins, oxidation of sulfides to sulfones and sulfoxides, and oxidation of substituted methylamines to substituted amines and formaldehyde. In general, these additional oxidation reactions proceed by mechanisms unique to each reaction type. Thus, an examination of the catalytic capabilities of the *R. glutinus* thymine hydroxylase seemed likely to provide insight into the non-heme iron chemistry utilized by the α-ketoglutarate dioxygenases.

5-Vinyluracil Oxidation. cyt P-450 (Guengerich & MacDonald, 1984), methane monooxygenase (Fox et al., 1990; Green & Dalton, 1989), and ω-hydroxylase (Katopodis et al., 1984) are known to catalyze the epoxidation of olefins. The α-ketoglutarate dioxygenase hyposcyamine hydroxylase also catalyzes an epoxidation reaction (Hashimoto & Yamada, 1987). To determine whether thymine hydroxylase catalyzes such a reaction, 5-vinyluracil was tested as an alternate substrate. As previously reported (Thornburg & Stubbe, 1989), both 5-vinyluracil and 5-(2-hydroxyethyl)uracil are converted to 5-(1,2-dihydroxyethyl)uracil by thymine hydroxylase. For each substrate, the stoichiometric production of CO_2 and 5-(1,2-dihydroxyethyl)uracil is observed, and one atom of oxygen from $^{18}O_2$ is incorporated into the product. With 5-vinyluracil, however, the label is located exclusively in the terminal hydroxyl group of the diol (Table II). Efforts to isolate the putative epoxide precursor to the dihydroxylated product have thus far failed. This failure is not suprising given the studies of Sakai and Santi (1973), which demonstrated that 5-(trifluoromethyl)uracil undergoes rapid hydrolysis in aqueous solution to produce 5-carboxyuracil. They propose a mechanism involving N-1-assisted elimination of fluoride ions, in each case followed by Michael addition of H_2O. These observations provide a reasonable explanation for our inability to isolate the epoxide as well as an explanation for the product produced. In our case, N-1-assisted ring opening of the epoxide, facilitated by the iron in the active site, would be followed by Michael addition of H_2O. It is

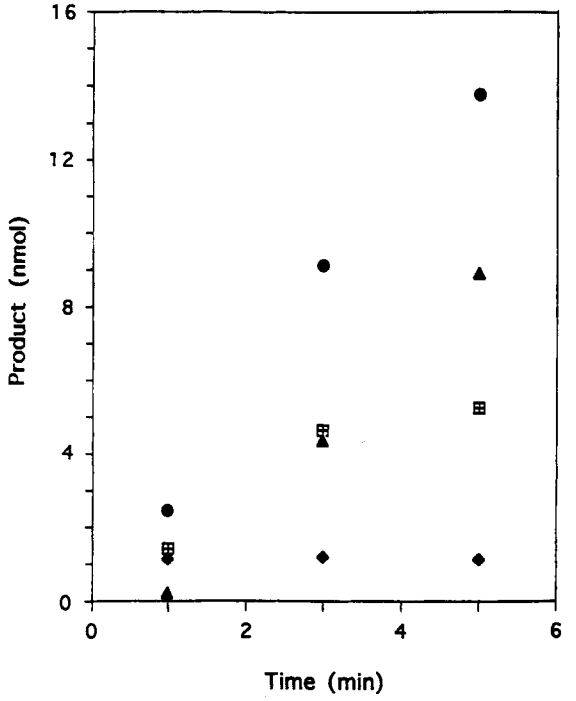


FIGURE 3: Time-dependent formation of products from 1-methylthymine: (●) 1-methyl-5-hydroxymethyluracil; (◆) thymine; (⊞) 5-(hydroxymethyl)uracil; (▲) 5-formyluracil.

interesting to note that N-1 methylation of 5-(trifluoromethyl)-uracil increased the stability of this molecule to fluoride elimination by approximately 1000-fold. Methylation of vinyluracil could facilitate isolation of the putative epoxide. Whether the epoxide is an actual intermediate in vinyluracil hydroxylation or whether some other type of intermediate is involved, perhaps a metallooxetane, remains to be established.

N-Demethylation of 1-Methylthymine. cyt P-450 is able to catalyze the oxidation of the methyl group of CH_3NR_2 to formaldehyde and an amine through an amine cation radical intermediate, followed by proton loss and hydration (Guengerich & MacDonald, 1984). Thymine hydroxylase from *N. crassa* has been shown to convert 1-methyluracil to uracil and to catalyze the decarboxylation of α-ketoglutarate in the presence of 1-methylthymine (Bankel et al., 1977). We have thus tested 1-methylthymine with *R. glutinis* hydroxylase. The HPLC assay demonstrated the formation of four separate products. No products were observed if enzyme, α-ketoglutarate, or O_2 was omitted from the assay mixture. Three of the products were readily identified as HMU (retention time, 5 min), 5-formyluracil (6.5 min), and thymine (12 min) by comigration with authentic compounds. The fourth product was postulated to be 1-methyl-5-(hydroxymethyl)uracil (**7**) (10 min). The observed distribution of products as a function of time (Figure 3) suggests that initially **7** and thymine are generated and that HMU and 5-formyluracil arise from subsequent enzyme reactions with thymine.

Compound **7** was isolated by HPLC from reactions with either $^{16}O_2$ or $^{18}O_2$ as cosubstrate. The UV spectrum of **7** has a λ_{max} of 270 ± 2 nm, as does that of 1-methylthymine. The GC–MS data on **7** are consistent with the proposed structure and indicate that one atom of ^{18}O has been incorporated (Table II). Thymine hydroxylase can thus bind 1-methylthymine productively in two orientations. Reaction at the C-5 methyl group gives **7**, while reaction at the N-1 methyl group gives 1-(hydroxymethyl)thymine. The latter is expected to be unstable and to decompose into formaldehyde and thymine. Subsequent turnovers of thymine by thymine hydroxylase would generate HMU and 5-formyluracil.

Kinetic data have been obtained for 1-methylthymine; the measured velocities include products from the hydroxylation of both methyl groups (Table I). The rate of reaction at N-1 relative to that at C-5 was 1.1 ± 0.1, demonstrating that there is essentially no discrimination between the two positions by the enzyme. The reported V_{max} represents a lower limit due to the fact that two effective competitive inhibitors, thymine and HMU, are being generated over the course of the enzyme reaction. The presence of these inhibitors does not affect V/K, since at very low substrate concentration the amount of inhibitors produced is also very low. The apparent V/K of 0.175 ± 0.004 mM^{-1} demonstrates a 100-fold decrease in enzyme specificity for 1-methylthymine relative to thymine. This suggests that the N-1 hydrogen of thymine may be involved in the normal binding of substrates.

5-(Methylthio)uracil Oxidation. cyt P-450 catalyzes the oxidation of sulfides to both sulfoxide and sulfones (Guengerich & MacDonald, 1984). The non-heme iron dependent ω-hydroxylase also converts sulfides to sulfoxides (Katopodis et al., 1988). (4-Hydroxyphenyl)pyruvate dioxygenase, a variant of the α-ketoglutarate dioxygenases for which the α-keto acid is a side chain of the substrate, has been shown to catalyze a similar reaction (Pascal et al., 1985). To determine whether thymine hydroxylase is capable of oxidizing sulfides, 5-(methylthio)uracil was tested as an alternate substrate. The $^{14}CO_2$ assay established that α-ketoglutarate was decarboxylated in the presence of this compound. The HPLC assay showed the formation of two products with retention times of 5.8 and 8.6 min. No products were observed if α-ketoglutarate, O_2, or enzyme was omitted from the reaction mixture. Samples of 5-(methylsulfonyl)uracil and 5-(methylsulfinyl)uracil prepared synthetically were found to have retention times of 5.8 and 8.6 min, respectively. The enzymatic products were isolated by HPLC, and their UV spectra were shown to have λ_{max}'s at 284 nm. Their EI mass spectra were identical to those of authentic sulfone and sulfoxide. Independent experiments have shown that thymine hydroxylase can convert the sulfoxide into the sulfone. To determine the source of oxygen in the product 5-(methylsulfinyl)uracil, reactions were run in the presence of $^{16}O_2$ and $^{18}O_2$. GC–MS analysis demonstrated that one atom of ^{18}O was incorporated into the product (Table II). These results establish that thymine hydroxylase can catalyze the oxidation of 5-(methylthio)-uracil to both a sulfone and a sulfoxide.

Hydroxylation of 5,6-Dihydrothymine. Unlike most of the α-ketoglutarate dioxygenases, thymine hydroxylase catalyzes the oxidation of an allylic C–H bond. To determine whether this enzyme can also catalyze the more difficult oxidation of an aliphatic C–H bond, 5,6-dihydrothymine was tested as an alternate substrate. The HPLC assay reveals formation of a product which migrates at 4.0 min. No reaction takes place in the absence of enzyme, α-ketoglutarate, or O_2. The product was isolated by HPLC, and the UV spectrum revealed no absorbance above 230 nm, indicating unambiguously that the 5,6-dihydro bond is intact and has not been oxidized. $^{18}O_2$-labeling experiments followed by GC–MS analysis (Table II) were unexpectedly consistent with the formation of HMU and incorporation of 1 equiv of ^{18}O. A sample of 5,6-dihydrothymine was, therefore, derivatized and analyzed by GC–MS under identical conditions to determine whether oxidation occurred during either of these processes. The mass spectrum obtained was that of the bis(trimethylsilyl) derivative of thymine, demonstrating that the 5,6-bond is oxidized during either the derivatization reaction or the GC–MS analysis.

These results establish, therefore, that thymine hydroxylase catalyzes the hydroxylation of 5,6-dihydrothymine. The surprising observation is that all of the racemic 5,6-dihydrothymine is consumed during this reaction. Thus, thymine hydroxylase can accommodate an sp^3-hybridized carbon at C-5 and does not appear to care about the stereochemistry at this position.

Using the $^{14}CO_2$ assay, preliminary kinetic constants were determined for the thymine hydroxylase reaction with 5,6-dihydrothymine (Table I). The apparent V_{max} of 0.064 ± 0.003 is only 5% that for the thymine reaction. However, comparison of the rate of CO_2 formation to the rate of 5,6-dihydrothymine consumption demonstrates that significant uncoupled decarboxylation occurs with this alternate substrate. As a result, the V_{max} determined here primarily reflects the rate of the uncoupled reaction. The magnitude of V_{max} for the hydroxylation reaction cannot be determined without an accurate assay for 5,6-dihydro-5-(hydroxymethyl)uracil. From the V_{max} isotope effect observed with (trideuteriomethyl)-thymine, we know that cleavage of the allylic C–H bond is partially rate-limiting. We would thus expect V_{max} to be significantly lower for the more difficult cleavage of the aliphatic C–H bond in 5,6-dihydrothymine. The extent of uncoupled decarboxylation observed with this substrate may result from a slow C–H bond cleavage, much like the isotope effect on the γ-butyrobetaine hydroxylase reaction results in increased uncoupled reaction (Holme et al., 1984). Alternatively, the uncoupled reaction may be due to distorted alignment between the substrate and the active site. Further kinetic studies are needed to distinguish between these possibilities.

SUMMARY

Evidence has been presented which strongly suggests that thymine hydroxylase is capable of catalyzing epoxidation, sulfur oxidation, and N-demethylation reactions, as well as the hydroxylation of unactivated C–H bonds. This α-ketoglutarate-dependent dioxygenase thus catalyzes a scope of reactions which is strikingly similar to that observed with liver microsomal cyt P-450 and the bacterial methane monooxygenase and ω-hydroxylase. The nature of the iron center responsible for these oxidations and the structural features of the protein which stabilize this reactive species remain to be elucidated. However, it is clear that there is nothing sacred about the heme of cyt P-450. Whether common mechanistic features exist among the various non-heme iron systems is the subject of ongoing investigations. The non-specificity of thymine hydroxylase from *R. glutinis* makes it an excellent prototype for further studies to unravel the detailed mechanism of the α-ketoglutarate dioxygenases.

ACKNOWLEDGMENT

We thank D. Bergstrom (Purdue University) for the gift of 5-(2-hydroxyethyl)uracil and G. Ashley (Northwestern University) for the Pascal versions of W. W. Cleland's kinetics programs.

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