Human cytochrome P450 (P450) 2A6 catalyzes 7-hydroxylation of coumarin, and the reaction rate is enhanced by cytochrome $b_5$ ($b_5$). 7-Alkoxycoumarins were O-dealkylated and also hydroxylated at the 3-position. Binding of coumarin and 7-hydroxycoumarin to ferric and ferrous P450 2A6 are fast reactions ($k_{on} \approx 10^6 \text{ M}^{-1} \text{ s}^{-1}$) and the $k_{off}$ rates range from 5.7-36 s$^{-1}$ (at 23°C). Reduction of ferric P450 2A6 is rapid (7.5 s$^{-1}$), but only in the presence of coumarin. The reaction of the ferrous P450 2A6 substrate complex with O$_2$ is rapid ($k \geq 10^6 \text{ M}^{-1} \text{ s}^{-1}$), and the putative Fe$^{2+}$·O$_2$ complex decayed at a rate of $\approx 0.3$ s$^{-1}$ at 23°C. Some 7-hydroxycoumarin was formed during the oxidation of the ferrous enzyme under these conditions, and the yield was enhanced by $b_5$. Kinetic analyses showed that $\approx 1/3$ of the reduced $b_5$ was rapidly oxidized in the presence of the Fe$^{2+}$·O$_2$ complex, implying some electron transfer. High intrinsic and competitive and non-competitive intermolecular kinetic deuterium isotope effects (6-10) were measured for O-dealkylation of 7-alkoxycoumarins, indicating the effect of C-H bond strength on rates of product formation. These results support a scheme with many rapid reaction steps, including electron transfers, substrate binding and release at multiple stages, and rapid product release, even though the substrate is tightly bound in a small active site. The inherent difficulty of chemistry of substrate oxidation and the lack of proclivity towards a linear pathway leading to product formation explain the inefficiency of the enzyme relative to highly efficient bacterial P450s.

P450 enzymes are involved in the oxygenation of a variety of natural products and xenobiotic chemicals in microbial systems (3, 4). Much is known about the structure, function, and catalytic features of some of the P450s, particularly the more extensively studied of the bacterial P450s (4, 5). In mammalian systems, P450s oxidize many drugs, steroids, carcinogens, fatty acids and eicosanoids, fat-soluble vitamins, and other endobiotic and xenobiotic chemicals (6). Less information is available about the biochemical details of most of the 57 human P450s (7). In particular, the basis of the inherently lower catalytic activities of these and other mammalian P450s relative to some of the microbial forms is not clear.

P450 2A6 is a low-to-medium abundance P450 in human liver (7-9) and is also expressed in some extrahepatic tissues (10). The history of this gene/protein goes back to Phillips et al. (11), who identified a human P450 cDNA as a relative of rat P450 2B1. The 7-hydroxylation of coumarin has long been used as an assay of P450 activity in.
animal and human liver microsomes (12, 13), and Yamano et al. (14) isolated a P450 2A6 cDNA (then termed 2A3) and first showed that the protein derived from heterologous expression had coumarin 7-hydroxylation activity. Miles et al. (15) also provided similar evidence for this particular sequence being associated with coumarin 7-hydroxylation. Our group purified a protein from human liver microsomes, identified it as P450 2A6, and showed it to be the major coumarin 7-hydroxylase in human liver (8). Subsequently P450 2A6 has been studied extensively, in large part because of its role in the metabolism of nicotine and carcinogenic N-alkyl nitrosamines (16, 17). Genetic polymorphisms have been identified (18, 19) and may be of relevance to cancer risk: (i) impaired metabolism of nicotine has been proposed to reduce cigarette smoking in P450 2A6-deficient individuals (20); (ii) impaired metabolism can yield reduced levels of activation of the N-nitrosamines found in tobacco (21). Some drugs are oxidized by P450 2A6 (7, 22). P450 2A6 also catalyzes the oxidation of indoles (23, 24), and we have used P450 2A6 mutants to synthesize new indirubins with activity as protein kinase inhibitors (25).

Recently X-ray crystal structures have been reported for P450 2A6, including forms with the substrates coumarin and nicotine bound (26). These structures, more than any of the other mammalian P450s solved to date, have a small binding site akin to that of bacterial P450 101A1 (27). The space for the substrate coumarin is very restricted, and the coumarin-bound structure has the C-7 atom located near the heme iron (26). A major conformational change is required to open and close the enzyme and allow the substrate (coumarin) to enter and leave (26).

We have been studying aspects of catalysis of mammalian P450s, including the rate-limiting steps in reactions (28-31). P450 2A6 was of interest because of the recently reported structure, the useful fluorescence properties and common use of coumarins as P450 substrates, and our inherent interest in the catalytic properties of P450 2A6 (8, 23-25, 32). Rates of several steps were measured (Scheme 1). Studies of O-dealkylation of 7-OR coumarins showed high kinetic hydrogen isotope effects and demonstrate the kinetic difficulty of C-H bond breaking. The 7-OR coumarins showed extensive formation of 3-OH products as well as 7-OH coumarin. Together the results provide a picture of a very dynamic catalytic cycle with considerably more flexibility than apparent with the more efficient bacterial P450s, providing some potential insight into rate differences.

**Experimental Procedures**

_Chemicals—Coumarin, 7-OH coumarin, 7-OMe coumarin, and 7-OEt coumarin were purchased from Aldrich Chemical Co. (Milwaukee, WI) and recrystallized from C6H12OH/H2O or CH3OH/H2O before use. 5-Deazaflavin was a gift of the late V. Massey (Univ. Michigan, Ann Arbor) (36)._ 

Deuterated 7-OMe coumarins were prepared by reaction of 7-OH coumarin with deuterated methyl iodides (Cambridge Isotopes, Cambridge, MA) in the usual manner (29, 37, 38) and recrystallized from CH3OH/H2O. [1-Ethyl-d2] 7-OEt coumarin was prepared in the same way from [1-d2] ethyl iodide (Cambridge). In the synthesis of [1-ethyl-d1] 7-OEt coumarin, CH3CHO was reduced with LiAlD4 in diethylene glycol diethyl ether to prepare [1-d1] C2H5OH (39) and the distilled product was reacted with tosyl chloride in dry pyridine to form the tosylate (72% yield, mp 27-30 °C (lit 21 °C (40)). The tosylate was then reacted with 7-OH coumarin in acetone/K2CO3 (under reflux), in the same manner as used with the alkyl iodides, to yield [1-ethyl-d1] 7-OEt coumarin, which was recrystallized from CH3OH/H2O (39% yield, mp 85-87 °C, lit 88-90 °C (41)). UV (CH3OH) ε323 1.23 × 104 M⁻1 cm⁻¹; electrospray MS, m/z 191.1 (MH⁺); NMR (CDCl3) δ 1.37 (dd, 3H, CH3), 4.03 (m, 1H, CHD), 6.22 (d, 1H, H-3), 6.69-6.82 (m, 2H, H-6, H-8), 7.33 (d, 1H, H-5), 7.60 (d, 1H, H-4). All deuterated coumarin derivatives were >98% isotopically enriched at the site of
modification as judged by MS and NMR spectroscopy.

The synthesis of 3-OH coumarins was done using the general procedure of Neubauer and Flatow (42), which involves condensation of salicylaldehyde or a 4-substituted salicylaldehyde with hippuric acid (N-benzyglycine) to form the N-benzoyl enamine, which was hydrolyzed in 10 N NaOH (100 °C, 45 min) to give the desired coumarin. 4-OEt salicylaldehyde was prepared by BCl₃ treatment of 2,4-(OE)₂ salicylaldehyde (Aldrich) in CH₂Cl₂ (80% yield) (43). The identities of the 3-OH coumarins were confirmed by their mp values and spectroscopy—3-OH coumarin: mp 151-154 °C (lit. 154 °C (44)); UV (CH₃OH) ε₃07 1.22 × 10⁴ M⁻¹ cm⁻¹, ε₂₉₄ 1.19 × 10⁴ M⁻¹ cm⁻¹, ε₂₃₅ 4.91 × 10³ M⁻¹ cm⁻¹; fluorescence (CH₃OH) λ excitation 310 nm, λ emission 395 nm; electrospray MS, m/z 163.1 (MH⁺); NMR (CDCl₃) δ 6.48 ( bs, 1H, H-4), 7.46-7.67 (m, 4H, H-5,6,7,9). 3-OH, 7-OMe coumarin: mp 179-182 °C (lit. 175.5-177.5 °C (45)); UV (CH₃OH) ε₃2₂ 1.4 × 10⁴ M⁻¹ cm⁻¹, ε₂₃₅ 5.6 × 10³ M⁻¹ cm⁻¹, ε₂₁₅ 8.4 × 10³ M⁻¹ cm⁻¹; electrospray MS, m/z 193.1 (MH⁺); NMR (CDCl₃) δ 3.79 (s, 3H, CH₃), 6.88 (dd, 1H, H-6), 6.94 (d, 1H, H-8), 7.08 (s, 1H, H-4), 7.43 (d, 1H, H-5). 3-OH, 7-OEt coumarin: mp 155-157 °C; UV (CH₃OH) ε₃2₂ 1.34 × 10⁴ M⁻¹ cm⁻¹, ε₂₃₅ 5.6 × 10³ M⁻¹ cm⁻¹, ε₂₁₅ 8.4 × 10³ M⁻¹ cm⁻¹; electrospray MS, m/z 206.9 (MH⁺); NMR (CDCl₃) δ 1.32 (t, 3H, CH₃), 4.05 (q, 2H, CH₂), 6.87 (dd, 1H, H-6); 6.89 (d, 1H, H-8), 7.08 (s, 1H, H-4), 7.42 (d, 1H, H-5).

Enzymes—P450 2A6 was expressed from a plasmid (originally obtained from P. Soucek, Natl. Inst. Public Health, Prague) in Escherichia coli, except that a (His)₅ tag was attached to the C-terminus (24, 46). Rat NADPH-P450 reductase was expressed in E. coli and purified as described (47). Recombinant human b₅ was expressed in E. coli JM109 cells from a plasmid (pSE420 (AMP) kindly provided by Satoru Asahi (Takeda Pharmaceutical, Osaka, Japan). The protein was solubilized and purified to electrophoretic homogeneity using modifications of the DEAE-cellulose and hydroxylapatite chromatography methods described elsewhere (48).

Spectroscopy—NMR spectra were recorded using Bruker 300 and 400 MHz instruments in the Vanderbilt facility.

UV-visible spectra were generally acquired using an OLIS/Cary 14 or a OLIS/Aminco DW2a instrument (OLIS, Bogart, GA).

Mass spectra were recorded using HPLC-MS methods (octadecylsilane columns, positive ion-electrospray or atmospheric pressure chemical ionization) in the Vanderbilt facility using a Thermo-Finnigan TSQ-7000 instrument (Thermo-Finnigan, Sunnyvale, CA).

Fluorescence measurements were made using either an SPEX Fluoromax-3 instrument (SPEC/Jobin Yvon, Edison, NJ) or an OLIS RSM-1000 instrument (OLIS), operating in the stopped-flow mode.

Stopped-flow kinetic UV-visible measurements were made using an OLIS RSM-1000 instrument (slit width 1.24-3.16 nm for absorbance beam). Some kinetic traces were obtained in the single wavelength mode; the rapid scanning mode was used with a 16 × 1 mm scanning disk to obtain spectra (16-1000 scans s⁻¹). In some cases the acquired spectra were used to derive kinetic traces at individual wavelengths, and fitting was done with the manufacturer’s software.

Assays—Typical steady-state coumarin oxidation reactions included 50 pmol P450 2A6, 100 pmol NADPH-P450 reductase, 50 pmol b₅ (when indicated), and 30 µg di-12:0 GPC in 0.50 ml of 50 mM potassium phosphate buffer (pH 7.4), along with a specified amount of the coumarin substrate. An aliquot of an NADPH-generating system was used to start reactions (final conc. 10 mM glucose 6-phosphate, 0.5 mM NADP⁺, and 1 IU yeast glucose 6-phosphate ml⁻¹ (49)). Stock coumarin solutions (5 mM) were made in H₂O, 7-OMe and 7-OEt coumarin stocks (50 mM) were made in CH₃CN and diluted into enzyme reactions.
with final organic solvent concentrations < 1% (v/v).

Incubations were generally done for 10 min at 37 °C, terminated with 0.10 ml of 17% HClO₄, and centrifuged (10³ × g, 10 min). CH₂Cl₂ (1.0 ml) was added to the supernatant to extract the products, followed by centrifugation at 10³ × g (process repeated one more time). The organic layers were combined, and the CH₂Cl₂ was removed under an N₂ stream. CH₂Cl₂ was added to the supernatant to extract the products, followed by centrifugation at 10³ × g (process repeated one more time). The organic layers were combined, and the CH₂Cl₂ was removed under an N₂ stream. The products were analyzed by HPLC using a Toso ODS-80™ octadecylsilane (C₁₈) column (4.6 mm × 150 mm, 5 µm) with the mobile phase H₂O:CH₃CN (55:45, v/v) containing 10 mM HClO₄, flow rate 1.0 ml min⁻¹, monitoring A₃₃₀. Kinetic parameters (Kₘ and kₗ) were determined using nonlinear regression analysis with Graph-Pad Prism software (Graph-Pad, San Diego, CA). In some cases (stopped-flow), 7-OH coumarin was monitored directly (F₃₉₀/₄₆₀).

Assays involving competitive deuterium isotope effects were done by HPLC-MS analysis of formaldehyde or acetaldehyde derived from [methyl-d₁] 7-OMe coumarin or [1-ethyl-d₂] 7-OEt coumarin, respectively, using the general approach described elsewhere (51,52). With [1-ethyl-d₁] 7-OEt and [1-ethyl-d₂] 7-OEt coumarin as substrates, the mass spectra were complicated due to the presence of contaminants in some of the reagents, particularly the solvents and glycerol. In order to minimize complications arising from residual aldehyde contamination in reagents and solvents, the following changes were made to the procedure. Reconstituted enzyme solutions (P450 2A6, NADPH-P450 reductase, and b₅) were dialyzed against glycerol-free 50 mM potassium phosphate buffer containing 0.2 mM EDTA and 0.1 mM dithiothreitol (two changes over 12 h, at 4 °C) prior to the addition of di-12:0 GPC. Dissolution of 7-OMe and 7-OEt coumarin in H₂O was achieved by sonication (Branson sonicator, microtip probe, 70% full power) (Branson, Danbury, CT) to avoid the introduction of organic solvents. Aqueous stock solutions (~0.5 mM) were then filtered and quantitated spectrophotometrically (vide supra). Hexanes and CH₂CN were heated with and distilled from 2,4-dinitrophenylhydrazine. 2,4-Dinitrophenylhydrazine (for use as a derivatization reagent) was recrystallized twice from CH₃OH/H₂O, dried in vacuo, dissolved in 6 N HCl (0.1%, w/v), and washed multiple times with a hexane/CH₂Cl₂ mixture (7:3, v/v) to remove hydrazone impurities prior to use for derivatization. Deuterium incorporation was determined using HPLC/negative ion atmospheric pressure chemical ionization MS of the resulting 2,4-dinitrophenylhydrazone derivatives (source temperature 550 °C; heated capillary voltage 20 V; heated capillary temperature 180 °C; ionization current 5 µA; sheath gas (N₂) pressure 70 psi; auxiliary gas (N₂) pressure 10 psi) (53).

Anaerobic experiments involved the use of glass anaerobic cuvettes and tonometers with a gas train connected to a manifold, alternating between vacuum and Ar. The basic systems are described elsewhere (54-56) and recent further modifications have been described (31). The OLIS RSM-1000 stopped-flow spectrophotometer has stainless steel lines leading up to the observation cell instead of Teflon, reducing the diffusion of O₂. As described earlier (31), the drive syringes were filled with anaerobic 0.10 M Na₂S₂O₄ (in 0.2 M potassium phosphate buffer, pH 7.4) overnight prior to use in order to scrub O₂ (31, 56). The drive syringes were then loaded with the contents of a tonometer containing 0.25 mM safranine T and 0.5 mM methyl viologen (photoreduced) in anaerobic 0.10 M Tris·HCl buffer (pH 7.7) containing 10 mM EDTA. The lack of O₂ in the system is indicated by blue color (methyl viologen radical cation), as opposed to red (oxidized safranine). Thus, the final displacement of the dye by the enzyme solution provides a reasonable check on the anaerobicity of the system.

Results

Oxidations Catalyzed by P450 2A6—Earlier work led to the characterization of P450 2A6 as the major coumarin 7-hydroxylase (8, 14, 15). Recently the structure of a P450 2A6 crystal has been reported (26),
with coumarin bound and positioned with the C-7 atom near the iron atom.

Assays of P450 2A6-catalyzed coumarin oxidation commonly utilize a sensitive fluorescence assay that reports 7-hydroxylation (57, 58). HPLC-UV assays indicated the formation of the single product 7-OH coumarin by chromatographic and spectral comparison to standard material (Table I, also see Supplementary Data). 7-OMe and 7-OEt coumarins were O-dealkylated to form 7-OH coumarin, but both of these substrates also formed the 3-hydroxy products, as judged by comparisons with synthetic materials. The identification of 3-OH, 7-OEt coumarin as a product of oxidation of 7-OEt coumarin has been reported previously with human liver microsomes (59, 60). The 3-hydroxylation of coumarin has been reported with human liver microsomes (61, 62). Neither we nor Born et al. (62) detected conversion of coumarin to 3-OH coumarin by P450 2A6 systems.

Subsequent analysis of steady-state kinetic parameters indicated that 3-hydroxylation was observed to a greater extent for 7-OEt coumarin than 7-OMe coumarin (Table I).3 The addition of \( b_3 \) stimulated the formation of 7-hydroxylation of coumarin 2-fold, as reported by others (46, 63). However, the activities with 7-OR coumarins were not stimulated, except for the decreased \( K_m \) for the 3-hydroxylation of 7-OEt coumarin.

Spectral Properties of P450 2A6 — The spectra of the ferric, ferric-coumarin, and ferrous-coumarin forms of P450 2A6 are shown in Fig. 1. The spectral properties were utilized in several subsequent experiments to measure rates of changes within the catalytic cycle (Scheme 1).

Second-derivative analysis (64, 65) of the spectra (Fig. 1) yielded estimates of 98% low-spin P450 iron in substrate-free ferric P450 2A6 and 88% high-spin iron with coumarin bound.

Substrate Binding (Step 1 of Scheme 1) — A large spectral difference is observed upon binding of the substrate coumarin (Fig. 2). Titration of P450 2A6 with coumarin yielded classic “Type I” difference spectra, with \( K_s = 0.38 \pm 0.03 \) \( \mu \)M (Figs. 2A,2B).

The rates of spectral changes could be monitored using stopped-flow spectroscopy (Fig. 2C). These traces were fit to single-exponential plots. Analysis of rates as a function of coumarin concentration (Fig. 2D) fit a relationship describing a 2-state system (\( Fe^{3+} + S \rightleftharpoons Fe^{3+} \cdot S \)), i.e. \( k_{on} = k_{off}[S] + k_{off} \) (67) and yielding \( k_{on} = 2.7 \times 10^6 \) \( M^{-1} \) s\(^{-1}\) and \( k_{off} = 5.7 \) s\(^{-1}\), with \( K_d = k_{off}/k_{on} = 2.1 \) \( \mu \)M, in reasonable agreement with the \( K_d \) values estimated by titration (Fig. 2B).

Product Release (Step 7 of Scheme 1) — Titration of ferric P450 2A6 with 7-OH coumarin yielded a “Type II” difference spectrum, which is probably indicative of ligation of the phenolic oxygen to the iron atom (Fig. 3A). The titration indicated binding as tight as for the substrate, \( K_d = 0.82 \pm 0.05 \) \( \mu \)M (Fig. 3B). The rate of binding could be measured using stopped-flow spectroscopy (Fig. 3C). Analysis of the rate as a function of 7-OH coumarin concentration (Fig. 3D) yielded \( k_{on} = 2.0 \times 10^6 \) \( M^{-1} \) s\(^{-1}\) and \( k_{off} = 6.8 \) s\(^{-1}\) (\( k_{off}/k_{on} \approx 3.4 \) \( \mu \)M).

P450 2A6-catalyzed oxidations of coumarin, 7-OMe coumarin, and 7-OEt coumarin were examined and did not show kinetic bursts (Fig. 4). On the basis of these results and the rates measured in the experiment of Fig. 4, steps following product formation are not rate-limiting in the formation of 7-OH coumarin from any of these substrates.

Reduction of Ferric P450 2A6 (Step 2 of Scheme 1) — Rates of reduction of ferric P450 2A6 were measured in an anaerobic CO environment, with ferrous P450 trapped as the CO complex (Fig. 5). The rate of binding of ferrous P450 and CO is much faster than reduction.

The rate of reduction of ferric P450 was slow in the absence of coumarin, with a fit to a single exponential of 0.13 s\(^{-1}\) (Fig. 5). In the presence of coumarin, a bi-exponential fit yielded \( k_1 = 7.5 \) s\(^{-1}\) and \( k_2 = 0.13 \) s\(^{-1}\) (Fig. 5).
About 50% of the P450 was reduced in the fast phase.

**Dissociation of Substrate from Ferrous P450 2A6 (Step 2a of Scheme 1)**—Although the binding of a substrate (coumarin) to ferric P450 2A6 produces a major spectral change, the changes observed upon addition of coumarin to ferrous P450 2A6 are much weaker. A difference spectra was observed with a trough at 438 nm and a peak at 460 nm (Fig. 6A). The rate of binding to NADPH-P450 reductase-reduced P450 2A6 under anaerobic conditions yielded $k_{\text{on}} = 1.5 \times 10^6 \text{ M}^{-1} \text{s}^{-1}$ and $k_{\text{off}} = 36 \text{ s}^{-1}$ ($k_{\text{off}}/k_{\text{on}} = 24 \mu\text{M}$) (Figs. 6B, 6C).

**Formation (Step 3 of Scheme 1) and Decomposition of Ferrous P450 2A6·O₂ Complex**—Ferrous P450 2A6 (in the presence of 50 μM coumarin) was introduced anaerobically into one syringe of the stopped-flow spectrophotometer and mixed with aerobic buffer (200 μM O₂). A rapid spectral change was observed, and the early spectra are shown in Fig. 7A. The rate of this reaction, as estimated from ΔA₃₉₀, was ~ 75 s⁻¹. This complex was not very stable and decayed to yield ferric P450 2A6 (Fig. 7B), with an estimated rate of 0.3 s⁻¹ ($t_{1/2} = 2.3 \text{ s}$) at 23 °C (Fig. 7C).

**Formation of Product in Limited Turnover Experiments**—The oxidation of coumarin was measured under conditions in which the electron input was limited to what should be a single cycle, e.g. as in Fig. 7. P450 2A6 was either photoreduced with 5-deazaflavin or reduced (by NADPH-P450 reductase) with a limited amount of NADPH (enough to fully reduce NADPH-P450 reductase and P450 once).³

The product 7-OH coumarin could be measured in all cases (Fig. 8), although the yields were low (Table II). The formation of product could be detected with only P450 present (Fig. 8A), which apparently indicates that two FeO₂²⁺ complexes can interact to provide the second electron for product formation (e.g. $2 \text{ FeO}_2^{2+} \rightarrow \text{FeO}_3^{2+}$ ($\rightarrow \rightarrow \text{FeO}_3^{2+} + \text{Fe}^{2+}$)). Product formation was more efficient when electrons were delivered from the reductase (Table II), although we do not know exactly how many electrons are delivered, i.e. it is possible that multiple 1-electron transfers could occur that are not relevant during steady-state catalysis.

In the cases of both photoreduced P450 2A6 and P450 2A6 that was reduced with NADPH/reductase, the presence of reduced $b_3$ raised the yield of product (Fig. 8, Table II). This result suggests that ferrous $b_3$ might be able to transfer electrons to the P450 2A6 FeO₂²⁺-substrate complex.

**Kinetic Deuterium Isotope Effects (Step 6 of Scheme 1)**—The experiments presented thus far have dealt with the binding of ligands and the activation of O₂ by P450. The relative rate of the step in which chemical transformation of the substrate occurs had not been addressed. This is a difficult question in the case of an aromatic or olefinic hydroxylation, unless an enzyme intermediate can be isolated and reacted directly to yield product. Such is not the case with P450s, so an alternate approach was used, that of measurement of kinetic deuterium isotope effects for reactions involving C-H bond cleavage of simple analogs. The work described in Table I showed that 7-OMe and 7-OEt coumarin O-dealkylations were catalyzed by P450 2A6 with catalytic efficiencies approaching that for coumarin 7-hydroxylation.

Several types of kinetic isotope effect experiments are possible (Scheme 3) and reveal different information. A so-called non-competitive intramolecular experiment approximates the intrinsic kinetic deuterium isotope effect, the isotope effect on the C-H bond breaking step itself (1, 2). In the case of a C-H bond-breaking step with a methyl group, the rapid rotation does not provide a kinetic barrier to attenuate the reaction, and the only limitation to the interpretation of this experiment is the potential contribution of a smaller geminal secondary kinetic isotope effect. P450 2A6-catalyzed 7-OMe coumarin O-demethylation showed a non-competitive intramolecular isotope effect of 9.8 (Table III).
A somewhat lower isotope effect was measured for the O-deethylation of 7-OEt coumarin (6.0) (Table III), although the point must be made that the O-deethylation involves a prochiral substrate, 7-OEt coumarin, which can preclude interpretation about intrinsic isotope effects.

We also measured competitive intermolecular isotope effects (Scheme 3B). Attenuation of the intrinsic isotope effect in such an experiment is suggestive of relatively slow exchange of substrate. The values for 7-OMe and 7-OEt coumarin in this experiment were still high (9.0 and 7.0, respectively) (Table III). The results are consistent with the rapid $k_{off}$ rate for the substrate coumarin (Fig. 2D).

The third type of experiment used was a non-competitive intermolecular system (Scheme 3C). This type of experiment can provide some information about the extent to which the C-H bond-breaking step is rate-limiting (in the steady-state) (1, 2). The value can be compared to the intrinsic isotope effect (Scheme 3A, Table III). High kinetic isotope effects (both $^{13}V$ and $^{17}V(V/K)$) were observed in these experiments (Fig. 9, Table IV) (regardless of whether $b_5$ was added or not, results not presented). These experiments provide strong evidence that the oxidation of the substrate by the active hypervalent oxygen species is difficult and can limit the rate of formation of these products, at least in the case of the O-dealkylation reactions. In the case of 7-OMe coumarin considerable “switching” to 3-hydroxylation was observed (Table IV, Fig. 9B). This result implies that the rotation of the substrate can occur to generate this product (68). Alternatively, the FeO$^{3+}$-deuterated substrate complex could decompose and the cycle can begin again with a protiated substrate (31). The switching effect (to 3-hydroxylation) was not significant in the case of 7-OEt coumarin (Fig. 9D, Table IV).

**Steady-state Spectral Measurements**—Steady-state spectra of the coumarin 7-hydroxylation reaction were recorded with the prospect of identifying an accumulating intermediate. An aerobic mixture of P450 2A6, NADPH-P450 reductase, $b_5$, di-12:0 GPC, coumarin, and an NADPH-regenerating system was mixed rapidly with NADPH and spectra were recorded. The first phase occurred within 1 s (Fig. 10A). The reduction of the flavins of the reductase was observed (e.g. 440-500 nm). Several steps occur during this time and a relatively stable spectrum accumulated by 1 s. The $\lambda_{max}$ was $\sim$ 415 nm and probably represents some iron-oxygen complex (or a mixture of more than one). It is not the ferric nor ferrous P450 2A6 (Fig. 1) and probably not the FeO$^{2+}$ complex (Fig. 7).

This spectrum persisted for several seconds and then the reduction of $b_5$ was observed (Fig. 10B). The reduction appeared to follow first-order kinetics (as judged by $\Delta A_{409}$ or $\Delta A_{424}$), with $k = 0.15$ s$^{-1}$. In other experiments with no P450 present, we measured the reduction of $b_5$ to be a bi-exponential process, with a first step (stoichiometric with 1 $b_5$ per NADPH-P450 reductase) of 18 s$^{-1}$ and a subsequent rate of 1.7 s$^{-1}$ (results not shown).

**Oxidation of Ferrous $b_5$**—One hypothesis for the enhancement of the catalytic activity of P450 2A6 by $b_5$ is the transfer of an electron from ferrous $b_5$ to the P450 FeO$^{2+}$ substrate complex (70, 71). This hypothesis has some support in the limited turnover experiments (Table II), where yields were enhanced in the presence of $b_5$. However,
alternate hypotheses could be valid (e.g. improvement of P450 efficiency through protein-protein interactions).

b$_3$ was photoreduced and mixed with air-saturated buffer (Fig. 11A). The first-order rate of oxidation was 0.13 s$^{-1}$ (Fig. 11B). The experiment was repeated in the presence of P450 2A6 and coumarin. Thus, ferrous (photoreduced) P450 2A6 reacts rapidly with O$_2$ to form an oxygenated complex (Fig. 7A); if reduced b$_3$ is present, it might transfer electrons to this FeO$_2^{2+}$ complex before the complex decomposes. In this experiment (Fig. 11C), reduction was faster than in the absence of P450 2A6. About 1/3 of the b$_3$ was oxidized rapidly (as judged by $\Delta A_{410}$ or $\Delta A_{424}$ measurements, and the data were fit to a biexponential plot with $k_1 = 3.6$ s$^{-1}$ and $k_2 = 0.04$ s$^{-1}$ (Fig. 11D).

We conclude that b$_3$ can participate in transferring electrons to the P450 2A6 FeO$_2^{2+}$ substrate complex, although this does not appear to be a well-coupled process.

**DISCUSSION**

The purpose of this work was to characterize individual steps in catalysis by a mammalian P450 enzyme, human P450 2A6. The enzyme has a small active site (260 Å$^3$), which is filled by the substrate coumarin (26), and binds the substrate reasonably tightly ($K_d$ ~ 1 µM, Fig. 2B). However, catalysis is still relatively slow and inefficient, in the context of the use of electrons for substrate oxidation (Tables I, V). Our results are interpreted in the context of a model with rapid exchange of ligands at multiple steps (requiring major conformational changes inferred from the X-ray crystallography work (26)), an unstable FeO$_2^{2+}$ complex, and a relatively difficult step for the chemistry of oxidation by the FeO$_3^{2+}$ complex.

Most of the work with P450 2A6 and coumarin substrates has been focused on the 7-hydroxylation reaction, which can be readily observed because of the strong fluorescence of the product 7-OH coumarin at neutral or alkaline pH (57, 58). HPLC-UV analysis also indicated the oxidation of a second site with the 7-OR coumarins (but not coumarin), which was identified as the 3-position by chromatographic and spectral comparison (Scheme 2, Fig. 9). Coumarin 3,4-epoxide has been reported not to convert to 3-OH coumarin (62), and therefore we presume that the chemical mechanism is one involving formation of a bond between (Fe)O and the C-3 atom of the coumarin (-CH$_2$CH-O-Fe), which collapses to form the enol product (favored over the keto tautomer). O-Dealkylation (at C-7) is a favorable reaction for 7-OR coumarins, presumably because the positioning approximates that observed in the P450 2A6·coumarin complex (26). The “shift” to 3-hydroxylation with 7-OEt coumarin was more extensive than for 7-OMe coumarin (Tables I, IV). Apparently the steric restriction imposed by the larger alkyl group shifts the equilibrium to an alternate form with the lactone carbonyl and the C-3 atom near the heme iron.

The molecular basis for the difference between oxidation at the 7- and 3-positions awaits the availability of the coordinates of the P450 2A6·coumarin crystal structure (26). The balance would appear to be sensitive not only to the bulk at the 7-position (Table I) but also the ease of oxidation of the C-7 substituent (Table IV). Thus, the explanation for the specificity will probably be both steric and dynamic. How difficult the prediction of regioselectivity and rates of oxidation will be for new putative ligands of P450 2A6 is unknown. In a general sense, the relatively low rates of coumarin oxidations by P450 2A6 (10 min$^{-1}$, Table I) would not have been expected if only a tight fit of the substrate and H$_2$O exclusion from the active site (as evidenced by a strong shift to high spin iron) (Figs. 1, 2) are the major factors involved in predicting catalysis, i.e. the properties of the P450 2A6·coumarin complex resemble those of P450 101A1·camphor, a system that turns over ~ 100-fold faster (5, 26).

The binding and dissociation rates were estimated for ferric P450 2A6 and two ligands, coumarin and the 7-OH product (Figs. 2, 3). The results can be fit reasonably well to a simple 2-state model and a more complex system may not be justified, at least at this
point. The \( k_{\text{on}} \) rates are near those reported for bacterial P450 101A1 (5, 72). Whether or not these are really a diffusion-limited rates is unclear, in that the second-order rates are still lower than for many enzymes, and theoretical calculations predict rates of \( \sim 10^7 \text{ M}^{-1} \text{ s}^{-1} \) (73, 74). A rapid rate of binding could be obscured by a slower, reversible transformation of the low-spin to the high-spin iron, yielding an artificially low apparent \( K_d \) (67). In principle, such a phenomenon should yield a hyperbolic plot of the apparent rate of binding (vs. substrate concentration) instead of a linear form (Figs. 2D, 3D), but more analysis is needed to address this possibility.

The \( k_{\text{off}} \) rates were 5.7 and 6.8 s\(^{-1}\) for the substrate coumarin and the product 7-OH coumarin, respectively (for ferric P450 2A6). The equation \( k_{\text{off}}/k_{\text{on}} = K_d \) yields parameters close to those estimated by steady-state spectral analysis (Figs. 2, 3). The rapid off-rates are consistent with other results presented here, including the lack of a burst of 7-OH coumarin formation (Fig. 4) and the lack of attenuation of the kinetic deuterium isotope effect in the competitive intermolecular experiment (Table III). The rapid \( k_{\text{off}} \) for coumarin (5.7 s\(^{-1}\) at 23 °C) is realistic in the context of the other parameters and is also competitive with the rate of reduction (Fig. 5).

Rapid reduction of ferric P450 2A6 (Fig. 5) was highly dependent upon the presence of the substrate coumarin, although only \( \sim \) one-half of the P450 2A6 was reduced in the fast phase (7.5 s\(^{-1}\) at 23 °C), even with a 2-fold excess of NADPH-P450 reductase. This rate is certainly much faster than overall catalysis (Table I) and should not be rate-limiting, even if fast reduction is only partial (Fig. 5). Our previous experience with (purified) microsomal P450s has been that about one-half of them show rapid reduction in the absence of substrate and the other half require substrate (75). Other P450s often show biphasic kinetics (75), probably due to spatial issues with reductase in the complexes (76). Although the iron of coumarin-saturated P450 2A6 is high-spin (Fig. 1), a conclusion that only high-spin P450 is rapidly reduced is unwarranted (75). Second-derivative analysis of the ferric Soret spectrum (Fig. 1) indicated \( \sim 88\% \) high-spin iron, but only 50% of the P450 2A6 was reduced rapidly (Fig. 5). We have previously presented evidence against a general linkage of substrate binding, low- to high-spin iron conversion, rapid reduction, and more positive redox potentials (\( E_{m/2} \)) in P450s (65, 75, 77), unlike the situation with bacterial P450 101A1 (78). Although we have not directly estimated the \( E_{m/2} \) of P450 2A6 ± substrate, consideration of the estimated \( K_d \) values of the Fe\(^{3+}\) and Fe\(^{3+}\) enzymes (Figs. 2, 6) and the Nernst equation would suggest that the \( E_{m/2} \) of the Fe\(^{3+}/Fe^{2+}\) couple would become somewhat more negative in the presence of coumarin, not more positive, applying a "thermodynamic box" analysis of substrate binding and reduction (73) and considering that the substrate is bound more tightly to the oxidized form of P450 2A6 (74) (Figs. 2, 6).

As discussed above, the \( k_{\text{off}} \) rate for coumarin from ferrous P450 2A6 is considerable (36 s\(^{-1}\)) (Fig. 6C) and may be an issue in the functionality of the Fe\(^{2+}\)-substrate complex. That is, some fraction might not be competent in that it could dissociate, bind O\(_2\), and then decompose. The possibility also exists that substrate might dissociate from the FeO\(_2\)^{2+}-substrate complex (or Fe-O complexes further in the catalytic cycle (Scheme 1)), although we do not have any measurements. In the case of rabbit P450 1A2, we recently presented evidence that dissociation of substrate from the FeO\(_3\)^{2+} complex itself (due to prevention of C-H bond breaking by deuterium substitution) was only accompanied by decomposition of the complex (31).

Reaction of the Fe\(^{2+}\) P450 2A6-coumarin complex with O\(_2\) produced two sets of spectral changes (Fig. 7). Although we did not examine the effect of varying O\(_2\) concentration on the rates, our view is that the rapid initial changes (Fig. 7A, e.g. \( A_{390} \) decrease) represent the formation of an FeO\(_2\)^{2+}(-substrate) complex and the succeeding changes (Fig. 7B, e.g. \( A_{390} \) increase) represent
the decay of the complex to regenerate Fe$^{3+}$P450 2A6. The estimated rate of the first reaction was ~ 75 s$^{-1}$ and the decay was 0.3 s$^{-1}$ (18 min$^{-1}$) at 23 °C. This complex appears to be much less stable than the Fe$^{2+}$-O$_2$ complexes reported for bacterial P450 101A1 (72, 80) and P450 108A1 (81) but has a stability similar to rabbit P450 1A2 (31, 82) and the heme domain of P450 102A1 (81); it is probably more stable than the complexes of rabbit P450 2B4 (83) and bacterial P450 119A1 (84). Analysis of 7-OH coumarin indicated low (but finite) yields in “limited cycle” experiments, e.g. when the kinetic reaction described above was analyzed for product formation (Fig. 8, Table II). Product formation apparently involves dismutation of two Fe$^{2+}$-O$_2$ complexes to achieve the requisite 2-electron stoichiometry (31). The amount of product formation in these experiments was increased when reduced NADPH-P450 reductase was present, indicating that electron transfer from reduced NADPH-P450 reductase to the P450 2A6 FeO$^{2+}$-substrate complex occurs under these conditions, although the efficiency was low (Table II).

As reported previously (46, 63, 85), the presence of Fe$^{3+}$ enhanced the steady-state $k_{cat}$ for coumarin 7-hydroxylation (Table I). One general hypothesis for the enhancement of P450 catalytic activities by Fe$^{3+}$ is transfer of an electron from ferrous Fe$^{2+}$ to the P450 FeO$^{2+}$-substrate complex (70, 71). The enhancement of yields of products derived from FeO$^{2+}$-coumarin by ferrous Fe$^{2+}$ (Fig. 8, Table II) is consistent with this hypothesis but does not necessarily prove electron transfer. Another set of experiments was done in which a mixture of Fe$^{2+}$ P450 2A6-coumarin-ferrous Fe$^{2+}$ was mixed with O$_2$ to form a FeO$^{2+}$-substrate-ferrous Fe$^{2+}$ complex and the rate of oxidation of the ferrous Fe$^{2+}$ was monitored (at 410 or 424 nm) (Fig. 11). About one-third of the Fe$^{2+}$ was oxidized rapidly (3.6 s$^{-1}$), while only the second, slower phase was observed for Fe$^{2+}$ oxidation in the absence of P450 2A6 (Fig. 11B). This result is consistent with transfer of an electron from reduced Fe$^{2+}$ to the P450 2A6 FeO$^{2+}$-substrate complex, although the process appears to be less than quantitative. The situation is further complicated by the results of the experiment presented in Fig. 10, where a mixture of oxidized P450 2A6, NADPH-P450 reductase, and Fe$^{3+}$ (plus coumarin and di-12:0 GPC) was mixed with NADPH. In the first part of the reaction, the Fe$^{3+}$ was not reduced (up to ~ 1 s) and then became reduced at a rate of ~ 0.15 s$^{-1}$ (at 23 °C). The Fe$^{3+}$ then stayed reduced for at least several minutes. This apparent rate was similar to the oxidation rate of ferrous Fe$^{2+}$ (Fig. 11B). For comparison, an experiment with only NADPH-P450 reductase and ferric Fe$^{3+}$ yielded a bi-exponential fit for reduction, with $k_1 = 18$ s$^{-1}$ and $k_2 = 1.7$ s$^{-1}$. The Fe$^{3+}$ experiments are complex and difficult to interpret, but our overall conclusion is that some electron transfer from ferrous Fe$^{2+}$ to the P450 2A6 FeO$^{2+}$-coumarin complex occurs but that this does not seem to be a particularly efficient process. In other work we have shown that P450 2A6 catalyzed coumarin 7-hydroxylation can be enhanced by apo-Fe$^{3+}$, devoid of heme and precluding electron transfer (85). We conclude that part of the enhancement may be attributable to the electron transfer (Figs. 8, 11, Tables I, II) but that Fe$^{3+}$ probably has another effect, probably some type of conformational effect on the P450.

Although the focus of this investigation was coumarin hydroxylation, the observed reaction (7-hydroxylation) was not amenable to the application of kinetic deuterium isotope effect studies (Scheme 2). However, 7-OMe and 7-OEt coumarin appear to be reasonable surrogates in that the P450 2A6 heme iron atom is apparently positioned in sites that yield O-dealkylation (C-7) or 3-hydroxylation. The apparent intrinsic deuterium isotope effect for 7-OMe coumarin was high (9.8) and the 7-OEt coumarin value was lower (6.0), possibly perturbed by the issue of prochirality (Table III). The observed isotope effects high and similar in both competitive and non-competitive intermolecular experiments (Table IV).

When oxidation of a C-7 alkyl group was inhibited by substitution with deuterium, much of the decrease was compensated for in terms of enhanced 3-hydroxylation (Table IV). The switch may be less obvious in the case of
7-OEt coumarin, in that the $^3V$ and $^3(V/K)$ values are not as high because of the already high rate of 3-hydroxylation. However, $\sim 1/2$ of the decrease in $k_{cat}$ due to deuterium substitution is manifested in the $k_{cat}$ for 3-hydroxylation for both 7-OMe and 7-OEt coumarin (Table IV). The lack of complete switching can presumably be attributed to the decomposition of the putative FeO$^{3+}$ complex, in the absence of substrate rotation or release followed by rebinding in the alternate orientation.

Collectively, these results (see Scheme 3) are interpreted to mean that (i) substrate exchange is rapid and (ii) the rate of C-H bond-breaking is an important contributor to the $k_{cat}$ for formation of the hydroxycoumarin products. The former conclusion is consistent with the measured $k_{on}$ and $k_{off}$ rates for coumarin with Fe$^{3+}$ and Fe$^{2+}$ P450 2A6 (Figs. 2, 6). The latter result is consistent with the rapid rates estimated for all the steps that could be measured. The spectra recorded in the steady state do not provide a clear description of the step most limiting in the reaction (Fig. 10). Two complications exist: (i) a definite spectrum of the putative hypervalent iron (perferryl) complex is unavailable (FeO$^{3+}$ spectra have only been recorded for P450s 101A1 (97) and 119A1 (98) and show $\lambda_{max} \sim 370$ nm (a region somewhat obscured by NADPH and the substrate coumarin), and (ii) the low efficiency of NADPH utilization (Table V) argues that a large fraction of iron-oxygen complexes will be non-productive.

Together the results provide a picture of P450 2A6 with a very dynamic reaction cycle and limited commitment to a dedicated course of reaction steps. The rates of ligand binding and dissociation we report here are not unusually high, in the context of measured binding rates for other enzymes (67) and accepted $K_d$ values. The knowledge that the substrate coumarin is well-enclosed in the protein in the crystal structure (26) argues that the protein is opening and closing as fast as the $k_{on}$ and $k_{off}$ rates. The $k_{off}$ rate will be competitive with reduction (Figs. 2D, 5). Substrate can dissociate rapidly from the Fe$^{2+}$ enzyme, as well as the Fe$^{3+}$ form (Fig. 6). The FeO$^{2+}$-substrate complex is unstable; it breaks down rapidly (Figs. 7B, 7C) and is relatively inefficient in accepting electrons from either NADPH-P450 reductase or $b_5$ (Table II, Figs. 11C, 11D). Thus, several steps in the cycle (Scheme 1) are inefficient. The isotope effect studies argue that it is the chemistry of substrate activation, not oxygen activation, that limits the rate of catalysis; however, steps preceding this step can be inefficient without necessarily being reflected in the isotope effect parameters.

One possible reason for the low rates of catalytic activity of most of the mammalian P450s is the existence of unproductive binding modes, which might be formed in large active sites (99-102). However, the crystal structure of P450 2A6 shows a tight active site (26) and the P450 2A6-coumarin complex has a low $K_d$ ($\sim 1 \mu M$) (Fig. 2B). Although a favorable alternate oxidation site (C-3) was found for the 7-OR substituted analogs (Table I, Scheme 2), the crystal structure can be used as an argument against the preponderance of non-productive binding modes. Reduction (of ferric P450) is reasonably fast with P450 2A6, although not as fast as in the P450 101A1 and 102A1 systems (5, 72, 103). An interesting feature of the P450 101A1 and 102A1 systems is that the binding of substrate has been demonstrated to lower the $E_m$ (78, 104, 105). As discussed (vide supra), this change indicates that the substrate binds more tightly to the ferrous enzyme (74, 79). With a 1-electron process, a change of +120 mV translates to a 10$^{2}$-fold decrease in $K_d$. Thus, the values for P450 101A1 are $k_{off} \approx 1$ s$^{-1}$ for the Fe$^{3+}$ enzyme (5) and should be $\sim 0.01$ s$^{-1}$ for Fe$^{2+}$ P450 101A1 or 102A1. Thus the bacterial P450(s) are committed to bind O$_2$, which is a very fast process ($k_{on} = 8 \times 10^7$ M$^{-1}$ s$^{-1}$) (5, 72). The P450 101A1 FeO$^{2+}$-camphor complex is more stable ($k_{decomposition} = 0.01$ s$^{-1}$ at 25 °C) (72) than the P450 2A6 FeO$^{2+}$-camphor complex (Fig. 7C). Apparently the rate of introduction of the second electron is rate-limiting in the P450 101A1 oxidation of camphor (5, 72). Deuterium isotope effects are low for camphor hydroxylation (106) but can
rise to 11-12 for slowly oxidized substrates (107). Thus, P450 101A1 can be viewed as having a very well-defined linear pathway through the catalytic cycle (e.g., Scheme 1) with clear intermediates (although all seem to change structures) (108). P450 2A6, even though binding the substrate tightly at the start of the cycle (Scheme 1), has a much “noisier” trajectory, with considerable ligand exchange, slower forward reaction rates, and more unstable oxygenated intermediates. It is not clear whether the inefficiency of these earlier oxygen activation steps contributes to the high kinetic isotope effects or whether this is largely influenced by inherently poor geometry of the FeO\textsuperscript{3+}-substrate complex (or intrinsically weaker chemical reactivity of the FeO\textsuperscript{3+} complex of some P450s). Some wobble does exist in the enzyme despite the low active site volume (26), as evidenced by the fraction of 3-hydroxylation observed for the 7-OR coumarins.

In concluding, a classical paradigm such as that shown in Scheme 1 may be too simplistic for an enzyme such as P450 2A6, which is very dynamic and introduces extra dimensions throughout the catalytic cycle, disrupting the smooth track normally assumed. We are interested in applying similar approaches to other human (and other) P450 enzymes to develop a better understanding of the factors that influence the rates and efficiencies of P450-catalyzed reactions. The demonstrated high rates of ligand exchange are of particular interest regarding substrates (e.g., several steroids) that appear to be sequentially utilized by P450s in multiple steps (33, 109).

Acknowledgments—We thank M. V. Martin and W. A. McCormick for preparing the enzymes used here and K. Trisler for her assistance in preparation of the manuscript.
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**FOOTNOTES**

*This work was supported in part by a Korea Research Foundation Grant (KRF-2000-015-FS002) and United States Public Health Service Grants R01 CA90426, P30 ES00267, and F32 ES12123. The costs of publication of this article were defrayed in part by the payment of page charges. This article must be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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The abbreviations used are: P450, cytochrome P450; OH, hydroxy; OMe, methoxy; OEt, ethoxy; OR, alkoxy; di-12:0 GPC, L-α-1,2-dilauroyl-sn-glycero-3-phosphocholine; MS, mass spectrometry; bs, cytochrome bs; Em, standard oxidation-reduction potential at pH 7.0. The conventions used for kinetic hydrogen isotope effects are $^{d}k_\text{cat}$ = intrinsic kinetic deuterium isotope effect, $^{d}V = ^{H}k_\text{cat}$/$^{D}k_\text{cat}$, and $^{d}(V/K) = (^{H}k_\text{cat}/^{D}K_\text{m})/(^{D}k_\text{cat}/^{H}K_\text{m})$ (1, 2).

3-Hydroxycoumarin was prepared (Experimental Procedures) and used as a standard for HPLC assays. A baculovirus-infected insect cell microsomal system was used in the earlier study (62) but no positive control for 7-hydroxylation was involved.

Higher rates of coumarin hydroxylation were observed when organic solvents were omitted from the reaction. Although microsomal coumarin 7-hydroxylation has been reported not to be very sensitive to CH3OH or dimethylsulfoxide (50), we found inhibitory effects of CH3OH and

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C$_2$H$_5$OH in our work with the reconstituted enzyme system. Coumarin stocks can be prepared at 5 mM in H$_2$O and stored at 4 °C without any difficulty.

When NADPH-P450 reductase and the limited amount of NADPH were mixed in the presence of a slight excess of ferric cytochrome c, the extent of reduction of the cytochrome c ($\Delta\varepsilon_{550} = 2.1 \times 10^4$ M$^{-1}$ cm$^{-1}$) corresponded to 97% of the amount expected based on two cytochrome c molecules being reduced/NADPH (i.e., 5.0 nmol NADPH and 5.0 nmol NADPH-P450 reductase yielded 9.7 nmol of cytochrome c reduced).

The above experiments were all done with coumarin, for which the 7-hydroxylation reaction is stimulated by $b_5$. However, the reason for the general lack of enhancement of the oxidations of the 7-OR coumarins is yet unexplained, and the above spectral experiments have not been extended to P450 2A6 in the presence of the other substrates.

The observed values we report here are not partitioned into primary and secondary kinetic isotope effects. Geminal secondary isotope effects are much lower than primary isotope effects ($\leq 1.4$) (73, 86). Relatively few secondary deuterium isotope effects have been measured in P450 reactions (87-89), and some of these were measured with crude microsomal systems, not individual enzymes (87, 88). The values ($\leq 1.3$) cannot be transposed to the P450 2A6 reactions under consideration here, i.e. 7-OR coumarin $O$-dealkylation. However, a reviewer has pointed out that, for [methyl-d$_3$]-7-OMe coumarin $O$-demethylation, $^{D}k_{obs} = ^{D}k^{1°}/^{D}k^{2°}$ (where $1°$ and $2°$ denote the true primary and secondary isotope effects). If $^{D}k^{2°}$ were 1.1, then with $^{D}k_{obs} = 9.8$ (Table III), $^{D}k^{1°} = 10.8$. However, in the intermolecular non-competitive experiment with [methyl-d$_3$]-7-OMe coumarin, the $^{D}(V/K)^2$ should be raised to the third power, or $1.1^3 = 1.3$, and $^{D}(V/K)_{obs} = ^{D}(V/K)^{1°}^{D}(V/K)^{2°}$. With $^{D}(V/K)_{obs} = 9.0$ (Table III), then $^{D}(V/K)^{1°} = 6.9$. Thus, some attenuation of the intramolecular isotope effect might not have been noticed in the comparisons, and the effect would be more dramatic if the secondary isotope effect is higher. In the absence of precise estimates of the (low) secondary isotope effects, we have accordingly avoided concluding that no suppression of the primary intrinsic isotope effect occurs in the intermolecular experiments. Nevertheless, the observed intermolecular $^{D}(V/K)$ values are still relatively high and are used in conclusions about the behavior of the enzyme reactions.

We have tried to avoid expressing a conclusion that the C-H bond-breaking step in 7-OR coumarin $O$-dealkylation is “rate-limiting.” The difficulties of reaching conclusions about rate-limiting steps in enzyme reactions have been addressed by Northrop (90, 91), demonstrating that similar perturbations of reaction steps occurring either prior to or after the C-H bond-breaking step can have varying and somewhat surprising effects (90). A further complication exists with P450-catalyzed oxidations in that multiple alternate pathways often follow the apparently irreversible step of O$_2$ activation, as opposed to a model in which the conversion of an enzyme-substrate complex to product is a rather linear pathway (90). The point has been raised that the observation of a $^{D}(V/K)$ isotope effect requires the existence of P450 branching after the irreversible step; i.e. decomposition or reduction of activated Fe-O complexes or “metabolic switching” to alternate oxygenations of the substrate (92, 93). This view has some validity (90) but does not offer a complete explanation for the observed high values of $^{D}(V/K)$ (or $^{D}V$) presented in this study. One issue is that most microsomal P450s have poor coupling efficiency (31, 33, 34), including P450 2A6 (Table V). However, in kinetic simulations with P450 1A2 reactions using a simple model that has an irreversible O$_2$ activation step followed by steps involving Fe-O complex decomposition (to H$_2$O$_2$ and H$_2$O) and production of a product derived from the substrate (C-H bond-breaking), $^{D}V$ and $^{D}(V/K)$ both shared an inverse hyperbolic relationship with the rate of the C-H bond-breaking step (31). Moreover, the observed kinetic
isotope effects can very considerably with different P450s and different reactions, even though all are of low efficiency (31, 34). The variability of observed kinetic isotope effects in various P450 reactions (28, 29, 31, 34, 94) is further extended in further work with human P450s 2E1 and 3A4 (95, 96). Thus, the branched nature of the P450 reactions can contribute to the higher expression of kinetic isotope effects (92, 93) but is not a sufficient explanation in the absence of further kinetic details about particular reactions.

**SCHEME LEGENDS**

Scheme 1. Catalytic cycle of P450. Adapted from previous reviews (30, 33, 34). Step 5 can be divided into two parts: (a) Fe$^{II}$$\cdot$O$_2$ + H$^+$ $\rightarrow$ Fe$^{III}$-OOH, and (b) FeOOH $\rightarrow$ Fe$^{III}$ although the dioxygen species have also been postulated to catalyze certain oxidations directly (35). Step 6 can also be divided into two parts: (a) Fe$^{III}$ + RH $\rightarrow$ [FeOH$^{III}$ R·], and (b) [FeOH$^{III}$ R·] $\rightarrow$ Fe$^{III}$ + ROH.

Scheme 2. Oxidations of coumarins catalyzed by P450 2A6.

Scheme 3. Outline of kinetic deuterium isotope effect experiments. A, Non-competitive intramolecular experiment for approximating the intrinsic isotope effect (with a methyl; prochirality is a potential confounder with a methylene). B, Competitive intermolecular experiment. C, Non-competitive intermolecular experiment (provides information about how rate-limiting the C-H bond breaking step is in the overall steady-state reaction. This experiment provides information about the relative rates of exchange of substrates, i.e. if attenuation of the intrinsic isotope effect is observed then exchange rates are partly rate-limiting (1, 2).

**FIGURE LEGENDS**

Fig. 1. Spectra of P450 2A6 complexes. A, Spectra were recorded in 50 mM potassium phosphate buffer (pH 7.4) with 5.2 µM P450 2A6, either with or without 50 µM coumarin. The ferrous form was produced by the addition of a few grains of Na$_2$S$_2$O$_4$. The inset (B) shows an expansion of the α,β-region. Ferric P450 2A6 (Fe$^{III}$), —; ferric P450 2A6-coumarin complex (Fe$^{III}$$\cdot$S), —·—·—; ferrous P450 2A6-coumarin complex (Fe$^{II}$$\cdot$S),  ————.

Fig. 2. Binding of coumarin to ferric (Fe$^{III}$) P450 2A6. A, Difference spectra obtained by titration of a 2 µM P450 solution (100 mM potassium phosphate, pH 7.4) with increasing concentrations (spectra shown with arrows) of coumarin (to 7.5 µM). B, Plot of data from part A; fit to a quadratic expression $K_d = ([E_t-S][S_t-E\cdot S])/ES$, where $E_t$ = total P450 concentration, $S_t$ = total coumarin concentration, and $ES$ = P450-coumarin complex (66) yielded $K_d = 0.38$ (± 0.03) µM. C, Rate of $\Delta A_{390}$ for a similar binding experiment with 8 µM coumarin, fit to a single exponential of 17 s$^{-1}$. D, $k_{obs}$ for traces obtained in Part C as a function of coumarin concentration. From $k_{obs} = k_{on}[S] + k_{off}$ (67), $k_{on} = 2.7 \times 10^6$ M$^{-1}$ s$^{-1}$ and $k_{off} = 5.7$ s$^{-1}$ ($k_{off}/k_{on} = 2.1$ µM).

Fig. 3. Binding of 7-OH coumarin to ferric (Fe$^{III}$) P450 2A6 (product release). A, Difference spectra obtained by titration of a 2 µM P450 solution (100 mM potassium phosphate, pH 7.4) with increasing concentrations (spectra shown with arrows) of 7-OH coumarin (to 8 µM). B, Plot of data from Part A; fit to a quadratic expression of $K_d = ([E_t-S][S_t-E\cdot S])/ES$, where $E_t$ = total P450 concentration, $S_t$ = total 7-OH coumarin concentration, and $ES$ = P450-7-OH coumarin complex (66) yielded $K_d = 0.82$ (± 0.05) µM. C, Rate of $\Delta A_{430}$ for a similar binding experiment with 16 µM 7-OH coumarin, fit to a single exponential of 18 s$^{-1}$. D, $k_{obs}$ for traces obtained in Part C as a function of 7-OH coumarin concentration. From $k_{obs} = k_{on}[S] + k_{off}$ (67), $k_{on} = 2.0 \times 10^6$ M$^{-1}$ s$^{-1}$ and $k_{off} = 6.8$ s$^{-1}$ ($k_{off}/k_{on} = 3.4$ µM).
**Fig. 4.** Lack of kinetic bursts in oxidations of coumarins to form 7-OH coumarin. In each case a reaction was done at 22 °C in the stopped-flow spectrofluorimeter (OLIS RSM-1000) by adding 22 µM NADPH and an NADPH-generating system (49) from one syringe to a typical mixture (final concentrations indicated) of P450 2A6 (0.5 µM), NADPH-P450 reductase (1.0 µM), b5 (0.5 µM), di-12:0 GPC (45 µM), and the substrate. Fluorescence excitation was at 390 nm and emission was monitored at 420 nm. The substrate concentrations were 50, 200, and 200 µM for coumarin, 7-OMe coumarin, and 7-OEt coumarin, respectively. The fluorescence corresponding to 0.5 µM 7-OH coumarin (indicative of one enzyme cycle) was determined by comparison with a conventional mixing experiment done with these preparations and coumarin in an OLIS DM45 spectrofluorimeter. The vertical line at t = 0 is the mixing artifact. The origins of the four traces are not intended to be identical, due to variations in the background fluorescence (as a result of both the coumarins and the NADPH).

**Fig. 5.** Reduction of ferric (Fe³⁺) P450 2A6. Experiments were done anaerobically, under a CO atmosphere, in an OLIS RSM-1000 stopped-flow spectrophotometer. One syringe contained NADPH (300 µM) in 100 mM potassium phosphate buffer (pH 7.4) The other tonometer contained a mixture of P450 2A6 (1.0 µM), NADPH-P450 reductase (4 µM), di-12:0 GPC (45 µM) in 100 mM potassium phosphate buffer (pH 7.4). Coumarin (40 µM) was also included when indicated. The inset shows an expansion of the plot with coumarin present. Rates were fit to exponential plots using the manufacturer’s software: without coumarin, 0.13 s⁻¹ (no correction for the apparent lag); + coumarin: bi-exponential fit of 7.5 s⁻¹ and 0.13 s⁻¹.

**Fig. 6.** Binding of coumarin to ferrous (Fe²⁺) P450 2A6. A, Difference spectrum obtained with 2.6 µM P450 2A6 with and without 50 µM coumarin (in 50 mM potassium phosphate buffer, pH 7.4). Both cuvettes were reduced with solid Na₂S₂O₄. B, Kinetics of ΔA₄₃₈ observed upon addition of 15 µM coumarin to ferrous P450 2A6. The experiment was done in a stopped-flow spectrophotometer. One syringe contained 100 µM coumarin (in 50 mM potassium phosphate buffer, pH 7.4) and the other contained P450 2A6 (4 µM), NADPH-P450 reductase (4 µM), di-12:0 GPC (45 µM), potassium phosphate buffer (50 mM potassium phosphate buffer, pH 7.4), and an NADPH-generating system composed of 10 mM glucose 6-phosphate, 0.5 mM NADPH, and IU yeast glucose 6-phosphate dehydrogenase ml⁻¹ (49). Both syringes were under an anaerobic environment (Ar). The data fit to a single exponential of 57 s⁻¹. C, k_obs for traces obtained in part B as a function of coumarin concentration. Fitting to the expression k_obs = k_on [S] + k_off yielded values of k_on = 1.5 x 10⁶ M⁻¹ s⁻¹ and k_off = 36 s⁻¹ (K_d = k_off/k_on = 24 µM).

**Fig. 7.** Reaction of ferrous P450 2A6 with O₂. P450 2A6 (4.9 nmol) was photoreduced under an Ar atmosphere (in the presence of 50 µM coumarin, 1.0 µM 5-deazaflavin, and 1.0 µM safranine T in 3.0 ml buffer as described under Fig. 7). Reduction was monitored (e.g. Fig. 2) in the OLIS/Cary 14 spectrophotometer and was complete after 3 min of

**Fig. 8.** Oxidation of coumarin to 7-OH coumarin in limited cycle experiments. A, P450 2A6 (5 nmol) was photoreduced under an Ar atmosphere (in the presence of 50 µM coumarin, 1.0 µM 5-deazaflavin, and 1.0 µM safranine T in 3.0 ml buffer as described under Fig. 7). Reduction was monitored (e.g. Fig. 2) in the OLIS/Cary 14 spectrophotometer and was complete after 3 min of
irradiation (36). The sample was mixed with air and quenched by the addition of 0.3 ml of 43% H$_3$PO$_4$ after 3 min. Products were extracted three times with 3.0 ml CH$_2$Cl$_2$ and the combined organic phase was concentrated to dryness under an N$_2$ stream and analyzed by HPLC using the fluorescence method of Soucek (58). The scale used in Part A is expanded 8-fold compared to the other parts. B, The experiment was as in Part A, except that 5 nmol of $b_5$ was included (and photoreduced before oxygenation). C, The experiment was as in Part A, except that 5 nmol of NADPH-P450 reductase was included and 12.5 nmol of NADPH was added anaerobically to achieve reduction (instead of photoreduction). D, The experiment was as in Part C except that 5 nmol of $b_5$ were also present and 15 nmol of NADPH were used for reduction.

**Fig. 9** Effect of deuterium substitution on oxidations of 7-OMe and 7-OEt coumarin by P450 2A6. Typical steady-state reaction conditions were used with $d_0$ (part A) or [methyl-$d_3$] 7-OMe coumarin (part B) or $d_0$ (part C) or [1-ethyl-$d_2$] 7-OEt coumarin (part D) and the products were analyzed by HPLC (100 µM substrate in each case).

**Fig. 10** Spectra of P450 2A6 coumarin-oxidizing reaction. One syringe of the stopped-flow instrument contained P450 2A6 (10 µM), NADPH-P450 reductase (20 µM), $b_5$ (10 µM), di-12:0 GPC (50 µM), coumarin (100 µM), glucose 6-phosphate (10 mM), yeast glucose 6-phosphate (1 IU ml$^{-1}$), and potassium phosphate buffer (100 mM, pH 7.4). The other syringe contained 100 µM NADPH in 100 mM potassium phosphate buffer (pH 7.4). The contents of the syringes were mixed and spectra were recorded (23 °C). A, The indicated spectrum was observed at 58 ms and the subsequent traces were recorded every 100 ms later, to an elapsed time of 858 ms. The decrease in $A_{390}$ was fit to a single exponential of 20 s$^{-1}$. B, The indicated spectra were recorded every 3 s after mixing and correspond to a slow reduction of the $b_5$ in the mixture. The decrease in $A_{409}$ was fit to a single exponential of 0.14 s$^{-1}$ and the increase in $A_{424}$ to 0.17 s$^{-1}$.

**Fig. 11** Oxidation of reduced $b_5$ by ferrous-oxy P450 2A6 (FeO$_2^{2+}$). $b_5$ (3.0 µM), in 50 mM potassium phosphate buffer (pH 7.4) containing 75 µM di-12:0 GPC, 100 mM Tris·HCl, 20 mM EDTA, 50 µM coumarin, and 1 µM 5-deazaflavin (36), was photoreduced and introduced into one side of the stopped-flow spectrophotometer. The sample was mixed with air-saturated 50 mM potassium phosphate buffer (pH 7.4), with a nominal O$_2$ concentration of 200 µM. A, Spectral traces of the reaction as described, with the first spectrum shown at 160 ms. Spectra were recorded every 3 s and progressed in the directions shown with the arrows. B, The data from Part A were fit to a single exponential with a rate of 0.13 s$^{-1}$. C, The experiment of Part A was repeated in the presence of 3.0 µM P450 2A6. The first spectrum shown was recorded at 800 ms and the subsequent spectra were recorded every 3 s. D, Data from Part C ($\Delta A_{410}$) were fit to a bi-exponential equation with $k_1 = 3.6$ s$^{-1}$ and $k_2 = 0.04$ s$^{-1}$. 
<table>
<thead>
<tr>
<th>Substrate</th>
<th>Without $b_5$</th>
<th></th>
<th></th>
<th>With $b_5$</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$k_{\text{cat}}$</td>
<td>$K_m$</td>
<td>$k_{\text{cat}}$</td>
<td>$K_m$</td>
<td>$k_{\text{cat}}$</td>
<td>$K_m$</td>
</tr>
<tr>
<td>Coumarin</td>
<td>0.075 ± 0.008</td>
<td>1.8 ± 0.7</td>
<td>0.148 ± 0.017</td>
<td>1.4 ± 0.6</td>
<td></td>
<td></td>
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<tr>
<td>7-OMe coumarin</td>
<td>0.040 ± 0.002</td>
<td>13 ± 2</td>
<td>0.016 ± 0.001</td>
<td>12 ± 3</td>
<td>0.037 ± 0.002</td>
<td>10 ± 2</td>
</tr>
<tr>
<td>7-OEt coumarin</td>
<td>0.045 ± 0.003</td>
<td>39 ± 7</td>
<td>0.133 ± 0.023</td>
<td>120 ± 30</td>
<td>0.033 ± 0.003</td>
<td>10 ± 4</td>
</tr>
</tbody>
</table>

TABLE I

Steady-state parameters for coumarin oxidation catalyzed by P450 2A6
<table>
<thead>
<tr>
<th>System</th>
<th>7-OH coumarin</th>
<th>( \text{nmol} )</th>
<th>% theo. yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>P450 2A6 (h( \nu ))(^a)</td>
<td></td>
<td>0.09</td>
<td>4(^b)</td>
</tr>
<tr>
<td>P450 2A6, ( b_3 ) (h( \nu ))(^a)</td>
<td></td>
<td>0.8</td>
<td>16(^c)</td>
</tr>
<tr>
<td>P450 2A6, reductase (NADPH)(^d)</td>
<td></td>
<td>0.66</td>
<td>7(^d)</td>
</tr>
<tr>
<td>P450 2A6, reductase, ( b_3 ) (NADPH)(^d)</td>
<td></td>
<td>1.40</td>
<td>14(^e)</td>
</tr>
</tbody>
</table>

\(^{a}\)h\( \nu \) = photochemical reduction.

\(^{b}\) Yield = 0.092 nmol 7-OH coumarin/5 nmol P450, \( \times 2 \) (correction for 2 e\(^-\) needed) = 0.04.

\(^{c}\) Yield = 0.80 nmol 7-OH coumarin/(5 nmol P450 + 5 nmol \( b_3 \)) = 0.16.

\(^{d}\) Yield = 0.66 nmol 7-OH coumarin/(5 nmol P450 + 5 nmol NADPH-P450 reductase + 12.5 nmol NADPH) = 0.66 nmol 7-OH coumarin/10 nmol reducing equivalents = 0.07.

\(^{e}\) Yield = 1.40 nmol 7-OH coumarin/(5 nmol P450 + 5 nmol \( b_3 \) + 5 nmol NADPH-P450 reductase + 15 nmol NADPH) = 1.40 nmol 7-OH coumarin/10 nmol reducing equivalents = 0.14.
<table>
<thead>
<tr>
<th>Substrate</th>
<th>Intramolecular&lt;sup&gt;a&lt;/sup&gt; (non-competitive)</th>
<th>Intermolecular&lt;sup&gt;b&lt;/sup&gt; competitive</th>
</tr>
</thead>
<tbody>
<tr>
<td>7-OMe coumarin</td>
<td>9.8 ± 0.1</td>
<td>9.0 ± 0.1</td>
</tr>
<tr>
<td>7-OEt coumarin</td>
<td>6.0 ± 0.4</td>
<td>7.0 ± 1.6</td>
</tr>
</tbody>
</table>

<sup>a</sup> See Scheme 3A. Estimated by analysis of formaldehyde (7-OMe coumarin) or acetaldehyde (7-OEt coumarin).

<sup>b</sup> See Scheme 3B. Estimated by analysis of formaldehyde (7-OMe coumarin) or acetaldehyde (7-OEt coumarin).
<table>
<thead>
<tr>
<th>Substrate</th>
<th>O-Dealkylation</th>
<th>3-Hydroxylation</th>
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<tbody>
<tr>
<td></td>
<td>$k_{\text{cat}}$</td>
<td>$K_m$</td>
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<tr>
<td>7-OMe coumarin</td>
<td></td>
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</tr>
<tr>
<td>$d_0$</td>
<td>2.4 ± 0.1</td>
<td>13 ± 2</td>
</tr>
<tr>
<td>$d_3$</td>
<td>0.25 ± 0.02</td>
<td>13 ± 3</td>
</tr>
<tr>
<td>7-OEt coumarin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$d_0$</td>
<td>2.7 ± 0.2</td>
<td>39 ± 7</td>
</tr>
<tr>
<td>$d_2$</td>
<td>0.34 ± 0.02</td>
<td>35 ± 4</td>
</tr>
<tr>
<td>Substrate</td>
<td>$b_s$</td>
<td>Rate</td>
</tr>
<tr>
<td>---------------------------</td>
<td>-------</td>
<td>----------------------------------------------------------------------</td>
</tr>
<tr>
<td></td>
<td></td>
<td>NADPH oxidation</td>
</tr>
<tr>
<td>Coumarin (20 $\mu$M)</td>
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<td>79 ± 3</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>40 ± 2</td>
</tr>
<tr>
<td>7-OMe coumarin (100 $\mu$M)</td>
<td>-</td>
<td>33 ± 1</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>17 ± 2</td>
</tr>
<tr>
<td>7-OEt coumarin (100 $\mu$M)</td>
<td>-</td>
<td>42 ± 2</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>21 ± 1</td>
</tr>
</tbody>
</table>

*Calculated by difference (69).*
Scheme 2

Coumarin

7-OMe Coumarin

7-OEt Coumarin

\[
\begin{align*}
\text{Scheme 2} & \\
\text{Coumarin} & \rightarrow \text{Coumarin} + \text{Coumarin} \\
7-\text{OMe} \text{ Coumarin} & \rightarrow \text{Coumarin} + \text{Coumarin}
\end{align*}
\]
Scheme 3

A

\[ R-\text{CHD}_2 \]

\[ \xrightarrow{k_1} R-\text{CHD-OH} \]

\[ \xrightarrow{k_2} R-\text{CD}_2\text{-OH} \]

\[ D_K = \frac{k_2}{k_1} \]

B

\[ R-\text{CH}_3 \]

\[ \xrightarrow{k_3} R-\text{CH}_2\text{-OH} \]

\[ \xrightarrow{k_4} R-\text{CD}_2\text{-OH} \]

\[ \text{comp}K = \frac{k_3}{k_4} \]

C

\[ R-\text{CH}_3 \]

\[ \xrightarrow{k_5} R-\text{CH}_2\text{-OH} \]

\[ \text{non-comp}K = \frac{k_5}{k_6} \]

\[ R-\text{CD}_3 \]

\[ \xrightarrow{k_6} R-\text{CD}_2\text{-OH} \]
Fig. 1

Absorbance

Wavelength, nm

A

B

Fe

Fe

Fe

Fe

Fe

Fe

Fig. 2C,D

![Graph C](image)

**Graph C**

![Graph D](image)

**Graph D**

- **ΔA_{390}** vs. **Time, s**
- **k_{obs}, s^{-1}** vs. **[Coumarin], μM**
Fig. 3C,D

C

\[ \Delta A_{430} \]

Time, s

D

\[ k_{\text{obs}}, \text{s}^{-1} \]

[7-OH Coumarin], \( \mu \text{M} \)
Fig. 4

F390/460, relative units

Time, s

0 50 100 150 200

0.5 µM

7-OH coumarin

No substrate

7-OEt coumarin

7-OMe coumarin

Coumarin
Fig. 5

\[ \Delta A_{450} \]

\[ \begin{align*}
\text{Time, s} \\
0 & 5 & 10 & 15 & 20 & 25 & 30 \\
\end{align*} \]

- substrate
+ coumarin

\[ 0.005 \]

Inset: 0.005
Fig. 6

A

Absorbance

Wavelength, nm

B

A_{438}

Time, s

C

k_{obs}, s^{-1}

[Coumarin], µM
Fig. 7

**A**

Absorbance vs. Wavelength, nm

- **t = 21 ms**

**B**

Absorbance vs. Wavelength, nm

- **t = 64 ms**

**C**

A390 vs. Time, s

- **Time, s**
  - 0 2.5 5.0 7.5 10 12.5
  - Absorbance
    - 1.00
    - 0.95
    - 0.90
Fig. 8

A. P450

B. P450, b5

C. P450, reductase

D. P450, reductase, b5

$F_{338/440}$

$t_R$, min
Fig. 9

A

B

C

D

\[ A_{330} \]

\[ t_R, \text{ min} \]
Fig. 10

Absorbance

Wavelength, nm

A

B

Wavelength, nm

Absorbance

t = 58 ms
Fig. 11

A

B

C

D

Absorbance

Wavelength, nm

Time, s

A424

A410
**Supplementary Data**

for M4-11019

Chul-Ho Yun, Keon-Hee Kim, M. Wade Calcutt, and F. Peter Guengerich

Kinetic Analysis of Oxidation of Coumarins by Human Cytochrome P450 2A6

*J. Biol. Chem.* **280**, 000-000 (2005)

**FIG. 1**

HPLC of P450 2A6-generated oxidation products of coumarins. The substrate concentration was 100 µM. The products were identified by chromatography with synthetic standards. The large peak eluting at 4.3 min is the substrate coumarin; 7-OH coumarin elutes at 2.8 min. Standard 3-OH coumarin eluted at 3.7 min in a separate run. See Table I of main section of paper.