Human cytochrome P450 (P450) 2A6 catalyzes 7-hydroxylation of coumarin, and the reaction rate is enhanced by cytochrome b5 (b5). 7-Alkoxyxycoumarins 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 ($K_{on} \sim 10^{6} \text{ M}^{-1} \text{ s}^{-1}$), and the $k_{on}$ rates range from 5.7 to 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 ($k \sim 10^{6} \text{ M}^{-1} \text{ s}^{-1}$), and the putative Fe$^{2+}$/O$_2$ complex decayed at a rate of $0.3 \text{ s}^{-1}$ at 23 °C. Some 7-hydroxyxycoumarin was formed during the oxidation of the ferrous enzyme under these conditions, and the yield was enhanced by $b_5$. Kinetic analyses showed that $-1/6$ 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 (values 6–10) were measured for O-dealkylation of 7-alkoxyxycoumarins, 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 toward a linear pathway leading to product formation explain the inefficiency of the enzyme towards highly efficient bacterial P450s.

P450$^1$ 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 2C6 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-alkylnitrosamines (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...
salicylaldehyde (Aldrich) in CH₂Cl₂ (80% yield) (43). The identities of the 3-OH coumarins were confirmed by their m.p. values and spectros-
copy, 3-OH coumarin, m.p. 151–154 °C (literature (44), 154 °C), UV
(CH₃OH) ε₂₃₀ 1.22 × 10⁴ M⁻¹ cm⁻¹, ε₂₅₃ 1.19 × 10⁴ M⁻¹ cm⁻¹, ε₂₆₈ 4.91 ×
10³ M⁻¹ cm⁻¹, fluorescence (CH₃OH) λₐ₅₆₅ 310 nm, λₐ₅₅₈ 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-OEt coumarin, m.p. 179–182 °C
(literature (45), 175.5–177.5 °C), UV (CH₃OH) ε₂₃₀ 1.4 × 10⁴ M⁻¹ cm⁻¹,
ε₂₅₃ 5.8 × 10³ M⁻¹ cm⁻¹, ε₂₆₈ 8.4 × 10³ M⁻¹ cm⁻¹, electrospray MS, m/z
193.1 (MH⁺), NMR (CDCl₃) δ 7.39 (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,
m.p. 155–157 °C, UV (CH₃OH) ε₂₃₀ 1.34 × 10⁴ M⁻¹ cm⁻¹, ε₂₅₃ 5.6 × 10³
M⁻¹ cm⁻¹, ε₂₆₈ 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, National Institute of Public Health, Prague) in
Escherichia coli, except that a His₅ 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 modifica-
tions of the DEAE-cellulose and hydroxylapatite chromatography meth-
ods 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-elect-
rospray, or atmospheric pressure chemical ionization) in the Vander-
bilt 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, Edin-
burgh, 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
Kinetics of P450 2A6 Coumarin Oxidation

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 Supplemental Fig. 1). 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.2

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 ofobox 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 Km 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

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.

3-Hydroxycoumarin was prepared (see "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 micromolar coumarin 7-hydroxylation has been reported not to be very sensitive to CH3OH or dimethylsulfoxide (50), we found inhibitory effects of CH3OH and C2H5OH in our work with the reconstituted enzyme system. Coumarin stocks can be prepared at 5 mM in H2O and stored at 4 °C without any difficulty.
Type I" difference spectra, with $K_s = 0.38 \pm 0.03$ $\mu$M (Fig. 2, A and B).

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 two-state system ($Fe^{3+} + S \rightleftharpoons Fe^{3+}S$), i.e. $k_{obs} = k_{on}[S] + k_{off}$ (67) and yielding $k_{on} = 2.7 \times 10^9$ $M^{-1}$ $s^{-1}$ and $k_{off} = 5.7$ $s^{-1}$ ($k_{off}/k_{on} = 2.1$ $\mu$M).

**FIG. 2.** Binding of coumarin to ferric ($Fe^{3+}$) P450 2A6. A, difference spectra obtained by titration of a 2 $\mu$M P450 solution (100 mM potassium phosphate, pH 7.4) with increasing concentrations (spectra shown with arrows) of coumarin (to 8 $\mu$M). B, plot of data from A, fit to a quadratic expression $K_y = ([E] - [E-S])([S] - [E-S])/[E][S]$, where $K_y$ total P450 concentration, $S$ = total coumarin concentration, and $ES = P450$-coumarin complex (66) yielded $K_y = 0.38 \pm 0.03$ $\mu$M. C, rate of $k_{obs}$ for a similar binding experiment with 8 $\mu$M coumarin, fit to a single exponential of 17 $s^{-1}$. D, $k_{obs}$ for traces obtained in C as a function of coumarin concentration. From $k_{obs} = k_{on} [S] + k_{off}$ (67), $k_{on} = 2.7 \times 10^9$ $M^{-1}$ $s^{-1}$, and $k_{off} = 5.7$ $s^{-1}$ ($k_{off}/k_{on} = 2.1$ $\mu$M).

**FIG. 3.** Binding of 7-OH coumarin to ferric ($Fe^{3+}$) P450 2A6 (product release). A, difference spectra obtained by titration of a 2 $\mu$M P450 2A6 solution (100 mM potassium phosphate, pH 7.4) with increasing concentrations (spectra shown with arrows) of 7-OH coumarin (to 8 $\mu$M). B, plot of data from A, fit to a quadratic expression yielded $K_y = 0.82 \pm 0.05$ $\mu$M. C, rate of $k_{obs}$ for a similar binding experiment with 16 $\mu$M 7-OH coumarin, fit to a single exponential of 18 $s^{-1}$. D, $k_{obs}$ for traces obtained in C as a function of 7-OH coumarin concentration. From $k_{obs} = k_{on} [S] + k_{off}$ (67), $k_{on} = 2.0 \times 10^9$ $M^{-1}$ $s^{-1}$ and $k_{off} = 6.8$ $s^{-1}$ ($k_{off}/k_{on} = 3.4$ $\mu$M).

"Type I" difference spectra, with $K_s = 0.38 \pm 0.03$ $\mu$M (Fig. 2, A and B).

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 two-state system ($Fe^{3+} + S \rightleftharpoons Fe^{3+}S$), i.e. $k_{obs} = k_{on}[S] + k_{off}$ (67) and yielding $k_{on} = 2.7 \times 10^9$ $M^{-1}$ $s^{-1}$ 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).
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).

**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, $K_{d} = 0.82 \pm 0.05$ μM (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$^{-1}$ ($k_{off}k_{on} \approx 3.4$ μM$^{-1}$).

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 after 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 ferrous P450 2A6 produces a major spectral change, the changes observed upon the addition of ferrous P450 2A6 are much weaker. A difference spectrum 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 $k_{on} = 1.5 \times 10^{6}$ M$^{-1}$ s$^{-1}$ and $k_{off} = 36$ s$^{-1}$ ($k_{off}k_{on} = 24$ μM$^{-1}$) (Figs. 6, B and C).

**Formation (Step 3 of Scheme 1) and Decomposition of Ferrous P450 2A6-O$_2$ 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 O$_2$). A rapid spectral change was observed, and

FIG. 5. Reduction of ferric (Fe$^{3+}$) 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 syringe contained a mixture of P450 2A6 (1.0 μM), NADPH-P450 reductase (2.0 μM), and 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); with coumarin, bi-exponential fit of 7.5 s$^{-1}$ and 0.13 s$^{-1}$.

The early spectra are shown in Fig. 7A. The rate of this reaction, as estimated from $A_{450}$, was $\approx 75$ 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$^{-1}$ ($t_{1/2} = 2.3$ 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 photo-reduced 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).

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 FeO$_2^{2-}$ complexes can interact to provide the second electron for product formation (e.g. $2$FeO$_2^{2-} \rightarrow$ FeO$_2^{4-}$ (--- FeO$^{3+}$) + Fe$^{3+}$). 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 photo-reduced P450 2A6 and P450 2A6 that was reduced with NADPH/reductase, the presence of reduced b5 raised the yield of product (Fig. 8, Table II). This result suggests that ferrous b5 might be able to transfer electrons to the P450 2A6 FeO$_2^{2-}$-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 O$_2$ 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

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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 ($\Delta A_{550}$ = 2.1 × 10$^{-5}$ 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 of NADPH and 5.0 nmol of NADPH-P450 reductase yielded 9.7 nmol of cytochrome c reduced).
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 (see 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 (see Scheme 3B). Attenuation of the intrinsic isotope effect in such an experiment is suggestive of a 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 koff rate for the substrate coumarin (Fig. 2D).

The third type of experiment used was a non-competitive intermolecular system (see 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 with the intrinsic
Fig. 8. Oxidation of coumarin to 7-OH coumarin in limited cycle experiments. A, P450 2A6 (5 nmol) was photo-reduced under an Ar atmosphere (in the presence of 50 μm coumarin, 1.0 μm 5-deazaflavin, and 1.0 μm safranin T in 3.0 ml of 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% H₂PO₄ after 3 min. Products were extracted 3 times with 3.0 ml of CH₂Cl₂, and the combined organic phase was concentrated to dryness under an N₂ stream and analyzed by HPLC using the fluorescence method of Soucek (58). The scale used in A is expanded 8-fold compared with the other parts. B, the experiment was as in A, except that 5 nmol of b₅ was included and photo-reduced before oxygenation. C, the experiment was as in 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 C except that 5 nmol of b₅ were also present, and 15 nmol of NADPH were used for reduction.

TABLE II
Yields of products formed from coumarin in limited cycle experiments hr, photochemical reduction.

<table>
<thead>
<tr>
<th>System</th>
<th>7-OH coumarin</th>
<th>nmol</th>
<th>% theoretical yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>P450 2A6 (hr)</td>
<td>0.09</td>
<td>4%</td>
<td></td>
</tr>
<tr>
<td>P450 2A6, b₅ (hr)</td>
<td>0.8</td>
<td>16%</td>
<td></td>
</tr>
<tr>
<td>P450 2A6, reductase (NADPH)</td>
<td>0.66</td>
<td>7%</td>
<td></td>
</tr>
<tr>
<td>P450 2A6, reductase, b₅ (NADPH)</td>
<td>1.40</td>
<td>14%</td>
<td></td>
</tr>
</tbody>
</table>

a Yield = 0.092 nmol of 7-OH coumarin/5 nmol of P450, × 2 (correction for 2e⁻ needed) = 0.04.
b Yield = 0.80 nmol of 7-OH coumarin/5 nmol of P450 + 5 nmol of b₅ = 0.16.
c Yield = 0.66 nmol of 7-OH coumarin/5 nmol of P450 + 5 nmol of NADPH-P450 reductase + 12.5 nmol of NADPH = 0.66 nmol of 7-OH coumarin/10 nmol of reducing equivalents = 0.07.
d Yield = 1.40 nmol of 7-OH coumarin/5 nmol of P450 + 5 nmol of b₅ + 5 nmol of NADPH-P450 reductase + 15 nmol of NADPH = 1.40 nmol of 7-OH coumarin/10 nmol of reducing equivalents = 0.14.

isotope effect (see Scheme 3A, Table III). High kinetic isotope effects (both ¹⁷V and ¹⁷(V/K)) were observed in these experiments (Fig. 9, Table IV) (regardless of whether b₅ 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-O-Me 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 FeO²⁺-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).

Stoichiometry of NADPH Utilization—Rates of NADPH oxidation, formation of products, and H₂O₂ formation were measured, and H₂O₂ formation was calculated by difference (69) (Table V). The results indicate that formation of coumarin products is a relatively inefficient process, with the bulk of the NADPH used to reduce O₂. As in the report of Tan et al. (63) with a baculovirus-based system, the addition of b₅ to P450 2A6 suppressed the formation of H₂O₂. The suppression of H₂O₂ formation was also noted in the presence of the 7-OR coumarin substrates, although formation of the coumarin product was not enhanced (except for 7-OEt coumarin 3-hydroxylation).

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, b₅, di-12:2 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 λₘₐₓ was ~415 nm and probably represents some iron-oxygen complex (or a mixture of more than one). It is not the ferric nor ferrous FeO²⁺ complex (Fig. 7). This spectrum persisted for several seconds, and then the reduction of b₅ was observed (Fig. 10B). The reduction appeared to follow first-order kinetics (as judged by ΔA₄₀₅ or ΔA₄₂₄, with k = 0.15 s⁻¹). In other experiments with no P450 present, we measured the reduction of b₅ to be a bi-exponential process, with a first step (stoichiometric with 1 b₅ per NADPH-P450 reductase) of 18 s⁻¹ and a subsequent rate of 1.7 s⁻¹ (results not shown).

Oxidation of Ferrous b₅—One hypothesis for the enhancement of the catalytic activity of P450 2A6 by b₅ is the transfer of an electron from ferrous b₅ to the P450 FeO²⁺-substrate complex (70, 71). This hypothesis has some support in the limited turnover experiments (Table II), where yields were enhanced in the presence of b₅. However, alternate hypotheses could be valid (e.g. improvement of P450 efficiency through protein-protein interactions).

b₅ was photo-reduced 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 (photo-reduced) P450 2A6 reacts rapidly with O₂ to form an oxygenated complex (Fig. 7A); if reduced b₅ is present, it might transfer electrons to this FeO²⁺ complex before the complex decomposes. In this experiment (Fig. 11C), reduction was faster than in the absence of P450 2A6. About ⅓ of the b₅ was oxidized rapidly (as judged by ΔA₄₁₀ or ΔA₄₂₄ measurements, and the data were fit to a bi-exponential plot with k₁ = 3.6 s⁻¹ and k₂ = 0.04 s⁻¹ (Fig.
We conclude that $b_5$ 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 (280 Å$^3$), which is filled by the substrate coumarin (26) and binds the substrate reasonably tightly ($K_d \approx 1 \mu M$, Fig. 2B). However, catalysis is still relatively slow and inefficient in the context of the use of electrons for substrate oxidation (Tables I and 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$^+$ 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 (FeO and the C-3 atom of the coumarin (COH$^2$.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 and 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.

**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 $d_1$ (A) or (methyl-CH$_3$)-7-OMe-coumarin (B) or $d_2$ (C), or 7-ethyl(1,4-d)_7-OEt-coumarin (D), and the products were analyzed by HPLC (100 μM substrate in each case).

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### Table IV

Intermolecular non-competitive kinetic deuterium isotope effects for 7-alkoxycoumarin O-dealkylation reactions catalyzed by P450 2A6

<table>
<thead>
<tr>
<th>Substrate</th>
<th>$k_{act}$ ($min^{-1}$)</th>
<th>$K_m$ (μM)</th>
<th>$V$ (nmol of product formed min$^{-1}$)</th>
<th>$V/K$ (nmol of product formed min$^{-1}$ (nmol of P450))$^{-1}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>7-OMe coumarin</td>
<td>$d_1$</td>
<td>2.4 ± 0.1</td>
<td>13 ± 2</td>
<td>9.8 ± 0.7</td>
</tr>
<tr>
<td>7-OMe coumarin</td>
<td>$d_2$</td>
<td>0.25 ± 0.02</td>
<td>13 ± 3</td>
<td>8.0 ± 0.7</td>
</tr>
<tr>
<td>7-OEt coumarin</td>
<td>$d_1$</td>
<td>2.7 ± 0.2</td>
<td>39 ± 7</td>
<td>8.0 ± 0.7</td>
</tr>
<tr>
<td>7-OEt coumarin</td>
<td>$d_2$</td>
<td>0.34 ± 0.02</td>
<td>35 ± 4</td>
<td>8.0 ± 0.7</td>
</tr>
</tbody>
</table>

---

### Table V

NADPH oxidation, product formation, and $H_2O_2$ formation by P450 2A6

<table>
<thead>
<tr>
<th>Substrate</th>
<th>$k_b$</th>
<th>Rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Coumarin (20 μM)</td>
<td>–</td>
<td>NADPH oxidation 7-OH coumarin formation 3-OH product formation $H_2O_2$ formation $H_2O$ formation$^a$</td>
</tr>
<tr>
<td>7-OMe coumarin (100 μM)</td>
<td>–</td>
<td>79 ± 3</td>
</tr>
<tr>
<td>7-OMe coumarin (100 μM)</td>
<td>+</td>
<td>40 ± 2</td>
</tr>
<tr>
<td>7-OEt coumarin (100 μM)</td>
<td>–</td>
<td>33 ± 1</td>
</tr>
<tr>
<td>7-OEt coumarin (100 μM)</td>
<td>+</td>
<td>17 ± 2</td>
</tr>
</tbody>
</table>

---

$^a$ Calculated by difference (69).
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 H₂O exclusion from the active site (as evidenced by a strong shift to high spin iron) (Figs. 1 and 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 and 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 kₘₐ 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 ∼10⁹ M⁻¹ 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 Kₐ (67). In principle, such a phenomenon should yield a hyperbolic plot of the apparent rate of binding (versus substrate concentration) instead of a linear form (Figs. 2D and 3D), but more analysis is needed to address this possibility.

The kₘₐ 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 kₘₐ/kₐ = Kₐ yields parameters close to...
ingly, the presence of this ligand lowers the ligand 4-cyanopyridine is bound 3 orders of magnitude Kd.

Very recently Ost et al. (74) (Figs. 2, 6). Indeed, it 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 approximately 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 requires 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 ~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,7)) in P450s (65, 75, 77), unlike the situation with bacterial P450 101A1 (78). Although we have not directly estimated the E(m,7) of P450 2A6 with and without substrate, consideration of the estimated E(m,7) values of the Fe O2 and Fe 2− enzymes (Figs. 2 and 6) and the Nernst equation would suggest that the E(m,7) 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). Very recently Ost et al. (79) reported that the ligand 4-cyanopyridine is bound 3 orders of magnitude more tightly to ferrous than ferric P450 102A1, and accordingly, the presence of this ligand lowers the E(m,7).

As discussed above, the koff rate for coumarin from ferrous P450 2A6 is considerable (36 s−1) (Fig. 6C) and may be an issue in the functionality of the FeO2−/substrate complex. That is, some fraction might not be competent in that it could dissociate, bind O2, and then decompose. The possibility also exists that substrate might dissociate from the FeO2−/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 FeO2+ 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 Fe2+ P450 2A6-coumarin complex with O2 produced two sets of spectral changes (Fig. 7). Although we did not examine the effect of varying O2 concentration on the rates, our view is that the rapid initial changes (Fig. 7A, e.g. A1390 decrease) represent the formation of an FeO2+−/substrate complex and the succeeding changes (Fig. 7B, e.g. A1390 increase) represent the decay of the complex to regenerate FeO2−/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 FeO2+−/O2 complexes reported for bacterial P450 101A1 (72, 80) and P450 108A1 (81) but has a similarity 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 FeO2− 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 FeO2+−/substrate complex occurs under these conditions, although the efficiency was low (Table II).

As reported previously (46, 63, 85), the presence of b5 enhanced the steady-state koff for coumarin 7-hydroxylation (Table I). One general hypothesis for the enhancement of P450 catalytic activities by b5 is transfer of an electron from ferrous b5 to the P450 FeO2+−/substrate complex (70, 71). The enhance-
Kinetics of P450 2A6 Coumarin Oxidation

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 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-$d_7$]-7-OMe coumarin O-demethylation, $k_{3\text{obs}}/k_3^\text{cat}$ (where 1 and 2 denote the true primary and secondary isotope effects). If $k_3^\text{nuc}$ were 1.1, then with $k_{3\text{obs}} = 9.8$ (Table III), $k_3^\text{cat} = 10.8$. However, in the intermolecular non-competitive experiments with [methyl-$d_7$]-7-OMe coumarin, the $k_{3\text{obs}}/k_3^\text{cat}$ would be raised to the third power, or 1.1$^3$ = 1.3, and $k_{3\text{obs}}/k_3^\text{cat} = 9.8/(k_{3\text{obs}})^2/(k_3^\text{cat})^2$, or $k_{3\text{obs}}/k_3^\text{cat} = (k_{3\text{obs}})^2/(k_3^\text{cat})^2$. With $k_{3\text{obs}}/k_3^\text{cat} = 9.0$ (Table III), then $k_{3\text{obs}}/k_3^\text{cat}$ = 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 $k_{3\text{obs}}/k_3^\text{cat}$ 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 before 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 $k_{3\text{obs}}/k_3^\text{cat}$ isotope effect requires the existence of P450 branching after the irreversible step; i.e. decomposition or reduction of activated Fe-O complex 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 $k_{3\text{obs}}/k_3^\text{cat}$ for the P450 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 2A6 reactions using a simple model that has an irreversible $O_2$ activation step followed by steps involving Fe-O complex decomposition (to $H_2O_2$ and $H_2O$) and product generation from the substrate (C–H bond-breaking), $k_{3\text{obs}}/k_3^\text{cat}$ value and $k_3^\text{cat}$ both shared an inverse hyperbolic relationship with the rate of the C–H bond-breaking step (31). Moreover, the observed kinetic isotope effects are able to be correlated 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 (29, 28, 31, 34, 94) is further extended in a further experiment 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.
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 and 5). The substrate can dissociate rapidly from the Fe$^{3+}$ enzyme as well as the Fe$^{3+}$ form (Fig. 6). The FeO$_2$$^{+}$-substrate complex is unstable; it breaks down rapidly (Figs. 7, B and C) and is relatively inefficient in accepting electrons from either NADPH-P450 reductase or $b_2$ (Table II, Figs. 11, C and D). Thus, several steps in the cycle (Scheme 1) are inefficient. The isotope effect studies argue that it is the chemistry of the $K_d$ or 102A1. Thus, the bacterial P450(s) are committed to bind O$_2$, and efficiencies of P450-catalyzed reactions. The demonstrated $k_{cat}$ and $k_{cat}/K_m$ for FeO$_2$$^{+}$-substrate complex (or intrinsically utilized by P450s in multiple steps (33, 109). Thus, P450 101A1 can be viewed as having a low active site volume (26), as evidenced by the fraction of substrates (107). Thus, P450 101A1 can be viewed as having a low active site volume (26), as evidenced by the fraction of substrates (107). Thus, P450 101A1 can be viewed as having a low active site volume (26), as evidenced by the fraction of substrates (107). Thus, P450 101A1 can be viewed as having a low active site volume (26), as evidenced by the fraction of substrates (107). Thus, P450 101A1 can be viewed as having a low active site volume (26), as evidenced by the fraction of substrates (107). Thus, P450 101A1 can be viewed as having a low active site volume (26), as evidenced by the fraction of substrates (107). Thus, P450 101A1 can be viewed as having a low active site volume (26), as evidenced by the fraction of substrates (107). Thus, P450 101A1 can be viewed as having a low active site volume (26), as evidenced by the fraction of substrates (107). Thus, P450 101A1 can be viewed as having a low active site volume (26), as evidenced by the fraction of substrates (107). 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