

## Short Communication

# Generation of Human Metabolites of 7-Ethoxycoumarin by Bacterial Cytochrome P450 BM3

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### ABSTRACT:

Recently, wild-type and mutant forms of bacterial cytochrome P450 BM3 (CYP102A1) have been found to metabolize various drugs through reactions similar to those catalyzed by human cytochromes P450 (P450s). Therefore, it has been suggested that CYP102A1 may be used to produce large quantities of the metabolites of human P450-catalyzed reactions. In this report, we show that the oxidation of 7-ethoxycoumarin, a typical human P450 substrate, is catalyzed by both wild-type and mutant forms of CYP102A1. Two major products

were produced as a result of *O*-deethylation and 3-hydroxylation reactions. These results demonstrate that CYP102A1 mutants catalyze the same reactions as human P450s. High noncompetitive intermolecular kinetic deuterium isotope effects were observed for 7-ethoxycoumarin *O*-deethylation in the CYP102A1 system. These results suggest that there is a common mechanism for the oxidation reactions catalyzed by both the bacterial CYP102A1 and human P450 enzymes.

Cytochrome P450 enzymes (P450s) constitute a large family of enzymes that are remarkably diverse oxygenation catalysts found throughout nature, from archaea to humans (<http://drnelson.utm.edu/CytochromeP450.html>). P450s are monooxygenases that introduce a single atom of molecular oxygen into an organic molecule. Because of their catalytic diversity and broad substrate range, P450s are attractive as biocatalysts in the production of fine chemicals, including pharmaceuticals (Guengerich, 2002a; Urlacher and Eiben, 2006; Lamb et al., 2007; Yun et al., 2007). Despite the potential use of mammalian P450s in various biotechnology fields, they are not suitable as biocatalysts because of their low stability, catalytic activity, and availability.

The P450 BM3 (CYP102A1) from *Bacillus megaterium* has strong similarity to eukaryotic members of the CYP4A (fatty acid hydroxylase) family. It is soluble but uses a mammal-type (class II) redox system: an FAD- and FMN-containing NADPH-P450 reductase (Narhi and Fulco, 1982, 1986). The P450 BM3 reductase domain is also soluble and is fused to the C terminus of the P450 domain in a single continuous 119-kDa polypeptide. Thus, P450 BM3 is an entire class II P450 system in a single polypeptide. The strategic importance of P450 BM3 was quickly recognized, and the model system has been extensively characterized subsequently (Munro et al., 2002). CYP102A1 was the first P450

discovered to be fused to its redox partner, a mammalian-like diflavin reductase. The fusion of these two enzymatic activities makes soluble CYP102A1 an ideal model for mammalian, particularly human, P450 enzymes. It was shown that engineered CYP102A1 mutants could oxidize several human P450 substrates to produce the authentic metabolites with higher activities (Otey et al., 2006; Yun et al., 2007, and refs. therein). Furthermore, CYP102A1 is a versatile monooxygenase with a demonstrated ability to work on a diversity of substrates (Bernhardt, 2006) and an established relevance to biotechnology (Di Nardo et al., 2007).

Recently, through rational design or directed evolution, wild-type CYP102A1 has been engineered to oxidize compounds showing little or no structural similarity to its natural substrate fatty acids (Lamb et al., 2007). Thus, CYP102A1 can be engineered to show specificity for such substrates as alkanes, short- and medium-chain fatty acids, drug-like molecules, and polycyclic aromatic hydrocarbons (Carmichael and Wong, 2001). A triple mutant of CYP102A1 (R47L/F87V/L188Q) can metabolize testosterone and several drug-like molecules that are known substrates of human P450s 1A1, 2C8, 2D6, and 3A4 (van Vugt-Lussenburg et al., 2006). These recent advances suggest that CYP102A1 mutants can be developed as biocatalysts for drug discovery and synthesis. If prodrugs are found to convert to biologically "active metabolites" by the liver P450s during the drug development process, large quantities of the pure metabolites are required to understand the drug's efficacy, toxic effect, and pharmacokinetics. Recently, a set of CYP102A1 mutants was shown to generate larger quantities of the authentic human metabolites of drugs, which may be difficult to synthesize (Otey et al., 2006; Landwehr et al., 2006). An alternative approach to preparing the metabolites is to use engineered CYP102A1 enzymes with desired properties.

7-Ethoxycoumarin has been used as a model substrate in numerous P450 studies. The oxidative *O*-dealkylation of coumarins can be catalyzed by a number of mammalian and bacterial P450 enzymes.

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**ABBREVIATIONS:** P450, cytochrome P450; 7-OH coumarin, 7-hydroxycoumarin; 3-OH 7-ethoxycoumarin, 3-hydroxy-7-ethoxycoumarin; PCR, polymerase chain reaction; TES, 2-[[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]amino]ethanesulfonic acid; HPLC, high-performance liquid chromatography; LC, liquid chromatography.

The human P450s 1A2 and 2E1 are the major catalysts of 7-ethoxycoumarin *O*-deethylation in human liver microsomes (Kim et al., 2006). The bacterial P450s 105A1 (Hussain and Ward, 2003), 105B1 (Hussain and Ward, 2003), and 107B1 (Ueno et al., 2005) can also catalyze the *O*-deethylation of 7-ethoxycoumarin.

Kinetic isotope effects are used to probe aspects of P450 kinetics. Kinetic hydrogen isotope effects, especially with deuterium, have been used to study details of the catalytic mechanisms of many P450s (Guengerich, 2002b). For example, the discovery of a high noncompetitive intermolecular kinetic deuterium isotope effect showed that the C-H bond-breaking step is at least partially rate-limiting (Guengerich, 2002b). We previously analyzed the isotope effects for human liver P450s implicated in 7-ethoxycoumarin *O*-deethylation, namely P450s 1A2, 2A6, and 2E1 (Yun et al., 2005; Kim et al., 2006). We found a high isotope effect even in the noncompetitive experiments performed with human liver microsomes and purified recombinant human P450s. Originally, the high kinetic isotope effects were shown on *O*-deethylation of 7-ethoxycoumarin catalyzed by rat and hamster P450s (Harada et al., 1984; Miwa et al., 1984).

Knowledge of the molecular mechanisms involved in the catalysis of *O*-dealkylation reactions is important in our understanding of the metabolism of several xenobiotics, including phenacetin (Yun et al., 2000), dextromethorphan (Keizers et al., 2005), and 3,4-methylenedioxymethylamphetamine (Keizers et al., 2005). In this study, we tested whether 7-ethoxycoumarin could be used as a prototype substrate for *O*-deethylation catalyzed by bacterial and human P450 enzymes. Based on the scientific literature, several amino acid residues in CYP102A1 were mutated to generate mutant enzymes showing increased activity toward human P450 substrates (Yun et al., 2007). The selected mutations enabled the CYP102A1 enzyme to catalyze *O*-deethylation and 3-hydroxylation of 7-ethoxycoumarin, which are the same reactions catalyzed by human P450s.

### Materials and Methods

**Chemicals.** 7-Ethoxycoumarin and 7-hydroxycoumarin (7-OH coumarin) were purchased from Sigma-Aldrich (St. Louis, MO) and recrystallized from ethanol-H<sub>2</sub>O mixtures before use. The deuterium-labeled substrate ([1-ethyl-*d*<sub>2</sub>]-7-ethoxycoumarin) was prepared and characterized as described elsewhere (Yun et al., 2005). The syntheses and characterization of 3-hydroxy-7-ethoxycoumarin (3-OH 7-ethoxycoumarin) were performed as described elsewhere (Yun et al., 2005). Other chemicals were of the highest grade commercially available.

**Construction of BM3 Mutants by Site-Directed Mutagenesis.** Seventeen different site-directed mutants of CYP102A1 were constructed. Most of the CYP102A1 mutants used in this study were selected based on earlier work showing their increased catalytic activity toward several human substrates. Some of them, however, were generated specifically for this study. Each mutant bears the following amino acid substitution(s) relative to wild-type CYP102A1: 1 (F87A) (Carmichael and Wong, 2001), 2 (A264G) (Carmichael and Wong, 2001), 3 (F87A/A264G) (Carmichael and Wong, 2001), 4 (R47L/Y51F) (Carmichael and Wong, 2001), 5 (R47L/Y51F/A264G) (Carmichael and Wong, 2001), 6 (R47L/Y51F/F87A) (Carmichael and Wong, 2001), 7 (R47L/Y51F/F87A/A264G) (Carmichael and Wong, 2001), 8 (A74G/F87V/L188Q) (Li et al., 2001), 9 (R47L/L86I/L188Q) (this work), 10 (R47L/F87V/L188Q) (van Vugt-Lussenburg et al., 2007), 11 (R47L/F87V/L188Q/E267V) (van Vugt-Lussenburg et al., 2007), 12 (R47L/L86I/L188Q/E267V) (this work), 13 (R47L/L86I/F87V/L188Q) (van Vugt-Lussenburg et al., 2007), 14 (R47L/F87V/E143G/L188Q/E267V) (this work), 15 (R47L/E64G/F87V/E143G/L188Q/E267V) (this work), 16 (R47L/F81I/F87V/E143G/L188Q/E267V) (this work), and 17 (R47L/E64G/F81I/F87V/E143G/L188Q/E267V) (van Vugt-Lussenburg et al., 2007).

The oligonucleotide primers used to introduce the BamHI/SacI restriction sites were: BamHI forward, 5'-AGC GGA TCC ATG ACA ATT AAA GAA ATG CCT C-3'; and SacI reverse, 5'-ATC GAG CTC GTA GTT TGT AT-3'. The following PCR primers were used for the mutations (the codon for the

amino acid substitution is in italics): R47L, 5'-GCG CCT GGT CTG GTA ACG CG-3'; Y51F, 5'-GTA ACG CGC TTC TTA TCA AGT-3'; E64G, 5'-GCA TGC GAT GGC TCA CGC TTT-3'; A74G, 5'-TA AGT CAA GGC CTT AAA TTT GTA CG-3'; F81I, 5'-GTA CGT GAT ATT GCA GGA GAC-3'; L86I, 5'-GGA GAC GGG ATT TTT ACA AGC T-3'; F87A, 5'-GAC GGG TTA GCG ACA AGC TGG-3'; F87V, 5'-GAC GGG TTA GTG ACA AGC TGG-3'; E143G, 5'-GAA GTA CCG GGC GAC ATG ACA-3'; L188Q, 5'-ATG AAC AAG CAG CAG CGA GCA A-3'; A264G, 5'-TTC TTA ATT GGG GGA CAC GTG-3'; E267V, 5'-T GCG GGA CAC GTG ACA ACA AGT-3'; and L86I/F87V, 5'-GGA GAC GGG ATT GTG ACA AGC TG-3'. The PCR primers were obtained from Genotech (Daejeon, Korea). The genes encoding CYP102A1 mutants were amplified from pCWBM3 by PCR using primers designed to facilitate cloning into the expression vector pCWori (Farinas et al., 2001). Oligonucleotide assembly was performed by PCR using the 22 sets of designed primers described above. The amplified genes were subsequently cloned into the pCWBM3 BamHI/SacI vector at the BamHI/SacI restriction sites. These plasmids were transformed into *Escherichia coli* DH5α F'-IQ, and this strain was also used to express the mutant CYP102A1 proteins. After mutagenesis, the presence of the desired mutations was confirmed by DNA sequencing (Genotech).

**Expression and Purification of CYP102A1 Mutants.** The plasmids of wild-type CYP102A1 (pCWBM3) and mutants were transformed into the *E. coli* strain DH5αIQ using standard procedures. The first culture was inoculated from a single colony into 5 ml of Luria-Bertani medium supplemented with ampicillin (100 µg/ml) and grown at 37°C. This culture was used to inoculate 250 ml of Terrific Broth medium supplemented with ampicillin (100 µg/ml). The cells were grown at 37°C with shaking at 250 rpm to an OD<sub>600</sub> ~ 0.8, at which time gene expression was induced by the addition of isopropyl-β-D-thiogalactopyranoside to a final concentration of 0.50 mM. 8-Aminolevulinic acid (1.0 mM) was also added. Following induction, the cultures were allowed to grow another 36 h at 30°C. Cells were harvested by centrifugation (15 min, 5000g, 4°C). The cell pellet was resuspended in TES buffer (100 mM Tris-HCl, pH 7.6, 500 mM sucrose, 0.5 mM EDTA) and lysed by sonication (Sonicator; Misonix, Inc., Farmingdale, NY). After the lysate was centrifuged at 100,000g (90 min, 4°C), soluble cytosolic fraction was collected and used for the activity assay. The cytosolic fraction was dialyzed against 50 mM potassium phosphate buffer, pH 7.4, and stored at -80°C. Enzymes were used within 1 month of manufacture. CYP102A1 concentrations were determined from the CO-difference spectra as described by Omura and Sato (1964) using ε = 91 mM/cm. For all of the wild-type and mutated enzymes, a typical culture yielding 300 to 700 nM P450 could be detected. The expression level of CYP102A1 wild-type and mutants was as follows:

TABLE 1

Rates of *O*-deethylation and 3-hydroxylation of 7-ethoxycoumarin by various CYP102A1 mutants

Assays were performed using 2.0 mM 7-ethoxycoumarin as described under *Materials and Methods*. Values are the mean ± S.D. of triplicate determinations.

Enzyme	<i>O</i> -Deethylation	3-Hydroxylation
nmol product/min/nmol P450		
Wild type	0.11 ± 0.01	0.14 ± 0.01
Mutant		
1	0.78 ± 0.01	1.7 ± 0.1
2	0.89 ± 0.01	0.86 ± 0.02
3	0.98 ± 0.04	1.1 ± 0.1
4	0.22 ± 0.01	0.35 ± 0.01
5	2.7 ± 0.1	2.5 ± 0.1
6	0.44 ± 0.02	1.3 ± 0.1
7	1.1 ± 0.1	1.6 ± 0.1
8	11 ± 1	18 ± 1
9	6.7 ± 0.1	12 ± 1
10	5.8 ± 0.4	17 ± 1
11	0.65 ± 0.01	2.3 ± 0.1
12	0.68 ± 0.01	1.6 ± 0.1
13	3.1 ± 0.1	13 ± 1
14	2.3 ± 0.1	7.7 ± 0.1
15	2.6 ± 0.1	8.6 ± 0.1
16	2.8 ± 0.1	15 ± 1
17	2.9 ± 0.2	16 ± 1

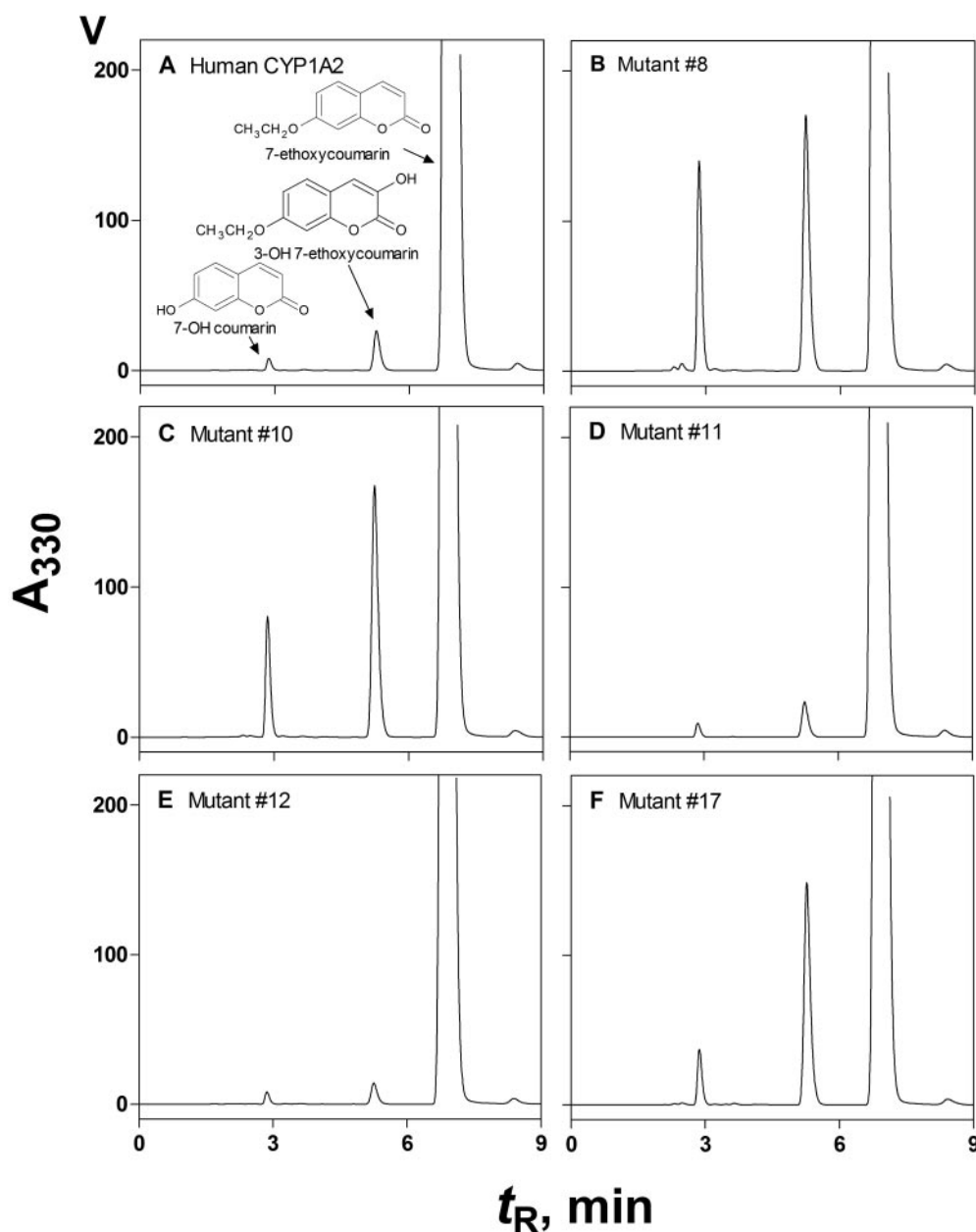


FIG. 1. HPLC chromatograms of 7-ethoxycoumarin metabolites produced by human CYP1A2 (A) and CYP102A1 mutants (B–F). Peaks were identified by comparing the retention times with those of standards. The peaks of the substrate and two major products are indicated. UV absorbance was monitored at 330 nm.

wild-type, 2.0 nmol P450/mg cytosolic protein; 1, 1.1 nmol P450/mg cytosolic protein; 2, 1.7 nmol P450/mg cytosolic protein; 3, 1.3 nmol P450/mg cytosolic protein; 4, 1.0 nmol P450/mg cytosolic protein; 5, 1.6 nmol P450/mg cytosolic protein; 6, 1.0 nmol P450/mg cytosolic protein; 7, 1.3 nmol P450/mg cytosolic protein; 8, 2.0 nmol P450/mg cytosolic protein; 9, 1.2 nmol P450/mg cytosolic protein; 10, 1.6 nmol P450/mg cytosolic protein; 11, 1.7 nmol P450/mg cytosolic protein; 12, 1.5 nmol P450/mg cytosolic protein; 13, 1.0 nmol P450/mg cytosolic protein; 14, 1.1 nmol P450/mg cytosolic protein; 15, 1.2 nmol P450/mg cytosolic protein; 16, 1.1 nmol P450/mg cytosolic protein; and 17, 1.8 nmol P450/mg cytosolic protein.

**Enzyme Assay.** Typical steady-state reactions for 7-ethoxycoumarin oxidation included 50 pmol CYP102A1 in 0.50 ml of 50 mM potassium phosphate buffer, pH 7.4, along with a specified amount of substrate. To determine the turnover numbers of several CYP102A1 mutants, we used 2.0 mM 7-ethoxycoumarin. An aliquot of an NADPH-generating system was used to initiate reactions (final concentrations: 10 mM glucose 6-phosphate, 0.5 mM NADP<sup>+</sup>, and 1 IU of yeast glucose 6-phosphate/ml). 7-Ethoxycoumarin stocks (50 mM) were prepared in CH<sub>3</sub>CN and diluted into the enzyme reactions such that the final organic solvent concentration was always less than 1% (v/v).

Reactions were generally incubated for 5 to 10 min at 37°C, terminated with 0.10 ml of 17% HClO<sub>4</sub>, and centrifuged (1000g, 10 min). CH<sub>2</sub>Cl<sub>2</sub> (1.0 ml) was added to the supernatant to extract the products, and the mixture was centrifuged at 1000g. (This process was then repeated once more.) The organic layers were combined, and the CH<sub>2</sub>Cl<sub>2</sub> was removed under an N<sub>2</sub> stream. The products, 7-OH coumarin and 3-OH 7-ethoxycoumarin, were analyzed by HPLC using a Gemini C18 column (4.6 × 150 mm, 5 μm; Phenomenex, Torrance, CA) with a mobile phase of H<sub>2</sub>O/CH<sub>3</sub>CN [55:45 (v/v)] containing 10 mM HClO<sub>4</sub>. The flow rate was 1.0 ml/min, and the absorbance at 330 nm was monitored (Yun et al., 2005). Kinetic parameters ( $K_m$  and  $k_{cat}$ ) were determined using nonlinear regression analysis with GraphPad PRISM software (GraphPad Software Inc., San Diego, CA). The data were fit to the standard Michaelis-Menten equation:  $v = k_{cat}[E][S]/([S] + K_m)$ . The equation is the standard Michaelis-Menten equation, where the velocity of the reaction is a function of the rate-limiting step in turnover ( $k_{cat}$ ), the enzyme concentration ([E]), substrate concentration ([S]), and the Michaelis constant ( $K_m$ ).

**LC/Tandem Mass Spectrometry Analysis of 7-Ethoxycoumarin and Its Metabolites.** The reaction residue was reconstituted into 100 μl of methanol by vortex mixing and sonication for 20 s. An aliquot (1 μl) of this solution was injected onto the LC column. A tandem quadrupole mass spectrometer (API

3000 LC/MS/MS; Applied Biosystems, Foster City, CA), coupled with an Agilent 1100 series HPLC system (Agilent Technologies, Palo Alto, CA) was used to identify 7-ethoxycoumarin and its metabolites. The separation was performed on a Luna C18 column (2-mm i.d.  $\times$  100 mm, 3.0  $\mu$ m; Phenomenex) using a mobile phase of acetonitrile and water [72:28 (v/v)] containing 0.1% formic acid at a flow rate of 0.2 ml/min. To identify the metabolites, mass spectra were recorded by electrospray ionization in positive mode. The turbo ion spray interface was operated at 4500 V and 400°C. The operating conditions were optimized by flow injection of a reference analyte and were as follows: nebulizing gas flow, 1.04 l/min; auxiliary gas flow, 4.0 l/min; curtain gas flow, 1.44 l/min; orifice voltage, 60 V; ring voltage, 400 V; collision gas (nitrogen) pressure,  $3.58 \times 10^{-5}$  Torr; and collision energy, 30 eV. Quadrupoles Q1 and Q3 were set on unit resolution.

**Kinetic Isotope Effect Determination.** Deuterium isotope effects using labeled ( $d_2$ ) 7-ethoxycoumarin were determined by a noncompetitive method, which is essentially the same as previous work (Kim et al., 2006). CYP1A2 was incubated with unlabeled ( $d_0$ ) 7-ethoxycoumarin or with labeled ( $d_2$ ) 7-ethoxycoumarin over a range of substrate concentrations (0.010–2.0 mM), and the products were analyzed by HPLC as described above.  $K_m$  and  $k_{cat}$  were determined by nonlinear regression (Yun et al., 2005; Kim et al., 2006). The conventions  $^D V = ^H k_{cat} / ^D k_{cat}$  and  $^D(V/K) = ^H(k_{cat}/K_m) / ^D(k_{cat}/K_m)$  of Northrop (1975) are used in the designation of kinetic isotope effects as described previously (Kim et al., 2006).

## Results and Discussion

**O-Deethylation and Hydroxylation of 7-Ethoxycoumarin by P450 BM3 and Its Mutants.** First, the abilities of all mutants to O-deethylate and 3-hydroxylate 7-ethoxycoumarin were measured at a fixed substrate concentration (2.0 mM). We found that wild-type P450 BM3 showed very low activity under the test conditions (Table 1) (0.11 and 0.14 min<sup>-1</sup> for O-deethylation and 3-hydroxylation, respectively). The turnover numbers for the entire set of 17 mutants for O-deethylation and 3-hydroxylation of 7-ethoxycoumarin varied over a 48- and 51-fold range, respectively. The identities of the major metabolites were verified by comparing the results of HPLC (Fig. 1) and LC/mass spectrometry (data not shown) with standard compounds that were prepared as described (Yun et al., 2005).

7-Ethoxycoumarin proved to be a good substrate, with high turnover numbers (up to 29 min<sup>-1</sup> in the case of mutant 8). Although 7-OH coumarin and 3-OH 7-ethoxycoumarin were the major metabolites, the relative ratio of these two products differed greatly depending on the mutant (Table 1). Only two mutants (2 and 5) showed a higher rate of O-deethylation than of 3-hydroxylation. For the most active mutants (8–10 and 13–17), the rate of 3-hydroxylation was higher than that of O-deethylation. The human P450 enzymes 1A2 and 2E1, the major enzymes for O-deethylation and 3-hydroxylation in human liver, also show a preference for the 3-hydroxylation reaction over the O-deethylation reaction (Yun et al., 2005; Kim et al., 2006). Thus, like the human P450 enzymes, most of the highly active CYP102A1 mutants favor the 3-hydroxylation reaction over the O-deethylation reaction. This preference is likely due to the orientation of the substrate in the active site.

Three high-activity and two low-activity mutants were chosen and used to measure kinetic parameters for O-deethylation and 3-hydroxylation of 7-ethoxycoumarin. Only mutants 8 and 10 showed significantly elevated  $k_{cat}$  values for O-deethylation and 3-hydroxylation reactions. However, the  $K_m$  values of both mutants also increased, such that the apparent values of  $k_{cat}/K_m$  were unchanged. The mutants displayed 25- and 15-fold variation in  $k_{cat}$  for O-deethylation and 3-hydroxylation reactions, respectively (Table 2). The  $K_m$  values varied to a similar extent as the  $k_{cat}$  values.

**Noncompetitive Intermolecular Kinetic Isotope Effect.** In this work, we examined the rate contribution of C-H bond cleavage to the CYP102A1 catalytic cycle for CYP102A1 mutants using deuterium-

TABLE 2  
Intermolecular isotope effects on O-deethylation and 3-hydroxylation of 7-ethoxycoumarin by CYP102A1 mutants

P450	7-Ethoxycoumarin	O-Deethylation				3-Hydroxylation			
		$k_{cat}$ min <sup>-1</sup>	$K_m$ $\mu$ M	$k_{cat}/K_m$	$^D V$	$^D(V/K)$	$k_{cat}$ min <sup>-1</sup>	$K_m$ $\mu$ M	$k_{cat}/K_m$
Mutant 8	$d_0$	17 $\pm$ 1	1050 $\pm$ 140	0.016 $\pm$ 0.002	11 $\pm$ 1	11 $\pm$ 2	25 $\pm$ 2	1050 $\pm$ 150	0.024 $\pm$ 0.004
	$d_2$	1.6 $\pm$ 0.1	1130 $\pm$ 160	0.0014 $\pm$ 0.0002			26 $\pm$ 2	1120 $\pm$ 150	0.023 $\pm$ 0.004
	$d_0$	10 $\pm$ 1	1220 $\pm$ 240	0.010 $\pm$ 0.002	10 $\pm$ 1	14 $\pm$ 4	29.5 $\pm$ 3.4	1340 $\pm$ 290	0.022 $\pm$ 0.005
	$d_2$	1.0 $\pm$ 0.1	1360 $\pm$ 230	0.00074 $\pm$ 0.00011			32.6 $\pm$ 3.2	1400 $\pm$ 260	0.023 $\pm$ 0.005
	$d_0$	1.7 $\pm$ 0.1	45 $\pm$ 5	0.038 $\pm$ 0.005	8.1 $\pm$ 0.6	13 $\pm$ 2	6.4 $\pm$ 0.2	81 $\pm$ 10	0.079 $\pm$ 0.010
	$d_2$	0.21 $\pm$ 0.01	72 $\pm$ 8	0.0029 $\pm$ 0.0004			8.1 $\pm$ 0.2	118 $\pm$ 10	0.069 $\pm$ 0.006
	$d_0$	0.68 $\pm$ 0.01	48 $\pm$ 3	0.014 $\pm$ 0.001	6.2 $\pm$ 0.6	17 $\pm$ 3	2.0 $\pm$ 0.1	427 $\pm$ 29	0.0047 $\pm$ 0.0004
	$d_2$	0.11 $\pm$ 0.01	131 $\pm$ 15	0.00084 $\pm$ 0.00012			2.8 $\pm$ 0.1	682 $\pm$ 45	0.0041 $\pm$ 0.0003
	$d_0$	2.9 $\pm$ 0.1	160 $\pm$ 12	0.0018 $\pm$ 0.0001	9.4 $\pm$ 0.4	12 $\pm$ 1	16.5 $\pm$ 0.4	225 $\pm$ 23	0.073 $\pm$ 0.008
	$d_2$	0.31 $\pm$ 0.01	203 $\pm$ 15	0.0015 $\pm$ 0.0001			17.6 $\pm$ 0.7	237 $\pm$ 35	0.074 $\pm$ 0.002
Mutant 10	$d_0$								
	$d_2$								
Mutant 11	$d_0$								
	$d_2$								
Mutant 12	$d_0$								
	$d_2$								
Mutant 17	$d_0$								
	$d_2$								

Kinetic parameters were calculated for the formation of the major products of 7-ethoxycoumarin oxidation by a panel of CYP102A1 mutants. Conditions are described under Materials and Methods. Values are means  $\pm$  S.D. of three independent experiments.



labeled substrates. The results were compared with those of human P450s (Yun et al., 2005; Kim et al., 2006). The intermolecular noncompetitive kinetic isotope effects were measured by performing assays with  $d_0$  and  $d_2$  substrates and comparing the kinetic parameters [ $^{D}V$  and  $^{D}(V/K)$ ] (Yun et al., 2000) (Table 2). Such assays provide the most direct information about the degree to which the C-H bond-breaking step is rate-limiting in the overall steady-state reaction. Several CYP102A1 mutants showed a strong isotope effect for 7-ethoxycoumarin *O*-deethylation [ $^{D}V = 6.2$ –11 and  $^{D}(V/K) = 11$ –17] (Table 2).

These results indicate that breaking of the C-H bond is strongly rate-limiting in the *O*-deethylation of 7-ethoxycoumarin. The previously reported isotope effects for *O*-dealkylation by the human P450s 2A6 (Yun et al., 2005), 1A2, and 2E1 (Kim et al., 2006) are also substantial. Thus, the rate of C-H bond breaking is a major factor in determining the rate of these reactions under any conditions. More importantly, these results reveal that the P450 profiles are remarkably similar to that for hydrogen atom abstraction. In fact, the isotope effects for the *O*-dealkylation of 7-ethoxycoumarin are nearly the same for all P450 enzymes characterized to date.

CYP102A1 mutants apparently did not increase the rate of 3-OH 7-ethoxycoumarin formation (*C*-hydroxylation) when rates of *O*-deethylation were reduced by deuterium substitution [ $^{D}V = 0.71$ –0.96 and  $^{D}(V/K) = 0.96$ –1.1]. Similarly, no “metabolic switching” was observed for human P450s 1A2 [ $^{D}V = 0.92$ ] (Kim et al., 2006) and 2A6 [ $^{D}V = 0.84$ ] (Yun et al., 2005) in the *O*-deethylation reaction of 7-ethoxycoumarin.

In summary, the work performed with this set of CYP102A1 mutants and 7-ethoxycoumarin, a typical human P450 substrate, revealed that bacterial CYP102A1 enzymes catalyze the same reactions as the human P450 enzymes. The similarities of oxidation profiles and the noncompetitive intermolecular kinetic deuterium isotope effects between CYP102A1 and human P450s suggests the possible application of CYP102A1 as a model system for studying the human enzymes.

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