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7-Ethoxycoumarin O-Deethylation Catalyzed by Cytochromes P450 1A2 and 2E1 in Human Liver Microsomes

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ABSTRACT. 7-Ethoxycoumarin O-deethylation has been used widely as a marker activity for assessing substrate specificities of cytochromes P450 (P450) in liver microsomes of mammals, and extensive studies have shown that in rats and mice the major catalysts are P450 1A1, 1A2, and 2B enzymes. In contrast to findings in experimental animal models, P450 2E1 has been reported to be a principal enzyme involved in 7-ethoxycoumarin O-deethylation in human livers. In this study, we further examined the roles of individual forms of human P450 involved in 7-ethoxycoumarin O-deethylation using microsomes from different human liver samples and from human lymphoblastoid cells expressing human P450 enzymes and purified P450 enzymes isolated from membranes of *Escherichia coli* expressing modified human P450 proteins. Kinetic analysis showed that there were at least two different enzymes involved in 7-ethoxycoumarin O-deethylation in different human samples. Samples that contained high amounts of P450 2E1 in liver microsomes showed biphasic curves for O-deethylation with relatively high turnover numbers, whereas P450 1A2-rich samples tended to have low K_m values with low V_{max} values. Anti-human P450 2E1 antibodies inhibited markedly ($P < 0.05$) the 7-ethoxycoumarin O-deethylation activities catalyzed by human liver microsomes particularly when examined at a high substrate concentration (200 μM). However, we also found that anti-P450 1A2 antibodies suppressed O-deethylation activities only at a low substrate concentration (10 μM). Recombinant human P450 1A2 was found to have a low K_m value for 7-ethoxycoumarin O-deethylation, whereas P450 2E1 showed a high K_m value. Of the P450 enzymes examined, P450 1A1 gave the highest O-deethylation activities with a low K_m value, although this enzyme is reported to be expressed extrahepatically in humans. Other human P450 enzymes, including P450 2A6, 2C10, 2D6, 3A4, and 3A5, did not show significant O-deethylation activities except that P450 2B6, a minor P450 component in human livers, was found to have a V_{max} value similar to that of P450 1A2 and a K_m value similar to that of P450 2E1. These results suggest that P450 1A2 is a low K_m enzyme for 7-ethoxycoumarin O-deethylation in human liver microsomes, although it has a lower V_{max} value than P450 2E1. BIOCHEM PHARMACOL 51;3:313–319, 1996.

KEY WORDS. 7-ethoxycoumarin; P450 1A2; P450 2E1; human P450; kinetic analysis

Recent studies have established that multiple forms of P450§ exist in human liver microsomes, and the individual P450 forms have considerable but overlapping substrate specificities to catalyze oxidation of a number of endobiotic and xenobiotic chemicals [1]. Since P450 has been shown to have key roles in drug biotransformation reactions, it is of great value to examine the catalytic specificities of P450 forms in drug oxidation reactions [2]. Recent studies have demonstrated that in several cases of two or more P450 enzymes participate in the oxidation of a drug with different K_m values (which are crude estimates of substrate affinity) [3–5]. For example, although bufuralol 1′-hydroxylation is catalyzed principally by P450 2D6 (one of the polymorphic enzymes in humans), P450 1A2 can also participate in the 4- and 6-hydroxylations as well as the 1′-hydroxylation of bufuralol [5]. Since the levels and composition of each P450 form differ among individuals, inter-individual variations in these P450 enzymes are one of the important determinants in assessing the different susceptibilities of humans towards drug actions and toxicities [6, 7].

7-Ethoxycoumarin O-deethylation has been used widely in characterizing substrate specificities of individual forms of P450 in liver microsomes of various animal species including humans [8–11]. In rat liver microsomes, P450 1A1, 1A2, and 2B1 have been shown to be the major enzymes involved in the reaction [9], but recently P450 2E1 has been reported to be a principal enzyme in human liver microsomes [10, 11]. In a preliminary account, however, we suggested that P450 1A2 also has a significant role in 7-ethoxycoumarin O-deethylation in human liver microsomes, based upon the reconstitution of the activities with recombinant P450 enzymes isolated from

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§ *Abbreviations:* P450, cytochrome P450; and IgG, immunoglobulin G.

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the membranes of *Escherichia coli* in which modified human P450 genes have been introduced.*

In this report, we examined the roles of various forms of human P450 enzymes, especially P450 1A2 and 2E1, in the O-deethylation of 7-ethoxycoumarin, using liver microsomes of several human samples and recombinant human P450 enzymes. We also used specific antibodies raised against purified P450 enzymes isolated from human liver microsomes or from membranes of *Escherichia coli*, which had been introduced as human P450 cDNAs. Kinetic analysis of 7-ethoxycoumarin O-deethylation catalyzed by human liver microsomal enzymes was also performed. The results presented in this work collectively indicate that P450 1A2 is a low K_m component of 7-ethoxycoumarin O-deethylation in human liver microsomes and has a lower V_{max} value than P450 2E1, which is a high K_m component in human liver microsomes.

MATERIALS AND METHODS

Materials

7-Ethoxycoumarin was obtained from the Aldrich Chemical Co., Milwaukee, WI, U.S.A., and 7-hydroxycoumarin from the Katayama Chemical Co., Osaka, Japan. Other chemicals used were from the same sources as described previously [6].

Enzyme Preparation

Human liver samples were obtained from organ donors or patients undergoing liver resection as described previously [6, 12]. Liver microsomes were prepared as described and suspended in 10 mM Tris-HCl buffer (pH 7.4) containing 0.10 mM EDTA and 20% glycerol (v/v) [13].

P450 2A6 and 2E1 were purified to homogeneity from human liver microsomes as described previously [14, 15]. Recombinant human P450 1A1, 1A2, 2C10, 2D6, 2E1, 3A4, and 3A5 proteins were purified to homogeneity from membranes of *E. coli* in which modified P450 genes had been introduced [16–22]. Microsomes of human lymphoblastoid cells expressing human P450 1A2, 2B6, and 2E1 were obtained from the GENETEST Co. (Woburn, MA, U.S.A.). Rabbit anti-P450 antibodies were prepared, and the IgG fractions were obtained as described [23]. NADPH-P450 reductase and cytochrome b_5 were purified from liver microsomes of phenobarbital-treated rabbits by the method of Yasukochi and Masters [24] as modified by Taniguchi *et al.* [25].

Enzyme Assays

7-ethoxycoumarin O-deethylation activities of P450 enzymes were determined as described with slight modifications [8, 13]. Incubation mixtures consisted of human liver microsomes (0.1 mg protein/mL) or microsomes (0.2 mg protein/mL) of human lymphoblastoid cells expressing human P450 enzymes with several concentrations of 7-ethoxycoumarin in a final volume of 1.0 mL of 100 mM potassium phosphate buffer (pH 7.4) containing an NADPH-generating system described previously [26]. Reconstituted P450 systems were composed of 10–20 pmol of purified human P450, 20–40 pmol of cytochrome b_5, 40 pmol of NADPH-P450 reductase, 20 μg of a phospholipid mixture consisting of L-α-dilauroyl-*sn*-glycero-3-phosphocholine, dioleoyl-*sn*-glycero-3-phosphocholine, and L-α-phosphatidyl-L-serine (1:1:1, by wt) per mL, and 0.25 mM sodium cholate [22, 27, 28]. Incubations were carried out at 37° for 15 min and terminated by adding 90 μL of aqueous 10% trichloroacetic acid (w/v); the metabolites were extracted with 1.0 mL of methylene chloride. The mixtures were centrifuged at 3000 *g* for 5 min, and aliquots (usually 0.5 mL) of the organic layer (lower layer) were re-extracted with 3.0 mL of 30 mM sodium borate (pH 9.0). The formation of 7-hydroxycoumarin was determined fluorometrically with a Shimadzu RF-5000 spectrophotometer (Shimadzu, Kyoto).

Other Assays

P450 was estimated spectrally by the method of Omura and Sato [29]. The contents of individual human P450 proteins in human liver microsomes were estimated by coupled sodium dodecyl sulfate-polyacrylamide gel electrophoresis/immunochemical development ("Western-blotting") [30]. Protein concentrations were estimated by the method of Lowry *et al.* [31].

Analysis of Data and Statistical Methods

Kinetic parameters for the hydroxylations of 7-ethoxycoumarin by human P450 enzymes were estimated using a computer program (KaleidaGraph, Synergy Software, Reading, PA) designed for nonlinear regression analysis of a hyperbolic Michaelis–Menten equation. In cases where there was more than one P450 enzyme contributing to the measured velocities, the kinetic parameters were estimated by fitting the data by the following equation [32]:

$$V = V_{max1} \cdot S/(K_{m1} + S) + V_{max2} \cdot S/(K_{m2} + S)$$

where V is the velocity of the product formations, S is the substrate concentrations, K_{m1} and K_{m2} are the constants for the low and high K_m components, and V_{max1} and V_{max2} are the maximum velocities for the apparent low and high K_m components, respectively.

RESULTS

Kinetic Analysis of 7-Ethoxycoumarin O-Deethylation by Human Liver Microsomes

Liver microsomes from five human samples were used for kinetic analysis (Eadie-Hofstee plots) of 7-ethoxycoumarin O-deethylation with various substrate concentrations (Fig. 1). The results showed the appearance of at least two P450 components in the O-deethylation of 7-ethoxycoumarin in liver microsomes of human samples HL-11, -13, and -56, because these human samples showed biphasic curves for the activities upon analysis of the Eadie–Hofstee plots. However, liver mi-

* Shimada T and Yamazaki H, unpublished results.

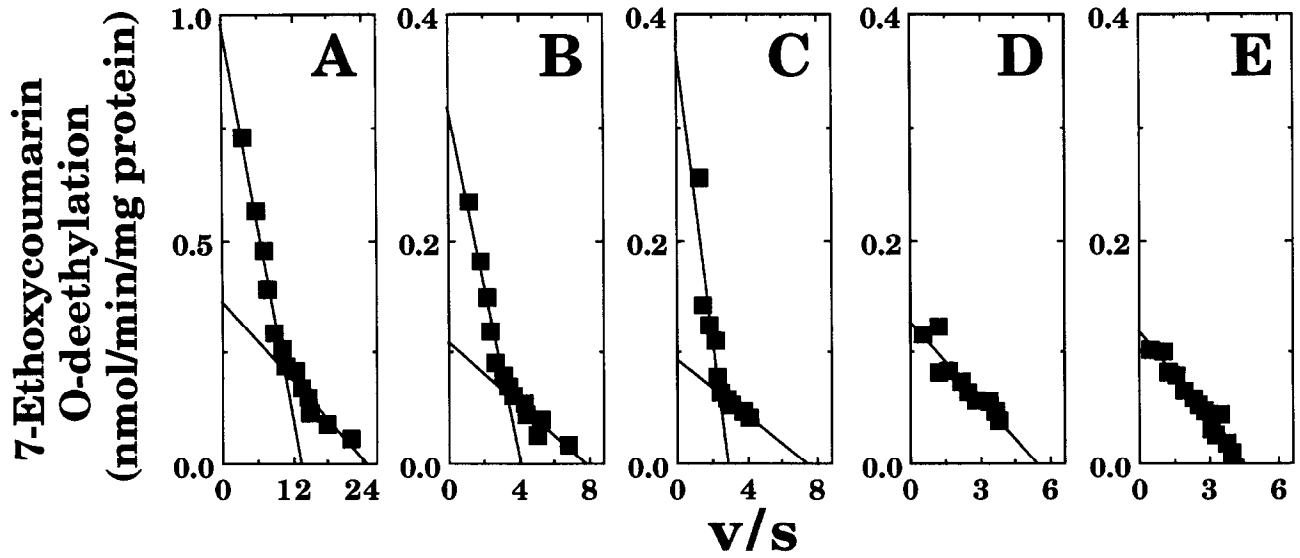

FIG. 1. Eadie-Hofstee plots of 7-ethoxycoumarin *O*-deethylation catalyzed by liver microsomes prepared from human samples HL-11 (A), HL-13 (B), HL-56 (C), HL-64 (D), and HL-67 (E). Experimental details are described in Materials and Methods. V, 7-ethoxycoumarin *O*-deethylation activity (nmol product formed/min/mg protein); S, 7-ethoxycoumarin (mM).

crosomes from samples HL-64 and -67 gave only one component for the 7-ethoxycoumarin O-deethylation activities. Of particular interest is the observation that a liver sample (HL-11) that contained relatively high levels of P450 2E1 in liver microsomes had the highest turnover numbers for 7-ethoxycoumarin O-deethylation activities, whereas two other human samples (HL-64 and -67) having relatively high levels of P450 1A2 showed only one apparent component for the O-deethylation activities (Table 1).

The K_m and V_{max} values for 7-ethoxycoumarin O-deethylation were calculated in these human samples using nonlinear regression analysis as described in the Materials and Methods, and these activities were compared with the contents of P450 1A2 and 2E1 in the liver microsomes (Table 1). Human samples HL-11, -13, and -56 showed two kinetic parameters for 7-ethoxycoumarin O-deethylation activities: the high K_m component (K_m values of about 0.15 mM and V_{max} values of 0.35–0.95 nmol/min/mg protein) contributed more to V_{max} than the low K_m component (K_m values of 11–16 μM and V_{max} values of 0.05–0.21 nmol/min/mg protein). As described above, human samples HL-64 and -67 that contained a relatively high amount of P450 1A2 gave only a low K_m component (~0.026 mM) for 7-ethoxycoumarin O-deethylation activity with V_{max} values of about 0.12 nmol/min/mg protein.

Effects of Anti-P450 Antibodies on 7-Ethoxycoumarin O-Deethylation Catalyzed by Human Liver Microsomes

Liver microsomes from two human samples (HL-11 and HL-67), that contained relatively high levels of P450s 2E1 and P450 1A2, respectively, were selected for further analysis of the effects of specific antibodies raised against purified P450 enzymes on 7-ethoxycoumarin O-deethylation activities using low (10 μM) and high (200 μM) substrate concentrations (Fig. 2). Two rabbit anti-P450 IgG preparations were used: anti-P450 1A2 IgG was obtained from rabbits immunized with purified recombinant (*E. coli*) P450 1A2 and anti-P450 2E1

TABLE 1. Kinetic analysis of 7-ethoxycoumarin *O*-deethylation by human liver microsomes of individual humans

	P450 content (nmol P450/mg protein)			7-Ethoxycoumarin *O*-deethylation			
				High K_m component		Low K_m component	
Human code no.	Total P450*	P450 1A2†	P450 2E1†	K_m (mM)	V_{max} (nmol/min/mg protein)	K_m (mM)	V_{max} (nmol/min/mg protein)
HL-11	0.52	0.038	0.069	0.148	0.95	0.016	0.21
HL-13	0.55	0.031	0.024	0.165	0.35	0.012	0.05
HL-56	0.88	0.046	0.049	0.152	0.43	0.011	0.06
HL-64	0.41	0.074	0.023			0.027	0.13
HL-67	0.68	0.113	0.029			0.026	0.12

* Spectrally determined P450.
† Immunochemically determined P450.

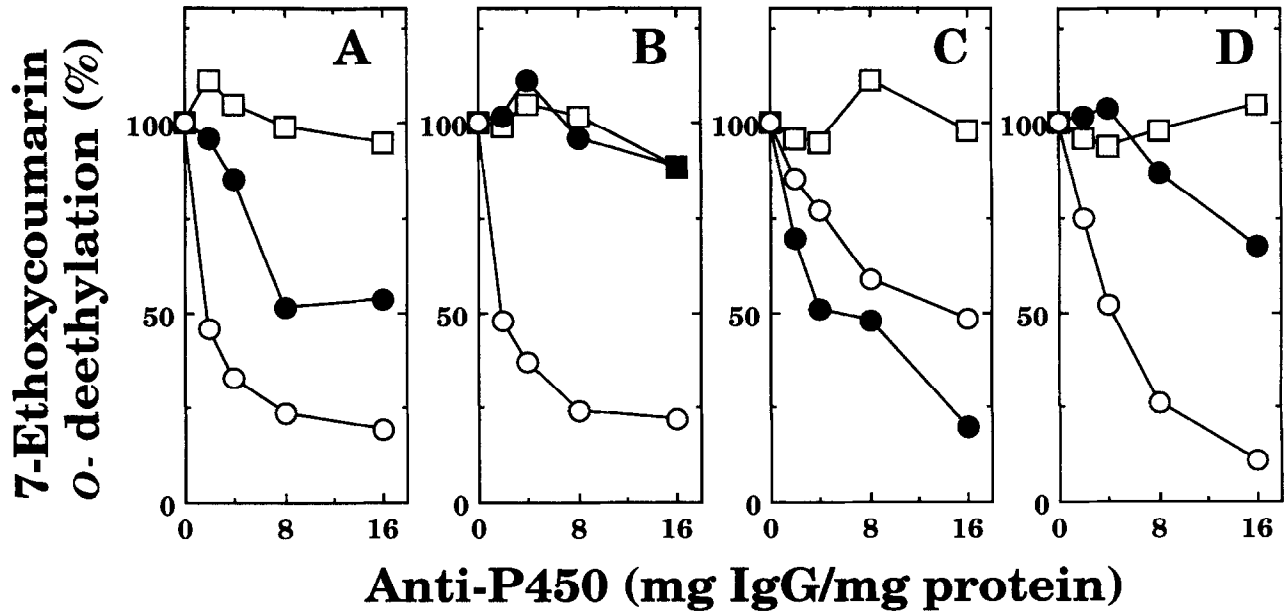

FIG. 2. Effects of preimmune IgG (□), anti-P450 1A2 IgG (●) and anti-P450 2E1 IgG (○) on 7-ethoxycoumarin O-deethylation activities catalyzed by liver microsomal samples HL-11 (A, B) and HL-67 (C, D). Two substrate concentrations (10 μM in A and C, and 200 μM in B and D) of 7-ethoxycoumarin were used for the assay with human liver microsomes (0.1 mg protein). Control activities in the absence of antibodies for A, B, C, and D were 0.16, 0.79, 0.03, and 0.14 nmol/min/mg protein, respectively.

was from rabbits immunized with purified P450 2E1 isolated from human liver microsomes [15]. Anti-P450 1A2 and anti-P450 2E1 IgGs have been found to be selective for inhibiting the ethoxyresorufin O-deethylation (a known reaction catalyzed by P450 1A2) and chlorzoxazone 6-hydroxylation (a typical P450 2E1 reaction), respectively, catalyzed by human liver microsomes [33, *]. In the human sample HL-11, anti-P450 2E1 was very inhibitory to 7-ethoxycoumarin O-deethylation at both low and high substrate concentrations (Fig. 2, A and B), whereas anti-P450 1A2 inhibited the activities by about 50% only at a low substrate concentration. In the human sample HL-67, anti-P450 1A2 inhibited to a marked extent ($P < 0.05$) the 7-ethoxycoumarin O-deethylation at a low substrate concentration, whereas the effects of anti-P450 2E1 were not as great especially at the low substrate concentration (Fig. 2, C and D). Other antibodies—raised against P450 2A6, 2C9/10, P450 2D6, and P450 3A4—did not affect 7-ethoxycoumarin O-deethylation activities catalyzed by liver microsomes of HL-11 and HL-67 at both low and high substrate concentrations (results not shown).

7-Ethoxycoumarin O-Deethylation by Purified P450 *Enzymes and by Recombinant Human* P450 *Enzymes*

Human P450 enzymes (P450 1A1, 1A2, 2C10, 2D6, 2E1, 3A4, and 3A5) isolated from *E. coli* membranes and P450 2A6 isolated from human liver microsomes were used to measure 7-ethoxycoumarin O-deethylation activities in reconstituted monooxygenase systems fortified with several components, as described in Materials and Methods. Among the P450 enzymes examined, P450 1A1 had the highest V_{max} value for 7-ethoxycoumarin O-deethylation, followed by P450 2E1 and P450 1A2 (Table 2). The K_m values of 7-ethoxycoumarin O-deethylation by P450 1A1 and 1A2 were determined to be 21 μM, much lower than the K_m value of 120 μM for P450 2E1. Other human P450 enzymes including P450 2A6, 2C10, 2D6, 3A4, and 3A5 did not have detectable 7-ethoxycoumarin O-deethylation activities.

Recombinant P450 2B6, as well as P450 1A2 and 2E1, in microsomes of human lymphoblastoid cells was also used to determine the catalytic specificities of these P450 enzymes for 7-ethoxycoumarin O-deethylation (Table 3). The K_m value

TABLE 2. Kinetic analysis of 7-ethoxycoumarin O-deethylation by human P450 enzymes expressed in *E. coli*

	7-Ethoxycoumarin O-deethylation	
	K_m (μM)	V_{max} (nmol/min/nmol P450)
CYP1A1	21 ± 2	13.8 ± 0.38
CYP1A2	21 ± 1	0.43 ± 0.01
CYP2A6*		<0.1
CYP2C10		<0.1
CYP2D6		<0.1
CYP2E1	120 ± 7	3.9 ± 0.13
CYP3A4		<0.1
CYP3A5		<0.1

Values are means ± SEM of the data set using a non-linear kinetic analysis from mean values obtained in duplicate at each substrate concentration.

* Purified P450 2A6 isolated from human liver microsomes.

* Soucek P, Guo Z, Sandhu P, Martin MV, and Guengerich FP, unpublished results.

TABLE 3. Kinetic analysis of 7-ethoxycoumarin O-deethylation by human P450 enzymes expressed in human lymphoblastoid cells

	7-Ethoxycoumarin O-deethylation	
	K_m (μM)	V_{max} (pmol/min/mg protein)
CYP1A2	18 ± 4	2.9 ± 0.2
CYP2B6	115 ± 24	4.0 ± 0.7
CYP2E1	104 ± 24	8.4 ± 1.0

Values are the means ± SEM of the data set using a non-linear kinetic analysis from mean values obtained in duplicate at each substrate concentration.

obtained in P450 2B6 was found to be very similar to that in P450 2E1, and the turnover number for the 7-ethoxycoumarin O-deethylation activity by P450 2B6 was determined to be lower than that by P450 2E1 and higher than that by P450 1A2.

DISCUSSION

Several recent studies have established that in many cases two or more P450 enzymes are involved in the oxidation of a drug with different apparent affinities in human liver microsomes [3–5, 34]. For example, we have reported recently that bufuralol 1′-hydroxylation is catalyzed principally by P450 2D6, while P450 1A2 can also participate in the 4- and 6-hydroxylation reactions as well as 1′-hydroxylation of bufuralol in humans [5]. Since the compositions of individual forms of P450 have been reported to differ in liver microsomes of different people, it is necessary to determine which human P450 enzymes have important roles in individual drug oxidation reactions [6, 7].

There are several reports indicating that species-related differences exist in the roles of P450 family enzymes in the metabolism of drugs, carcinogens, and environmental contaminants in humans and experimental animals [1, 35]. Although S-mephenytoin 4′-hydroxylation has been identified recently as being catalyzed primarily by P450 2C19 in humans, P450 3A family enzymes have been reported to be dominant in metabolizing S-mephenytoin in rats [36–38]. It has also been reported that activation of aflatoxin B_1 to the ultimate *exo*-8,9-epoxide metabolite is catalyzed mainly by P450 3A4 in humans; however, in rats P450 2C subfamily enzymes are suggested to have important roles in the activation [39–41]. Such species-related differences in P450-catalyzed drug oxidation reactions are important factors in understanding the basis for different susceptibilities towards drug action and toxicities in different animal species.

7-Ethoxycoumarin O-deethylation has been reported to show species-related differences in the roles of P450 enzymes in rats and humans [11]. Our preliminary kinetic studies* suggested that P450 1A1 had the lowest K_m value as compared with those by P450 1A2, 2B1, and 2E1, based on the results of induction of P450 isoforms by specific inducers in rats. It is of particular interest to note that although treatment of rats with isoniazid, a P450 2E1 inducer, caused induction of 7-ethoxycoumarin O-deethylation by about 7-fold, the observed K_m value was similar to that of liver microsomes from untreated rats. These results support the previous view that P450s 1A1, 1A2, and 2B1 are more active in 7-ethoxycoumarin O-deethylation than P450 2E1 in rats [9].

In contrast to rats, human P450 2E1 has been reported to be a major enzyme involved in the O-deethylation of 7-ethoxycoumarin [6, 12]. However, it is suggested here that P450 1A2 is also involved in the 7-ethoxycoumarin O-deethylation reaction in human liver microsomes. This suggestion comes from the following lines of evidence. First, kinetic analysis showed that there are at least two different enzymes involved in 7-ethoxycoumarin O-deethylation in different human samples. Samples containing relatively high amounts of P450 2E1 in liver microsomes showed biphasic curves for O-deethylation, with relatively high turnover numbers, whereas P450 1A2-rich samples tended to have only a low K_m component with low V_{max} values. It should be mentioned that Boobis *et al.* [42] and Bayliss *et al.* [43] have also reported that there exist two components for 7-ethoxycoumarin O-deethylation activities in human liver microsomes and human hepatocyte suspensions, respectively. Second, anti-P450 1A2 inhibited 7-ethoxycoumarin O-deethylation catalyzed by liver microsomes of P450 1A2-rich samples only at a low substrate concentration (10 μM), while at a high substrate concentration (200 μM) anti-P450 2E1 was more effective. Finally, recombinant P450 1A2 had a low K_m and P450 2E1 had a high K_m value in reconstituted 7-ethoxycoumarin O-deethylation systems, although the turnover number (V_{max}) was 9-fold higher for P450 2E1 than for P450 1A2.

Difficulty sometimes exists in determining which P450 enzymes are dominant when drugs or other chemicals are administered *in vivo* to humans. In some cases, the results obtained in *in vitro* studies do not match the *in vivo* results. For example, it has been reported that the erythromycin breath test is not useful for determination of P450 3A4 *in vivo*, although a number of reports have suggested that erythromycin metabolism is catalyzed mainly by P450 3A4 [44, 45]. To extrapolate from *in vitro* results to *in vivo* settings, more detailed studies are required in cases in which two or more P450 enzymes participate in the oxidation reactions.

Recombinant P450 1A1 was found to have the highest activity for 7-ethoxycoumarin O-deethylation, with a low K_m. However, since contents of P450 1A1 in human liver microsomes are reported to be generally below the limit of detection [46], it is suggested that the contribution of this enzyme for 7-ethoxycoumarin O-deethylation in humans might be minor. Of several human P450 enzymes examined in this study, recombinant human P450 2B6 in human lymphoblastoid cells was also found to have considerable activity. The K_m value obtained in P450 2B6 was found to be very similar to that in P450 2E1 and the turnover number for the 7-ethoxycoumarin O-deethylation activities by P450 2B6 was deter-

* Shimada T and Yamazaki H, unpublished results.

mined to be lower than that by P450 2E1 and higher than that by P450 1A2. Our previous studies, however, have suggested that the level of P450 2B6 in human liver microsomes is extremely low as compared with those of P450 1A2 and 2E1 [6, 12], and therefore the contribution of this enzyme is probably also minor. Contributions of other P450 enzymes—including P450s 2A6, 2C9/10, 2D6, 3A4, and 3A5—in 7-ethoxycoumarin O-deethylation were excluded because these enzymes did not have detectable activities in reconstituted systems.

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