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High Catalytic Activity of Human Cytochrome P450 Co-expressed with Human NADPH-Cytochrome P450 Reductase in *Escherichia coli*

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ABSTRACT. Forms of human cytochrome P450 (P450 or CYP), such as CYP1A1, CYP1A2, CYP2A6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1, and CYP3A4, were expressed or co-expressed together with human NADPH-P450 reductase in *Escherichia coli*. When P450 was expressed alone in *E. coli*, the expression level of holo-P450 ranged from 310 to 1620 nmol/L of culture. The expression level of holo-P450 decreased by co-expression with the reductase, and the level ranged from 66 to 381 nmol/L of culture. The expression level of the reductase varied depending on the forms of P450 co-expressed, and ranged from 204 to 937 U/L of culture. We assayed the catalytic activity of P450 using *E. coli* cells disrupted by freeze–thaw. When co-expressed with the reductase, human P450 catalyzed the oxidation of representative substrates at efficient rates. The rates appeared comparable to the reported activities of P450 in a reconstituted system containing purified preparations of P450 and the reductase. BIOCHEM PHARMACOL **55**;8:1315–1325, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. human P450; heterologous expression; *E. coli*; drug metabolism

P450 or CYP§ is a heme-containing enzyme that catalyzes the oxidation of a wide variety of endogenous and exogenous compounds, including drugs, carcinogens, and other xenobiotic chemicals [1–3]. Catalyzing the bio-oxidation reactions, P450 enzymes play roles in the detoxification and the activation of chemicals to modify the actions of chemicals [4]. P450 superfamilies are composed of families, and four of the families have been identified as having the ability to catalyze the oxidation of foreign chemicals. Since the catalytic properties of P450 even in the same family vary among animal species, it is necessary to use human P450 to predict human drug metabolism that will affect drug actions and toxicities. Human liver specimens have been used as tools to predict human drug metabolism, but the use of these preparations is limited by several factors, including ethical reasons. In addition, the population of each form of P450 varies according to the medical background of donor patients. Another disadvantage of the use of human livers is the low levels of P450 in these biological materials, which are, in some cases, insufficient to analyze the metabolite of a new drug.

The use of P450 preparations expressed in heterologous expression systems has become more popular for examining human drug metabolism, partly because a preparation possessing the same properties can be supplied constantly. Thus, efforts in recent years have realized the expression of several P450 isoforms in bacteria [5–10], yeast [11, 12], and cultured mammalian cells [13–16]. Among the heterologous expression systems, *E. coli* expression systems have advantages compared with other expression systems in terms of the low cost to maintain them, the ease of use, and the high yield of P450 with a relatively short period of incubation. However, the *Escherichia coli* expression system also has a disadvantage compared with other expression systems, since high-level expression of P450 cannot be achieved without modifications of the N-terminal amino acid sequence of P450 protein [17], and no clear evidence has been reported on the change of catalytic properties by modification of N-terminal amino acid sequences. Many reports have appeared indicating that the P450 enzyme expressed in *E. coli* after modification of the N-terminus still shows considerable catalytic activities in reconstituted systems containing NADPH-P450 reductase purified from liver microsomes from appropriate animals [18–24]. *E. coli* has an endogenous electron transport system to support the low level of P450 activity [25]. Since this activity is low, the addition of a purified preparation of the reductase is needed

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§ *Abbreviations:* P450 or CYP, cytochrome P450; reductase, NADPH-cytochrome P450 reductase; LB, Luria-Bertani; TB, Terrific Broth; IPTG, isopropyl β-D-thiogalactopyranoside; HRP, horseradish peroxidase; and $NADP^+$, nicotinamide adenine dinucleotide phosphate-oxidized form.

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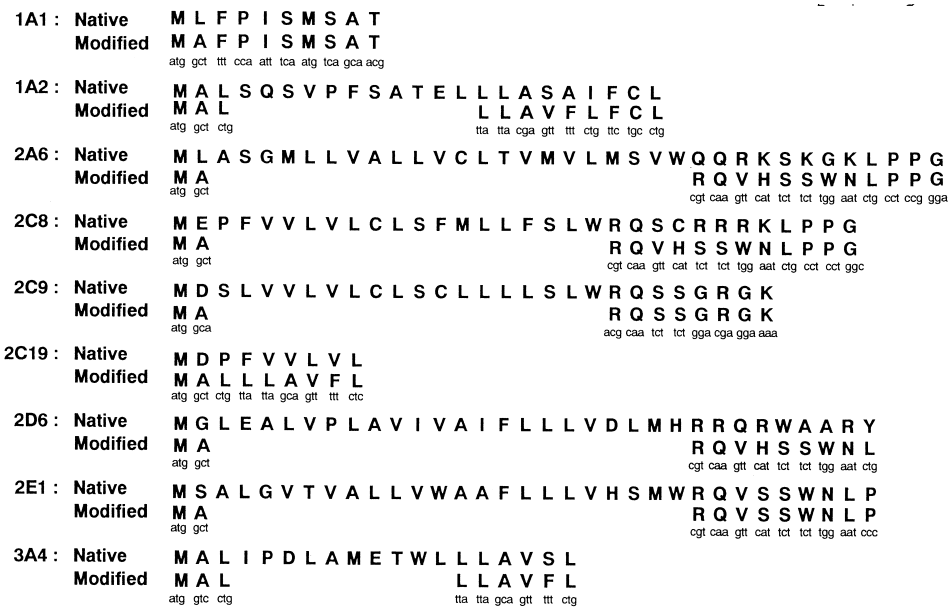

FIG. 1. Modification of N-terminal amino acid and nucleotide sequences for the expression of P450 in *E. coli*.

to reconstitute the system for sufficient activity. To make it easy to predict human drug metabolism using the *E. coli* expression system, the co-expression of both P450 and the reductase is assumed to be the best way, since no purification steps for the enzymes are necessary to incubate the bacteria with a drug to be tested.

In the present paper, we report the successful co-expression of both human P450 and the reductase in *E. coli*, enabling the establishment of genetically engineered *E. coli* strains that have a high metabolic activity for analyzing human drug metabolism.

MATERIALS AND METHODS

Materials

$NADP^+$, glucose-6-phosphate, and glucose-6-phosphate dehydrogenase were obtained from Oriental Yeast; 7-ethoxyresorufin, 7-hydroxyresorufin, 7-ethoxycoumarin, 7-hydroxycoumarin, coumarin, and propranolol from the Aldrich Chemical Co.; 11β- and 6β-hydroxytestosterone from Steraroid, Inc.; tolbutamide and 4-hydroxytolbutamide, 4-nitrophenol, 1,2-dihydroxy-4-nitrobenzene, *o*-hydroxybenzamide, aniline hydrochloride, *p*-aminophenol, taxol, taxotere, testosterone, and phenobarbital sodium from Wako Pure Chemicals; and (*S*)-mephenytoin, 4-hydroxymephenytoin, bufuralol, and 1′-hydroxybufuralol from the Gentest Corp. All other chemicals and solvents were of the highest grade commercially available.

The expression vector pCW was a gift from Dr. Hiroko Sato, Hoffmann–La Roche, and the cDNAs for CYP2D6, CYP1A2, and CYP2E1 were provided by Dr. Frank J. Gonzalez, National Cancer Institute. cDNAs for CYP1A1, CYP2A6, CYP2C19, and the human NADPH-P450 reductase were obtained from human liver total RNA by reverse transcriptase-polymerase chain reaction (RT-PCR) methods in this study. cDNA clones coding for CYP2C8, CYP2C9, and CYP3A4 were isolated from the human adult liver cDNA library prepared in this laboratory [26–28].

Construction of Expression Plasmids

The 5′-termini of nine P450 cDNAs were modified to achieve a high expression level following a method described previously [18–24]. All modifications were introduced by PCR mutagenesis. Modified N-terminal amino acid sequences and cDNA sequences for each of the nine isoforms of P450 are shown in Fig. 1. These modified cDNAs were ligated into a pCW vector. A plasmid carrying a P450 cDNA was digested by *Bst* 1107I and ligated with the reductase cDNA. When necessary, cDNAs for a P450 and the reductase joined to a *tac* promoters and a terminator were introduced into a pCW plasmid, and were linked tandemly. The structure of the co-expression plasmid is shown in Fig. 2.

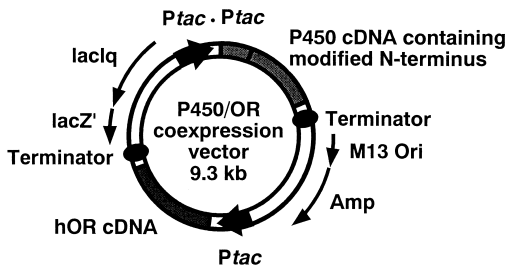

FIG. 2. Structure of plasmid for the co-expression of P450 and the reductase. The 9.3 kb plasmid contains cDNAs for P450 and the reductase, which were linked to the *tac* promoter and terminator, respectively.

Determination of the Expression Level of P450 and the Reductase

E. coli DH5α cells transformed with a plasmid DNA were grown overnight at 37° in LB medium containing 100 μg of ampicillin/mL. A 1-mL aliquot was used to inoculate 100 mL of modified TB medium [18] containing 0.5 mM of δ-aminolevulinic acid in a 500-mL flask. In the case of a system expressing P450 alone, induction of the *tac* promoter was initiated by the addition of 1.0 mM of IPTG, and the mixture was allowed to incubate for 18–48 hr at 30° with vigorous shaking (120 rpm). In the case of a system expressing both P450 and the reductase, induction was initiated by the addition of 1.5 mM of IPTG after preincubation for 8 hr and then the mixture was incubated at 30° for 12 hr with vigorous shaking (120 rpm). Cells were harvested and resuspended in 10 mL of 100 mM of Tris–acetate buffer (pH 7.6) containing 0.5 M of sucrose and 0.5 mM of EDTA, and then frozen at −80° until used. The expression level of P450 in the whole cells of *E. coli* was determined by $Fe^{2+} \cdot CO$ vs Fe^{2+} difference spectra, according to the method of Omura and Sato [29] after dilution of the suspension with 100 mM of potassium phosphate buffer (pH 7.4) containing 20% (v/v) glycerol and 0.2% (w/v) Emulgen 911. The difference spectra were recorded by using a Shimadzu UV-Vis spectrophotometer model MPS-2000. The yield of NADPH-P450 reductase in the sonicated fraction of *E. coli* whole cells was estimated by measuring the rate of cytochrome *c* reduction [30]. The unit of the reductase was defined as the amount of the enzyme that reduced 1 μmol of cytochrome *c*/min.

Immunodetection of P450 and the Reductase

The expression of P450 and the reductase proteins in the *E. coli* whole cells was confirmed by Western blot analysis. SDS–PAGE was performed using a 10% acrylamide gel according to the method of Laemmli [31]. The separated proteins were transferred onto an Immobilon membrane (Waters–Millipore). Immunoblot detection was performed using the following antibodies: goat antibodies to rat CYP1A1, rat CYP2C6, rat CYP2E1, and the rat reductase, and rabbit antibodies to rat CYP1A2, rat CYP2A1, human CYP2D6, and rat CYP3A2. When the goat antibodies for P450 were used as a primary antibody, HRP conjugated with rabbit anti-goat immunoglobulin (Vector Laboratories) was used as a secondary antiserum. When rabbit antibodies against P450 were used as a primary antibody, goat anti-rabbit immunoglobulin (Daiichi Pure Chemicals) and HRP conjugated rabbit anti-goat immunoglobulin were used as a secondary and tertiary antiserum, respectively. The presence of P450 and the reductase proteins was visualized by staining with 3′-diaminobenzidine.

Assays for Catalytic Activity of P450

All assays were carried out with *E. coli* whole cells expressing both P450 and the reductase. The bacterial preparations obtained by a culture were kept frozen until used. An appropriate amount of bacterial preparation was added to the incubation mixture. The amount of bacterial preparation will be given as the amount of P450 in the following experimental procedure. Except for the assay of 4-nitrophenol hydroxylase activity by CYP2E1, a typical incubation mixture consisted of 100 mM of potassium phosphate buffer (pH 7.4), 50 μM of EDTA, an NADPH-generating system (0.5 mM of $NADP^+$, 5 mM of $MgCl_2$, 5 mM of glucose-6-phosphate, and 1 U/mL of glucose-6-phosphate dehydrogenase), and 6.6 to 50 pmol of P450 in a final volume of 1.0 mL. All reactions were initiated by the addition of a substrate.

7-Ethoxyresorufin O-deethylase [32], 7-ethoxycoumarin O-deethylase [33] and coumarin 7-hydroxylase [34] activities were assayed by fluorometric determination of metabolites as described elsewhere. The amount of P450 added to the incubation mixture was 50 pmol for the assay of 7-ethoxyresorufin and 7-ethoxycoumarin O-deethylations, and 10 pmol for the assay of coumarin hydroxylation. Incubations were carried out at 37° for 10 min for the assay of 7-ethoxyresorufin O-deethylation, and 15 min for 7-ethoxycoumarin and coumarin oxidations. The final volume was 1.0 mL for all assays. Aniline hydroxylation was assayed by a colorimetric method as described elsewhere [35]. The amount of P450 added to the incubation mixture was 25 pmol, and the final incubation volume was 1.0 mL. Incubations were carried our at 37° for 15 min.

The assay of (*S*)-mephenytoin 4-hydroxylation was performed as described by Yasumori *et al.* [36]. For this assay, the final volume of the incubation mixture was 0.25 mL, and to the mixture was added 10 pmol of P450. Incubations were carried out at 37° for 60 min. Analysis of the metabolite was performed by HPLC (Hitachi) equipped with a Capcell Pak C18 column (4.6 × 250 mm; SG120 Å; 5 mm; Shiseido).

The activity of 4-nitrophenol hydroxylase was assayed at pH 6.8 according to the method reported by Tassaneeyakul *et al.* [37]. Briefly, the incubation mixture contained 3.3 pmol of P450 in a final volume of 0.5 mL, and incubations were performed at 37° for 60 min. Analysis of the metabolite was performed by HPLC, as mentioned above.

The assay of tolbutamide 4-hydroxylation was carried out as described by Chen *et al.* [38]. Briefly, the incubation mixture (0.25 mL) contained 12.5 pmol of P450, and the mixture was incubated at 37° for 30 min. Analysis of the metabolite was performed by HPLC equipped with a Novapak C18 column (3.9 × 150 mm; 60 Å; 4 mm; Waters).

Taxol 6-hydroxylation was assayed as described by Cresteil *et al.* [39] with minor modifications. Quantification of metabolites was achieved by comparing the peak area of the metabolite with that of an internal standard, taxotere. Since a pure reference standard for 6-hydroxytaxol was not available, we presented the reaction velocity as the peak

area ratio of the metabolite to the internal standard. The incubation mixture (0.25 mL) contained 15.4 pmol of P450, and incubations were carried out at 37° for 30 min. Analysis of the metabolite was performed by HPLC equipped with a Capcell Pak C18 column as mentioned above.

The assay of testosterone 6β-hydroxylation was performed essentially according to the method described by Arlotto *et al.* [40] with minor modifications. The incubation mixture consisted of 11 pmol of P450 expressed in *E. coli* in a final volume of 1.0 mL. Incubations were carried out at 37° for 5 min and terminated by the addition of 4 mL of diethyl ether, and to the mixture was added 1 nmol of 11β-hydroxytestosterone as an internal standard. Metabolites were extracted with diethyl ether. The mixtures were centrifuged at 3000 *g* for 5 min, and the organic layer was evaporated. The residue was dissolved in 200 μL of a solvent used as an initial HPLC mobile phase. A 100-μL aliquot was subjected to HPLC equipped with a Capcell Pak C18 column, as mentioned above. The mobile phase was a mixture of methanol, water, and acetonitrile at 39:60:1 (solvent A) and at 80:18:2 (solvent B). A linear gradient from 98% (v/v) solvent A (0 min) to 20% (v/v) solvent A (30 min) was achieved at a flow rate of 1.0 mL/min.

The assay of bufuralol 1′-hydroxylation was performed as described by Nakamura *et al.* [41], except that the incubation mixture (0.15 mL) contained 7.5 pmol of P450.

All determinations were performed in duplicate. K_m and V_{max} values were calculated from Lineweaver–Burk plots.

RESULTS

Expression of P450 and the Reductase

We constructed expression plasmids with an insert of P450 cDNA alone for nine forms of P450 or together with the reductase cDNA. The plasmids thus constructed were introduced into *E. coli* DH5α. The expression level of holo-P450 determined by carbon monoxide difference spectra is shown in Table 1. Introduction of the plasmid into *E. coli* resulted in the expression of large amounts of the hemoprotein, while a wide variation in the expression level was observed. The expression level ranged from 310 to 1620 nmol/L of culture, and the time to obtain the highest expression level also differed, depending on the forms of P450. CYP2C8 was expressed in *E. coli* at the highest level, and the amount of CYP2C8 in *E. coli* was greater than 1600 nmol/L of culture. The lowest expression was seen with CYP1A1, in which the level of expression was 310 nmol/L of culture.

We also constructed an additional nine expression plasmids carrying cDNAs for each form of P450 together with the P450 reductase, and introduced them into *E. coli* DH5α. The expressions of P450 and the reductase were not necessarily associated with each other. Figure 3 shows periodic changes in the expression levels of CYP2D6 and the P450 reductase after addition of IPTG to the culture medium. As can be seen clearly in the figure, the expression of CYP2D6 was decreased, while the level of the reductase was increased within the culture period. Similar results were observed in the expression of other forms of P450 (data not shown).

TABLE 1. Expression level of P450 in *E. coli* transformed with plasmids carrying cDNA for human P450

Form	Expression level (nmol/L culture)	Incubation time (hr)
CYP1A1	310	24
CYP1A2	520	42
CYP2A6	870	24
CYP2C8	1620	48
CYP2C9	1210	48
CYP2C19	750	48
CYP2D6	580	18
CYP2E1	1250	36
CYP3A4	410	36

E. coli whole cells were suspended in 100 mM of potassium phosphate buffer (pH 7.4) containing 20% (v/v) glycerol and 0.2% (v/v) Emulgen 911. P450 content was measured by $Fe^{2+} \cdot CO$ vs Fe^{2+} difference spectra. The specific values for the expression level are presented as the means of two experiments.

Figure 4 shows the results of an immunoblot analysis of *E. coli* whole cells co-expressing each of the nine P450s and the reductase. Antibodies raised to P450 prepared from liver microsomes from rats as described above cross-reacted specifically with the corresponding human P450s expressed in *E. coli*, except for P450s in CYP2C and CYP1A subfamilies. Antibodies to rat CYP2C6 reacted not only with human CYP2C but also with CYP2A6, CYP2E1, and other forms of P450 (Fig. 4C). However, antibodies to rat CYP2A1 and rat CYP2E1 did not cross-react with CYP2C proteins (Fig. 4, B and E). It has been shown that antibodies to rat CYP1A1 and CYP1A2 cross-react with CYP1A2 and CYP1A1, respectively [42]. As expected, antibodies to rat CYP1A2 reacted with both CYP1A1 and CYP1A2 (Fig.

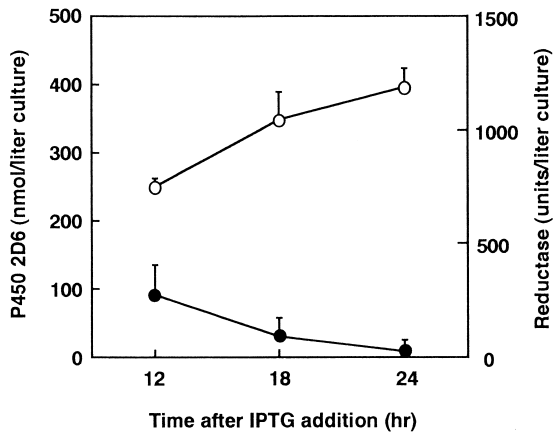

FIG. 3. Time-course of the expression level of P450 (●) and the reductase (○) after the addition of IPTG in *E. coli* co-expressing CYP2D6 and the reductase. The bacterial cells were harvested from 100 mL of culture. The reductase activity of a sonicated whole cell fraction was calculated by measuring the rate of cytochrome *c* reduction.

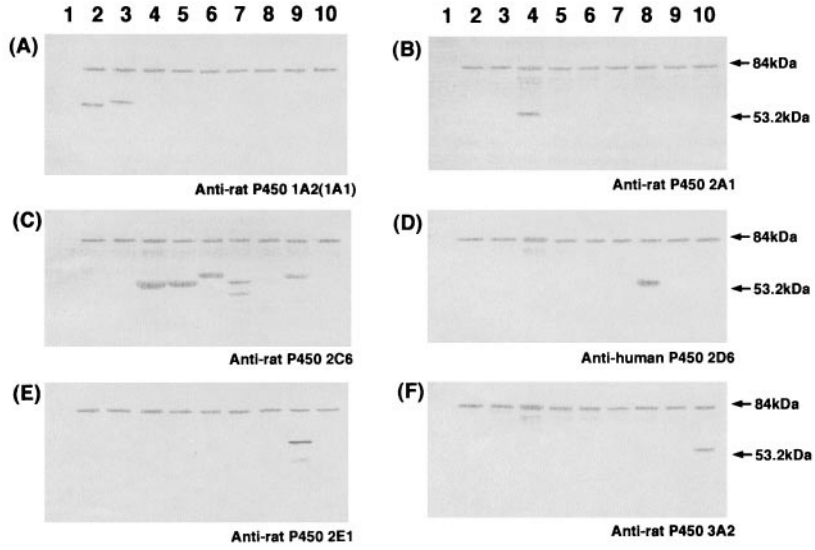

FIG. 4. Immunoblot analysis for each form of P450 in *E. coli* co-expressing P450 and the reductase. In all cases, bacterial whole cells (10 μg protein) were applied for immunoblotting analysis. Lane 1: *E. coli* carrying pCW plasmid alone. Lane 2: CYP1A1 and the reductase. Lane 3: CYP1A2 and the reductase. Lane 4: CYP2A6 and the reductase. Lane 5: CYP2C8 and the reductase. Lane 6: CYP2C9 and the reductase. Lane 7: CYP2C19 and the reductase. Lane 8: CYP2D6 and the reductase. Lane 9: CYP2E1 and the reductase. Lane 10: CYP3A4 and the reductase. Antibodies to rat CYP1A2 (or rat CYP1A1) (A), rat CYP2A1 (B), rat CYP2C6 (C), human CYP2D6 (D), rat CYP2E1 (E), or rat CYP3A2 (F) were applied. In all cases, antibodies to the rat reductases were applied. The upper and lower bands correspond to the reductase and the P450 protein, respectively.

4A). In addition, antibodies to rat NADPH-P450 reductase cross-reacted with the human reductase expressed in *E. coli*. *E. coli* cells that were transformed with the pCW plasmid were applied as a negative control (Fig. 4, lane 1). These results of the immunoblot analysis indicated the expression of significant amounts of P450 and the reductase in *E. coli*. The carbon monoxide difference spectra of P450 expressed in *E. coli* are shown in Fig. 5. The peak wavelength in the difference spectra characteristic of each form of P450 was almost the same as that seen with purified preparations of P450. As an example, a peak at 447 nm was seen in CYP1A.

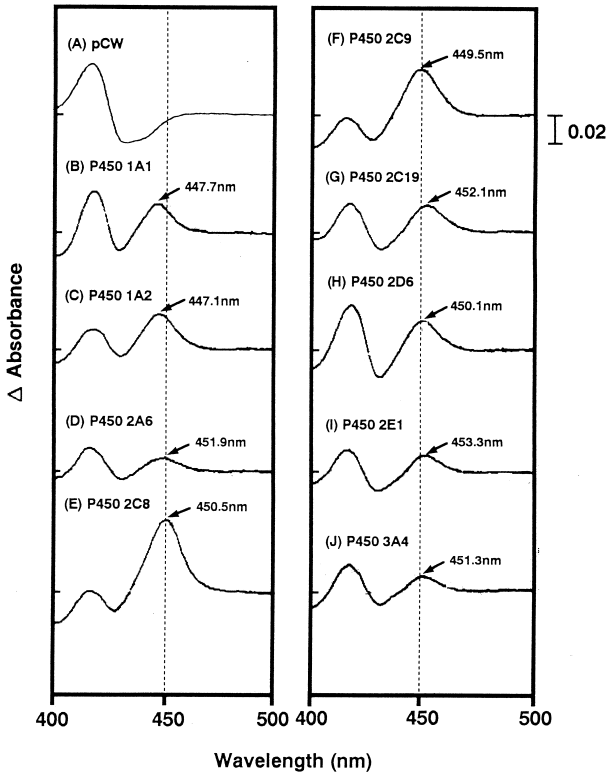

FIG. 5. Typical CO-difference spectra of P450 in genetically engineered *E. coli* co-expressing P450 and the reductase.

The expression level of P450 and the reductase in *E. coli* transformed with a plasmid carrying both P450 and the reductase cDNAs is summarized in Table 2. Compared with the results shown in Table 1, the expression level of P450 was decreased by co-expression with the reductase. The highest expression level of P450 was achieved for CYP2C8, and the level was 381 nmol/L of culture. The lowest expression level was seen for CYP2A6, and the level was 66 nmol/L of culture. The expression levels of the reductase ranged from 204 to 937 U/L of culture.

Catalytic Activities of P450 *expressed in* E. coli

The catalytic activities of P450 expressed in *E. coli* were examined using whole cells of *E. coli* transformed with plasmids carrying both P450 and the reductase cDNAs. *E. coli* whole cells were used after disruption by freeze–thaw.

TABLE 2. Expression level of P450 and the reductase in *E. coli* transformed with plasmids carrying cDNAs for human P450 and the reductase

Form	Expression level	
	P450 (nmol/L culture)	Reductase (U/L culture)
CYP1A1	121 ± 15	397 ± 73 (132)*
CYP1A2	172 ± 54	204 ± 76 (68)
CYP2A6	66 ± 11	266 ± 39 (89)
CYP2C8	381 ± 17	937 ± 146 (312)
CYP2C9	165 ± 77	381 ± 103 (127)
CYP2C19	121 ± 29	568 ± 56 (189)
CYP2D6	91 ± 44	754 ± 37 (251)
CYP2E1	92 ± 13	341 ± 7 (114)
CYP3A4	84 ± 54	436 ± 18 (145)

Specific values are presented as means ± SD ($N = 3$).

*Numbers in parentheses are the amount of the reductase (nmol/L culture) calculated by assuming that 1 nmol of the reductase corresponds to 3 U.

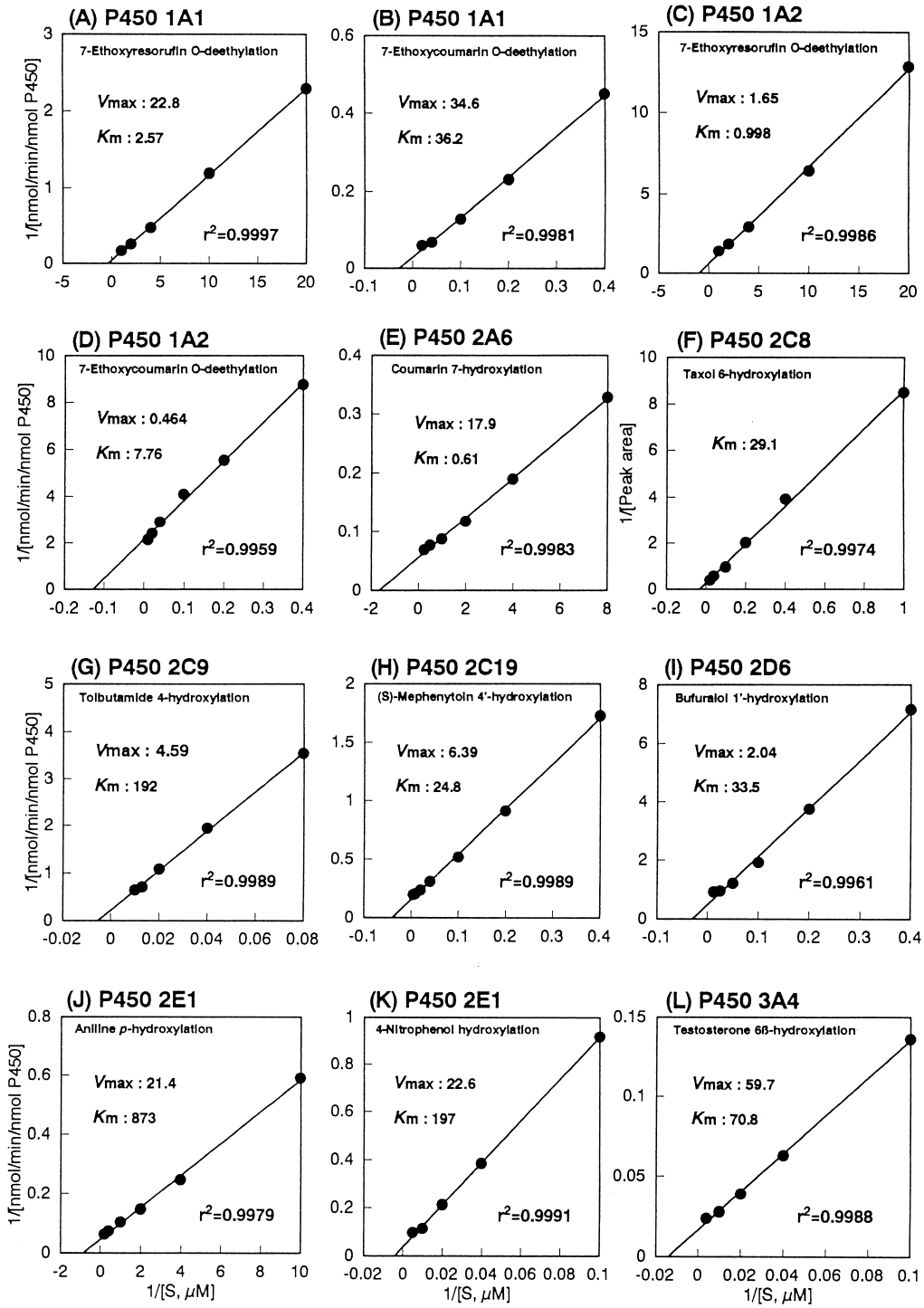

FIG. 6. Lineweaver–Burk plots for the metabolism of each substrate by each of the nine forms of P450 expressed in *E. coli*. The values are the means of duplicate determinations. The K_m and V_{max} values are expressed as μM and nmol/min/nmol P450, respectively.

The apparent K_m and V_{max} values for the catalytic activities were calculated from Lineweaver–Burk plots (Fig. 6). 7-Ethoxyresorufin and 7-ethoxycoumarin were used to evaluate the catalytic activities of CYP1A1 and CYP1A2. *E. coli* DH5α cells that were transformed with CYP1A1 or CYP1A2 together with the reductase were found to metabolize these substrates at efficient rates. The apparent K_m and V_{max} values of CYP1A1 for 7-ethoxyresorufin O-deethylation were 2.57 μM and 22.8 nmol/min/nmol P450, and the values for 7-ethoxycoumarin O-deethylation were 36.2 μM and 34.6 nmol/min/nmol P450, respectively. Similarly, the apparent K_m and V_{max} values of CYP1A2 for 7-ethoxyresorufin O-deethylation were 0.998 μM and 1.65 nmol/min/nmol P450, and the values for 7-ethoxycoumarin O-deethylation were 7.76 μM and 0.464 nmol/min/nmol P450, respectively.

Coumarin has been shown to be hydroxylated at the 7-position to yield 7-hydroxycoumarin specifically by CYP2A6 [43]. *E. coli* DH5α cells transformed with a plasmid carrying CYP2A6 and the reductase cDNAs were also capable of metabolizing this substrate. The apparent K_m and V_{max} values of CYP2A6 for coumarin hydroxylation were 0.61 μM and 17.9 nmol/min/nmol P450, respectively.

Taxol has been reported to be metabolized by CYP2C8 to yield 6-hydroxytaxol [44]. *E. coli* DH5α cells expressing CYP2C8 together with the reductase metabolized taxol with an apparent K_m of 29.1 μM. Because an authentic standard was not available, the V_{max} value of this reaction could not be obtained.

Tolbutamide was used to evaluate the catalytic activity of CYP2C9 [45]. *E. coli* DH5α cells transformed with a plasmid containing inserts of CYP2C9 and the reductase cDNAs metabolized this substrate. The apparent K_m and V_{max} values for tolbutamide 4-hydroxylation were 192 μM and 4.59 nmol/min/nmol P450, respectively.

(*S*)-Mephenytoin is widely known to be a representative drug that shows genetic polymorphism in metabolism in humans [46, 47]. *E. coli* cells expressing CYP2C19 metabolized (*S*)-mephenytoin to yield the 4′-hydroxylated metabolite with apparent K_m and V_{max} values of 24.8 μM and 6.39 nmol/min/nmol P450, respectively.

Bufuralol as well as debrisoquine is a typical substrate of CYP2D6 and also shows genetic polymorphism in its metabolism in humans [48]. The apparent K_m and V_{max} values of bufuralol 1′-hydroxylation for CYP2D6 expressed in *E. coli* were determined to be 33.5 μM and 2.04 nmol/min/nmol P450, respectively.

Aniline and 4-nitrophenol have been used as typical substrates for the examination of the activity of CYP2E1 [49, 50]. *E. coli* cells transformed with a plasmid carrying CYP2E1 and the reductase metabolized these substrates. The apparent K_m and V_{max} values for aniline *p*-hydroxylation were calculated to be 873 μM and 21.4 nmol/min/nmol P450, respectively. The apparent K_m and V_{max} values of CYP2E1 for 4-nitrophenol hydroxylation were 197 μM and 22.6 nmol/min/nmol P450, respectively.

Testosterone has been reported to be metabolized by the CYP3A family to yield 6β-hydroxytestosterone [51]. The activity of CYP3A4 expressed in *E. coli* was examined by determination of testosterone 6β-hydroxylation. The apparent K_m and V_{max} values were 70.8 μM and 59.7 nmol/min/nmol P450, respectively.

DISCUSSION

In the present study, we were able to express human P450 together with the reductase in *E. coli*, and found that P450s expressed in *E. coli* showed catalytic activities. Compared with the K_m and V_{max} values of human P450s reported thus far, P450s expressed in *E. coli* did not necessarily show values identical to those obtained using human liver microsomes (Table 3). The reasons for the discrepancy may be explained as follows. First, liver microsomes contain many forms of P450, and the K_m and V_{max} values were reflected by the presence of other forms. Second, P450 expressed in *E. coli* possesses modified N-terminal amino acid sequences. Thus, the modified N-terminal amino acids might change the catalytic properties of P450. Lastly, the lipid or membrane environments of human liver microsomes and *E. coli* are different, and the difference caused the alteration of the catalytic properties of P450.

When only P450 was expressed in *E. coli* according to methods reported previously [18–24], the expression level of holo-P450 was high and the level ranged from 310 to 1620 nmol/L of culture. However, when the reductase cDNA was introduced in addition to P450 cDNA, the expression level of P450 decreased below one-third the level seen in the expression of P450 alone. The cause of this decrease in the expression level of P450 may be accounted for as follows. First, the capacity of protein synthesis in *E. coli* is not enough for the synthesis of the additional two proteins. Second, P450 might be degraded, probably by an active oxygen produced by the reductase. Supporting this idea, the expression of holo-P450 reached a maximum level 12 hr after the addition of IPTG and then decreased, while the level of apo-P450, determined by Western blot, increased even after incubation for 12 hr. This increase of the P450 level was nearly parallel with the increase of the expression of the reductase.

In Western blot analysis using antibodies to seven forms of P450, antibodies to rat CYP2C6 cross-reacted with human CYP2A6, CYP2E1, and three other hemoproteins, while antibodies to rat CYP2E1 and CYP2A1 specifically recognized human CYP2E1 and CYP2A6, respectively. It is likely that antibodies to rat CYP2C6 recognized the same epitope(s) present in human CYP2C, CYP2A6, and CYP2E1.

E. coli cells expressing P450 alone showed low but significant catalytic activities in the oxidation of a substrate, indicating that there are some factors resembling the reductase in the ability to transfer electrons to P450 (not shown). However, the introduction of the reductase cDNA to express the enzyme in the same cells resulted in a large enhancement of the catalytic activity of P450. Thus, we showed in the present paper that all *E. coli* strains co-expressing P450 and the reductase efficiently catalyzed typical substrates without addition of the reductase in whole cells.

Human liver microsomes contain multiple forms of P450. Since a substrate can be metabolized by multiple forms of P450 with different affinities, a nonlinear curve in Lineweaver–Burk plots is often obtained, reflecting the affinity of more than two enzymes for a substrate. In the *E. coli* system, only one form of P450 is expressed in the cells, which should lead to a linear curve in the plots. As was expected, we obtained a high linearity in the plots using the *E. coli* whole cells ($r^2 > 0.9959$) (Fig. 6).

Dong and Porter [63] observed that no substrate was metabolized by *E. coli* whole cells expressing both CYP2E1

TABLE 3. Catalytic activities or kinetic parameters

P450 form	Catalytic reaction	Source	Enzyme preparation	Activity	K_m (μM)	Reference
1A1	7-Ethoxyresorufin O-deethylation	*E. coli*	Whole cells	22.8*†	2.57	Present study
		Human liver	Microsomes	0.06†	0.19	52
		E. coli	Purified	8.0†		19
		Hep G2	Cell lysates	1.36†		53
	7-Ethoxycoumarin O-deethylation	*E. coli*	Whole cells	34.6*†	36.2	Present study
1A2	7-Ethoxyresorufin O-deethylation	*E. coli*	Whole cells	1.65*†	0.998	Present study
		Human liver	Microsomes	0.185, 0.060*‡	0.39, 0.40	54
		E. coli	Purified	0.72†		18
	7-Ethoxycoumarin O-deethylation	*E. coli*	Whole cells	0.464*†	7.76	Present study
		Human liver	Microsomes	0.122*‡	5.84	55
2A6	Coumarin 7-hydroxylation	*E. coli*	Whole cells	17.9*†	0.61	Present study
		Human liver	Microsomes	1.33, 1.51‡	0.2–0.6	34
		Human liver	Microsomes	0.031–1.350‡	2.3	43
		Human liver	Purified	2.09†		56
		Hep G2	Cell lysates	13.0†		53
2C8	Taxol 6-hydroxylation	*E. coli*	Whole cells	NC§	29.1	Present study
		Human liver	Microsomes	0.12*‡	15.0	39
		E. coli	Purified	4.1*‡	23.7	24
2C9	Tolbutamide 4-hydroxylation	*E. coli*	Whole cells	4.59*‡	192	Present study
		Human liver	Microsomes	0.273*‡	120	57
		Hep G2	Cell lysates	2.48†		53
		E. coli	Purified	1.4†		24
		Yeast	Microsomes	2.07†,0.035‡	260	38
2C19	(*S*)-Mephenytoin 4′-hydroxylation	*E. coli*	Whole cells	6.39*†	24.8	Present study
		Human liver	Microsomes	0.189*‡	21.6	58
		Human liver	Microsomes	0.205*†	16.5	59
		Human liver	Purified	0.323*†	65.4	59
		E. coli	Purified	3.3†		24
2D6	Bufuralol 1′-hydroxylation	*E. coli*	Whole cells	2.04*†	33.5	Present study
		Human liver	Microsomes	0.134*‡ (HL-18)	44	60
		Hep G2	Cell lysates	1.69†		53
		E. coli	Purified	1.23†		23
		Human liver	Purified	6.37†		61
		Human liver	Purified	0.58†		62
2E1	Aniline *p*-hydroxylation	*E. coli*	Whole cells	21.4*†	873	Present study
		E. coli	Solubilized membranes	1.8†		63
		Human liver	Microsomes		6.3–24.2	52
		E. coli	Solubilized membranes	35.1†	440	64
		Hep G2	Cell lysates	14.8†		53
		Human liver	Purified	7.30†		49
		Human liver	Purified	13.6†		65
	4-Nitrophenol hydroxylation	*E. coli*	Whole cells	22.6*†	197	Present study
		E. coli	Solubilized membranes	2.0†		63
		Human liver	Microsomes	0.99*‡	30	50
		Human liver	Microsomes	1.1*‡	26.3	37
		E. coli	Solubilized membranes	9.4†		64
3A4	Testosterone 6β-hydroxylation	*E. coli*	Whole cells	59.7*†	70.8	Present study
		E. coli	Whole cells	17.3†		66
		Human liver	Microsomes	9.6, 4.8, 8.9*‡	68, 229, 89	67
		Human liver	Microsomes	0.50‡		68
		Human liver	Purified	2.24†		51
		Hep G2	Cell lysates	57.2†		53
		E. coli	Purified	9.8†		22

All determinations were performed in duplicate.

*Apparent V_{max} value.

†Unit of metabolic activity (nmol/min/nmol P450).

‡Unit of metabolic activity (nmol/min/mg protein).

§Not calculated.

and the reductase, while the activities of 4-nitrophenol and *N*-nitrosodimethylamine oxidations became detectable after solubilization of the membranes of the *E. coli*. In their study, it was proposed that a most likely reason for the lack of the activity in whole cells was the fact that the expression level of P450 was very low. Furthermore, they described, as the reason for the phenomenon, that substrates could not gain access into the inside of cell membranes where the enzymes locate. In contrast to the data mentioned above, *E. coli* DH5α co-expressing CYP2E1 and the reductase possessed high catalytic activities for aniline and 4-nitrophenol. In this study, we used cells kept frozen at −80° until used as an enzyme source to determine K_m and V_{max} values. In our preliminary experiments using *E. coli* cells expressing CYP2E1, CYP2A6, or CYP2D6, which were freshly prepared after culture, we obtained the same K_m and V_{max} values as seen using cells after freeze–thaw (not shown). It is unlikely that the activity appeared due to the freeze–thaw, which causes the degradation of cell membranes. In accord with our hypothesis, Blake *et al.* [66] demonstrated that typical substrates of CYP3A4, such as testosterone and nifedipine, were metabolized in *E. coli* whole cells that co-expressed CYP3A4 and the reductase. It appears that the permeability of the membranes of *E. coli* XL1 Blue employed by Dong and Porter [63] is different from that of *E. coli* JM109 and DH5α employed by Blake *et al.* [66] and by us, respectively. This hypothesis should be examined in further studies.

The amount of the reductase was calculated by assuming that 1 nmol of reductase reduces 3 μmol of cytochrome *c*/min [69]. Thus, the molar ratio of the reductase to P450 was calculated to vary from 0.4:1 (*E. coli* co-expressing CYP1A2 and the reductase) to 1.73:1 (*E. coli* co-expressing CYP3A4 and the reductase). In our previous studies, we reported that P450 requires 2- to 3-fold excess amounts of the reductase to show its full activity in a reconstituted system [70]. Thus, the expression level of the reductase in *E. coli* strains established in the present study was close to the level yielding the highest activity of P450. In addition, it may also be possible that the reductase efficiently interacts or functionally associates with P450 in *E. coli* inner membranes.

It has been well known that cytochrome b_5 is required depending on the form of P450 and substrate used. Among the forms of P450, CYP2E1 and CYP3A4 are forms of P450 that require cytochrome b_5 with many substrates [51, 71]. The enhancement of P450 activity by co-expression of cytochrome b_5 has been reported in other systems [72, 73]. In this study, however, high catalytic activities towards typical substrates were seen in whole cells without cytochrome b_5. It would be interesting to know if the activity of these forms of P450 is further enhanced by co-expression of cytochrome b_5 in *E. coli*.

In other studies using living whole cells that were not frozen, we confirmed that some isoforms of P450 (CYP2A6, CYP2D6, and CYP2E1) catalyzed the oxidation of substrates without externally added NADPH as an NADPH-generating system (not shown). We have not examined if the same can be seen for other isoforms of P450. The activity of P450 was about the same as that seen with whole cells used after freeze–thaw in the presence of an NADPH-generating system. These results indicate that the amounts of NADPH being generated in *E. coli* cells are sufficient to metabolize drugs.

In summary, we have successfully expressed each of the nine forms of human P450 together with the human reductase in *E. coli*. These nine strains showed high catalytic activities for typical substrates in whole cells. Therefore, the genetically engineered strains of *E. coli* will be useful tools to predict drug metabolism in humans.

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