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Recombinant Enzymes Overexpressed in Bacteria Show Broad Catalytic Specificity of Human Cytochrome P450 2W1 and Limited Activity of Human Cytochrome P450 2S1[S]

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ABSTRACT

Human cytochromes P450 2S1 and 2W1 have received only limited attention with regard to characterization of function. Both cytochromes P450 have been reported to be overexpressed in human tumors, and cytochrome P450 2S1 is induced by carcinogenic polycyclic hydrocarbons. We report methods for high-level expression and purification of both cytochromes P450 from *Escherichia coli*, with the goal of establishing function. The level of expression of human cytochrome P450 2W1 achieved using codon optimization for *E. coli* was 1800 nmol of cytochrome P450 per liter of culture, the highest level achieved in this laboratory to date. Assays with a number of the typical cytochrome P450 substrates showed no detectable activity, including some for which qualitative reports have appeared in the literature. Cytochrome P450 2W1 catalyzed benzphetamine *N*-demethylation (k_{cat}, 3.8/min) and arachidonic acid oxidation, albeit at a very low rate (~0.05/min). In a *umu* genotoxicity screen, cytochrome P450 2W1 catalyzed the activation of several procarcinogens, particularly polycyclic hydrocarbon diols, but cytochrome P450 2S1 did not. The bioactivation of procarcinogens by cytochrome P450 2W1 may be of significance in the context of reports of preferential expression of the enzyme in tumors, in that activation of procarcinogens could lead to the accumulation of mutations and enhance the carcinogenic process.

P450 enzymes are important catalysts involved in the oxidation of sterols, eicosanoids, fat-soluble vitamins (Ortiz de Montellano, 2005), and a large fraction of drugs used today (Guengerich, 2005). The human genome has 57 P450 or "*CYP*" genes. One way to classify these genes is based on the substrates oxidized by the P450s (Guengerich, 2005). The P450s involved only in the oxidation of xenobiotics are rather dispensable (in the absence of exposure to particular drugs), as judged by the results of the studies involving human polymorphisms and transgenic mice (Gonzalez and Kimura, 2003). However, dysfunctional (mutant) genes for the P450s involved in the metabolism of sterols and vitamins can be debilitating or lethal (Nebert and Russell, 2002; Guengerich, 2005).

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At least 13 of the human P450 genes can be considered "orphans" in the sense that they presently have no ascribed function in terms of metabolism of either endogenous or xenobiotic chemicals (Guengerich et al., 2005). One of these P450s is P450 2S1, which was first identified in databases of expressed sequence tags (Rylander et al., 2001) and subsequently cloned by another group (Rivera et al., 2002). The mRNA is inducible by polycyclic hydrocarbons, including dioxins (Rivera et al., 2002), and a role in the metabolism of carcinogens was suggested. The protein is expressed in human skin, with up-regulation by UV light (Smith et al., 2003). All-*trans*-retinoic acid was also reported to induce P450 2S1, and the same compound was reported to be a substrate, being converted to the 4-hydroxy and 5,6-epoxide products (Smith et al., 2003), although no quantitation was reported. Another qualitative report of the ability of P450 2S1 to catalyze oxidation of naphthalene (to unidentified products) has appeared (Karlgren et al., 2005a). Up-regulation of expression has been reported in tumors (Downie et al., 2005). Although only very limited results on function have

ABBREVIATIONS: P450, heme-thiolate protein P450 or cytochrome P450 (Palmer and Reedjik, 1992); CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; PCR, polymerase chain reaction; HPLC, high-performance liquid chromatography; SA buffer, potassium phosphate, glycerol, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate, dithiothreitol, and EDTA; buffer SB, potassium phosphate, glycerol, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate, dithiothreitol, EDTA, and imidazole; buffer WA, potassium phosphate, glycerol, dithiothreitol, EDTA, sodium cholate, and Tergitol Nonidet P-10; buffer WB, potassium phosphate, glycerol, EDTA, and dithiothreitol.

been published (and negative evidence has been presented for roles in the metabolism of benzo[*a*]pyrene, the tobacco-specific nitrosamine 4-(methylnitrosoamino)-1-(3-pyridyl)-1-butanone, and the heterocyclic arylamine 2-amino-1-methyl-6-phenylamidazo[4,5-*b*]pyridine (Wang et al., 2005)), efforts to link allelic variants with cancer have nevertheless been initiated (Saarikoski et al., 2004).

Another of the orphan P450s is P450 2W1. Relatively little information about human P450 2W1 has been published. A cDNA (AK000366.1) was reported in a Japanese project, but this has been suggested by Nelson (http://drnelson.utmem.edu/CytochromeP450.html) to be missing two exons based on the genomic sequence. One report of mRNA analysis indicates that P450 2W1 is transcribed in several adult human tissues, including spleen, testis, lung, pancreas, placenta, ovary, thymus, prostate, colon, and small intestine (Choudhary et al., 2005). The mRNA was also expressed at a relatively high level in fetal lung tissue. However, another laboratory has reported that P450 2W1 mRNA is not expressed in any of these adult or fetal human tissues (Karlgren et al., 2005a). The latter laboratory reported that P450 2W1 was expressed in HepG2 cells (of human liver origin) but was not found in human liver microsomes (Gomez et al., 2005; Karlgren et al., 2005b). One report indicated that P450 2W1 was not expressed in B16A2 or human embryonic kidney 293 cell lines because of differences in DNA methylation (Gomez et al., 2005), but other reports from the same laboratory indicate the expression of P450 2W1 in human embryonic kidney 293 cells and the production of protein variants (Karlgren et al., 2005b). Karlgren and coworkers (2006) recently reported expression in 54% of human tumors examined at much higher levels than in fetal and adult tissues. Another group reported the up-regulation of P450 2W1 in patients with gastric cancer (Aung et al., 2005).

One of the limitations of the study of the functions of these two family 2 P450s and others is the limited availability of recombinant protein. Only limited information regarding heterologous expression systems has been reported with these two P450s (Smith et al., 2003; Karlgren et al., 2005a, 2006; Wang et al., 2005). Furthermore, none of the reported catalytic activities have been quantified. Although questions about the roles of both P450 2S1 and 2W1 have been raised in regard to cancer, only limited assays with carcinogens have been reported (Wang et al., 2005). We present high-level bacterial systems for expression and purification and some analyses of proposed and other catalytic activities.

Materials and Methods

Chemicals and Reagents. P450 2S1 cDNA was a gift of O. Hankinson (University of California, Los Angeles, Los Angeles, CA) (Rivera et al., 2002). Oligonucleotides for cDNA synthesis and *N*-terminal modifications were purchased from Operon (Huntsville, AL) in either 96-well format (at 10 nmol scale in, in wet form, normalized to 100 μM each) or tube format (at 50 nmol scale). All oligonucleotides were of salt-free quality and were used directly without further purification.

Naphthalene was recrystallized from diethyl ether, and all-*trans*-retinoic acid (Sigma Chemical Co., St. Louis, MO) was recrystallized from ethanol (in amber glass). 1-Naphthol and 2-naphthol, used as standards for product searches, were recrystallized from ethanol/H_2O mixtures. Arachidonic acid, [1-^{14}C]arachidonic acid (50 μCi/μmol), and [1-^{14}C]oleic acid (54 μCi/μmol) were purchased from Sigma-Aldrich (St. Louis, MO). Benzo[*a*]pyrene and benz[*a*]anthracene were purchased from Sigma and Aldrich Chemical Co. (Milwaukee, WI), respectively. Benzo[*a*]pyrene-7,8-diols [(±)-, (+)-, and (−)-], 3-hydroxybenzo[*a*]pyrene, and benz[*a*]anthracene-*trans*-3,4-diol were purchased from the National Cancer Institute's Chemical Carcinogen Repository/Midwest Research Institute (Kansas City, MO). 7,12-dimethylbenz[*a*]anthracene, 7,12-dimethylbenz[*a*]anthracene-*trans*-3,4-diol, chrysene-1,2-diol, 5-methylchrysene-1,2-diol, 5,6-dimethylchrysene-1,2-diol, and dibenzo[*a,l*]pyrene-11,12-diol were kindly donated by S. S. Hecht (University of Minnesota, Minneapolis, MN). Other chemicals and reagents used in this study were obtained from sources described previously or were of the highest qualities commercially available (Shimada et al., 1996, 2001; Kim and Guengerich, 2004).

Optimization of the P450 2W1 Nucleotide Sequence for Heterologous Expression and PCR-Based Gene Synthesis. Automatic codon optimization to suit the codon preference bias of *Escherichia coli* and oligonucleotide design for PCR-based gene synthesis were performed using DNAWorks 2.4 (Hoover and Lubkowski, 2002) as described previously (Wu et al., 2006). The amino acid sequence and the native cDNA sequence information for human P450 2W1 were obtained from the cytochrome P450 homepage of D. R. Nelson (http://drnelson.utmem.edu/human.P450.seqs.html) and assembled from GenBank sequence AC073957. A PstI restriction site was selected from the native cDNA sequence and was used to separate the full-length gene into two synthons encoding amino acids 1 to 214 (Synthon 1) and 215 to 490 (Synthon 2). In addition, a $(His)_5$ tag was added to the C-terminal to facilitate purification. A series of overlapping 40- to 50-mers were prepared for each synthon (see Supplemental Data) based on the DNA Works results (as described above).

The assembly and amplification of Synthon 1 and Synthon 2 were performed in the same manner as that for the synthons of P450 27C1 (Wu et al., 2006). Positive clones containing both Synthon 1 and Synthon 2 in a "monocistronic" pCW vector [not coding for NADPH-cytochrome P450 reductase (Parikh et al., 1997)] were selected by following the standard molecular biology protocols and were sequenced in both the sense and antisense strands in the Vanderbilt facility on an Applied Biosystems model 3700 fluorescence sequencing unit using a Taq dye terminator kit (PE Applied Biosystems, Foster City, CA).

N-Terminal Modifications for P450 2S1 and 2W1. N-Terminal mutations were introduced into the native construct (pTARGET 2S1 and pCW 2W1) by PCR-based mutagenesis. A C-terminal $(His)_5$ tag was introduced into the P450 2S1 cDNA at the same time using a 3′-primer containing sequences encoding histidine residues and XbaI site (see Supplemental Data). The N-terminal segment of P450 2W1 was amplified between the NdeI and the PstI sites using 5′-PCR primers containing the desired mutations and the 3′ outmost oligonucleotide of Synthon 1 (see Supplemental Data). PfuUltra High-Fidelity DNA polymerase was used for the PCR amplification at an annealing temperature of 55°C for P450 2S1 and 60°C for P450 2W1. The products were purified, double-digested, and ligated with the monocistronic pCW vector (together with the 843-base pair fragment derived from the digestion of construct 1 for P450 2W1). The modifications were confirmed by sequencing the open reading frame region of the new constructs. Bicistronic plasmids of 2S1 construct 1 and 2W1 construct 3 were later constructed by ligating the corresponding cDNA with a bicistronic pCW vector (Parikh et al., 1997) using the NdeI and XbaI restriction sites.

Expression of P450s 2S1 and 2W1. The expression trials of P450 2S1 constructs 1 to 5 were performed at 29°C in *E. coli* DH5α F′IQ using methods described elsewhere (Gillam et al., 1993). Construct 1 of P450 2S1 was also coexpressed with chaperon groEL/ES in *E. coli* DH5α at 29°C, as well as P450 2W1 constructs at 27°C in the same manner as for P450 27C1 (Wu et al., 2006).

Large-scale expressions for P450 2S1 construct 1 and P450 2W1 construct 3 were performed in 2.8-liter Fernbach flasks with 500 ml of media for 40 h at 29°C (for P450 2S1) and 27°C (for P450 2W1)

essentially in the same manner as used for P450 27C1(Wu et al., 2006) in a New Brunswick Innova 4300 shaker (New Brunswick Scientific, Edison, NJ).

Purification of Recombinant P450s 2S1 and 2W1. All purification steps were performed at 4°C. *E. coli* membranes were prepared as described previously (Gillam et al., 1993; Guengerich and Martin, 2006). The P450 2S1 membrane fractions from a 1-l culture were diluted in buffer SA [300 mM potassium phosphate, pH 7.4, containing 20% glycerol (v/v), 1.25% CHAPS (w/v), 0.1 mM dithiothreitol, and 0.1 mM EDTA). The mixture was stirred for 2 h and centrifuged at 10^5g for 30 min. The resulting supernatant was loaded onto a Ni^{2+}-nitriloacetic acid agarose column (QIAGEN, Valencia, CA; 5-ml packed-bed volume), which had been equilibrated with 10 column volumes of buffer SA and 20 mM imidazole (buffer SB). After washing with 10 column volumes of buffer SB, P450 2S1 was eluted at a flow rate of 0.5 ml/min with buffer SA containing 200 mM imidazole, and the eluted orange fraction was collected as ~0.7-ml aliquots into tubes containing 10 μl of 100 mM EDTA to bind any eluted Ni^{2+} ions. The eluted P450 2S1 was pooled and dialyzed three times against 100 volumes of 100 mM potassium phosphate buffer, pH 7.4, containing 20% glycerol (v/v) and 0.1 mM EDTA to remove CHAPS.

The P450 2W1 membrane fractions were diluted in buffer WA [same components as buffer SA except containing 1.0% sodium cholate (w/v) and 1.0% Tergitol Nonidet P-10 (w/v) substituted for CHAPS] and purified on a Ni^{2+}-nitriloacetic acid agarose column in the same manner as that for P450 2S1 using buffer WA with imidazole. The eluted orange fraction was applied to prepacked PD-10 desalting columns (Amersham Biosciences, Piscataway, NJ) to remove imidazole using buffer WA with an increased concentration of EDTA (1 mM) in the elution buffer.

Detergent-free P450 2W1 was prepared by applying the eluted enzyme to a Biogel HTP hydroxylapatite column (10-ml packed-bed volume; Bio-Rad, Richmond, CA), which had been equilibrated and then was washed with buffer WB [40 mM potassium phosphate, pH 7.7, containing 20% glycerol (v/v), 0.5 mM EDTA, and 0.1 mM dithiothreitol] until the A_{280} of the effluent was <0.02. P450 2W1 was then eluted by increasing the potassium phosphate concentration to 0.7 M, with sodium cholate added to a final concentration of 1.0% (w/v). The eluted P450 2W1 was dialyzed three times against 100 volumes of 300 mM potassium phosphate buffer, pH 7.4, containing 20% glycerol (v/v), 0.1 mM dithiothreitol, and 1 mM EDTA to remove cholate.

Activation of Procarcinogens by P450s 2S1 and 2W1 Using a *Salmonella typhimurium* NM2009 Tester Strain (*umu* Assay). The procedures have been described in detail elsewhere (Oda et al., 1985; Shimada et al., 1989, 1994), and the basis of the system is described as follows. *S. typhimurium* TA1535 is used as a host (but mutations are not scored as in the typical Ames test protocol). The bacteria are incubated with the P450 system, procarcinogen, and NADPH, and the activated carcinogens modify the bacterial DNA. The DNA damage leads to polymerase stalling and the induction of the "SOS response" (Friedberg et al., 2006). The protein RecA binds to single-stranded regions of DNA produced by polymerase stalling and cleaves the repressor LexA, starting a cascade leading to the activation of >30 genes. Among these is *umuC/D*, which codes for a DNA polymerase that can bypass DNA damage and allow survival of the bacteria. Oda and coworkers (1985) linked the *umuC* upstream region to a *lacZ* reporter gene on a plasmid, which was added to *S. typhimurium* TA1535 (to produce the tester strain pSK1002, subsequently modified with an *N*-acetyltransferase to produce NM2009). In this system, then, DNA damage resulting from P450 activation of procarcinogens induces the SOS response, the *umu*-based reporter system, and the production of β-galactosidase, which is monitored in a colorimetric assay.

In brief, bicistronic membrane fractions containing P450 1B1 (Shimada et al., 1996) (10 pmol, as positive control), 2S1, or 2W1 (25 pmol each) were incubated at 37°C with 2.5 μM concentrations of the procarcinogens and a 750-μl fresh culture of *S. typhimurium* NM2009 (Shimada et al., 1994) in 1.0 ml of 50 mM potassium phosphate buffer, pH 7.4, and an NADPH-generating system for 2 h. The resulting mixtures (100 μl) were then remixed with a mixture of 25 μl of 0.1% SDS (w/v), 3 μl of $CHCl_3$, and 100 μl of *o*-nitrophenyl-β-D-galactopyranoside (4 mg/ml stock dissolved in 0.1 M sodium phosphate buffer, pH 7.0) and 900 μl of 60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$, and 50 mM β-mercaptoethanol to determine β-galactosidase activity. After 15-min incubation at 37°C, the reactions were terminated by adding 0.5 ml of a 1 M Na_2CO_3 solution. A_{420} readings of the final mixtures were recorded. Relative bacterial growth rate was determined by measuring the optical density at 600 nm. The activation of *umu* gene expression is presented as units of β-galactosidase activity per minute per nanomole of P450 (Shimada et al., 1994).

Enzyme Assays. Substrate concentrations are indicated in the relevant table (Table 2). [1-^{14}C]Oleic acid (54 μCi/μmol, stock 0.1 mCi/ml in ethanol) and [1-^{14}C]arachidonic acid (50 μCi/μmol, stock 0.1 mCi/ml in ethanol) were each used in 1.0 ml of 100 mM potassium phosphate buffer, pH 7.4, containing bicistronic membranes of P450 2S1 or 2W1 or with human liver microsomes (0.5–0.9 μM P450). The reactions (at 37°C) were initiated by the addition of an NADPH-generating system composed of (final concentrations) 0.5 mM NADPH, 10 mM glucose 6-phosphate, and 1.0 IU yeast glucose 6-phosphate dehydrogenase per milliliter (Guengerich, 2001) and terminated after 30 min by the addition of 0.04 ml of 2.0 M NaCl/1.0 M HCl, with mixing in a vortex device. After extraction with 2 × 1.0 ml of ethyl acetate, the combined organic extracts were dried under an N_2 stream, dissolved in CH_3OH, and subjected to HPLC. HPLC was done on a 4.6 × 150 mm Prodigy octadecylsilane (C18) column (3 μm; Phenomenex, Torrance, CA) for oleic acid and arachidonic acid products [with an 80–100% CH_3CN linear gradient over 20 min in 0.2% aqueous CH_3CO_2H (v/v) solution, at a flow rate of 1 ml/min]. Detection was with a 2B β-RAM flow counter (IN/US Systems, Tampa, FL) for ^{14}C-labeled materials.

Similar incubation conditions were used for the other assays (except *umu* assays, see above), using reaction volumes of 0.75 to 1.5 ml, with human liver microsomes (sample HL 114) as a positive control. Benzphetamine and *N*,*N*-dimethylnitrosamine *N*-demethylation were measured colorimetrically (Nash, 1953; Guengerich, 2001). Testosterone hydroxylation and benzo[*a*]pyrene oxidation were measured using HPLC (on a 6.2 × 80 mm Zorbax ODS column, 3 μm; Agilent Technologies, Palo Alto, CA) as described previously (Bauer et al., 1995; Krauser and Guengerich, 2005). The details and results of the naphthalene and all-*trans*-retinoic acid oxidation assays (HPLC), done according to the general procedures described by Cho et al. (2006) and Marill et al. (2000), respectively, are described in the Supplemental Data. HPLC detection was done with a Thermo UV-1000 rapid-scanning UV detector (Thermo Electron Corporation, Waltham, MA) and with benzo[*a*]pyrene and naphthalene with a M^{ac}Pherson FX-750 fluorescence detector (M^{ac}Pherson, Danvers, MA).

Results

Construction and Expression of N-Terminal Variants. The P450 2W1 cDNA was synthesized with the codons optimized to fit the codon bias of *E. coli* using the same approach established in previous work (Wu et al., 2006) with an overall error rate of 0.45 errors per kilobase (two errors from three plasmid sequences). The alignment of the final optimized cDNA sequence compared with the native sequence of P450 2W1 is shown in Fig. 1. The N-terminal hydrophobic sequences of P450s 2S1 and 2W1 were truncated or modified before the well-conserved proline-rich region. The modifications were based on reported examples on

successful expressions of P450s, family 2 enzymes in particular, in *E. coli* (Table 1).

The expression of five P450 2S1 constructs in *E. coli* DH5α F′IQ was initially only modest. Only construct 1 was able to generate ~120 nmol P450/l. An increased expression level of up to 600 nmol P450/l could be achieved for this construct when it was coexpressed with the molecular chaperon *gro*ES/EL at 29°C for 40 h (Fig. 2A).

The expressions of the three P450 2W1 constructs (coexpressed with molecular chaperon *gro*EL/ES in *E. coli* DH5α) differed dramatically, with construct 3 having the highest expression level (1800 nmol P450/l) and constructs 1 and 2 <100 nmol P450/l (Fig. 2B) after incubation for 40 h at 27°C. A direct comparison of construct 3 expression with and without coexpression of *gro*ES/EL showed a dramatic increase of P450 expression (1800 versus 350 nmol P450/l culture) when arabinose was added to initiate the induction of *gro*ES/EL (Fig. 2B).

The expression of bicistronic P450 2S1 (construct 1) and P450 2W1 (construct 3) yielded approximately half of the amount of P450 compared with the corresponding monocistronic version. The human NADPH-P450 reductase contents in membranes were 88 and 68% of that of P450 for 2S1 and 2W1, respectively, as judged by a cytochrome *c* reduction assay, similar to previous results (Parikh et al., 1997).

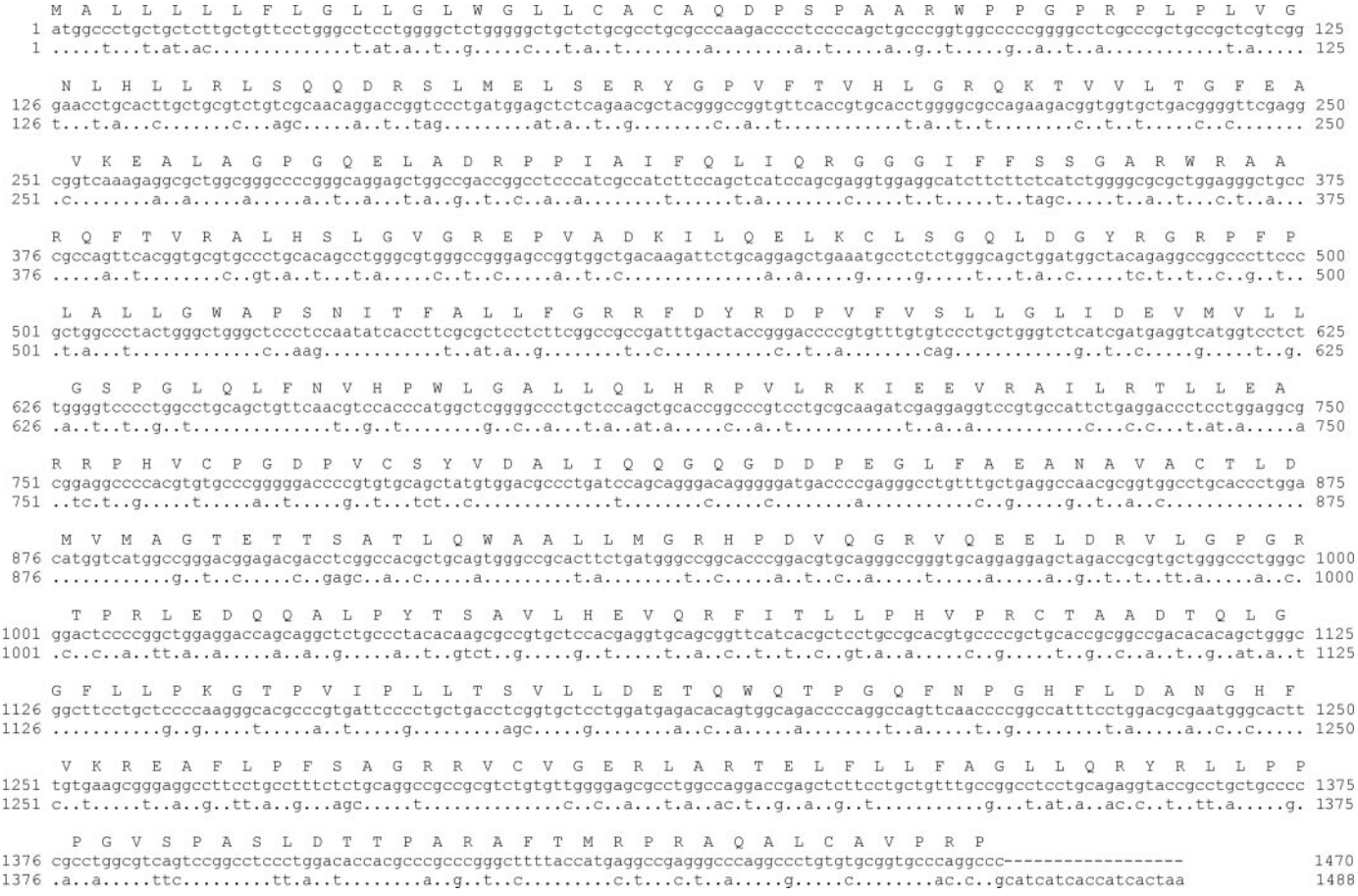

Fig. 1. Modifications introduced into P450 2W1 cDNA to optimize codon usage in *E. coli*. Top line, predicted amino acid sequence; middle line, nucleotide sequence predicted from genomic DNA; bottom line, nucleotide sequence optimized for *E. coli* expression.

TABLE 1

Selection of *N*-terminal sequences of P450s 2S1 and 2W1 for heterologous expression

Amino acid changes are in italics and underlined.

Construct	Basis of Selection of N Terminus	*N*-Terminal Amino Acid Sequence
2S1 Native		MEATGTWALLLALALLLLLTLALSGTRARGHL PPGPTPLP
2S1 #1	P450 2C3 rabbit[a]	*MAKKTSSKGK*L PPGPTPLP
2S1 #2	P450 2C11 rat[b]	*MARQSFGRGK*L PPGPTPLP
2S1 #3	P450 2E1 human[c]	*MARQVHSSWN*L PPGPTPLP
2S1 #4	P450 17A1 bovine[d]	*M*ALLLA*VF*LLLLLTLALSGTRARGHL PPGPTPLP
2S1 #5	Full-length	M*A*ATGTWALLLALALLLLLTLALSGTRARGHL PPGPTPLP
2W1 #1	Synthesized native	MALLLLLFLGLLGLWGLLCACAQDPSPAARW PPGPRPLP
2W1 #2	P450 17A1 bovine[d]	M*A*LLL*AVFL*GLLGLWGLLCACAQDPSPAARW PPGPRPLP
2W1 #3	P450 2C3 rabbit[a]	*MAKKTSSKGKL* PPGPRPLP

[a] Richardson et al. (1995).
[b] Licad-Coles et al. (1997).
[c] Gillam et al. (1994).
[d] Barnes et al. (1991).

Purification of P450s 2S1 and 2W1. Solubilization of *E. coli* membranes containing P450 2S1 was achieved in the presence of 1.25% CHAPS (w/v) with an efficiency of 65%. After the Ni^{2+}-nitriloacetic acid column, the $(His)_5$-tagged P450 2S1 was eluted in apparent quantitative yield as judged by P450 content. Purified P450 2S1 was quite stable after removal of the detergent by dialysis, but P450 2W1 tended to aggregate under similar conditions. Thus, P450 2W1 was finally solubilized and purified in the presence of 1.0% sodium cholate (w/v), 1.0% Tergitol (w/v), and a high concentration of phosphate buffer, with a solubilization efficiency of 95% and a quantitative Ni^{2+} column yield. Attempts to remove Tergitol using a hydroxylapatite column resulted in ~60% loss of the protein, however, largely because of aggregation at the top of the column.

The purified P450 2S1 and 2W1 proteins showed a major band at ~50 kDa (calculated 54 and 52 kDa for P450s 2S1 and 2W1, respectively, based on sequences) using SDS-polyacrylamide gel electrophoresis (Fig. 3). The specific content of P450 2S1 was estimated to be 13.4 nmol/mg protein and that of P450 2W1 to be 16.8 nmol/mg protein, based on a bicinchoninic acid colorimetric protein estimation method.

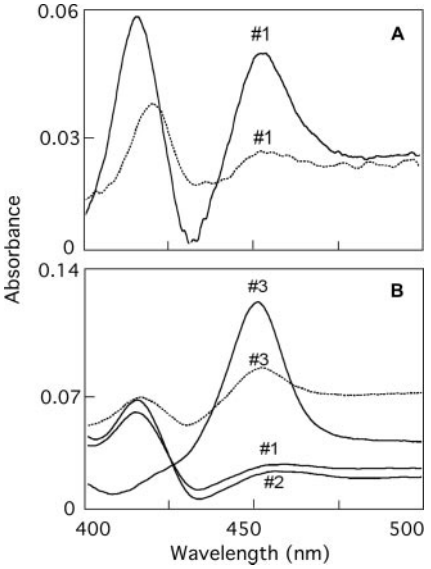

Fig. 2. Expression of P450 2S1 construct 1 (A) and 2W1 constructs 1, 2, and 3 (B) in *E. coli*. Constructs were expressed in strain DH5α (with pGro12 EL/ES). Expression of P450 2S1 construct 1 and 2W1 construct 3 (without coexpression of *gro*EL/ES) is shown with broken lines. Fe^{2+}·CO versus Fe^{2+} difference spectra were recorded using 1:2 dilutions of whole-cell extracts.

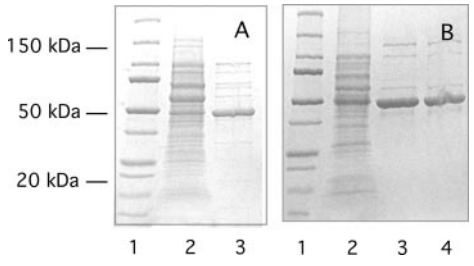

Fig. 3. SDS-polyacrylamide gel electrophoresis of recombinant P450 2S1 (A) and 2W1 (B). Lane 1, protein M_r markers; lane 2, solubilized fraction; lane 3, Ni^{2+}-nitriloacetic acid agarose-purified fraction; lane 4, PD-10-desalted fraction.

Spectral Properties of Purified P450s 2S1 and 2W1. The spectra of purified P450s 2S1 and 2W1 were recorded (Fig. 4) and are very typical of most P450s, with both ferric P450s in the low-spin iron form (A and D), as confirmed using second-derivative analysis (O'Haver and Green, 1976). The P450s were essentially free of cytochrome P420 (C and F). One unusual observation for which we have no explanation at this time is the presence of a small band at 450 nm in the ferrous spectra (A and D) that is not due to carbon monoxide. Several of the potential substrates (10 μM coumarin, 100 μM naphthalene, 100 μM lauric acid, and 100 μM arachidonic acid; Table 2) were added to the ferric enzymes but did not cause the conversion of low- to high-spin iron, except (very weakly) in the case of naphthalene with P450 2W1 ($K_{d,app}$ 2.5 ± 1.9 mM).

Search for Catalytic Activities of P450s 2S1 and 2W1. Several reactions were examined, including some known to be catalyzed by multiple P450s and those for which qualitative evidence has been presented with these two proteins. All studies were done with membranes from "bicistronic" constructs, in which NADPH-P450 reductase was coexpressed (Table 2). No oxidation of *N*,*N*-dimethylnitrosamine, testosterone, benzo[*a*]pyrene, or oleic acid could be detected. *d*-Benzphetamine *N*-demethylation was catalyzed by P450 2W1 but not by P450 2S1 (Table 2). Further analysis of the P450 2W1 reaction yielded K_m = 380 (± 40 μM) and k_{cat} = 3.8 (± 0.2)/min. P450 2W1 also catalyzed arachidonic acid oxidation to a mixture of several products that have not been defined (see Supplemental Data); the reaction was NADPH-dependent but very slow, with the total of all products still only giving a turnover number of ~0.05/min. Karlgren and coworkers (2006) recently reported trace conversion of arachidonic acid to the 8,9-, the 11,12-, and the 14,15-epoxides but did not quantify rates.

Naphthalene has been reported to be oxidized by P450 2S1 to two unidentified products, M1 and M2 (Karlgren et al., 2005a). In our systems, neither P450 2S1 nor 2W1 formed

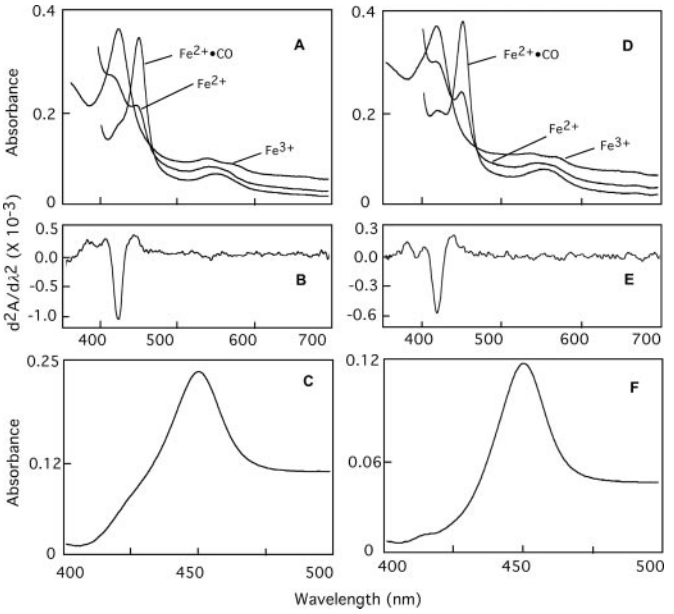

Fig. 4. Spectra of purified P450 2S1 (A–C) and 2W1 (D–F). A and D, absorbance spectra (absolute). B and E, second derivatives of absorbance spectra (ferric state). C and F, Fe^{2+}·CO versus Fe^{2+} difference spectra.

any detectable products from naphthalene, when reactions were carried out in either the absence or presence of added rat epoxide hydrolase (Table 2 and Supplemental Fig. S1). Exactly what other primary oxidation products could be formed from naphthalene, aside from 1- and 2-naphthol, naphthalene-1,2-oxide, and naphthalene-1,2-dihydrodiol, is unclear, except for the possibility of the 2,3-epoxide and its dihydrodiol. It would seem that these compounds would have been detected in the HPLC-UV analysis. No quantitative information was presented in the analysis by Karlgren and coworkers (2005a). The limit of detection in our study was ~0.05/min (Table 2 and Supplemental Data).

TABLE 2

Reactions examined with P450s 2S1 and 2W1

Each reaction was catalyzed, with the indicated substrate concentration, using *E. coli* membranes in which the P450 was coexpressed with human NADPH-P450 reductase. The limits of detection are indicated.

Reaction	P450 2S1	P450 2W1
	nmol product formed/min/nmol P450	
d-Benzphetamine *N*-demethylation (1.0 mM)	<0.02	2.9
N,*N*-Dimethylnitrosamine *N*-demethylation (1.0 mM)	<0.02	<0.02
Naphthalene oxidation (300 μM), (± excess epoxide hydrolase; to 1- and 2-naphthol, 1,2-dihydronaphthol, or any other products)	<0.05	<0.05
Testosterone hydroxylation (100 μM)	<0.08	<0.08
Benzo[*a*]pyrene oxidation (100 μM)	<0.3	<0.05
All-*trans*-retinoic acid oxidation (10 μM)	<0.003	<0.003
Oleic acid hydroxylation (9 μM)	<0.003	<0.03
Arachidonic acid hydroxylation (50 μM)	<0.01	~0.05

P450 2S1 has also been reported to convert all-*trans*-retinoic acid to the 4-hydroxy and 5,6-epoxide products (Smith et al., 2003), but our analyses did not show the formation of any products with a turnover number ≥0.003/min (Table 2 and Supplemental Fig. S2). No quantitation of retinoic acid products was done in the report by Smith and coworkers (2003), so a direct comparison is not possible (as with all assays in Table 1, products were detected with human liver microsomes as a positive control). One conceivable possibility for the lack of P450 2S1 activity is the modified N terminus, although the same substitution was used with the active P450 2W1 (Table 1).

Another search involved chemical carcinogens that might be activated by these P450s, given the potential relationships postulated for cancer (Saarikoski et al., 2004; Aung et al., 2005; Downie et al., 2005). As mentioned earlier, P450 2S1 is known to be induced by polycyclic aromatic hydrocarbons and, because of this induction, proposed to also activate carcinogens (Rivera et al., 2002). We used a bacterial *umu* assay that has previously been established as a useful means of monitoring the activation of procarcinogens by P450s (Oda et al., 1985; Shimada et al., 1989, 1994, 1996) (Fig. 5). P450 1B1, which has high activity toward many procarcinogens (Shimada et al., 1996), was used as a positive control. The results (Fig. 5) showed very limited, if any, activation of the 22 various carcinogens tested. However, P450 2W1 had significant activity toward at least half of those tested. The group includes several polycyclic aromatic hydrocarbon dihydrodiols ("diols") (which are generally more positive than the parent hydrocarbons in these systems because of the lack of

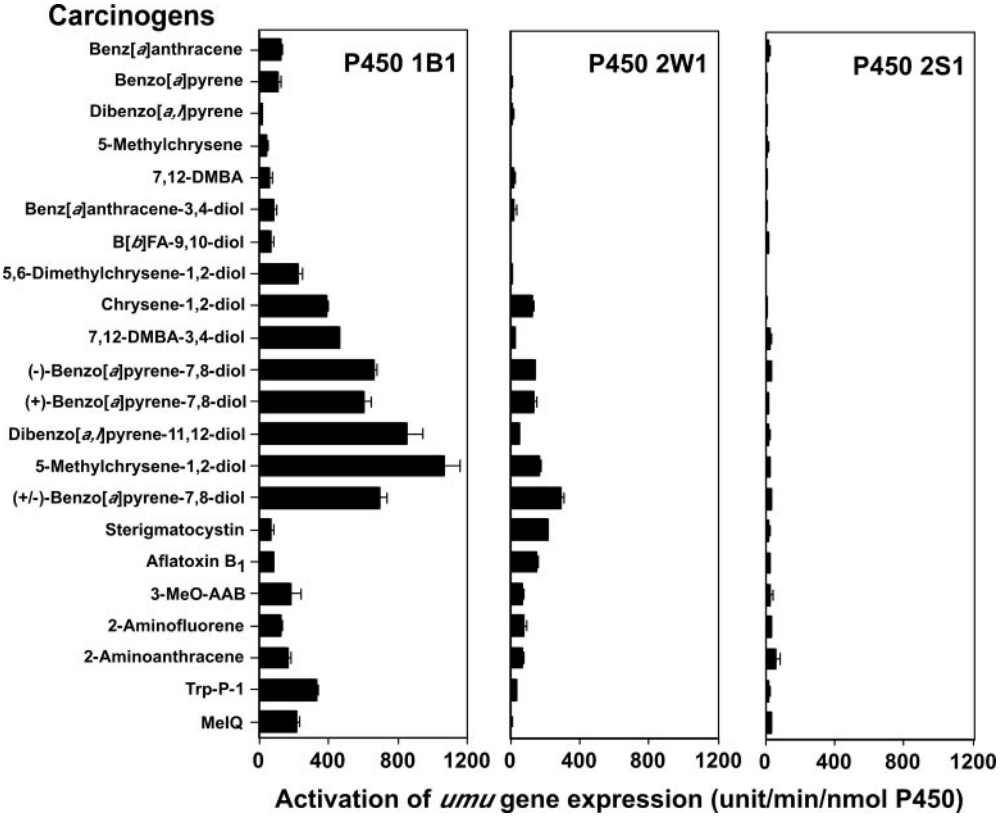

Fig. 5. Activation of procarcinogens by P450s 1B1, 2S1, and 2W1 using *S. typhimurium* NM2009 *umu* system.

epoxide hydrolase), aflatoxin B_1 and sterigmatocystin, and some arylamines.

Bicistronic cultures of P450 2W1 yield some blue color, which we believe is generally indicative of indole 3-hydroxylation (Gillam et al., 2000). However, in preliminary in vitro assays (with bicistronic membranes), activity was not detected in a simple colorimetric assay (Wu et al., 2005), although the sensitivity is probably not great enough to preclude conclusions about the reaction with P450 2W1. P450 2S1 cultures did not produce any blue color.

Discussion

High-level expression systems have been developed for two orphan human P450s, 2S1 and 2W1. Both P450s have been purified to homogeneity and have rather typical spectral properties. Several potential reactions of these P450s have been examined. P450 2W1 has some broad capability to oxidize exogenous chemicals, including the drug benzphetamine, arachidonic acid (albeit slow), and 11 of the 22 procarcinogens examined. The oxidative capability of P450 2W1 contrasts with that of P450 2S1, which did not oxidize any of the potential substrates tested at measurable rates, including naphthalene and all-*trans*-retinoic acid. If either of these reactions is catalyzed by P450 2S1, the rates seem to be very low. The availability of methods to obtain large amounts of these two Family 2 P450s should permit the application of broader searches for function. The activation of a broad range of procarcinogens is of potential relevance in light of the reported preferential expression of P450 2W1 in tumors (Karlgren et al., 2006) but not in normal human tissues, in that P450 2W1 might predispose the tumors to the accumulation of mutations and accelerate cancer development.

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