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Functional Characterization of Human Cytochrome P450 2S1 Using a Synthetic Gene-Expressed Protein in *Escherichia coli* [S]

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ABSTRACT

Human cytochrome P450 2S1 was recently identified and shown to be inducible by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin and hypoxia. It is highly expressed in epithelial cells of tissues that are exposed to the environment and in many tumors of epithelial origin. The biological function of CYP2S1 has not yet been determined, although its possible role in carcinogen metabolism has been suggested. In this report, we investigated its ability to metabolize carcinogens. To obtain a large quantity of active enzyme for substrate screening, we overexpressed CYP2S1 in *Escherichia coli* (200 nM culture), using a synthetic gene approach. High-level expression allowed us to achieve purification of CYP2S1 with high specific content and purity (16 nmol/mg). Despite high-level expression, we found that CYP2S1 was not readily reduced by cytochrome P450 reductase, and thus no activity was found using NADPH. However, the oxidative activity of CYP2S1 was supported by cumene hydroperoxide or H_2O_2, such that CYP2S1 oxidized many important environmental carcinogens, including benzo[*a*] pyrene, 9,10-dihydro-benzo[*a*]pyrene, 7,12-dimethylbenz[*a*]anthracene, benzo[*a*]pyrene-7,8-dihydrodiol, aflatoxin B1, naphthalene, and styrene, with high turnover. Most substrates tested were converted to detoxification products, except in the case of benzo[*a*]pyrene-7,8-dihydrodiol, which was converted into the very potent carcinogenic metabolite 7,8-dihydrodiol-*trans*-9,10-epoxide at a relatively efficient rate ($K_m = 12.4 \pm 2\ \mu M$, turnover = $2.3\ min^{-1}$). This metabolite formation was also supported both in vitro and in vivo by fatty acid hydroperoxides described in the accompanying report (p. 1044). Together, these data indicate that CYP2S1 contributes to the metabolism of environmental carcinogens via an NADPH independent activity.

Cytochrome P450s (P450s) are heme-thiolate enzymes functionally classified as monooxygenase (EC 1.14.14.1), which catalyzes a wide spectrum of reactions (e.g., heteroatom oxygenations, carbon hydroxylations, epoxidations, and dehalogenations). P450s play critical roles in bioactivation and detoxication of a wide variety of xenobiotic substances, including environmental toxicants and therapeutic compounds, as well as participating in the endogenous metabolism of fatty acids, steroids, eicosanoids, bile acids, and fat-soluble vitamins. With the sequencing of the human genome, 57 human cytochrome P450s and 58 pseudogenes have been discovered (http://drnelson.utmem.edu/CytochromeP450.html). Of the 57 human cytochrome P450s, 13 remain classified as "orphans," because their functions are still largely unknown (Guengerich et al., 2005). These are P450s 2A7, 2S1, 2U1, 2W1, 3A43, 4A22, 4F11, 4F22, 4V2, 4Z1, 20A1, and 27C1. The putative roles of these cytochrome P450s in carcinogenesis and toxicology warrant further study (Stark and Guengerich, 2007).

The *CYP2S1* gene was identified by a homology search in the human genome (Rylander et al., 2001) and also by a representational difference analysis subtractive hybridization procedure to isolate novel 2,3,7,8-tetrachlorodibenzo-*p*-dioxin-inducible genes (Rivera et al., 2002). Dioxin induction of CYP2S1 is mediated by the aromatic hydrocarbon receptor and its dimerization partner, the aromatic hydrocarbon re-

ABBREVIATIONS: P450, cytochrome P450; IPTG, isopropyl-β-D-thiogalactopyranoside; hNPR, human P450 reductase; BaP, benzo[*a*]pyrene; 9,10-H_2-BaP, 9,10-dihydro-benzo[*a*]pyrene; BaP-7,8-diol, benzo[*a*]pyrene-*trans*-7,8-dihydrodiol; r7,t8,t9,c10-tetrol, benzo[*a*]pyrene-*r*-7,*t*-8,*t*-9,*c*-10-tetrahydrotetrol; DMBA,7,12-dimethylbenz[*a*]anthracene; 3,4-diol-DMBA,7,12-dimethylbenz[*a*]anthracene-*trans*-3,4-diol; 7-OH-DMBA,7-hydroxy methyl-12-methylbenz(*a*)anthracene; 12-OH-DMBA, 12-hydroxymethyl-7- methylbenz[*a*]anthracene; HPLC, high-performance liquid chromatography; KP$_i$, potassium phosphate buffer; CHP, cumene hydroperoxide; HR, human reductase; ΔG, Gibbs free energy of a secondary structure of any given single-stranded DNA or RNA.

ceptor translocator (Hankinson, 1995). Members of families 2 through 4 are not generally inducible by 2,3,7,8-tetrachlorod-ibenzo-*p*-dioxin and PAHs. CYP2S1 was also shown to be inducible by hypoxia via hypoxia-inducible factor 1, and by all-*trans*-retinoic acid (Smith et al., 2003; Rivera et al., 2007). Moreover, elevated expression of CYP2S1 has been detected in the smokers (Thum et al., 2006). The human *CYP2S1* gene, the sole member of the *CYP2S* subfamily, is located in a cluster of *CYP2* family members on chromosome 19q. This cluster includes the *CYP2A6*, *CYP2A13*, *CYP2B6*, and *CYP2F1* genes. The proteins encoded by these genes are the closest relatives to human CYP2S1, with 47 to 49% sequence identity to CYP2S1 (Saarikoski et al., 2005b).

CYP2S1 is highly expressed in epithelial cells of tissues that are exposed to the environment (e.g., skin, respiratory, urinary, and gastrointestinal tracts), and expression seems to be higher than for other P450s. In addition, CYP2S1 is highly expressed in many tumors of epithelial origin (Saari-koski et al., 2005b). Thus, the level of CYP2S1 protein is increased in colorectal cancer, and high expression was associated with poor prognosis (Kumarakulasingham et al., 2005). Elevated levels of CYP2S1 protein also occur in metastasic ovarian cancer (Downie et al., 2005). CYP2S1 is primarily an extrahepatic enzyme, because it is expressed in many extrahepatic tissues but very poorly in liver (Bieche et al., 2007).

Based on its mode of regulation and sites of expression, CYP2S1 was suggested to have a potential role in metabolism of carcinogens (Saarikoski et al., 2005a). However, only very limited data on the function of CYP2S1 have been published. Smith and coworkers reported that human CYP2S1 expressed in *Escherichia coli* metabolized all-*trans*-retinoic acid to 4-hydroxy- and 5,6-epoxy-retinoic acid. However, no quantitation was reported (Smith et al., 2003). Wang et al. (2005) expressed human CYP2S1 in Chinese hamster ovary cells and insect cells, and reported that there was no increase in 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone or benzo-[*a*]pyrene toxicity in the transfected Chinese hamster ovary cells, and that the heterologously expressed CYP2S1 protein expressed in insect cells failed to metabolize nicotine and 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine. Karlgren et al. (2005) expressed human CYP2S1 in yeast and reported that it can metabolize naphthalene, but no identification or quantitation of products was presented. Wu et al. (2006), however, could not detect metabolism of naphthalene or all-*trans*-retinoic acid using *E. coli* recombinant human CYP2S1, even though they obtained high-level bacterial expression of CYP2S1 using chaperon GroEL/ES. Thus, questions about the role of CYP2S1 have been raised in regard to carcinogen and xenobiotic metabolism, but the results have been ambiguous. In this report, we present an improved method for heterologous expression of human CYP2S1 in *E. coli*. The high-level expression that we obtained allowed us to achieve protein purification with a high yield, and provided us a reliable enzyme preparation source for analyzing and characterizing the function and catalytic activity of CYP2S1.

Materials and Methods

Chemicals and Reagents. *E. coli* LMG 194 was purchased from Invitrogen (Carlsbad, CA). The human *CYP2S1* cDNA was described previously (Rivera et al., 2002). The pBAD plasmid was purchased

from Invitrogen. pCW′/1A2:hNPR, a pCW bicistronic IPTG-inducible plasmid containing the human P450 reductase cDNA (hNPR) was kindly provided by Dr. Fred Guengerich (Vanderbilt University, Nashville, TN). All restriction digestion enzymes were purchased from New England Biolabs (Ipswich, MA). Recombinant human CYP1A1, CYP2C9, CYP2C8, CYP3A4 (all containing cytochrome P450 reductase), and P450 reaction buffer mixtures containing NADP(+)/H regeneration enzymes were obtained from Biocatalytics, Inc. (Pasadena, CA). Cumene hydroperoxide (CHP), hydrogen peroxide, testosterone, 6-β-hydroxytestosterone, harmaline, all-*trans*-retinoic acid, naphthalene, coumarin, and styrene were obtained from Sigma-Aldrich (St. Louis, MO). The carcinogens aflatoxin B1, benzo[*a*]pyrene (BaP), 9,10-dihydro-benzo[*a*]pyrene (9,10-H_2-BaP), benzo[*a*]pyrene-*trans*-7,8-dihydrodiol (BaP-7,8-diol), benzo[*a*]pyrene-*r*-7,*t*-8,*t*-9,*c*-10-tetrahydrotetrol (r7,t8,t9,c10-tetrol), 4-hydroxy benzo-[*a*]pyrene, 7,12-dimethylbenz[*a*]anthracene (DMBA), 7,12-dimethyl-benz[*a*]anthracene-*trans*-3,4-diol, 7-hydroxy methyl-12-methylbenz(*a*)an-thracene (7-OH-DMBA), 12-hydroxymethyl-7- methylbenz[*a*]anthracene (12-OH-DMBA) were purchased from the National Cancer Institute's Chemical Carcinogen Repository (Kansas City, MO). IPTG and L-arabi-nose and the HPLC solvents (HPLC grade) were obtained from Sigma Aldrich (St. Louis, MO). Oligonucleotides were purchased from IDT DNA (San Diego, CA). All the carcinogens were weighed out in a glove box and handled with extreme care.

Design of a Synthetic Gene for CYP2S1 to Optimize Heterologous Expression in *E. coli*. Codon and mRNA structure optimization for the *CYP2S1* gene to suit heterologous expression in *E. coli* was as follows. The amino acid sequence of CYP2S1 with N-terminal modification 4 and a C-terminal His-4 tag (Supplementary Table S1) was reverse translated by DNA2.0 software into DNA with the most abundant *E. coli* codons. The DNA sequence was then submitted to the DNA mfold server (http://mfold.bioinfo.rpi.edu/cgi-bin/dna-form1.cgi) to theoretically predict the secondary structure of single stranded DNA (which is used in place of single-stranded RNA), and its ΔG was calculated. Based on the structure and the ΔG information, the *CYP2S1* DNA sequence was further optimized by replacing codon(s) in the region where base paring of more than seven consecutive bases occurred. The DNA was then refolded. The final DNA sequence (Fig. S1) was sent to Gene Oracle (Mountain View, CA) for full-length gene synthesis. After the synthetic *CYP2S1* DNA was synthesized and its sequence was confirmed, it was transferred to the pBAD vector using the NcoI and HindIII sites at the 5′ and 3′ ends, respectively. The synthetic *CYP2S1* DNA was also transferred into the dual-expression vector containing the human P450 reductase (*hNPR*) gene to generate pBdtacHR-CYP2S1 vector (Supplementary Fig. S2).

Expression and Processing of CYP2S1. All plasmids were transformed into *E. coli* LMG 194 (Invitrogen) for expression. The expression trials of various constructs were carried out in 400 ml of terrific broth medium plus 100 μg/ml ampicillin in 2-liter flasks. The conditions were as followed. A 1-ml overnight culture at 30°C in Luria-Bertani medium + 100 μg/ml ampicillin was transferred into 400 ml of terrific broth + 100 μg/ml ampicillin. The culture was grown in a shaker (New Brunswick Scientific, Edison, NJ) at 34°C at 150 RPM. After 5 h, 0.5 mM Δ-aminolevulinic acid (heme precursor) and 0.5 mM IPTG (inducer for the P450 reductase) were added, and the temperature was reduced to 30°C. One hour later, 0.02% of L-arabinose was added, and the growth conditions were changed to 30°C at 115 rpm, and continued for 24 to 36 h before harvest. The culture was harvested by centrifugation at 5000*g* for 15 min at 4°C. The cell pellet was then resuspended in 10% of the original culture volume in 20 mM potassium phosphate buffer (KP_i), pH 7.5, containing 20% glycerol, 1 mM phenylmethylsulfonyl fluoride, and 0.1 mM dithiothreitol. Speroplasts were produced as described previously (Wester et al., 2002). The speroplasts were then resuspended in 100 mM KP_i, pH 7.5 containing 6 mM $MgSO_4$, 1 mM phenylmethylsul-fonyl fluoride, and 0.1 mM dithiothreitol (4 ml for every gram of cell pellet). The resuspended spheroplasts were lysed with a homoge-

nizer at 13,000 psi followed by centrifugation, to collect the CYP2S1-containing lysate. The concentration of CYP2S1 in each culture (nanomoles per liter of culture) was measured using the Fe^{2+} CO versus Fe^{2+} difference spectrum assay of the lysate as described by Omura and Sato (1964b), using a Shimadzu dual-beam UV-Vis spectrophotometer. The CYP2S1 content was calculated using an extinction coefficient of 91 $mM^{-1}cm^{-1}$. P450 reductase activity was estimated using cytochrome c as a substitute electron acceptor, and yields (nanomoles of reductase per liter of culture) were calculated assuming a specific activity of 3200 nmol of cytochrome c reduced/min/nmol reductase (Yasukochi and Masters, 1976).

NADPH-Reduced Fe^{2+} CO versus Fe^{2+} Difference Assay. Bacterial cell membranes containing P450 and P450 reductase were diluted to 0.2 μM in 100 mM KP_i, pH 7.25, 20% glycerol, and 1-ml aliquots were suspended in each of two cuvets. One of the two cuvets was bubbled with CO for 30 s. A baseline spectrum of equal light absorption from 400 to 500 nm was recorded between the cuvets using a dual-beam spectrophotometer (Shimadzu). NADPH (1 mM) was added to both cuvets, and the difference spectrum from 400 to 500 nm was recorded. Substrate (50 μM) was then added to both cuvets, and the difference spectrum was repeated. A small amount (<1 mg) of dithionite was then added as a positive control, and the spectrum was recorded again.

Enzyme Assay. All assays were conducted in 1.5-ml Eppendorf tubes in duplicate. For the assays using NADPH to support P450 activities, 1000-μl reactions contained 1× reaction buffer mixture (100 mM KP_i, pH 7.5, NADPH/NADP + regenerating enzymes), 50 to 200 nM recombinant CYP3A4 + P450 reductase, CYP1A2 + P450 reductase, CYP2C8 + P450 reductase, or 200 nM CYP2S1 + reductase (either nonsynthetically or synthetically encoded CYP2S1), and various concentrations of substrates. The reactions were carried out at 37°C for 30 min to 2 h before being terminated with 1000 μl of acetonitrile contained 2% acetic acid. The reactions were frozen in dry-ice methanol for 5 min, followed by centrifugation at 13,000g for 15 min to remove proteins. The supernatants were then analyzed by HPLC with diode array and fluorescence detectors. For assays using CHP or H_2O_2, 150-μl reactions contained 100 mM KP_i, pH 7.5, 0.1 to 0.2 μM CYP3A4, CYP1A1, or purified synthetically encoded CYP2S1 (see Supplementary Figure S5), various concentrations of CHP (0.1 μM–1 mM) or H_2O_2 (0.1 μM–10 mM), and various concentration of substrates. The reactions were carried out at 37°C for 5 min before being stopped with 150 μl of acetonitrile (2% acetic acid). The samples were centrifuged at 13,000g for 15 min to remove protein. Supernatants were then analyzed using HPLC.

K_m and Turnover Determinations. To determine the K_m of recombinant CYP2S1 for various substrates, each substrate was progressively diluted 1:3 eight times, and the dilutions were incubated in 150-μl reaction mixtures containing 200 nM purified CYP2S1 and 1 mM CHP. To determine the K_m of CHP and H_2O_2 for CYP2S1 supporting various substrate oxidations, seven progressive 1:3 dilutions of 1 mM CHP and 10 mM H_2O_2 were used. All the reactions were terminated after 5 min at 37°C by the addition of 150 μl of ice-cold acetonitrile (2% acetic acid), except in the case of aflatoxin B1, where 150 μl of 100% acetonitrile was used. All samples were analyzed in duplicate. The rates of product formation were measured by HPLC using authentic standards. Turnovers were calculated as nanomoles of product per nanomole of CYP2S1 per minute using data from samples with the highest concentration of substrates and CHP or H_2O_2. The K_m of each substrate was calculated using Prism software (San Diego, CA) with nonlinear regression and the Michaelis-Menten equation [$V = V_{max} \cdot S/(K_m + S)$].

Analytical Procedures and Product Identification. All samples were analyzed using HPLC. The HPLC system consisted of the Shimadzu prominence series, including the LC-20 AT prominence LC pump, DGU-20A5 degasser, CBM-20 prominence communications bus module, SPD-20A prominence UV/VIS detector, and RF-10AXL fluorescence detector (Shimadzu, Kyoto, Japan). A C18 reversed-phase column (4.6 × 250 mm, 5 μm; Shimadzu) was used to

separate the metabolite products. To separate and detect 6-β-OH-testosterone, the solvents were held at 10% B from 0 to 10 min, increased to 90% B from 10 to 25 min, and then held for another 5 min before returning to the starting condition (solvent A, H_2O; solvent B, 100% acetonitrile; flow rate, 1 ml/min). The testosterone metabolite 6-β-OH-testosterone was monitored at a wavelength of 240 nm. To separate BaP, DMBA, or BaP-7,8-diol and their metabolites, solvents were held at 40% B from 0 to 3 min, increased to 95% B from 3 to 30 min, and then held for another 10 min, before returning to the starting condition (solvent A, H_2O; solvent B, 100% acetonitrile; flow rate, 1 ml/min). The products were monitored from 200 to 500 nm with the diode array detector and with the fluorescence detector setting at 340 nm/402 nm (excitation/emission). To separate 9,10-H_2-BaP, the conditions were set as described by Kim et al. (1998). To separate aflatoxin B1 and its metabolites, solvents were held at 20% B from 0 to 10 min, increased to 60% B from 10 to 23 min, and then held for another 5 min before returning to the starting condition (solvent A, H_2O and 0.1% acetic acid; solvent B, 100% acetonitrile; flow rate, 1 ml/min). The products were monitored with the diode array detector and fluorescence detector setting at 370 nm/440 nm (excitation/emission). To separate all-$trans$-retinoic acid and its metabolite, the solvents were held at 50% B from 0 to 3 min, increased to 90% B from 3 to 20 min, and then held for another 10 min, before returning to the starting condition (solvent A, H_2O; solvent B, 100% acetonitrile; flow rate, 1 ml/min). The products were monitored at a wavelength of 340 nm.

Most products were identified using standard metabolites. However, in some cases, because of the unavailability of standards, we had to deduce the identity of products by comparing their UV spectra to those of similar metabolites, or by using information from previous reports. In the case of 9,10-H_2-BaP metabolism, we identified its product based on the comparison of its UV absorbance spectrum with that of the corresponding metabolite of BaP. In the case of aflatoxin B1's products M1 and Q1, we used information from a previous report, in which M1 was produced exclusively by CYP1A2 and Q1 was exclusively produced by CYP3A4 (Gallagher et al., 1996). In the case of all-$trans$-retinoic acid's 4-OH metabolite, we used information from Nadin and Murray (1999), in which CYP2C8 produces 4-OH-retinoic acid as a major product.

Results

Expression of N-Terminally Modified Variants of CYP2S1. Heterologous expression of recombinant human P450 enzymes in *E. coli* has proved to be challenging. Modification of the N-terminal amino acids of many human P450 enzymes is necessary to express them in *E. coli* (Barnes et al., 1991; Guengerich et al., 1996). However, no consensus N-terminal modified sequence guarantees successful expression of human P450s in *E. coli*. Thus, we tested various N-terminal modifications that have been shown to give successful expression of P450s, with human CYP2S1 (Supplementary Table S1). CYP2S1 expression was assessed using the reduced Fe^{2+} CO versus Fe^{2+} difference assay, which measures not only the amount of P450 but also the amount of P420, the denatured form of the P450 protein. All constructs were coexpressed with cytochrome P450 reductase, and P450 measurements were performed with lysates rather than whole cells, because the former gave more accurate product identification and quantification as a result of lower background noise. Among the different modifications, only modification 4 was able to generate modest amounts of P450 (20–30 nM culture), and this modification had a higher P450 to P420 ratio compared with the other modifications (Supplementary Fig. S3), although it still had a high level of P420,

representing more than 70% of the total CYP2S1 protein. The incubation time and temperature of the growth conditions after inducer treatment can have great effects on the expression of P450s in *E. coli*. Various time and temperature parameters were tested with all the N-terminally modified constructs. Growth at 30°C for 24 h provided the best conditions for modification 4 (Supplementary Fig. S3). The amount of cytochrome P450 reductase that was coexpressed with CYP2S1 was also measured using cytochrome *c* as substrate, and its molar concentration was at least 2-fold higher than that of CYP2S1 (data not shown).

Construction and Expression of Semisynthetic and Synthetic CYP2S1. Because modification 4 had the best expression among the modification constructs, it was chosen for further optimization of expression. The expression of several human P450s, including that of CYP2S1, has been shown to be improved upon coexpression with chaperon groEL/ES (Mitsuda and Iwasaki, 2006; Wu et al., 2006). However, in our hands, there was little improvement in expression when modification 4 was coexpressed with groES/EL (data not shown). A synthetic gene strategy that

overcomes the codon bias of the *E. coli* host has commonly been used to improve heterologous expression (Wu et al., 2006; Potenza et al., 2007; Wardenga et al., 2008). In an attempt to improve expression of CYP2S1, we applied both semisynthetic and synthetic gene strategies. The semisynthetic cDNA (semi-CYP2S1-dh) and full synthetic cDNA (syn2S1-dh) were designed based on the amino acid sequences of CYP2S1 construct 4 (CYP2S1-dh) (Supplementary Table S1 and Fig. S4). The above three cDNAs encode the same protein. The semi-CYP2S1-dh cDNA was designed to reduce the ΔG of mRNA folding, by introducing changes only in the first 100 bases of the gene. Whereas, the syn2S1-dh cDNA was designed to have optimized codons that fit the codon bias of *E. coli,* as well as having minimized mRNA secondary structure (Fig. 1A). The syn2S1-dh construct has the highest ΔG (-189.52 kcal/mol), and the most open secondary structure compared with native CYP2S1-dh (-245.82 kcal/mol) and semi-CYP2S1-dh (-240.13 kcal/mol) (Fig. 1A). The syn2S1-dh construct also yielded the highest P450 content (>200 nM culture) and the least amount of P420 (Fig. 1D). The semi-CYP2S1-dh construct expressed better (50–60
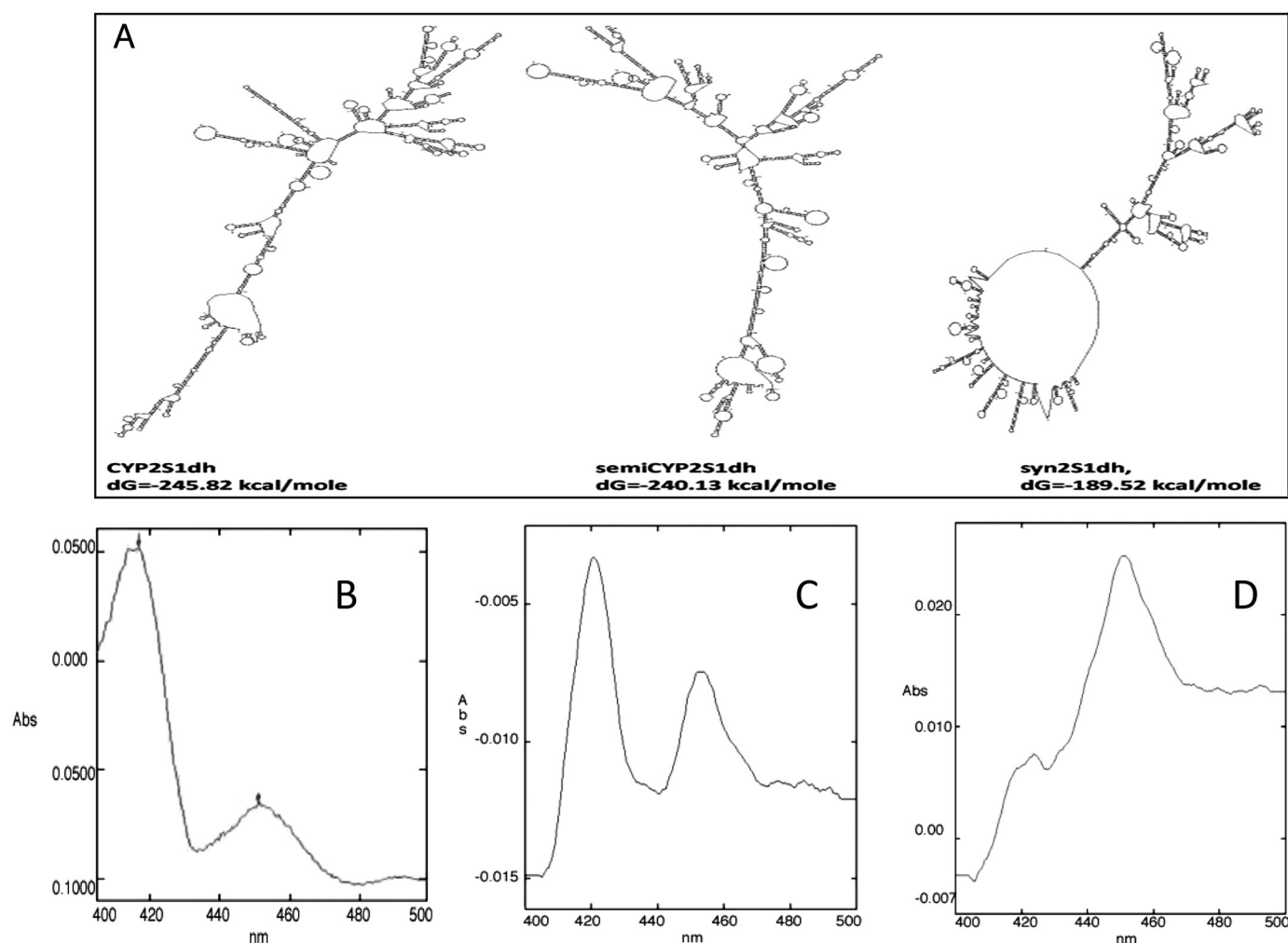
Fig. 1. Comparison of the hypothetical mRNA secondary structures, ΔG values, and P450 spectral characteristics expression of native CYP2S1dh, semiCYP2S1dh, and syn2S1dh. A, structures and ΔG values were calculated at a 37°C folding temperature and 1 M Na$^+$ using mfold (http://mfold.bioinfo.rpi.edu/cgi-bin/dna-form1.cgi). Expression of the native CYP2S1 construct (B), semiCYP2S1-dh construct (C), and syn2S1-dh construct (D) in *E. coli* LMG 194. All constructs were coexpressed with P450-reductase. Fe^{2+} CO versus Fe^{2+} difference spectra were measured using 1:5 dilutions of lysate supernatants. The expression levels of CYP2S1, semiCYP2S1-dh, and syn2S1-dh were estimated to be 20 to 30, 50 to 60, and 200 nM culture, respectively.
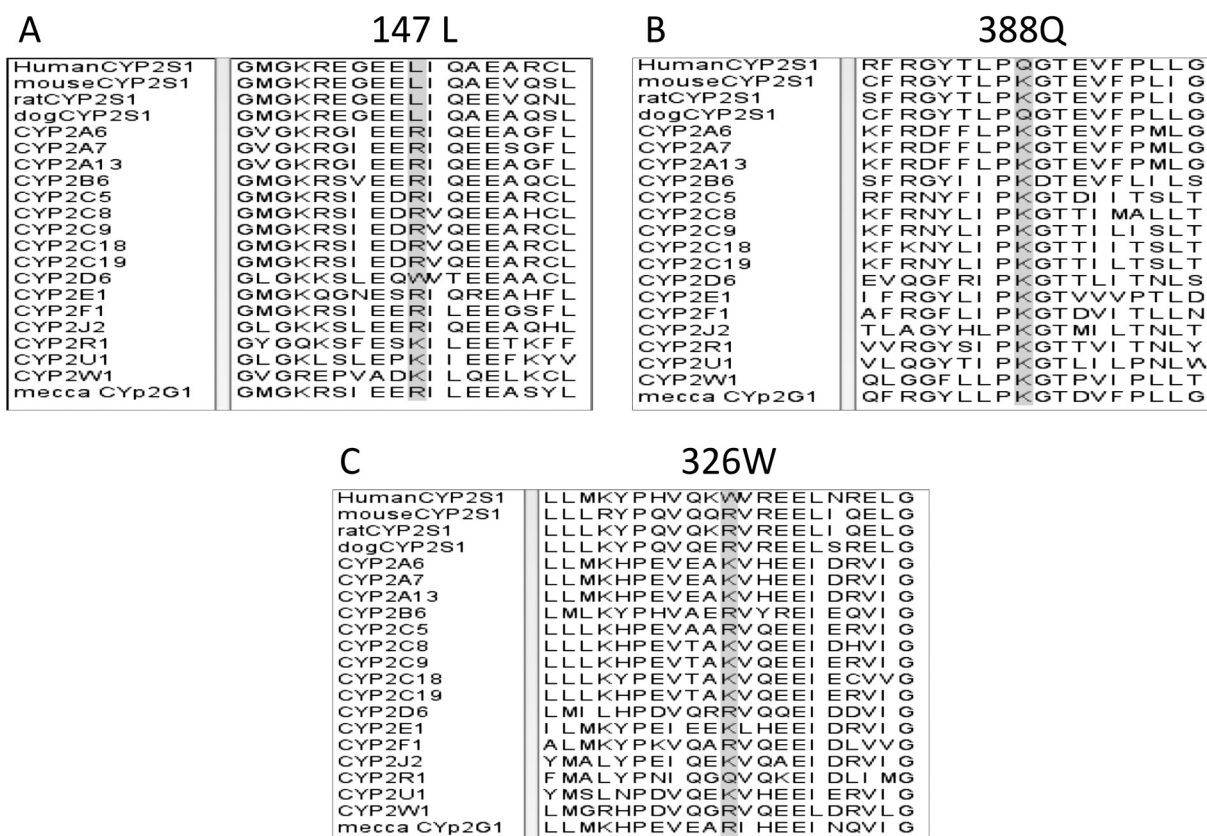
Fig. 2. Amino acid sequence alignment of human CYP2S1 with other CYP2 family enzymes. The highlighted amino acids are thought to be important in the interaction between the cytochromes P450 and P450 reductase.
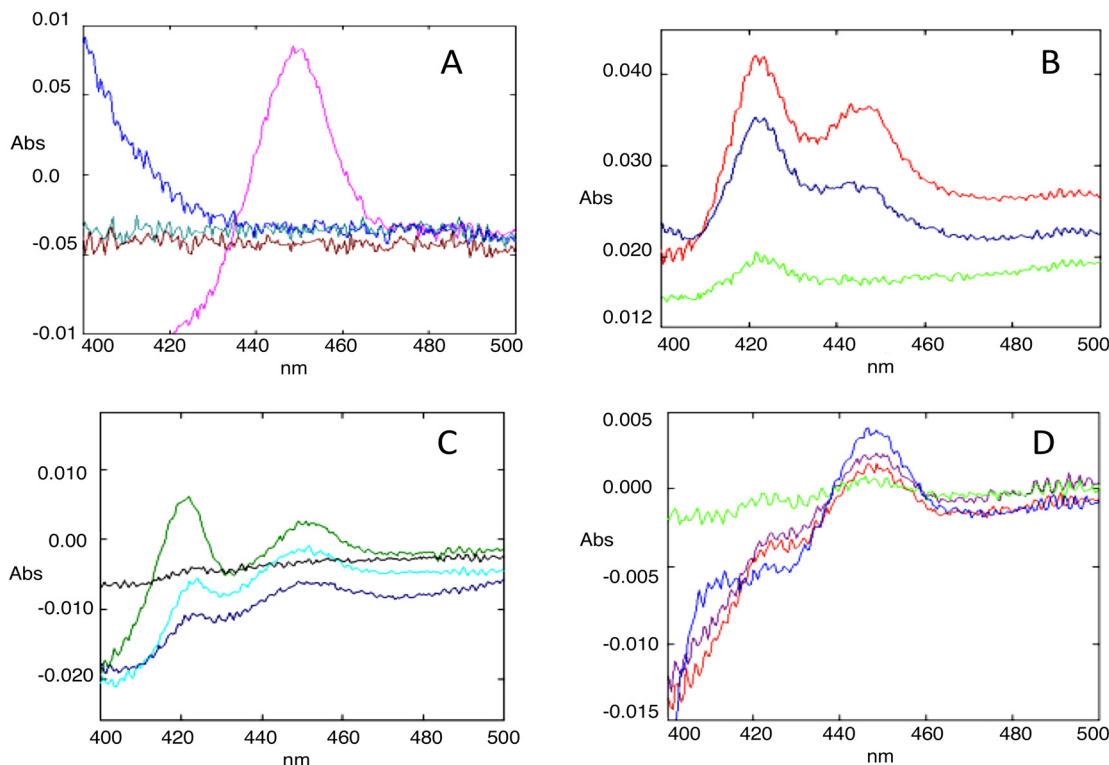

Fig. 3. NADPH reduced Fe²⁺ CO versus Fe²⁺ difference spectra of CYP2S1 + P450 reductase (A). Light blue, CYP2S1 + CO; brown, CYP2S1 + CO + NADPH; blue, CYP2S1 + CO + NADPH + naphthalene; purple, CYP2S1 + CO + NADPH + naphthalene + dithionite. Spectra of CYP1A1 + P450 reductase (B): green, CYP1A1 + CO; blue, CYP1A1 + CO + NADPH; red, CYP1A1 + CO + NADPH + harmaline. Spectra of CYP2C9 + P450 reductase (C): black, CYP2C9 + CO; blue, CYP2C9 + CO + NADPH; light blue, CYP2C9 + CO + NADPH + diclofenac; green, CYP2C9 + CO + NADPH + dithionite. Spectra of CYP3A4 + P450 reductase (D), green, CYP3A4 + CO; red, CYP3A4 + CO + NADPH; purple, CYP3A4 + CO + NADPH + diclofenac; blue, CYP3A4 + CO + NADPH + testosterone.

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nM culture) than the native CYP2S1-dh construct (Fig. 1, B and C). Our results suggest that codon usage and mRNA secondary structure should both be considered when designing a synthetic gene for heterologous expression.

Examination of CYP2S1 Catalytic Activity using NADPH. Several compounds were examined, including some that have been reported to be substrates for CYP2S1, such as all-*trans*-retinoic acid, naphthalene, and aflatoxin B1 (Smith et al., 2003; Karlgren et al., 2005; Wang et al., 2005). Other potential substrates tested included, benzo[a]pyrene, DMBA, testosterone, methoxyresorufin, ethoxyresorufin, and methoxy-4-trifluoromethylcoumarin. All the reactions were performed with membranes containing CYP2S1 derived from various constructs (Supplementary Table S1), including the synthetic construct, plus cytochrome P450 reductase, or with membranes containing only cytochrome P450 reductase as a

negative control. Even in the presence of NADPH, CYP2S1 did not form any detectable products with any of these compounds, after 2 h of incubation (data not shown). Our failure to observe activity of CYP2S1 with NADPH agrees with the results of Wu et al. (2006), who reported that CYP2S1 did not oxidize any substrate tested, including naphthalene and all-*trans*-retinoic acid.

Lack of Interaction between CYP2S1 and P450 Reductase. A plausible explanation for the lack of CYP2S1 activity in vitro is deficient interaction between the P450 reductase and CYP2S1. The interaction between P450 and P450 reductase has been suggested in many studies as being due to electrostatic interaction between charged amino acids, mainly involving arginine/lysine of P450 and glutamate/aspartate of P450 reductase (Shen and Strobel, 1993; Voznesensky and Schenkman, 1994; Nikfarjam et al., 2006).
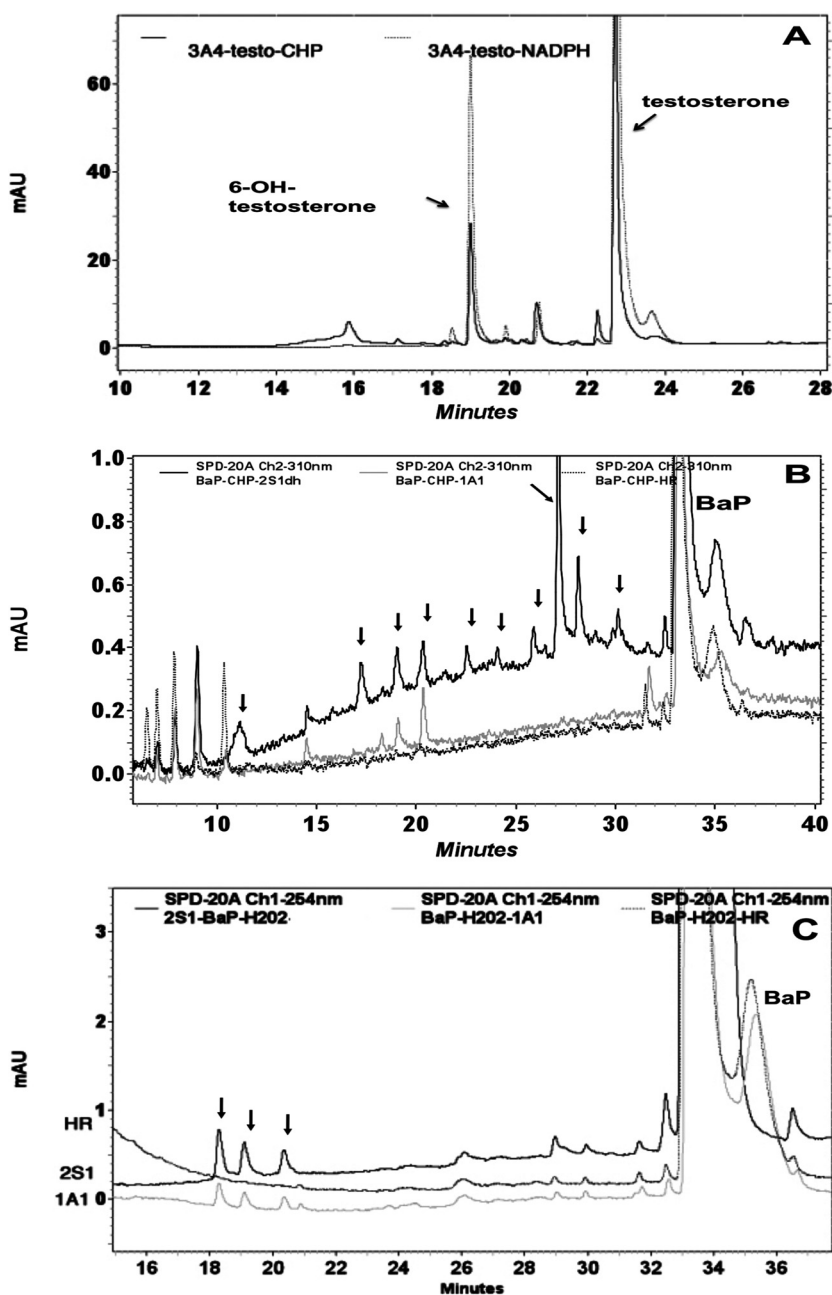

Fig. 4. Cumene hydroperoxide and hydrogen peroxide support oxidation by CYP2S1. HPLC chromatograms of 0.15 μM CYP3A4 + reductase (A) incubated with 100 μM testosterone and 1 mM cumene hydroperoxide (dark line), or with 200 μM testosterone and a NADP/NADPH regeneration mixture (gray line) for 10 min at 37°C. The 19-min peak corresponds to a 6-β-OH-testosterone, detected at 245 nm. HPLC chromatograms (B) comparing activities of 0.15 μM CYP2S1 (solid black line), 0.15 μM CYP1A1 (gray line), and human reductase sample (HR, dotted line) with 100 μM benzo[a]pyrene in the presence of 1 mM cumene hydroperoxide for 10 min at 37°C. HPLC chromatograms (C) of 0.15 μM CYP2S1, 0.15 μM CYP1A1, and human reductase sample (HR) toward 100 μM benzo[a]pyrene in the presence of 10 mM H_2O_2 for 10 min at 37°C. Products, indicated by arrows, were detected at 254 nm.

Figure 2, A to C, shows the alignment of CYP2S1 to various CYP2 family enzymes. At amino acid position 147, the human, mouse, canine, and rat CYP2S1 have leucine, a neutral amino acid, whereas other CYP2s have either lysine or arginine, which are positively charged amino acids. Crespi and Miller (1997) reported that CYP2C9*2, a slow drug metabolism allele of CYP2C9 that possesses a R144C mutation (equivalent to amino acid 147 in CYP2S1), exhibited slower kinetics compared with the wild-type enzyme because of altered interaction of CYP2C9*2 with the P450 reductase. In addition, human and canine CYP2S1 possess glutamine at position 388, whereas other CYP2s have lysine (Fig. 2B). Bernhardt et al. (1984) showed that when fluorescein isothiocyanate was selectively bound to the ε-amino group of lysine-382 of CYP2B4 (equivalent to amino acid 388 in CYP2S1), the activity of CYP2B4 was inhibited because of impaired electron transfer from the P450 reductase. Finally, Nikfarjam et al. (2006) showed that Lys326 and/or Lys327 in the J-helix of P450 17α play an important role in interaction with P450 reductase. The 326th amino acid of CYP2S1 is a tryptophan rather than the lysine or arginine occurring in other CYP2 family members (Fig. 2C). These differences in the

CYP2S1 protein suggested that CYP2S1 may interact weakly or not at all with the P450 reductase.

In the presence of NADPH, P450 reductase transfers one electron from NADPH to heme Fe^{3+} of P450 and reduces it to Fe^{2+}. The latter has high affinity for CO as well as for oxygen. If the heme Fe^{2+} is bound to CO, there is an increase in absorbance at 450 nm (Omura and Sato, 1964a). We used this characteristic of the P450 reaction cycle to examine whether the P450 reductase can transfer an electron from NADPH to CYP2S1. When NADPH was added to the CYP2S1 + P450 reductase sample in the presence of CO, there was no absorbance increase at 450 nm (Fig. 3A). Electron transfer is usually more readily observed in the presence of substrate (Lewis and Pratt, 1998). However, when naphthalene [which is capable of binding CYP2S1, as shown by the fact that it elicits a type I substrate-induced difference spectrum (Bui and Hankinson, data not shown)] was added, there was also no increase in absorbance at 450 nm (Fig. 3A). Adding sodium dithionite, which directly reduces CYP2S1 independently of P450 reductase, increased absorbance at 450 nm (Fig. 3A). The assay was validated with CYP1A1, CYP2C9, and CYP3A4. All these P450s exhibited increased absorbance at 450 nm when NADPH was added. The absorbance at 450 nm increased even further in each case in the presence of substrate (Fig. 3, B–D). These data indicate that P450 reductase cannot transfer an electron from NADPH to CYP2S1 as a result of the lack of interaction between the two proteins, suggesting that this may be the reason that there was no detectable activity of CYP2S1 in vitro using P450 reductase and NADPH.

All the remaining studies detailed in this article were performed with purified synthetically encoded CYP2S1.

Catalytic Activity of CYP2S1 with Hydroperoxides. Many P450s can use organic hydroperoxides to carry out oxidation of their substrates in the absence of cytochrome P450 reductase. This peroxide-dependent pathway is known as the "peroxide shunt" (Barr et al., 1996). Cumene hydroperoxide can support several P450-catalyzed reactions, including aromatic and aliphatic hydroxylation, N- or O-dealkylation, and alkene epoxidation (Nordblom et al., 1976; Griffin et al., 1980; Barr and Mason, 1995). When CYP3A4 + P450 reductase was incubated with 1 mM cumene hydroperoxide in the presence of 100 μM testosterone, 6-β-OH-testosterone
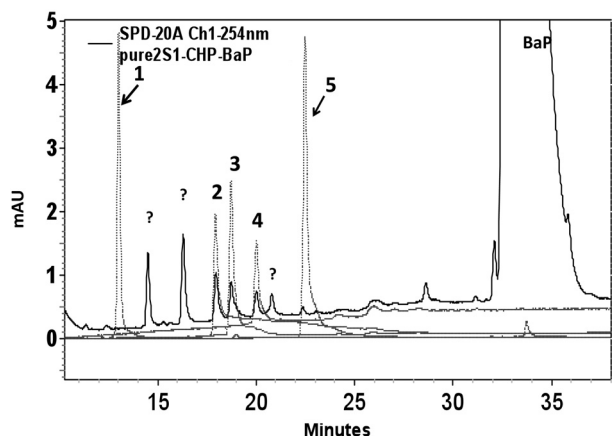

Fig. 5. Identification of CYP2S1-catalyzed products of BaP. HPLC chromatogram of 0.2 μM CYP2S1 incubated with 200 μM BaP and 1 mM CHP (solid black line). Products were identified at 254 nm using authentic standards (dotted lines), 6,12-BaP-quinone (4); 3,6-BaP-quinone (3); 1,6 benzo[a]pyrene (2), 4-hydroxy benzo[a]pyrene (4-OH-BaP) (5), and BaP-7,8-diol (1). Other products, indicated with question mark, were not identified.

TABLE 1

K_m values and turnover numbers of various substrates using purified synthetically encoded CYP2S1 (0.2 μM) and CHP (1 mM)

Data are represented as averages $\pm$ S.E. of duplicate determinations. K_m values were determined with Prism software using nonlinear regression with one site binding mode and the $V = V_{max} \cdot S/(K_m + S)$ equation. Turnovers were determined using the highest concentration of substrate.

Substrate	Product	K_m	Turnover
		μM	$nmol\ product/nmol\ CYP2S1/min$
Benzo[a]pyrene	1,6-Dione	94 ± 22	0.83
	3,6-Dione	48.6 ± 8	1.85
	6,12-Dione	72.7 ± 14	3.25
7,12-Dimethylbenz[a]anthracene	7-OH-DMBA	219 ± 4	15.7
	12-OH-DMBA	34 ± 9	26
9,10-Dihydro-benzo[a]pyrene	9,10H$_2$-BaP-7,8-dihydrodiol	22.6 ± 6	1.6
Benzo[a]pyrene-trans-7,8dihydrodiol	r7,t8,t9,c10-tertol	12.43 ± 2	2.3
Aflatoxin B1	Unknown	266 ± 40	N.D.
all-trans-Retinoic acid	4-OH-retinoic acid	20.9 ± 0.9	N.D.
Naphthalene	Unknown	N.D.	N.D.
Styrene	Unknown	N.D.	N.D.
Testosterone	No activity		
Coumarin	No activity		

N.D., not determined.

was detected just as it was with CYP3A4 + P450 reductase incubated with testosterone and NADPH (Fig. 4A). Likewise, when CYP2S1 or CYP1A1 + P450 reductase was incubated with 1 mM cumene hydroperoxide and benzo[a]pyrene, multiple products were detected (Fig. 4B), whereas when the P450 reductase-only sample (HR) was incubated with cumene hydroperoxide and benzo[a]pyrene, no product was detected (Fig. 4B). In addition, when CYP2S1 was heat-treated at 80°C for 20 min, product formation was reduced by at least 80% (data not shown), thus indicating that product formation is enzymatic and CYP2S1-dependent. H_2O_2 (3 mM) also supported metabolism of BaP by CYP2S1 and CYP1A1 but not P450 reductase to multiple products (Fig. 4C). Together, these data clearly show that CYP2S1 can use cumene hydroperoxide and H_2O_2 to oxidize benzo[a]pyrene.

In the presence of cumene hydroperoxide, CYP2S1 oxidized benzo[a]pyrene to the 1,6-quinone, 3,6-quinone, and 6,12-quinone, with K_m values and turnovers of 94 ± 22 μM and 0.83 min^{-1}, 49 ± 8 μM and 1.85 min^{-1}, and 73 ± 4 μM and

3.25 min^{-1}, respectively (Fig. 5 and Table 1). At least two unknown products with absorbances at 254 nm were also detected (Fig. 5). However, no 4-OH-BaP or BaP-7,8-diol was formed. The latter is a precursor of the carcinogenic metabolite 7,8-diol-trans-9,10-epoxide, which is extremely reactive and can bind to macromolecules such as DNA, RNA, and protein (Gelboin, 1980). This suggests that CYP2S1 does not covert BaP into this carcinogenic metabolite. CYP2S1 (in the presence of cumene hydroperoxide) converted BaP to the above quinones at much higher rates than those reported for CYP1A1, -1A2, and -1B1 with P450 reductase and NADPH (Kim et al., 1998). Quinone metabolites are thought to represent substrates for conjugation and detoxification pathways (Wood et al., 1975). However, some evidence suggests that the quinones can form DNA adducts when they undergo one election reduction by P450 reductase to form unstable semiquinone species (Joseph and Jaiswal, 1994).

In addition to benzo[a]pyrene, CYP2S1 could oxidize many procarcinogens in the presence of cumene hydroperoxide,
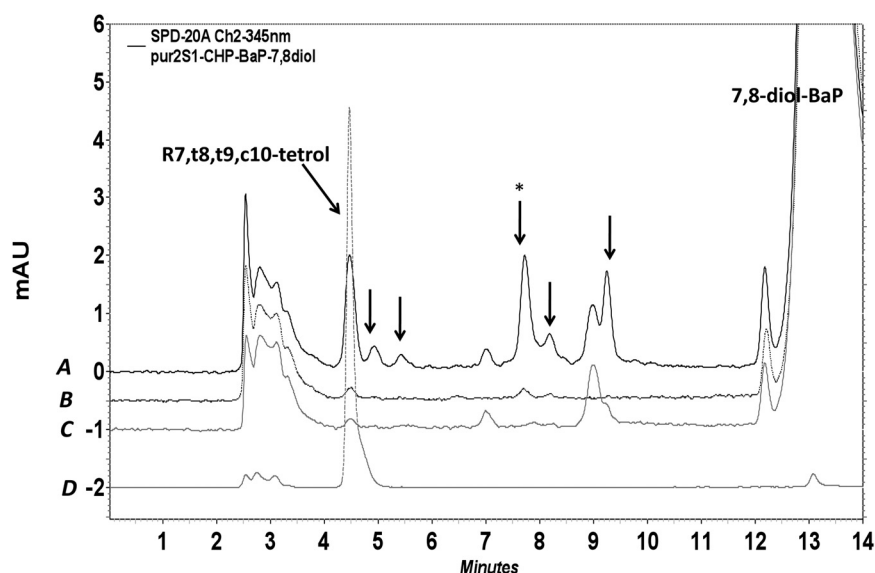

Fig. 6. Identification of CYP2S1-catalyzed products of BaP-7,8-diol. HPLC chromatograms of 0.2 μM CYP2S1 incubated with 133 μM BaP-7,8-diol and 1 mM CHP (A), no CHP control (B), and no CYP2S1 control (C). Detection was set at 345 nm. r7,t8,t9,c10-tetrol was identified using an authentic standard (D). Other unknown products, indicated by arrows, were also detected. The product marked with * has the same UV spectrum as r7,t8,t9,c10-tetrol, suggesting that it is its enantiomer.
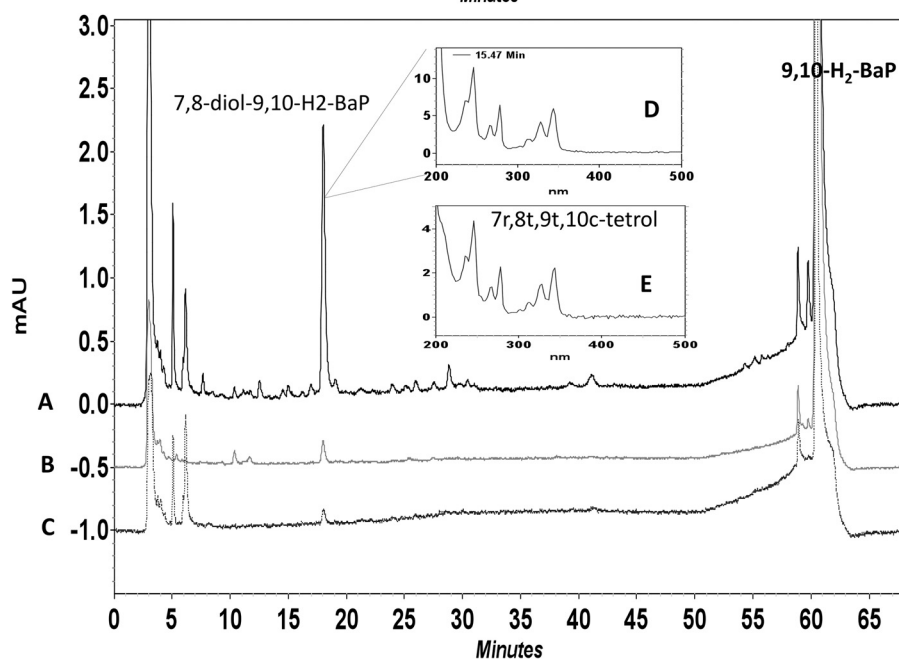

Fig. 7. Identification of CYP2S1-catalyzed products of 9,10-H_2-BaP. HPLC chromatogram of 0.2 μM CYP2S1 incubated with 133 μM 9,10-H_2-BaP and 1 mM CHP (A), without CYP2S1 (C) control, or without CHP (B) control. Detection was set at 310 nm. The 7,8-diol-9,10-H_2-BaP product was identified based on the comparison of its UV-spectrum (D) to that of r7,t8,t9,c10-tetrol (E).

including BaP-7,8-diol, 9,10-H$_2$-BaP, DMBA, and aflatoxin B1. It could also metabolize all-*trans*-retinoic acid and other compounds listed in Table 1. BaP-7,8-diol is an intermediate product of the BaP bioactivation pathway leading to the mutagenic epoxide 7,8-diol-*trans*-9,10-epoxide. It is formed mainly by CYP1A1 and -1B1, and these same enzymes can also metabolize it to the above epoxide (Kim et al., 1998; Shimada et al., 1999). In the presence of cumene hydroperoxide, CYP2S1 could convert BaP-7,8-diol to at least six products (Fig. 6). The major product was identified as BaP-*r*-7,*t*-8,*t*-9,*c*-10-tetrahydrotetrol (r7,t8,t9,c10-tetrol) (K_m = 12.4 ± 2 μM, 2.3 min^{-1}), which is indicative of 7,8-diol-*trans*-9,10-epoxide formation (Fig. 6, and Table 1) (Kim et al., 1998). The turnover of r7,t8,t9,c10-tetrol formation by CYP2S1 is higher than that catalyzed by CYP1A1, -1A2, and -1B1 (Kim et al., 1998). This suggests that CYP2S1 could be important in the bioactivation of BaP-7,8-diol in vivo.

When 9,10-H$_2$-BaP (a BaP derivative) was incubated with cumene hydroperoxide and CYP2S1, a major product at 15-min retention time was detected (Fig. 7). This product has the same UV spectrum as r7,t8,t9,c10-tetrol, indicating that the 8,9-double bond was oxidized to the 8,9-diol. Thus the product is likely to be 7,8-diol-9,10-H$_2$-BaP (Fig. 7), which would be derived from the hydrolysis of 9,10-H$_2$-BaP-7,8-epoxide. Whether 9,10-H$_2$-BaP-7,8-epoxide is carcinogenic is not known, although a similar compound, 9,10-diol-*trans*-7,8-epoxide, has weak mutagenic activity (Thakker et al., 1978). The K_m and rate of 7,8-diol-9,10-H$_2$-BaP formation were calculated based on the r7,t8,t9,c10-tetrol standard: K_m, 23 ± 6 μM; turnover, 1.6 min^{-1} (Table 1).

DMBA is a potent carcinogen that induces many types of cancer, adrenocorticolytic, and immunosuppression in mice and other species (Lindhe et al., 2002; Gao et al., 2005). Its toxicity and carcinogenicity depends on CYP1 family en-
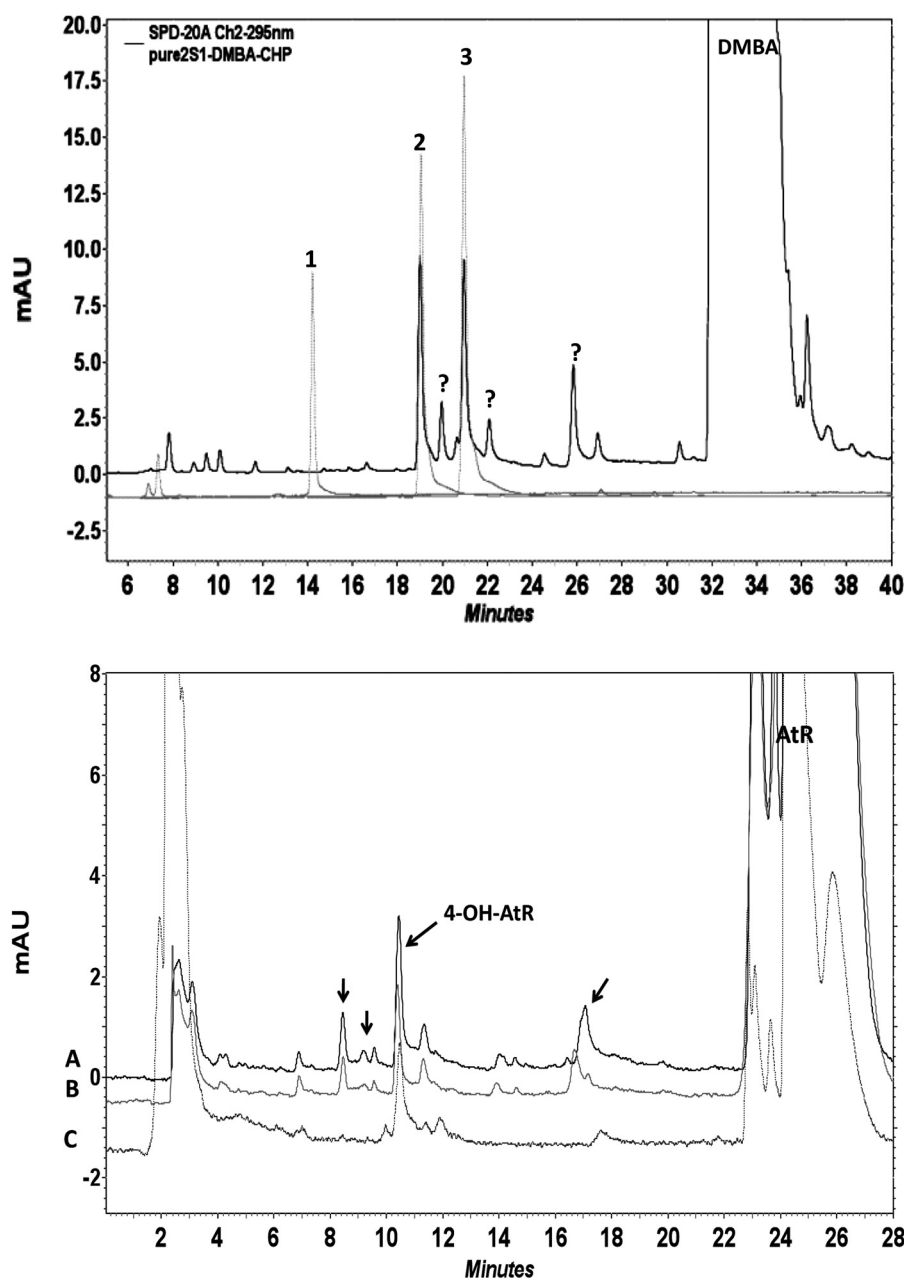

Fig. 8. Identification of CYP2S1-catalyzed products of DMBA. HPLC chromatogram of 0.2 μM DMBA incubated with 500 μM DMBA and CHP (solid black). Products were identified at 295 nm using authentic standards, 12-OH-DMBA (3), 7-OH-DMBA (2), and 3,4-diol DMBA (1). Other unknown products are indicated by question marks.

Fig. 9. Identification of CYP2S1-catalyzed products of all-*trans*-retinoic acid (AtR). HPLC chromatograms of 0.2 μM CYP2S1 incubated with 20 μM AtR and 1 mM CHP (A) or 1 mM H$_2$O$_2$ (B), and of 0.1 μM CYP2C8 + reductase incubated with 5 μM AtR and NADPH (C). Products were detected at 350 nm. 4-OH-AtR was identified based on a previous report in which CYP2C8 produced 4-OH-AtR from AtR (Nadin and Murray, 1999).

TABLE 2

K_m values and turnover numbers of CHP and H_2O_2 for various substrates, determined by incubation of 0.2 μM CYP2S1 and fixed concentration of substrates

Data are represented as averages $\pm$ S.E. of duplicate determinations.

Substrate	Product	CHP		H_2O_2	
		K_m	Turnover	K_m	Turnover
		μM	nmol product/nmol CYP2S1/min	μM	nmol product/nmol CYP2S1/min
Benzo[a]pyrene (167 μM)	1,6-Dione	16.1 $\pm$ 3.2	0.58	107 $\pm$ 21	0.44
	3,6-Dione	29.4 $\pm$ 5	1.36	215 $\pm$ 42	0.8
	6,12-dione	30.8 $\pm$ 6	2.24	232 $\pm$ 46	1.46
7,12-Dimethylbenz[a]anthracene (133 μM)	7-OH-DMBA	58.6 $\pm$ 11	15.7	4800 $\pm$ 950	15
	12-OH-DMBA	62.0 $\pm$ 12	26	> 5000	14.8
9,10-Dihydro-benzo[a]pyrene (50 μM)	9,10H$_2$-BaP-7,8-dihydrodiol	188 $\pm$ 37	0.55	60 $\pm$ 12	0.13
Benzo[a]pyrenetrans7,8dihydrodiol (133 μM)	r7,t8,t9,c10-tertol	31.6 $\pm$ 5	1.1	1906 $\pm$ 350	0.3
Aflatoxin B1 (243 μM)	Unknown	8.8 $\pm$ 2	N.D.	3500 $\pm$ 700	N.D.
Retinoic acid (20 μM)		1.1 $\pm$ 0.4	N.D.	1.2 $\pm$ 0.4	N.D.

N.D., not determined.

zymes (Nebert and Russell, 2002). When DMBA was incubated with CYP2S1 and cumene hydroperoxide, 7-OH-DMBA, 12-OH-DMBA, and other unknown metabolites were detected (Fig. 8). CYP2S1 converted DMBA to 7- and 12-OH-DMBA at faster rates than for any other substrate tested, 15.7 min^{-1} for 7-OH-DMBA and 26 min^{-1} for 12-OH-DMBA (Table 1). Although 7-OH and 12-OH DMBA are considered detoxification products of DMBA, these two products can be further oxidized by P450, leading to tumor induction in mice (Chou et al., 1981; Wislocki et al., 1981). Neither the carcinogenic metabolite 1,2-epoxide-3,4-diol-DMBA nor its precursor 7,12-dimethylbenz[a]anthracene-trans-3,4-diol was detected (Fig. 8). Thus, CYP2S1 may play an important role in metabolism of DMBA.

All-trans-retinoic acid was reported by Smith et al. (2003) to be metabolized by CYP2S1 into the 4-hydroxy and 5,6-epoxide products, but we and others (Wu et al., 2006) could not repeat this observation using CYP2S1 with P450 reductase and NADPH. However, when CYP2S1 was incubated with all-trans-retinoic acid in the presence of cumene hydroperoxide or H_2O_2, the 4-hydroxy and other products were detected (Fig. 9), as determined by comparing CYP2S1's reaction to that of CYP2C8, because the latter has been shown to convert all-trans-retinoic acid to the 4-hydroxy product (Nadin and Murray, 1999). The K_m of all-trans-retinoic acid for CYP2S1 was determined to be 21 $\pm$ 0.9 μM (Table 1). Other chemicals found to have metabolized by CYP2S1 using cumene hydroperoxide, included aflatoxin B1, naphthalene, and styrene, but no activity was found with testosterone, and coumarin (Table 1).

Hydrogen peroxide has also been shown to be able to support P450-catalyzed reactions (Otey et al., 2006). We investigated whether hydrogen peroxide could support CYP2S1 activity and compared its oxidant activity to that of cumene hydroperoxide (Table 2). As described above, H_2O_2 supported oxidation of benzo[a]pyrene (Fig. 4C) and all-trans-retinoic acid by CYP2S1 (Fig. 9). Aflatoxin B1 (a mycotoxin whose mutagenicity and toxicity is dependent on P450) was also oxidized by CYP2S1 in the presence of H_2O_2 with a K_m of 3500 $\pm$ 700 μM (Table 2), to products other than aflatoxin M1 and Q1, as determined by comparison of the reaction of CYP2S1 with those of CYP1A2 and -3A4 (Fig. 10A). At least four fluorescent products were detected in the CYP2S1 but not in the CYP1A2 and -3A4 samples (Fig. 10B). The principal carcinogenic product of aflatoxin B1 metabolism is the exo-8,9-epoxide (Gallagher et al., 1996). We could not determine whether CYP2S1 converts aflatoxin B1 to this epoxide. In general, hydrogen peroxide could support CYP2S1 metabolism of substrates to the same products as cumene hydroperoxide, but with considerably higher K_m values and slower turnovers (Table 2). The K_m for hydrogen peroxide ranged from 1 μM to 5000 μM, the lowest K_m being that for all-trans-retinoic acid and the highest, that for DMBA (Table 2). For cumene hydroperoxide, the K_m values ranged from 1 to 187 μM, the lowest K_m being that for all-trans-retinoic acid and the highest, that for 9,10-H$_2$-BaP.

Discussion

One of the many challenges to functionally characterizing CYP2S1 and other orphan P450s is the availability of good enzyme preparations. Wu et al. (2006) used a system of high

expression of CYP2S1 in *E. coli* that incorporated chaperone GroEL/ES. However, 50% of the CYP2S1 was still in the form of P420, which, when present in a preparation, can undermine substrate screening. In this report, we successfully applied a synthetic gene approach to achieve a high level expression of human CYP2S1 in *E. coli* with at least 90% purity and containing very little if any P420. The high-level of CYP2S1 expression achieved in this report will be necessary for subsequent crystallization. The synthetic gene approach for heterologous expression in *E. coli* has been used successfully in several other cases (Wu et al., 2006; Potenza et al., 2007; Wardenga et al., 2008). However, in our approach, we not only replaced the native gene's codons with the most abundant *E. coli* codons, but we also increased the ΔG free energy of the mRNA structure. We demonstrated that the combination of these two factors should be considered in achieving successful heterologous expression. This synthetic gene approach could be applied to other P450s that have not been successfully expressed in *E. coli*, such as CYP2F1 (Baldwin et al., 2005).

Although high-level expression of CYP2S1 was achieved, we were unable to detect activity toward any potential substrate using NADPH and P450 reductase. We obtained evidence that the lack of NADPH-dependent CYP2S1 activity was probably due to the lack of its interaction with P450 reductase when the two proteins were coexpressed in *E. coli*. Even when we attempted to reconstitute the purified CYP2S1 and P450 reductase using L-α-1,2-dilauroyl-*sn*-glycero-3-phosphocholine according to a method described previously (Brian et al., 1990), we failed to demonstrate any interaction between the two proteins or NADPH-dependent activity (data not shown). The lack of CYP2S1 and P450 reductase interaction can be explained, at least in part, by the fact that CYP2S1 does not contain certain lysine and arginine amino acids at the important positions 147, 326, and 388, which have been proposed to be important for the interactions of various P450s with P450 reductase (Crespi and Miller, 1997; Schulze et al., 2000; Nikfarjam et al., 2006). Although it is possible that N-terminal modification of the enzyme negates its interaction with the P450 reductase, this is unlikely, because many mammalian P450s have been expressed in *E. coli* and none of their activities have been reported to be affected by the N-terminal modifications that are always included (Barnes et al., 1991; Guengerich et al., 1996).

The prototypical cytochrome P450 enzymes are monooxygenases that catalyze the hydroxylation or epoxidation of their substrates. To achieve this, they use molecular oxygen
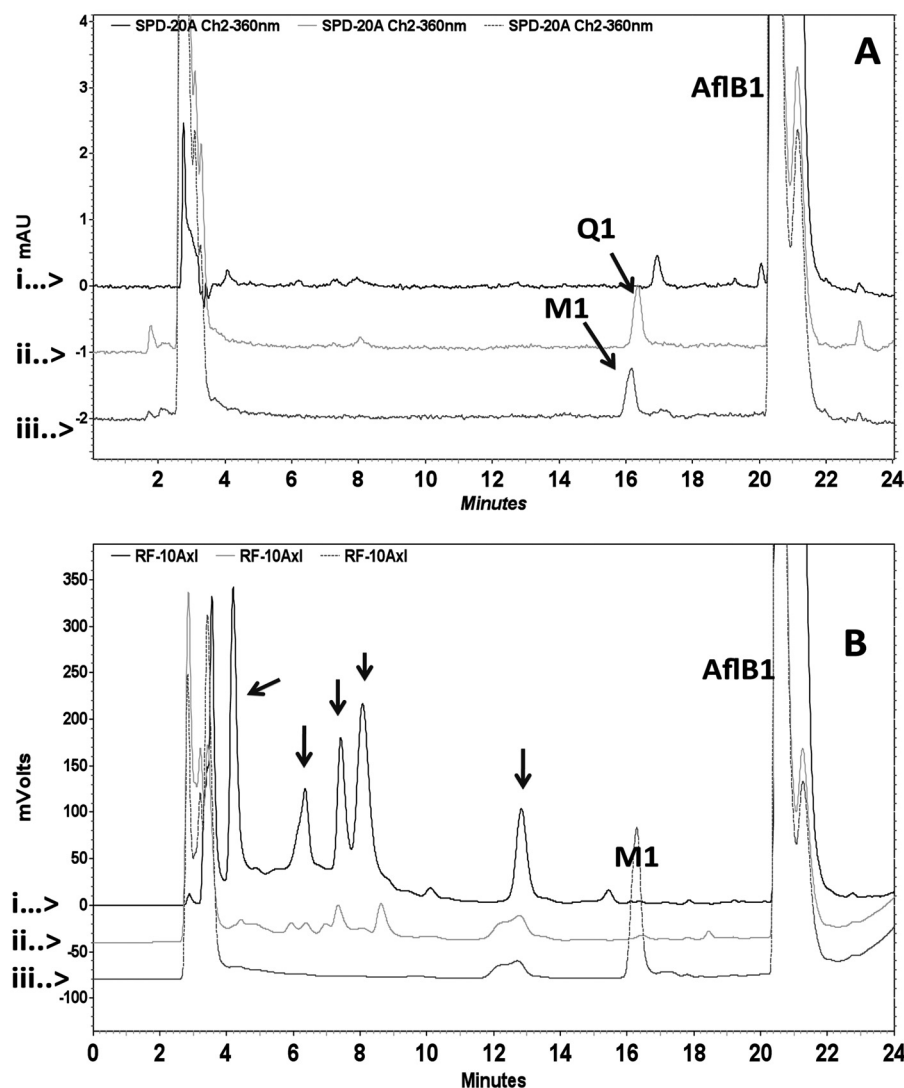

Fig. 10. Identification of CYP2S1-catalyzed products of aflatoxin B1. HPLC chromatograms of 0.2 μM CYP2S1 incubated with 243 μM aflatoxin B1 and 3 mM H_2O_2 (i), of 0.2 μM CYP3A4 + reductase incubated with 243 μM aflatoxin B1 and NADPH (ii), and of CYP1A2 + reductase incubated with 243 μM aflatoxin B1 and NADPH (iii). Products were detected with UV-Vis detector at 360 nm (A) and with a fluorescence detector (B). M1 and Q1 were identified based on a previous report (Gallagher et al., 1996). Unknown products are indicated by arrows.

and reducing equivalents from NADPH, where one of the oxygen atoms is reduced to H_2O and the other is inserted into the substrate. However, there is not an absolute requirement for molecular oxygen and NADPH. The activities of some P450s, including certain microsomal P450s, do not depend on P450 reductase and NADPH. Examples include allene oxide synthase, thromboxane synthase (CYP5A), and prostacyclin synthase (CYP8A) (Brash and Song, 1995; Yeh et al., 2007). These enzymes have been shown not to interact with P450 reductase. In addition, some P450s exhibit peroxygenase-like activity. That is, they can use peroxides as oxygen surrogates to support the oxidation of substrates. In this report, we show that CYP2S1 catalytic activity is well supported by peroxides such as hydrogen peroxide and cumene hydroperoxide. In the presence of peroxides, CYP2S1 was found to metabolize a wide variety of substrates, including many important procarcinogens, such as BaP, DMBA, BaP-7,8-diol, and aflatoxin B1, at relatively fast rates compared with other P450 enzymes involved in the metabolism of the same substrates.

In general, CYP2S1 seems to convert most substrates tested to less toxic metabolites. Our data suggest that human CYP2S1 may play an important role in detoxification of DMBA, BaP, and aflatoxin B1. On the other hand, CYP2S1 converts BaP-7,8-diol to a very reactive metabolite, a 7,8-diol-*trans*-9,10-epoxide, which is a potent tumor initiator. The conversion of this substrate by recombinant human CYP2S1 is faster than the rate of conversion by human recombinant CYP1A1, CYP1A2, or CYP1B1 in the presence of P450 reductase and NADPH (Kim et al., 1998). This metabolism by CYP2S1 is consistent with our observation of in vivo toxicity of BaP-7,8-diol in mammalian cells overexpressing human CYP2S1 (Bui et al., 2009).

The mechanism by which P450s use cumene hydroperoxide to oxidize substrates is often quite complex. Cumene hydroperoxide can undergo both hemolytic cleavage and heterolytic cleavage in the presence of P450 (Ortiz de Montellano, 1995). When CYP2S1 was incubated with the antioxidant butylated hydroxyanisole, its BaP-7,8-diol epoxidation activity was reduced by 50% (data not shown). This suggests that CYP2S1-catalyzed epoxidation proceeds by both a peroxidative reaction and a peroxygenative reaction, because the former incorporates an oxygen of peroxide into the substrate via heterolytic cleavage of peroxide that is not inhibitable by antioxidant, whereas the latter occurs via a free radical mechanism that can be inhibited by antioxidant (Ortiz de Montellano, 1995).

Other heme proteins such as hemoglobin and myoglobin have been shown to have peroxygenase-like activity in vitro. For instance, hemoglobin, and myoglobin were reported to oxidize BaP-7,8-diol using hydrogen peroxide (Catalano and Ortiz de Montellano, 1987). However, their turnover numbers (calculated using the data from the above report) are between 3×10^{-5} and 3×10^{-4} min^{-1}, which are 1000 to 2000 times lower than that of CYP2S1. In addition, the concentrations of hemoglobin and myoglobin used in the study of Catalano and Ortiz de Montellano (1987) were 50 to 100 times that of CYP2S1 used in our study. In addition, adding detergent (100 μM Tween 20) was necessary to achieve the oxidation of BaP-7,8-diol in the above cases, making the conditions not physiologically relevant. Thus, although peroxide-dependent activity may not be physiologically relevant for myoglobin and hemoglobin, it is probably

relevant for CYP2S1 and other P450s, as we demonstrate in our accompanying manuscript (Bui et al., 2009).

Although cumene hydroperoxide can support the oxidative activity of CYP2S1 in vitro, it is not present in cells and thus cannot serve as a cofactor for CYP2S1 in vivo. Hydrogen peroxide, on the other hand, occurs in vivo and could serve as a biological oxidizing agent to support CYP2S1 activity. Although the K_m of H_2O_2 for some substrates is in the millimolar range, probably exceeding the concentration occurring under normal physiological conditions, H_2O_2 concentrations surge during oxidative stress or inflammatory responses. Because these conditions are associated with smoking-related cancer, elevated H_2O_2 could be an important contributor to BaP-7,8-diol carcinogenesis in vivo. It is noteworthy that the K_m values of H_2O_2 for all-*trans*-retinoic acid is relatively low, corresponding more closely to those present under physiological conditions. Biological peroxides such as lipid hydroperoxides, cholesterol hydroperoxide, pregnenolone 17α-hydroperoxide, and progesterone 17α-hydroperoxide occur in vivo. Lipid hydroperoxides are capable of serving as oxygen surrogates for many P450-dependent mono-oxygenations (Wang and Liehr, 1994; Anari et al., 1997). We show in our accompanying report (Bui et al., 2009) that various lipid hydroperoxides can support CYP2S1-catalyzed oxidation of BaP-7,8-diol at faster rates than cumene hydroperoxide and hydrogen peroxide, and at the range of concentrations of fatty acid hydroperoxides that closely resemble physiological conditions. It is possible that CYP2S1 can also use these biological peroxides to oxidize other substrates in vivo. Thus, the peroxide-dependent oxidative activity of CYP2S1 could be physiologically relevant. In summary, our data suggest that CYP2S1 can play an important role in the metabolism of various important xenobiotics as well as endogenous compounds in a peroxide-dependent fashion.

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