

# Characterization of Pulmonary CYP4B2, Specific Catalyst of Methyl Oxidation of 3-Methylindole

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## ABSTRACT

The selective toxicity of chemicals to lung tissues is predominantly mediated by the selective expression of certain pulmonary cytochrome P450 enzymes. This report describes the purification, cloning, and characterization of a unique enzyme, CYP4B2, from goat lung. The purified P450 enzyme was isolated by multistep ion exchange chromatography to electrophoretic homogeneity with an apparent molecular mass of 55,000 Da. Western blotting studies demonstrated that CYP4B enzymes were selectively expressed in lung tissues of rabbits, rats, and mice. Two cDNAs, CYP4B2 and CYP4B2v, were cloned from goat lung tissue. CYP4B2 was predicted to be 511 amino acids and approximately 82% similar to the four known CYP4B1 proteins. Concurrently, a variant of the known human CYP4B1 cDNA, that contained a S207 insertion, was cloned from human lung tissue. The modified recombinant goat CYP4B2 was expressed in *Escherichia coli* and the enzyme

catalyzed the *N*-hydroxylation of the prototypical substrate 2AF. CYP4B2 preferentially dehydrogenated, rather than hydroxylated, the pneumotoxicant 3-methylindole (3MI) ( $V_{\max}$  = 4.61 versus 0.83 nmol/nmol of P450/min, respectively). To investigate the relevance of covalent heme binding of CYP4 enzymes in CYP4B2-mediated metabolism of 3MI, a site-directed mutant (CYP4B2/A315E) was evaluated. The mutation had little effect on the  $V_{\max}$  of either dehydrogenation or hydroxylation but increased the  $K_m$ , which decreased the catalytic efficiency ( $V/K$ ) for 3MI. The A315E mutation shifted the absorbance maximum of the enzyme from 448 to 451 nm, suggesting that the electron density of the heme was altered. These results demonstrate that CYP4B2 is highly specific for methyl group oxidation of 3MI, without formation of ring-oxidized metabolites, and seems to be predominately responsible for the highly organ-specific toxicity of 3MI in goats.

The selective expression of cytochrome P450 (P450) genes in organs other than liver has received considerable attention in the recent literature (Buters et al., 1999; Koskela et al., 1999; Ding and Kaminsky, 2003). Much of the research has focused on P450 enzymes that catalyze the bioactivation of toxicants that selectively cause respiratory damage. Many lung toxicants, such as trichloroethylene, 3-methylindole (3MI), and naphthalene, require metabolic activation to form the ultimate toxic species (Yost, 1997). Pneumotoxicity can be correlated to the expression of certain P450 genes, coupled with the bioactivation of the toxic agents in cells where the enzymes are expressed (Gram, 1997).

The rabbit CYP4B1 isozyme, which comprises more than 50% of the total cytochrome P450 protein in rabbit lung (Serabjit-Singh et al., 1979; Domin et al., 1986), has been shown to catalyze the bioactivation of the pneumotoxins

4-ipomeanol and *N*-nitrosobutylamine (Boyd, 1984; Schulze et al., 1990), in addition to the selective dehydrogenation of valproic acid to form the putative hepatotoxin, 2-*n*-propyl-4-pentenol acid (Rettie et al., 1988, 1995). Thus, CYP4B1 seems to be a likely cytochrome P450 candidate that could be responsible for bioactivation of several selective pneumotoxins (Yost, 1997) in animals. Recent studies have implicated human CYP4B1 as the primary culprit in the production of mutagenic and carcinogenic metabolites of aromatic amines in bladder tissues (Imaoka et al., 2000, 2001). These studies, concerning the catalytic competency of human 4B1, are contradicted by results of Zheng et al. (1998), which demonstrated that heme was not incorporated into the apoprotein of human 4B1 unless the serine at residue 427 in the "meander" region of the protein was mutated to a proline residue, a residue present in all the other 4B1 enzymes. Conversely, Imaoka et al. (2001) expressed a fusion protein of P450 reductase and a cytochrome P450 enzyme they cloned from human bladder that contained an additional serine insertion at position 207. The fusion protein catalyzed the bioactivation of bladder carcinogens.

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**ABBREVIATIONS:** P450, cytochrome P450; 3MI, 3-methylindole; 3MEI, 3-methyleneindolenine; I3C, indole-3-carbinol; 3MOI, 3-methyloxindole; PAGE, polyacrylamide gel electrophoresis; 2AF, 2-aminofluorene; RT, reverse transcription; PCR, polymerase chain reaction; RACE, rapid amplification of cDNA ends; NAC, *N*-acetylcysteine; bp, base pair(s); EST, expressed sequence tag.

3MI is a systemic pneumotoxin that produces acute bovine pulmonary edema and emphysema in cattle (Carlson and Yost, 1989; Yost, 1989). Pneumotoxicity of 3MI is thought to be mediated through bioactivation by pulmonary cytochrome P450 enzymes (Yost, 1997). Toxicity caused by 3MI is also highly specific to pulmonary tissues in a number of species including goats, mice (Turk et al., 1984), and rats (Adams et al., 1988). Human toxicity caused by 3MI has not been firmly established, but human exposure to 3MI from cigarette smoke (Hoffmann and Rathkamp, 1970) is significant. Several studies have demonstrated that cytochrome P450 enzyme(s) bioactivate 3MI to reactive electrophilic metabolites that ultimately cause pulmonary edema and damage to bronchial and alveolar epithelial cells (Carlson et al., 1968; Huijzer et al., 1989; Nichols et al., 1990), and to nasal olfactory epithelial cells (Turk et al., 1986; Adams et al., 1988).

The predominant toxic electrophilic intermediate produced by P450-mediated dehydrogenation of 3MI is 3-methyleneindolenine (3MEI) (Skiles and Yost, 1996), although other reactive intermediates have been identified (Skordos et al., 1998a,b). Certain P450s (e.g., CYP1B1, CYP2A6, CYP2C19, and CYP2D6) catalyze oxygenation of 3MI to the unreactive metabolites indole-3-carbinol (I3C) and 3-methyloxindole (3MOI), whereas others (CYP2B6, CYP3A4/5, and CYP2E1) form only 3MOI, and CYP1A1/2 catalyze the production of all three metabolites I3C, 3MOI, and 3MEI (Lanza and Yost, 2001). The goat CYP2F3 (Wang et al., 1998) and human CYP2F1 (Lanza et al., 1999; Lanza and Yost, 2001) enzymes are unique because they produce only the dehydrogenated electrophilic intermediate 3MEI. None of the enzymes investigated before this study catalyze both dehydrogenation (3MEI) and oxygenation (I3C) at the methyl position, without ring oxygenation (3MOI). A recombinant rabbit CYP4B1, expressed in HepG2 cells, produced the highest rate of 3MEI formation and covalent binding to cellular proteins (Thorn-ton-Manning et al., 1996). To fully understand the catalytic mechanisms of the CYP4B enzymes, and to elucidate the molecular mechanism(s) by which 3MI exerts toxicity, it is essential to characterize this enzyme from the target organ of the most susceptible species, goat lung. This manuscript describes the purification and cloning of goat pulmonary CYP4B2, which seems to be predominantly responsible for the bioactivation of 3MI in goat lung (Wang et al., 1998).

The mechanisms of 3MI oxidation by CYP4B2 may be controlled by a recently identified structural feature of the CYP4 family enzymes. Several members of the CYP4 family have a unique property of covalent linkage of the prosthetic heme to the apoprotein (Henne et al., 2001; LeBrun et al., 2002a). This fascinating characteristic is shared by several CYP4A and CYP4F enzymes, in addition to rabbit and human CYP4B1, where it has been shown that a highly conserved glutamic acid is linked, presumably via an ester bond to a modified heme (Henne et al., 2001; LeBrun et al., 2002a,b). Speculation in the recent literature (Henne et al., 2001) associates the unique  $\omega$ -hydroxylase catalytic function, shared by most of these enzymes, with the requirement for covalent heme attachment and corresponding conformationally restricted structure of the enzymes. Mutation of the glutamic acid to an alanine abolished covalent heme attachment and reduced the  $k_{\text{cat}}$  of CYP4A enzymes for lauric acid hydroxylation (LeBrun et al., 2002a). Therefore, an additional objective of this study was to determine whether covalent

attachment of the heme in the goat enzyme might restrict the metabolism of 3MI to methyl group oxidation.

## Materials and Methods

**Animals.** Adult female goats were obtained from local farmers in Utah or Idaho. Half an hour before surgery, goats were administered (i.v.) 20 mg of Rompun (muscle relaxant) followed by 6000 U of heparin. About 30 min later, 500 mg of pentobarbital was administered. When the goat became unresponsive to stimuli, the chest cavity was opened, plastic tubing was inserted through the trachea to inflate the lungs, and another plastic tube was inserted and secured into the pulmonary artery through an incision in the right ventricle. While maintaining an inflated lung, 10 liters of ice-cold solution of 0.9% NaCl/1.0 mM EDTA was used to perfuse the lungs through the pulmonary artery. Well-perfused lungs appeared blanched after this procedure. Lungs were immediately sliced into 1- to 2-inch cubes, frozen with liquid nitrogen, and stored at  $-80^{\circ}\text{C}$ . Rabbits used for antibody production were purchased from R and R Rabbitry (Stanwood, WA).

**Microsomes.** Frozen goat lung slices were suspended in a volume of homogenization buffer (0.1 M Tris-acetate buffer, pH 7.4, containing 1 mM EDTA and 20  $\mu\text{M}$  butylated hydroxytoluene) equal to three times the tissue weight. Semifrozen tissue was minced with scissors into small pieces, and connective tissue and visible airways were removed and discarded. The minced tissue was homogenized for 1 min in a blender, then disrupted with Tissuemizer (Tekmar, Cincinnati, OH) using two bursts of 20 s each and finally homogenized in a tightly-fitted Potter-Elvehjem homogenizer using three strokes. The homogenate was centrifuged at 10,000g for 20 min, and the pellet was suspended in one third of the homogenization buffer initially used and recentrifuged. The supernatant fractions were combined and recentrifuged at 100,000g for 75 min. Microsomal pellets were washed in 0.1 M potassium pyrophosphate, 1 mM EDTA, and 20  $\mu\text{M}$  butylated hydroxytoluene, centrifuged at 100,000g, then suspended to approximately 20 mg of protein per ml in 10 mM potassium phosphate, pH 7.4, 20% glycerol, 1.0 mM EDTA, and 0.1 mM dithiothreitol (buffer A) and stored at  $-20^{\circ}\text{C}$ .

**Purification of CYP4B2.** A modification of the procedure used to purify P450 isozyme 5 (CYP4B1) from rabbit lung or liver (Parandosh et al., 1987) was used. Goat pulmonary microsomes (2200 mg, 1150 nmol P450) were diluted in buffer A containing potassium phosphate (10 mM, pH 7.4), dithiothreitol (0.1 mM), EDTA (1.0 mM), and glycerol (20%) to give a final protein concentration of 10 mg/ml. Emulgen 911 and recrystallized sodium cholate were added dropwise while stirring the mixture at  $4^{\circ}\text{C}$  to yield a final detergent concentration of 2 and 1%, respectively. The mixture was stirred for an additional 30 min then centrifuged at 100,000g for 60 min and the pellet was discarded. The supernatant was applied to a column ( $2.6 \times 35$  cm) of DEAE-Sepharose pre-equilibrated in buffer A containing 0.2% Emulgen 911. The column was washed with 1 liter of buffer A and eluted with 2.4 liters of a linear gradient of 0 to 0.2 M KCl in the same buffer. Fractions were analyzed by SDS-PAGE, for P450 spectral content, and by measuring 3MI substrate disappearance (using Ehrlich's reagent to detect indoles; see Assays). The enzyme activities were determined after stripping off detergent from each fraction and reconstitution with goat liver NADPH-cytochrome P450 reductase and dilauroyl phosphatidylcholine.

Fractions with the highest 3MI turnover were combined and dialyzed against three changes of buffer A (2 liters) and then applied to a column of CM-Sepharose ( $2.6 \times 12$  cm) pre-equilibrated with 1 liter of buffer B (similar to buffer A except that the pH was 7.1 instead of 7.4) containing 0.2% Emulgen 911. About one fourth of the total P450 that was applied to the CM-Sepharose column eluted in the void volume. Approximately one half of the P450 was eluted with 0.1 M KCl in buffer C (this P450 is referred to as 0.1 M KCl fraction). Each of these P450 fractions (the void fraction and the 0.1M KCl fraction) were dialyzed against two changes of buffer C (similar to buffer A

above except that its pH was 7.7 instead of 7.4) and then diluted with two volumes of buffer C. Each P450 fraction was then chromatographed over a column (0.5 × 16 cm) of DEAE-cellulose (DE-52) pre-equilibrated in buffer C containing 0.2% Emulgen 911 and 0.1% sodium cholate. In each case, the void fraction (column effluent during P450 loading) was devoid of any protein. Further washing of each DE-52 column with buffer C in the presence of 0.05% sodium cholate and 0.1% Emulgen 911 preferentially eluted a highly purified P450 fraction with a subunit molecular mass of approximately 55,000 Da. Fractions of highest purity were combined, dialyzed against 1 change (8-fold excess) of buffer A, then applied to a second column of CM-Sepharose (1.5 × 7 cm) that was pre-equilibrated with buffer B containing 0.2% Emulgen 911. P450 was bound to the column, which, after washing with 0.2% Emulgen 911 in buffer A, was eluted with a linear gradient of 0 to 0.1 M KCl in the same washing buffer. Fractions containing no other proteins besides CYP4B2 were combined, concentrated, dialyzed against 10 mM Tris-acetate, pH 7.4, 20% glycerol, 1.0 mM EDTA and then treated with SM-2 biobeads for 2.5 h at 3°C. This final preparation (2.7 mg, 45 nmol of P450) of P450 showed a single protein by silver-stained SDS-polyacrylamide gel electrophoresis. An aliquot of the purified protein was submitted to the University of Utah Peptide Sequencing Facility for N-terminal sequencing.

**Antibody Production and Preparation of IgG.** Antibody against purified goat lung P450 was raised in a female New Zealand White rabbit, and the IgG fraction was subsequently prepared from complement-inactivated serum by ammonium sulfate fractionation followed by DE-52 chromatography (Kaminsky et al., 1981). A pre-immune serum sample was collected from the same animal before immunization with the purified P450 protein, and preimmune IgG was prepared from this serum sample.

**Purification of NADPH-Cytochrome P450 Reductase.** Liver microsomes from female goats were used to purify NADPH-P450 reductase according to the one-step procedure using 2',5'-ADP-Sepharose which was used for purifying the rat liver enzyme (Shepherd et al., 1983). Enzyme activity was measured by cytochrome C reduction (Phillips and Langdon, 1962).

**Assays.** Assay of 3MI turnover (substrate disappearance) was measured as described previously (Huijzer et al., 1989), using Ehrlich's reagent (2% *p*-dimethylaminobenzaldehyde in glacial acetic acid/perchloric acid, 84:16) to measure the concentration of 3MI in the incubation mixture. *N*-Hydroxylation of 2AF was measured colorimetrically (Belanger et al., 1981). Other assays, including gel electrophoresis, used standard literature methods (Guengerich et al., 1982). Incubations of polyclonal anti-4B2 with lung microsomes or reconstituted CYP4B2 included a concentration (10 mg of IgG/nmol of P450) of anti-4B2 that produced maximal inhibition. Antibodies were preincubated with the enzyme(s) for 30 min at 4°C before enzyme activities were determined.

**Preparation and Screening of Goat Lung cDNA Library.** The cDNA library was made from goat lung total RNA by Stratagene (La Jolla, CA). The cDNAs were synthesized using random primers and were placed in a λZAP II vector that contained the phagemid pBluescript SK<sup>-</sup>. The library was amplified to 1 × 10<sup>6</sup> plaque-forming units. Plaque lifts were performed by plating the amplified library on Luria broth plates and lifting the plaques onto nitrocellulose membranes. The membranes were neutralized, denatured, and baked at 80°C for 2 h under vacuum. Screening of the library was done by plaque lift hybridizations using the full-length human <sup>32</sup>P-labeled cDNA of CYP4B1 (a generous gift from Dr. Frank Gonzalez, National Institutes of Health). The positive clones from the primary screen were screened twice subsequently to eliminate any false positives and to purify the positive clones. Positive clones that were isolated at the end of the tertiary screen were stored at 4°C as glycerol (50%, v/v) stocks.

**Identification and Isolation of Positive Clones.** The phagemid pBluescript SK<sup>-</sup> containing the cDNA inserts of the positive clones of CYP4B2 were isolated from the λZAP II vector by *in vivo*

excision protocols. The rescued phagemid was combined with 200 ml of XL-1 Blue *Escherichia coli* cells and incubated at 37°C for 15 min. The cells were spread on a culture plate (Luria broth, 20 g/l with ampicillin, 50 mg/ml) which was spread with Blue-Gal (halogenated indolyl-β-D-galactoside) and isopropylthio-β-galactoside and incubated at 37°C overnight. The white clones were isolated and stored as glycerol (50% v/v) stocks at -70°C.

**Southern Blot Hybridization of GL4B76.** The plasmid DNA of CYP4B2 was digested with the restriction endonuclease *Eco*RI and separated on a 1% agarose gel. The digested DNA was transferred onto a nylon membrane and was hybridized with the <sup>32</sup>P-labeled CYP4B1 cDNA probe. The hybridization was done at 42°C and washes were performed twice at 42°C and once at 55° and 65°C with a solution containing 15 mM NaCl and 1.5 mM Na<sub>3</sub> citrate, pH 7.0, and 0.2% SDS. The washed membrane was then exposed to X-ray film for 3 h at -70°C.

**Sequencing and Comparison of Positive Clones.** The plasmid DNA from positive clones was isolated and used to sequence both the sense and the antisense strands of the cDNA. The sequencing gel was run at a constant power of 80 W and a voltage not exceeding 2000 V with electrophoresis buffer containing 89 mM Tris base, 2 mM boric acid, and 2 mM EDTA, pH 8.0. The manual sequencing was confirmed by automatic sequencing at the DNA/Peptide Facility at the University of Utah. The amino acid sequence was predicted from the nucleotide sequence using the TRANSLATE program of GCG (Genetics Computer Group, University of Wisconsin) software. The predicted amino acid sequence was compared with documented sequence information of different cytochrome P450 proteins present in the SWISS-PROT library. All sequence alignments were made with ClustalW (<http://www.ebi.ac.uk/clustalw/>).

**Northern Blot Analysis using Positive Clones.** Total RNA from lung and liver tissues of goat were isolated using single-step extraction procedures (Chomczynski and Sacchi, 1987). The extracted total RNA was electrophoresed on a 2.2 M formaldehyde, 1% agarose gel. Northern blots were prepared by transferring the RNAs onto a nylon membrane and were hybridized with <sup>32</sup>P-labeled cDNA probes of either the positive clones or human CYP4B1 at 42°C and washed twice at 42°C and once at 51°C in solution containing 15 mM NaCl and 1.5 mM Na<sub>3</sub> citrate, pH 7.0, and 0.2% SDS. The membranes were exposed to X-ray film overnight at -70°C.

**cDNA Synthesis Using RT-PCR.** Total cellular RNA was isolated from goat or human lung tissues using an RNeasy Kit and QIAshredder microspin homogenizer (QIAGEN, Valencia, CA). RNA samples were stored at -70°C. First-strand cDNA synthesis for RT-PCR was performed using Superscript II RNase H<sup>-</sup> reverse transcriptase (Invitrogen, Carlsbad, CA) according to manufacturer's specifications. All common molecular biology enzymes and reagents were obtained from Invitrogen unless otherwise stated. Oligo (dT)<sub>12-18</sub> (0.5 ng) or the cDNA cloning primer (Integrated DNA Technologies, Coralville, IA) (5'-GAA GAA TTC TCG AGC GGC CGC (dT)<sub>18</sub>-3'), and 5 μg of total RNA was used in each synthesis. The cDNA was stored at -20°C for subsequent PCR amplification. PCR primers were designed to amplify goat CYP4B2 based on the incomplete CYP4B2 clone sequence obtained from the library screen. To acquire the complete sequence, 3'-rapid amplification of cDNA ends (RACE) was performed. Briefly, first-strand cDNA synthesis using reverse transcription was performed using the cDNA cloning primer. PCR was carried out using platinum *Pfx* DNA polymerase according to manufacturer's instructions with the following primers: IDT 3'-RACE reverse (5'-GAA GAA TTC TCG AGC GGC CGC-3') and forward (5'-CCC CTT ACG CTT TTC TCC TGA-3') primer. The PCR reactions were executed using 2 μl of cDNA (of 20 μl total), 2.5 units of platinum *Pfx* DNA polymerase, 5 μl of 10× PCR buffer, 1 μl of 50 mM MgSO<sub>4</sub>, 1 μl of 10 mM dNTP mix, 1 μl of each 10 μM primer, and water to a final volume of 50 μl. PCR reactions were denatured at 94°C for 3 min, followed by 30 cycles of melting at 94°C for 1 min, annealing at 55°C for 1 min, extending at 72°C for 2 min, and a 10-min final extension. Products were visualized by electrophoresis



on a 1% (v/v) agarose gel stained with ethidium bromide. The PCR product was gel excised and subcloned using Zero Blunt TOPO PCR cloning kit for sequence identification, according to manufacturer's recommendations. Using the sequence information acquired from the 3'-untranslated end of the goat CYP4B2 cDNA, a primer set was designed that permit amplification of the entire coding region of the goat CYP4B2 cDNA: forward, 5'-CAG CCA TGG TGC CTG TTT TGC-3'; reverse, 5'-AGC CCA GCT TGG AGA GGG CAG-3'. The full-length amplified cDNA product was again subcloned using Zero Blunt TOPO PCR cloning kit and prepared for sequencing. All plasmid preparations were performed using QIAGEN alkaline-lysis procedure and affinity column purification kits. Sequencing was performed at the University of Utah DNA sequencing facility.

In addition, a full-length coding region from the human CYP4B1 cDNA was amplified using specific primers designed from the published cDNA and genomic sequences. First-strand synthesis using oligo (dT)<sub>12-18</sub> was performed as before except with 5 µg of total RNA from human lung tissue. PCR was performed as before with platinum *Pfx* DNA polymerase and the primer set: forward, 5'-GGA ACT GCA ACC ATG GTG CCC AGC T-3'; reverse, 5'-CCA TCT GGG ACC CCA TTC TCA TCA G-3'. The amplified PCR product was subcloned using Zero Blunt TOPO PCR cloning kit for sequence verification.

**Construction of CYP4B Bicistronic Expression Vector.** The pCW bicistronic expression vector (Parikh et al., 1997), which was a generous gift from Dr. Fred Guengerich (Vanderbilt University, Nashville, TN), was used in the construction of the CYP4B2 expression plasmids. All the following PCR manipulations were performed using platinum *Pfx* DNA polymerase using the conditions described previously. Three expression plasmids were constructed using PCR with the following goat CYP4B2 expression primers. The first forward primer (5'-CAT ATG GCT CTT CTT TGG GCC GTC TTT AGT CTC TCC CAC CTG AG-3') engineered the well established sequence modification (Barnes et al., 1991) and an *NdeI* restriction site for cloning (as underlined). The second primer introduced a silent mutation because it only engineered an *NdeI* restriction site (5'-GCC CTT CAT ATG GTG CCT GTT TTG-3') and the third primer (5'-CAT ATG GCT AGA GCT ATG GAC AGA TTC TCA GGC CC-3') removed the membrane anchoring signal and also introduced an *NdeI* site. All three primers were paired with the same reverse primer (5'-TTG GGC TGT CTA GAG CTC TCA TCT-3'), which introduced an *XbaI* site just outside of the stop codon. A site-directed mutant that contained a glutamate-315 (Henne et al., 2001) instead of alanine-315 was produced using Quik-Change site-directed mutagenesis kit (Stratagene, La Jolla, CA), according to the manufacturer's recommendations, with the mutated primer (5'-CAT TCA TGT TCG AAG GCC ATG ACA C-3') and its complement. The resulting PCR products were subcloned into the Zero Blunt TOPO vector for storage and future digests. The inserts were removed from the TOPO vectors and subcloned into the bicistronic pCW vector using the restriction enzymes *NdeI* and *XbaI*. These expression vectors were used to transform DH5α cells for subsequent P450 expression studies. All vectors were sequenced to confirm orientation and sequence of inserts.

The human CYP4B1 expression vectors were prepared essentially as described above. The PCR primers included a forward primer (5'-ACT GCA CAT ATG GCT CTG TTA TTA GCA GTT TTT AGC TTC TCC TCC-3') containing the CYP17 modification (Barnes et al., 1991), a wild-type primer (5'-ACT GCA CAT ATG GTG CCC AGC TTC CTC TCC-3'), and a reverse primer (5'-CAT TCT CTC TAG AGC TAC TTC CCA GAC CCA GG-3'). The sequence and orientation of the expression vectors was confirmed by sequencing. In addition, the human CYP4B1 expression plasmids containing the wild-type and CYP17 sequence modification were mutated to convert the meander region serine-427 to a proline (Zheng et al., 1998) using Stratagene's Quik-Change site-directed mutagenesis kit and appropriate primers: 5'-GGT CTT TGA CCC TCT GCG CTT TTC C-3' and complement. Sequence of the mutated vectors was confirmed with a suitable primer (5'-GCA GAC CTC CGG GCT GAA GTG GAC ACA TTC-3'). Additional site-directed mutants included changing the con-

served CYP4 glutamate-315 (Henne et al., 2001) to alanine-315, as present in the goat, using a primer pair (5'-CAT TCA TGT TGG CAG GCC ATG ACA C-3' and 5'-GTG TCA TGG CCT GCA AAC ATG AAT G-3'). The double mutants proline-427 and alanine-315, of the human CYP4B1 expression vector with and without the CYP17 sequence modification, were also generated.

**Expression of Bicistronic Expression Vectors in *E. coli*.** Protein was produced by inoculation from stocks, a 5 ml culture of Luria broth-ampicillin (100 µg/ml), growing bacteria at 37°C at 250 rpm overnight, and then adding this to a 500-ml culture of Terrific broth-ampicillin containing trace salts and 1.0 mM thiamine (Barnes, 1996) the following day. Bacterial growth was monitored until A<sub>600</sub> of 1.0 was obtained. The heme precursor α-amino levulinic acid was added (0.5 mM) and incubated for 30 min before induction with 1.0 mM isopropylthio-β-galactoside. The expression cultures were then grown at 30°C with shaking at 150 rpm for 15 h. Cells were pelleted at 4000g for 15 min and stored at -20°C for membrane preparations.

**Preparation of Bacterially Expressed CYP4B2.** P450 membranes were purified essentially as described elsewhere (Barnes, 1996). All tubes and glassware was prechilled, and the entire procedure was performed at 4°C. Pelleted bacterial cells were resuspended in 100 mM Tris-acetate buffer, pH 7.6, containing 500 mM sucrose and 0.5 mM EDTA at ~70 mg of cells (wet weight)/ml. The suspension was diluted with an equal volume of H<sub>2</sub>O, containing 0.10 mg of lysozyme/ml, and gently shaken for 30 min to hydrolyze the outer cell membrane. The resulting spheroplasts were pelleted at 100,000g for 15 min and resuspended at 0.5 g/ml in 100 mM potassium phosphate buffer, pH 7.4, containing 6 mM magnesium acetate, 20% glycerol (v/v), 0.10 mM dithiothreitol, 1.0 mM phenylmethylsulfonyl fluoride, and 100 µl/liter protease inhibitor cocktail (Sigma, St. Louis, MO) containing 4-(2-aminoethyl)benzenesulfonyl fluoride, aprotinin, leupeptin, bestatin, pepstatin A, and E-64. The spheroplasts were lysed by sonication with two 20-s bursts (70% power) in a prechilled container held in an ice-salt bath (-10°C). The resulting lysate was centrifuged at 10,000g for 15 min. The supernatant was then centrifuged at 100,000g for 60 min to pellet the membranes. The final pellet was resuspended in 50 mM Tris-acetate buffer, pH 7.6, containing 0.25 mM EDTA and 0.25 M sucrose and stored at -70°C. The pellets and supernatants were kept from each step and P450 spectra were determined for quality control. Bovine serum albumin protein determinations were determined according to manufacturer's protocol (Pierce, Rockford, IL). P450 content was determined by measuring the reduced CO-absorbance spectra (Omura and Sato, 1964).

**Metabolism of 3MI.** Each reaction mixture consisted of 200 pmol (0.2 µM) of goat CYP4B2, 2 mM NADPH, 4 mM NAC, 50 mM sodium phosphate buffer, pH 7.4, and varying amounts of 3MI (in 50 mM phosphate buffer) to a final volume of 1000 µl. Incubations were performed for 30 min at 37°C in a shaking water bath. The reaction was stopped by addition of 1 ml of ice-cold acetonitrile. The precipitate was separated from the mixture by centrifugation at 21,000g for 10 min. The resulting supernatant was removed, and 20 µl of 1 mM 3-phenyloxindole (in acetonitrile) was added as an internal standard. The samples were concentrated using a SpeedVac (Thermo Savant, Holbrook, NY) to remove the acetonitrile and reduce the volume to 200 µl. Liquid chromatography was performed using an Agilent 1100 System (Agilent Technologies, Palo Alto, CA). Metabolites were separated by injecting samples onto a 5 µm, 250 × 4.6 mm reversed-phase Luna C18 column (Phenomenex, Torrance, CA) using a gradient system that began at 10% acetonitrile and 90% ammonium acetate (1 mM, pH 6.0) with a flow rate of 1 ml/min. The gradient preceded to 35% acetonitrile over 5 min, 50% over 9 min, then 55% over the next 4 min, followed by a final change to 95% acetonitrile over 5 min. Metabolites were monitored at 254 and 280 nm using an Agilent 1100 series diode array detector. The data were analyzed using HP ChemStation software (ver. 0.08.03; Hewlett Packard, Palo Alto, CA). The peak ratio of each metabolite to internal standard in duplicate was used along with a previously determined standard

curve to quantify amounts of the different metabolites. The data were fit using the Michaelis-Menten equation ( $V = V_{\max}[S]/[S] + K_m$ ) and Kaleidagraph software (Synergy Software, Reading, PA) to calculate the constants  $V_{\max}$  and  $K_m$ .

## Results

**Purification of Goat Lung Microsomal CYP4B2.** Female goat lung microsomes were used to purify goat CYP4B2, using a series of chromatographic columns including DEAE-Sepharose, CM-Sepharose, and DEAE-cellulose. The lobes of the lungs were cut into 1- to 2-inch cubes and frozen until microsomes were prepared. Typically, a 300 g (wet weight) portion of lung tissue provided 2.20 g of microsomal protein with a P450 content of 0.53 nmol/mg of protein (total P450, 1150 nmol). Fractions collected after each step were assayed for P450 content and for 3MI turnover (using Ehrlich's reagent to determine the disappearance of 3MI) after reconstituting with NADPH-cytochrome P450 reductase. The average P450 content of purified CYP4B2 (from several different goats) was approximately 17 nmol/mg of protein. The purified CYP4B2 enzyme had an activity of 34.5 nmol of 3MI metabolized/nmol of P450/min, which was a 39-fold purification from microsomes as measured by enzyme activity and a 34-fold purification as measured by P450 content. From approximately 1150 nmol of P450 (2200 mg) from a goat lung microsomal preparation, we typically obtained 45 nmol (2.7 mg) of purified enzyme. Thus, the overall yield of purified CYP4B2 protein averaged about 4% from goat lung microsomes. SDS-PAGE analysis demonstrated the presence of a purified protein with an apparent molecular mass of 55,000 Da (Fig. 1) at the end of the second CM-Sepharose chromatography.

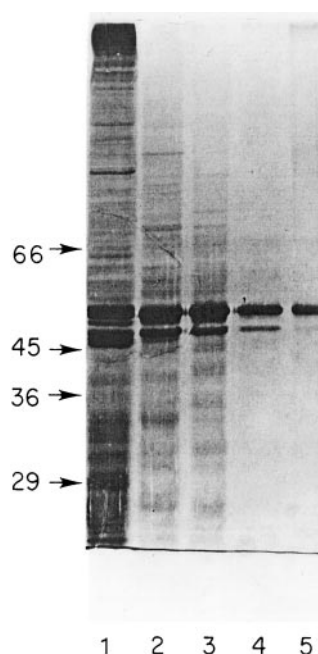
**Western Blots and Amino Acid Sequencing.** Microsomal proteins from lung and liver tissues of goat, sheep, rabbit, rat, mouse, and human were run on a 7.5% SDS-PAGE gel and transferred to a nylon membrane and the blot was probed with anti-4B2 (Fig. 2). The antibodies recognized proteins in both the liver and lung tissues of all species, but the levels of immunoreactivity were much higher in lung than liver in all species except human, where immunoreactivity was much higher in liver tissue. This result was surprising, because human mRNA for 4B1 has been localized to lung, colon, and bladder but not to liver tissues (Nhamburo et al., 1989; Czerwinski et al., 1994; Windmill et al., 1997; Imaoka et al., 2000). The antibodies to goat 4B2 may have bound to proteins other than 4B1 in human liver tissues. Anti-4B2 was also immunoreactive with liver proteins in goats and rats that seemed to have lower molecular masses than the 4B1 enzymes from these species.

Twenty-four amino acids ( $^1\text{MVPVLLSLSLSHLSLWAFGLVSVL}^{24}$ ) were identified by N-terminal sequencing of the purified goat lung enzyme. When this sequence was compared with the human and rabbit N-terminal sequences, it had 66% similarity to the human 4B1 and 47% similarity to the N terminus of the rabbit 4B1. However, the 24-amino acid sequence was identical to the translated amino acid sequence from the cloned CYP4B2 cDNA. Interestingly, this region of all the CYP4B enzymes is hypervariable, so there is a high probability that the purified enzyme is the product of the cloned CYP4B2 gene.

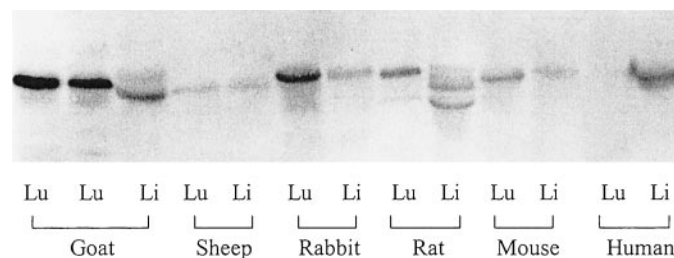
**Goat Lung cDNA Library Screening.** A  $\lambda$ ZAP II cDNA library was prepared from goat lung tissue. The library was

amplified to give  $1 \times 10^6$  plaque-forming units. Clones ( $1.1 \times 10^6$ ) were screened, and 236 were positive in the primary screen. Twenty-one clones were selected randomly and were purified by secondary and tertiary screening. At the end of tertiary screening, 16 clones positively hybridized to the probe. In vivo excision was performed on eight of the 16 clones to excise the pBluescript SK-phagemid that contained the cDNAs. Plasmid DNA preparations were made from the phagemids.

**Characterization of cDNA Clones.** Three clones, GL4B76, GL4B33, and GL4B39, were selected for further characterization because they had unique restriction digest patterns (data not shown). Southern blot hybridizations were performed on the *Eco*RI restriction enzyme-digested plasmid DNA of GL4B76. A DNA fragment with an approximate size of 1.5 kilobases hybridized to the human CYP4B1 cDNA, which was not present in the undigested plasmid DNA of GL4B76. Thus, the presence of a cDNA insert that was orthologous to human CYP4B1 was determined in the positive clone, GL4B76.



**Fig. 1.** SDS-PAGE analysis of purified goat lung CYP4B2 (lane 1, supernatant of solubilized microsomes; lane 2, DEAE-Sepharose 4B eluate; lane 3, initial CM-Sepharose eluate; lane 4, eluate after DEAE-cellulose (DE-52) chromatography; lane 5, final CM-Sepharose eluate). The approximate molecular masses in kilodaltons are indicated. The proteins were visualized by silver staining.



**Fig. 2.** Western blot analysis of microsomal protein (10  $\mu$ g/lane) from lung (Lu) and liver (Li) tissues of goat, sheep, rabbit, rat, mouse, and human using polyclonal antibodies raised against the purified goat lung CYP4B2.



Northern blot hybridizations were performed with the radiolabeled *EcoRI* fragments of the cDNA clones GL4B33, GL4B39, and GL4B76, against goat lung and liver total RNA. All fragments hybridized to a transcript of 2.0 kilobases in goat lung but not in liver (data not shown). A lung-specific 2.0-kilobase mRNA was also identified in goat lung when the human CYP4B1 cDNA was hybridized to the transcripts (Ramakanth et al., 1994). Ribosomal RNA (18S and 28S) was observed with approximately equal intensity in all lung and liver samples, which demonstrated relatively consistent loading in all lanes. The size and position of the transcript was identified with reference to the 18S and 28S ribosomal RNAs.

**Analysis of Library Clones Sequences.** Inspection of the sequencing data from the λZAP II library screen revealed that clones GL4B33 and GL4B39 were incomplete yet overlapped and contained the 5' and 3' portions, respectively, of a variant cDNA based on other published CYP4B1s. The GL4B33/GL4B39 variant contained a 30-bp insertion producing a premature stop codon. The premature stop codon is at a position that removes the requisite cysteine residue near the C-terminal end, which is a feature of all functional P450 holoenzymes. Clone GL4B76 was compared with the human, rabbit, rat, and mouse CYP4B1 sequences using ClustalW. Clone GL4B76 did not contain the full-length cDNA and seemed to be an artifact of poor library construction, probably because of incomplete cDNA transcription. GL4B76 contained a transcript from 6 bp of the 5'-untranslated region to position 1446 of the coding region, which removed the C-terminal 27 amino acids, truncating the cDNA to 484 amino acids instead of the putative 511 amino acids based on other CYP4Bs.

**Generation and Analysis of CYP4B cDNAs from Human and Goat Lung Tissues.** Using the sequence information obtained from the truncated GL4B76, primers were designed to amplify the remaining 3' C-terminal sequence from goat total RNA. The 3'-RACE technique amplified a product that contained the remaining 3'-coding and 3'-untranslated region of the CYP4B2 cDNA, determined by sequence comparison with the published rabbit and human CYP4B1 sequences. A primer set that amplified the entire coding region of goat CYP4B2 produced a cDNA that was 1671 bp (Fig. 3). CYP4B2 coded for a 511-amino acid protein with 82% sequence identity to the human protein and 85% nucleotide identity to the cDNA. Amino acid and nucleotide percentage identity comparisons of CYP4B2 with other published sequences from rabbit, rat, and mouse are depicted in Table 1.<sup>3</sup> A search of the GenBank EST database identified two genes in cattle (*Bos taurus* EST, accession numbers AW347853 and BF602740) that correspond by sequence identity to CYP4B1 and CYP4B2, respectively. The goat CYP4B2 reported in this manuscript is 94% identical to the BF602740 sequence, which supports the nomenclature we have adopted (David Nelson, University of Tennessee, Memphis, personal communication). This conclusion means that there is probably a CYP4B1 gene in goats.

The variant GL4B33/39 was also identified in the amplification in nearly one third of clones that were screened. This variant CYP4B2v, identified both in the library screen and

RT-PCR procedure, contained a 30-bp insertion at position 1351 relative to the start site that produced a premature stop at position 1354 (Fig. 3). A single nucleotide difference was observed at position 1074 that produced a missense glutamate-to-aspartate mutation. Based on the locations of the 30-bp insertion and the single base pair mutation relative to the intron-exon junctions of human CYP4B1 (Fig. 3), it would seem to be an alternate splice transcript and not a pseudo-gene.

Based on literature ambiguities regarding the sequence of human CYP4B1, we also decided to obtain CYP4B1 cDNA

CYP4B2v	1	ATGGTGGCTGTTTGGCTCTCCCTGAGTCTCTCCACCTGAGCGTTGGGGCTTGGGCTGCTCAGCTCTAGCTCTCTCT
CYP4B2		MetValProValLeuLeuSerLeuSerHisLeuSerLeuTrpAlaPheGlyLeuValSerValLeuValPheLeu
	81	TAACTCTACTCGCTGCTGCTGCGGAGGAGAGTATGCCAGAGTATGACAGAGTCTCAGGCGCCCGCCACCTGCTGG
		uLysLeuThrArgLeuLeuLeuArgArgGlnMetLeuAlaArgAlaMetAspArgPheSerGlyProTrpThrHisTrpL
	161	TTTTTGGGCTGCTGAGATCGAGACGAGCTGGGGTTGGCAAGGCTGGTACTGGACCTACACAGTTCCTCTAGCC
		ePheGlyHisAlaLeuGluIleGlnGlnThrGlyGlyLeuAspLysValValTyrTrpThrHisGlnPheProTrpAla
	241	CACCACTCTGGCTGGACGATTTCTGGCTCTGACATCTATGATCTGAATATGCCAAGCTGTATACAGTCGAGG
		HisGlnLeuTrpLeuGlyArgPheLeuGlyPheLeuAsnIleTyrAspGluTyrAlaLysAlaValPheAlaGlySer
	321	AGACCCGAAGGCCGAGATGTGTGATGACTTCTCTCCAGTGGATTGGGAAGGCTTGGTCTTCCCAAGGCGCTAAGT
		yAspProLysAlaProAspValTyrAspPheLeuGlnTrpIleGlyLysGlyLeuValLeuGlnGlyProLysT
	401	GGTTCAGCATGCCAAGCTGCTCACACTGGCTTCACTATGATGTGCTGAAGCCCTACGTCGGCCGCTGTTGGCAGTCC
		rPheGlnHisArgLysLeuLeuThrProGlyPheHisTyrAspValLeuLysProTyrValAlaValPheAlaGlySer
	481	GCACCTGCTATGCTGACAAAGTGGGAAGAAAGGCTCTGAGCAAAAGAGCTTGACATCTTCAGCGATGGGCGACAT
		AlaArgValMetLeuAspLysTrpGluLysLysAlaArgGluGlnLysSerPheAspIlePheSerAspValGlyHisMe
	561	GGGCTGGAGCTGCTATGAGTGCCTTTGGCAAGGAACAGCGGCTGTAAAGCAGGACCACTACTTACCTGGT
		tAlaLeuAspSerLeuMetLysCysThrPheGlyHisTyrSerGlyLeuAsnAspArgAspAsnAspTyrTyrLeu
	641	CGTTCAGCGAGCTCACACTGCTAATCGACGAGCGATGCTTCTCCAGTACCAATGACTTCATCTACTTCTACCC
		iaValSerGluLeuThrLeuLeuMetGlnClnArgIleAspSerPheGlnTyrHisAsnAspPheIleTyrPheLeuThr
	721	CCGATGGCCGCGCTTCTGCGGGCTGCCAGTGGCCCATGACACAGACAGGCTCATCAGGGAACGGAAGCAGC
		ProHisGlyArgArgPheLeuArgAlaCysGlnValAlaHisAspHisThrAspGlnValIleArgGluArgLysAlaAl
	801	CCTTCAGTATAGAAAGCGGGAAGAGATACAGCAAGAGGACCTGAGCTTCTGGACATCTTCTGGGCGCTGGG
		AlaLeuGlnAspLysLysGluArgGluLysIleGlnSerLysArgHisLeuAspPheLeuAspIleLeuLeuGlyAlaArg
	881	ATGAAGAAGGCTACAGCTATCGGATGAAGACCTCGGCGCCAGGTGAACACATTATGTTCCAGGCGCATGACACACC
		spGluGluGlyIleLysLeuSerAspGluAspLeuArgAlaGluValAlaThrPheMetPheAlaGlyHisAspThrThr
	961	ACCAGGCGCATCTCATGGGTTCTCTACTGCATGCTCCCTGATCCCGGAGCAGCAGGCTGTTGTCGGGAAGATTCAGA
		ThrSerAlaIleSerTrpValLeuTyrCysMetSerLeuTyrProGluHisGlnArgCysArgGluGluIleGlnG
	1041	GATTCTGGGAGCGGACTCTTTGAAGTGGGAAGATCTGGCAGAGATGACCTACCTGACATGATCAAGGAGAGCT
		uLeuLeuGlyAspArgAspSerLeuLysTrpGluAspLeuAlaGluMetTyrTyrLeuThrMetCysIleLysGluSerP
	1121	TCGCGCTGTACCGCTGTGCGCCAGGTGTACCGCAGCTCAGCAAGGCTGTCAACTTTTGGATGGTGGCTCTTACT
		heArgLeuTyrProProValProGlnValTyrArgGlnLeuSerLysProValAsnPheValAspGlyArgSerLeuPro
	1201	GCAGGAGGCTGATTTCTCTGACATCTATGCCCTCCATAGGAATAGTACAGTGTGACAGTACCTGTGAGGCTTCGACCC
		AlaGlySerLeuIleSerLeuHisIleTyrAlaLeuHisArgAsnSerThrValTrpThrAspProGluValPheAspPr
	1281	CTTACGCTTTTCTCTGAGAAATGTGGCGGAGCGCCAGCTTTGGCTTCATCCCTCTCTGCTGGGCGC
		oLeuArgPheSerProGluAsnValAlaGlyArgHisSerPheAlaPheIleProPheSerAlaGlyPro
	1351	ATCCCGACCTCCCTGTCCAG
		ArgAsnCysIleGlyGlnGlnPheAlaMetAsnGluValLysValValThrAlaLeuCys
	1411	TTGCTCGCTTTTGGATTTTCCCGGATCCCAAGCTACCCATCCAGTGGCCGAGCTGGTCTCGCTCCCAAGATGG
		LeuLeuArgPheGluPheProAspProSerLysLeuProIleGlnMetProGlnLeuValLeuArgSerLysAsnG
	1491	CATCCACCTCCATCTGAGCCAGTGGGCGGCTCTGAGAATAG
		yIleHisLeuHisLeuLysProValGlyProGlySerGluLysT

**Fig. 3.** The nucleotide and predicted amino acid sequence of the complete translated regions of the goat CYP4B2 and CYP4B2v cDNAs. The amino acids are represented by their three-letter codes beneath each codon. The sequence of CYP4B2v is shown only where it differs from the sequence of goat CYP4B2. Stop codons are in bold, and the 30-bp insertion into CYP4B2v is indicated above the dashed marks. Putative intron-exon junctions (based on the genomic sequence of human CYP4B1) are indicated with arrowheads.

**TABLE 1**

Sequence comparisons among CYP4B2 and other P450s from the CYP4B subfamily

The amino acid sequence of CYP4B2 was aligned with other members of the CYP4B subfamily and the percent identity was calculated using ClustalW. Amino acid sequences for the various CYP4B1 enzymes were obtained from the database search and retrieval system at the National Center for Biotechnology Information (<http://www.ncbi.nlm.nih.gov>).

Cytochrome P450	Species	Amino Acid	Nucleic Acid
% identity			
CYP4B1	Human	82	85
CYP4B1	Rabbit	83	82
CYP4B1	Rat	81	82
CYP4B1	Mouse	82	83

<sup>3</sup> Accession numbers (GenBank) for the CYP4B enzymes in this article are: goat CYP4B2, AY151046; goat CYP4B2 variant, AY151047; human CYP4B1, AY151048; human CYP4B1 variant, AY151049.

from human tissue. RT-PCR was performed with primers designed from the 5'- and 3'-untranslated sequence of the *CYP4B1* gene (GenBank accession number NT\_004386) and the products were cloned into a TOPO-Blunt vector. Five clones were sequenced and analyzed by comparison with the sequences from published CYP4B1s. Two sequences were identified,<sup>3</sup> one native (Fig. 4) and one variant (data not shown), that were both different from the published human CYP4B1 cDNAs (GenBank accession numbers XP001598 and P13584). The native human amino acid sequence was compared with the GenBank accession CYP4B1 sequences and found to contain two amino acid differences; the first was at position 37, which is probably correct in our sequence and an error in sequence accession P13584. The second difference, an insertion at position 207, is probably the result of an imperfect intron-exon splice junction (position 207 is exactly at the intron-exon junction); however, no other transcript without the serine 207 addition was identified in any of the five clones sequenced. The transcript with serine 207, although not deposited in GenBank, was reported by Bylund et

al (1999) and later by Imaoka et al. (2001). The variant human cDNA is missing exon 3 (data not shown). Further analysis of GenBank revealed four other variants CYP4B1\*2, \*3, \*4, and \*5 (Lo-Guidice et al., 2002) that are different from the sequences we identified and are the result of polymorphic alleles. It remains to be determined whether the variant we identified is another allelic variant or an alternate splice product.

There is a highly conserved glutamate in the rabbit, rat, mouse, and human CYP4B1 enzymes at amino acid position 315 that is an alanine in the goat enzyme (Fig. 4). This acidic glutamate residue, which resides in the I-helix, is present in all other CYP4 isozymes and required for covalent heme attachment. This attachment is believed to be an ester linkage, producing a "monohydroxy heme derivative" that is presumably derived from self-catalyzed oxidation of one of the protoporphyrin IX methyl groups (Henne et al., 2001; LeBrun et al., 2002b). The lack of an acidic residue may indicate that the goat CYP4B2 apoprotein is not covalently attached to the heme prosthetic group.

**Expression of CYP4B2 in *E. coli*.** The goat and human CYP4B cDNAs were subcloned into the pCW bicistronic expression plasmid (Parikh et al., 1997), which contains the redox partner NADPH P450-reductase, using PCR to generate a 5'-*Nde*I site and a 3'-*Xba*I site as described under *Materials and Methods*. In addition to the wild-type goat CYP4B2 and human CYP4B1 vectors generated, modification of the N terminus to contain CYP17 sequence (Barnes et al., 1991) was also engineered into both the goat and human expression vectors. A goat CYP4B2 expression plasmid was also produced that lacked the first 40 amino acids. This modification removes the membrane-anchoring signal and possibly facilitates expression of a cytosolic enzyme. To investigate the role of the covalently linked glutamate-315 in the metabolism of 3MI, a site-directed mutant of the goat CYP4B2 expression vector containing CYP17 sequence (CYP4B2/A315E) was generated. The human expression vectors were generated to investigate whether the human cDNA identified in this study, containing the additional serine-207, would be active in a bacterial expression system. The human expression vectors, containing the wild-type and CYP17 sequence modification, were used to generate two more mutated expression vectors that contain a proline in the mean-der region instead of the wild-type serine-427 (Zheng et al., 1998). The proline-427 to serine-427 difference in the mean-der region of the human CYP4B1 enzyme renders it inactive (Zheng et al., 1998). Another set of site-directed mutants included CYP4B1/E315A, a human CYP4B1 expression vector with CYP17 sequence modification that contained an alanine-315 instead of a glutamate-315, and a double mutant that contained both the proline-427 and alanine-315 substitutions.

Only the two goat constructs CYP4B2 and CYP4B2/A315E produced a significant amount of spectrally active P450 protein. The typical  $\lambda_{\max}$  for CYP4B1 enzymes that contain glutamate-315 is 448 nm. Surprisingly, the absorbance maximum of CYP4B2, which contained an alanine-315, was also 448 nm. When we mutated alanine-315 to glutamate-315 in CYP4B2/A315E, the  $\lambda_{\max}$  shifted to 451 nm. An opposite shift in  $\lambda_{\max}$  from 450 to 448 nm was observed by LeBrun et al. (2002a) with CYP4A1 when they mutated the orthologous glutamate-320 to alanine-320. It can be speculated, based on

Goat CYP4B2	1	MVPSLLSLSLSLSLWAFGLVSVLFLKLRLLRRQLARAMDSPGPTTHWLFHGALE
Human CYP4B1	1	MVPSLLSLSLSLSLWAFGLVSVLFLKLRLLRRQLARAMDSPGPTTHWLFHGALE
Rabbit CYP4B1	1	-MLGFLS---RLGLWAGSLILILGLFKLRLRLRRQLARAMDSPGPTTHWLFHGALE
Rat CYP4B1	1	MVNLFLSFLSLRLGLWASVVLIMVILKLLSLRLRRQLARAMDSPGPTTHWLFHGALE
Mouse CYP4B1	1	MALSLFLSFLSLRLGLWASVVLIMVILKLLSLRLRRQLARAMDSPGPTTHWLFHGALE
Goat CYP4B2	61	IQQTGLDKVSVVWTHQFYAHQLWLRFLGLFNLIDPEYAKAVYSRGDPKAPDVYDFFLQ
Human CYP4B1	61	IQETGSLDKVSVWHAQFYAHPLWFGQFGLFNLIDPEYAKAVYSRGDPKAPDVYDFFLQ
Rabbit CYP4B1	56	IQRTGSLDKVVTWQQFYAHPLWFGQFGLFNLIDPEYAKAVYSRGDPKAPDVYDFFLQ
Rat CYP4B1	61	IQKLGSLDKVSWAQFYAHPLWFGQFGLFNLIDPEYAKAVYSRGDPKAPDVYDFFLQ
Mouse CYP4B1	61	IQRTGSLDKVVTWQQFYAHPLWFGQFGLFNLIDPEYAKAVYSRGDPKAPDVYDFFLQ
Goat CYP4B2	121	WIGKGLLVLOGKFWQHRKLLTPGFHYDVLKPYVAVFAESARVMDKWEKAKREKQSFDI
Human CYP4B1	121	WIGKGLLVLOGKFWQHRKLLTPGFHYDVLKPYVAVFAESARVMDKWEKAKREKQSFDI
Rabbit CYP4B1	116	WIGKGLLVLOGKFWQHRKLLTPGFHYDVLKPYVAVFAESARVMDKWEKAKREKQSFDI
Rat CYP4B1	121	WIGKGLLVLOGKFWQHRKLLTPGFHYDVLKPYVAVFAESARVMDKWEKAKREKQSFDI
Mouse CYP4B1	121	WIGKGLLVLOGKFWQHRKLLTPGFHYDVLKPYVAVFAESARVMDKWEKAKREKQSFDI
Goat CYP4B2	181	FSDVGHMALDSLMKCTFGKGTSGNLN-RDNNYVLAVSELTLLMQQRIDSFQYHNDFIYFL
Human CYP4B1	181	FCDVGHMALDLMKCTFGKGTSGNLN-RDNNYVLAVSELTLLMQQRIDSFQYHNDFIYFL
Rabbit CYP4B1	176	FSDVGHMALDLMKCTFGKGTSGNLN-RDNNYVLAVSELTLLMQQRIDSFQYHNDFIYFL
Rat CYP4B1	181	FCDVGHMALDLMKCTFGKGTSGNLN-RDNNYVLAVSELTLLMQQRIDSFQYHNDFIYFL
Mouse CYP4B1	181	FCDVGHMALDLMKCTFGKGTSGNLN-RDNNYVLAVSELTLLMQQRIDSFQYHNDFIYFL
Goat CYP4B2	240	TPHGRRLFRACQVAHDHDDQVIRERKAAQDEKREKIQSRRLHDFDILLGARDEEGIK
Human CYP4B1	241	TPHGRRLFRACQVAHDHDDQVIRERKAAQDEKREKIQSRRLHDFDILLGARDEDDIK
Rabbit CYP4B1	235	TPHGRRLFRACRAAHDDHDDVIRQRKAAQDEKREKIQSRRLHDFDILLGVRSQVQ
Rat CYP4B1	240	TPHGRRLFRACRAAHDDHDDVIRQRKAAQDEKREKIQSRRLHDFDILLGVRSQVQ
Mouse CYP4B1	240	TPHGRRLFRACRAAHDDHDDVIRQRKAAQDEKREKIQSRRLHDFDILLGARDESGIK
Goat CYP4B2	300	LSDEDLRAEVNTFMFGCHDDTTTSAISWVLYCMLYPEHQRCREEVILGDRDSLKWED
Human CYP4B1	301	LSADLRAEVNTFMFGCHDDTTTSGISWFLYCMALYPEHQRCREEVILGDDQFQWED
Rabbit CYP4B1	295	LSDTDLRAEVNTFMFGCHDDTTTSGISWFLYCMALYPEHQRCREEVILGDDQFQWED
Rat CYP4B1	300	LSADLRAEVNTFMFGCHDDTTTSGISWFLYCMALYPEHQRCREEVILGDDQFQWED
Mouse CYP4B1	300	LSADLRAEVNTFMFGCHDDTTTSGISWFLYCMALYPEHQRCREEVILGDRDSQWED
Goat CYP4B2	360	LAEMTYLTMCIKESFRLYPVQVYRQLSKPVTFVDRSLPAGSLISLHIALHRNSTVW
Human CYP4B1	361	LGKMTYLTMCIKESFRLYPVQVYRQLSKPVTFVDRSLPAGSLISLHIALHRNSAVV
Rabbit CYP4B1	355	LAKMTYLTMCIKESFRLYPVQVYRQLSKPVTFVDRSLPAGSLISLHIALHRNSDVV
Rat CYP4B1	360	LAKMTYLTMCIKESFRLYPVQVYRQLSKPVTFVDRSLPAGSLISLHIALHRNSTVW
Mouse CYP4B1	360	LAQMTYLTMCIKESFRLYPVQVYRQLSKPVTFVDRSLPAGSLISLHIALHRNSAVV
Goat CYP4B2	420	TDPEVDFLRFSPENVAGRHSAFIFPSAGPRNCIGQGFAMNEVKVVTALCLLRFESPD
Human CYP4B1	421	PDPEVDFLRFSTENASRRHFAFMPFSAFPRNCIGQGFAMNEKVVVTALCLLRFESPD
Rabbit CYP4B1	415	PDPEVDFLRFSPENSSRRHFAFIFPSAGPRNCIGQGFAMNEKVVVTALCLLRFESVD
Rat CYP4B1	420	PDPEVDFLRFSPENAGRHSAFMPFSAFPRNCIGQGFAMNEKVVVTALCLLRFESPD
Mouse CYP4B1	420	PDPEVDFLRFSPENMTGRHFAFMPFSAFPRNCIGQGFAMNEKVVVTALCLLRFESPD
Goat CYP4B2	480	PSKLPQMPQLVLRKNGIHLHLKPVGPGSEK*
Human CYP4B1	481	PSRLPIKMPQLVLRKNGIHLHLKPLGPGSGK*
Rabbit CYP4B1	475	PLRLPIKLPQLVLRKNGIHLHLKPLGPGKAEK*
Rat CYP4B1	480	PSKMPKVPQLVLRKNGIHLHLKPLASRSK*
Mouse CYP4B1	480	PSKIPKVPQLVLRKNGIHLHLKPLGPGSGK*

**Fig. 4.** The amino acid sequence of CYP4B2 was aligned with other members of the CYP4B subfamily using ClustalW (<http://www.ebi.ac.uk/clustalw/>). Critical amino acids that are discussed in the text are highlighted and marked with arrows. The following symbols denote the degree of conservation observed: \*, residues or nucleotides are identical in all sequences in the alignment; ., conserved substitutions have been observed; ., semiconserved substitutions are observed.

this observation, that there is a covalent interaction of mutated glutamate-315 with the heme prosthetic group that is not present with the native alanine-315.

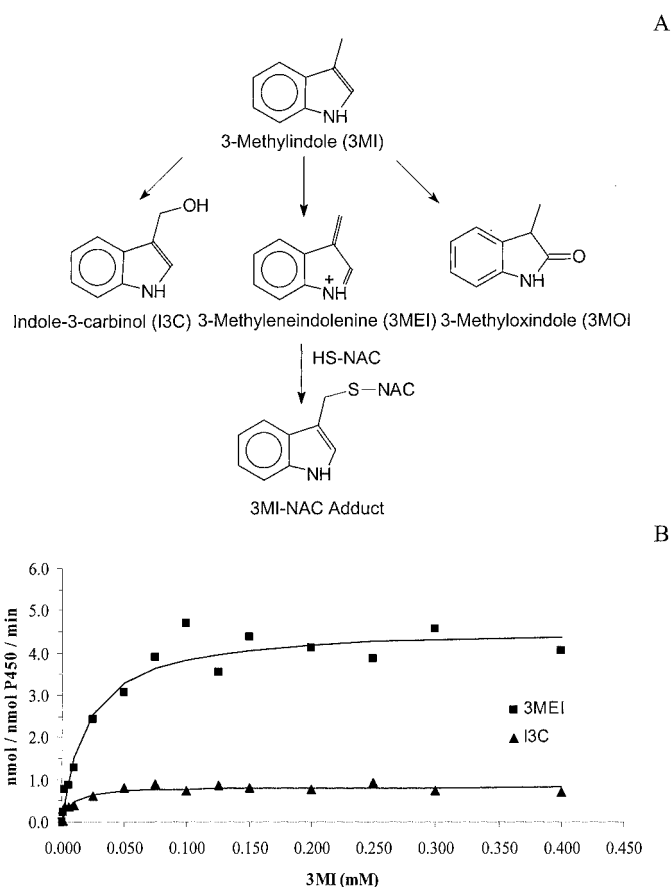
The conditions used for the expression of the other constructs were the same as those conditions used for CYP4B2 expression. Expression of CYP4B2 produced a modest 20 nM of P450, possibly because expression of this enzyme seemed detrimental to bacterial health. The goat CYP4B2 mutant without the membrane signal anchor produced a slight but detectable P450 spectrum. However, no other investigations were performed with this mutant. Despite extensive attempts using various conditions and multiple mutations, none of the human CYP4B1 expression constructs produced any spectrally detectable P450 in *E. coli*. Similarly, expression of human CYP4B1 in insect cells with a baculovirus expression system did not produce the holoenzyme, only spectrally inactive P420 (Zheng et al., 1998). Recently, in contradiction to these findings, the human sequence containing serine-207 was successfully expressed in a transgenic mouse model and in yeast as a fusion protein with NADPH-reductase (Imaoka et al., 2001).

**N-Hydroxylation of 2-Aminofluorene by CYP4B2.** Catalytic activity of goat lung microsomes, purified CYP4B2, and the bacterially expressed CYP4B2 membrane preparations were determined using 2AF, a prototypical CYP4B substrate. Turnover of 2AF to *N*-hydroxylaminofluorene was determined using a simple colorimetric assay. This assay is based on the reduction of ferric iron by the hydroxylamino moiety, with the resulting ferrous iron being quantified by coupling with 2,4,6-tripyridyl-*s*-triazine (TPTZ) to form a purplish color with a maximum absorbance at 595 nm (Belanger et al., 1981). CYP4B2 efficiently catalyzed the *N*-hydroxylation of 2AF (Table 2). This activity was inhibited by preincubation with anti-4B2 antibody in incubations with goat lung microsomes and purified CYP4B2. Surprisingly, the bacterially expressed CYP4B2 enzyme was more efficient than either goat lung microsomes or purified CYP4B2 at *N*-hydroxylation of 2AF. Recombinant CYP4B2 was inhibited by preincubation with the P450 "suicide substrate" 1-aminobenzotriazole. However, the recombinant enzyme did show *N*-hydroxylation activity comparable with that of rabbit and guinea pig pulmonary microsomal preparations (Vanderslice et al., 1987).

**Metabolism of 3-Methylindole by CYP4B2.** Catalytic turnover of 3MI was determined for CYP4B2 membrane preparations from bacteria. Incubation mixtures of varying 3MI concentrations, 200 pmol of CYP4B2 or CYP4B2/A315E,

NADPH, and NAC were analyzed by HPLC. NADPH-cytochrome P450 reductase was coexpressed with the CYP4B2. Activity, measured by cytochrome *c* reduction (data not shown), showed the bacterial membrane preparations had ample reductase activity for efficient electron donation (Iwata et al., 1998), based on comparisons of nine coexpressed P450s with reconstituted systems.

Analysis of the metabolites revealed that CYP4B2 catalyzed the production of only the dehydrogenated reactive intermediate 3MEI, which was trapped by NAC, and the hydroxylated product I3C but not the ring-oxygenated metabolite 3MOI. A metabolic scheme showing the structures of these three products is shown in Fig. 5A. The rates of production of these metabolites is plotted against substrate concentration in Fig. 5B. No other known NADPH-dependent 3MI metabolites were produced in these reactions or in incubations with control bacterial membrane preparations (data not shown). Figure 5B demonstrates that CYP4B2 was much more efficient at catalyzing the production of 3MEI than I3C. Incubations with 0.1 mM 3MI and 200 pmol of CYP4B2 catalyzed the production of 3-fold higher levels of 3MEI than I3C (24 versus 8 nmol, respectively) in 30 min. The relative ratio of 3MEI to I3C was ~3 at all substrate concentrations.



**Fig. 5.** A, metabolites of 3MI [HS-NAC, NAC; 3MI-NAC adduct, 3-[(*N*-acetylcystein-S-yl)-methyl]indole]. B, rates of CYP4B2-mediated dehydrogenation (3MEI) versus oxygenation (I3C). Bacterial membrane preparations containing 200 pmol of CYP4B2 were incubated for 30 min with 2 mM NADPH, 4 mM NAC, and varying concentrations of 3MI in 50 mM sodium phosphate buffer at 37°C. The production of 3MEI-NAC adduct and I3C were determined by HPLC and the rates of product formation were calculated using a standard curve with 3-phenyloxindole as an internal standard. Each point represents the mean of two incubations.

TABLE 2

*N*-Hydroxylation of 2-aminofluorene by goat lung microsomes and CYP4B2

Incubations of 2-AF with goat lung microsomal P450, purified, or expressed CYP4B2 are described under *Materials and Methods*. Rate is expressed as  $A_{595\text{nm}}/\text{nmol}$  of P450/min. Remaining activity after preincubation with preimmune serum (10 mg/nmol), anti-4B2 (10 mg/nmol), or 250  $\mu\text{M}$  1-aminobenzotriazole (ABT) for 30 min at 4°C is indicated.

Enzyme Incubations	Rate	Remaining Activity		
		Preimmune	Anti-4B2	ABT
	nmol of P450/min		%	
Goat Lung microsomes	0.396	103	6	N.D.
Purified CYP4B2	0.410	123	42	N.D.
Expressed CYP4B2	1.256	N.D.	N.D.	24

N.D., not determined.



Higher concentrations of NAC did not increase the detectable amounts of the trapped 3MEI adduct (data not shown). We have demonstrated previously (Skiles and Yost, 1996) that I3C does not spontaneously dehydrate to 3MEI at pH 7.4 or react with NAC to form an NAC adduct. Finally, I3C is not formed from 3MEI through the addition of water, as long as the NAC thiol nucleophile is present at high concentrations. A separate experiment showed that preincubation with 0.25 mM 1-aminobenzotriazole abolished the production of the 3MEI and I3C metabolites (data not shown).

An intriguing feature of the CYP4B2 enzyme is its apparent specificity for methyl group oxidation to produce either 3MEI or I3C. No ring oxygenation to form 3MOI was detected. This result is particularly remarkable compared with products formed by the rabbit CYP4B1, which efficiently produced all three major metabolites (Thornton-Manning et al., 1996). No other P450 enzyme tested (including CYP1A1, CYP1A2, CYP1B1, CYP2E1, CYP2F1, CYP2F3, CYP2A6, CYP2D6, CYP2B6, CYP3A4, or CYP2C19) (Lanza and Yost, 2001) exhibited this specificity.

CYP4B2 seems to be the principal P450 enzyme in goat lung that catalyzes the bioactivation of 3MI to 3MEI. Catalytic efficiencies were reported as  $V/K = 143$  (Skiles and Yost, 1996) for goat lung microsomes, and  $V/K = 231$  for expressed CYP4B2 (Table 3). The importance of covalent heme binding in the catalytic turnover of 3MI by the site-directed mutant CYP4B2/A315E was investigated. The mutant CYP4B2/A315E enzyme was approximately half as efficient at dehydrogenation as the wild-type enzyme ( $V/K = 150$  versus 231, respectively). Hydroxylation of 3MI by CYP4B2/A315E was also nearly three times less efficient ( $V/K = 40$  versus 119). Both effects are attributable predominately to decreased binding affinity of 3MI, as shown by the increases in  $K_m$  of the mutant enzyme. Mutation of the alanine residue at 315 to glutamate did not change the catalytic specificity for methyl group oxidation of 3MI; no ring-oxidized products (e.g., 3MOI) were produced. Thus, if the mutant 315 glutamate was covalently attached to a modified heme, the perturbation was not sufficient to alter substrate orientation in the catalytic site.

## Discussion

In this study, we purified and characterized a goat lung-specific P450 protein. From N-terminal amino acid analysis of the protein, we assumed it was a member of the 4B subfamily because it was similar to the human sequence. The protein has metabolic activities that are characteristic of the other CYP4B enzymes, from rabbits or guinea pigs, as is

evident from its metabolism of 2-aminofluorene (Vanderslice et al., 1987). The goat CYP4B2 enzyme is most probably the principal lung-specific protein that bioactivates 3MI in goat lung (Skiles and Yost, 1996).

Our previous studies indicated that human 2F1 metabolizes 3MI (Thornton-Manning et al., 1991; Lanza et al., 1999; Lanza and Yost, 2001), and when the same goat lung library described in this manuscript was screened with the human CYP2F1 cDNA, the goat lung CYP2F3 was cloned and characterized (Wang et al., 1998). In fact, the antibody to the goat 4B enzyme was preincubated with goat lung microsomes, and production of the putative electrophilic intermediate, 3MEI, was inhibited by 85% (Wang et al., 1998). Thus, approximately 80 to 85% of 3MI turnover to the toxic metabolite in goat lung microsomes seems to be catalyzed by the CYP4B enzyme and approximately 20% by CYP2F3, as demonstrated by inhibition with anti-2F2.

A CYP4B2 cDNA, with an open reading frame of 1533 bp, was also identified in this study. The cDNA translates into a protein of 511 amino acids, which had a similarity of approximately 81 to 83% to all known CYP4B subfamily proteins. Northern blot studies demonstrated the lung-specific transcription of the *CYP4B2* gene in the goat (Ramakanth et al., 1994). The CYP4B2 predicted amino acid sequence was identical to the first 24 N-terminal residues of the purified goat lung microsomal protein. Therefore, the CYP4B2 cDNA seems to code for the purified goat lung enzyme.

The human CYP4B1 protein sequence that was identified in this study differed from orthologous CYP4B1 proteins from other species in two important residues (Fig. 4). The human enzyme had a serine at position 427 and a serine insertion at position 207. These differences were identified in all five of our clones and in all 50 individuals in another study (Imaoka et al., 2001). We could not determine the functional significance of the Ser207 insertion because we were unable to express an active human CYP4B1 enzyme (with or without the Ser207 insertion), using the same vectors and *E. coli* strain that were used to express the active CYP4B2 enzyme. Multiple attempts, including the mutation of the Ser427 to a proline residue, a change that was previously successful (Zheng et al., 1998), proved unsuccessful in our attempts. All species other than humans have a requisite proline at the 427 position that seems to be required for heme incorporation into the apoenzyme (Zheng et al., 1998). Therefore, enzyme turnover by CYP4B1 in human lung is doubtful. However, reports that vaccinia-expressed human CYP4B1 contributes to the metabolism of other substrates (Waxman et al., 1991) and that human CYP4B1 metabolizes aromatic amines to carcinogenic electrophiles in human bladder tissues (Imaoka

TABLE 3

Enzyme kinetics constants for the dehydrogenation versus hydroxylation of 3-methylindole

Incubation of 3MI with goat lung microsomal P450, expressed CYP4B2, or CYP4B2/A315E mutant as described under *Materials and Methods*. Apparent kinetic constants were determined by curve fitting analysis of substrate concentration versus product formation rate using the Michaelis-Menton equation ( $V = V_{max}[S]/[S] + K_m$ ) with KaleidaGraph software (Synergy Software, Reading, PA).

Enzymes	3MEI (Dehydrogenation)			I3C (Hydroxylation)		
	$V_{max}$	$K_m$	$V/K$	$V_{max}$	$K_m$	$V/K$
	nmol of product/nmol of P450/min	mM		nmol of product/nmol of P450/min	mM	
Goat lung microsomes <sup>a</sup>	8.70	0.061	143	1.30	0.100	13
Expressed CYP4B2	4.61	0.020	231	0.83	0.007	119
Expressed CYP4B2/A315E	4.81	0.032	150	0.83	0.021	40

<sup>a</sup> Values from Skiles and Yost (1996).

et al., 2001) argue for the catalytic integrity of the human enzyme.

The most significant sequence difference between CYP4B2 and the CYP4B1 enzymes was an alanine at position 315 that is a glutamate in most other members of the CYP4 family, and all of the CYP4B1 enzymes. The highly conserved acidic residue serves as an anchor for covalent heme attachment. Studies are being performed to investigate the role of covalent heme binding in the catalytic preferences of CYP4 enzymes. The functional significance of this difference was investigated for 3MI metabolism in this study. We observed that CYP4B2/A315E had a higher  $K_m$  for 3MI, which reduced the overall efficiency of dehydrogenation and hydroxylation. Other studies have shown the opposite effect of covalent attachment; normally, efficiency is reduced when covalent binding is ablated by mutation of glutamate to alanine (LeBrun et al., 2002a). However, we have no direct evidence that the CYP4B2 apoenzyme doesn't bind covalently to heme, other than the unusual absorbance maximum shift from 448 to 451 nm, upon mutation of the alanine 315 residue.

In this study, a cDNA coding for a protein with 451 amino acids, that is complementary to a putative pseudogene of *CYP4B2*, was identified. The occurrence of this cDNA was high; nearly one third of clones sequenced contained the 30-bp insertion. The insertion sequence is present upstream to the requisite cysteine, which acts as the fifth ligand to the heme porphyrin. The second codon of the insertion is a stop codon, thus terminating the translation before production of the apoprotein with a functional cysteine. Hence, no holoenzyme would be formed. It is still possible that this cDNA is merely an alternate splice product, because alignment of CYP4B2 with the human *CYP4B1* gene demonstrates that the insertion is very near the putative intron-exon junction of CYP4B1.

In conclusion, we have identified a new CYP4B enzyme, which we designated CYP4B2 based on amino acid similarity and comparisons with the EST database. This enzyme, which can be expressed in reasonable amounts in bacteria, is capable of metabolizing the prototypical CYP4B substrate 2-aminofluorene and the pneumotoxicant 3-methylindole to its reactive intermediate 3-methyleneindolenine. Given its likely abundance in lung tissues and its apparent efficiency at generating the reactive intermediate of 3MI, CYP4B2 is probably the principal player in the P450-mediated bioactivation of 3MI in goats. The enzyme's apparent specificity for methyl group oxidation without ring oxygenation is unique among P450s, making it ideal for characterizing the mechanism of 3MI dehydrogenation. CYP4B2 also presents a good model to assess the importance of covalent heme binding in catalyzing the intriguing  $\omega$ -hydroxylation of lipophilic substrates, as well as the dehydrogenation of such substrates as valproic acid and lauric acid.

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