

## HETEROLOGOUS EXPRESSION OF CYP2K1 AND IDENTIFICATION OF THE EXPRESSED PROTEIN (BV-CYP2K1) AS LAURIC ACID ( $\omega$ -1)-HYDROXYLASE AND AFLATOXIN B1 EXO-EPOXIDASE

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### ABSTRACT:

LMC2 is the most abundant constitutively expressed hepatic cytochrome P450 found in sexually immature rainbow trout (*Oncorhynchus mykiss*) and is also the isozyme that activates the carcinogen aflatoxin B1 (AFB1). This P450 has been cloned, sequenced, and designated as CYP2K1. The present report describes the heterologous expression of enzymatically active CYP2K1 (BV-CYP2K1) in baculovirus *Spodoptera frugiperda* (Sf9) insect cells and its catalytic and immunoreactivity characterization in comparison with that of the previously purified LMC2 P450. Homogenates of Sf9 cells expressing the CYP2K1 enzyme and LMC2 both catalyzed the hydroxylation of lauric acid and the epoxidation of AFB1 in the presence of rat NADPH-cytochrome P450 reductase. Both LMC2 and BV-CYP2K1 catalyzed the oxidation of

lauric acid primarily at the ( $\omega$ -1) position plus small amounts at the ( $\omega$ -2) position. Formation of AFB1 epoxide was shown indirectly by the appearance of an AFB1 epoxide-glutathione conjugate when P450 incubation mixtures contained AFB1, glutathione (GSH) together with mouse liver cytosol or purified rat GSH-transferase. When the AFB1 epoxide-GSH conjugate produced by BV-CYP2K1 and purified LMC2 was analyzed by HPLC using a chiral column, it had a retention time identical to that produced by CYP3A4, a human P450 known to form exclusively the AFB1 *exo*-epoxide. These results, therefore, confirm that the cDNA-expressed CYP2K1 protein is catalytically and immunologically identical to purified trout LMC2 and that these two enzymes produce primarily the highly carcinogenic stereoisomeric *exo*-epoxide form of AFB1.

Rainbow trout (*Oncorhynchus mykiss*) express multiple cytochrome P450 (CYP)<sup>1</sup> forms that are involved in the metabolism of endogenous and exogenous chemicals (Buhler and Wang-Buhler, 1998; Williams et al., 1998). At least five P450s (LMC1-LMC5) have been purified from liver of juvenile rainbow trout (Miranda et al., 1989). Three of the constitutive hepatic P450s LMC1, LMC2, and LMC5, have been cloned, sequenced, and designated as CYP2K1 (Buhler et al., 1994), CYP2 M1 (Yang et al., 1998), and CYP3A27 (Lee et al., 1998), respectively, by the P450 Nomenclature Committee. CYP2K1 has been the most intensively studied trout P450 enzyme, not only because it is the most abundant P450 isoform expressed in trout liver but also due to its ability to activate the carcinogen AFB1. The rainbow trout is used extensively as a model

system for studying carcinogenesis, in part because of its unusually high sensitivity to carcinogens such as AFB1 (Bailey et al., 1996). Using polyclonal and monoclonal antibodies raised against the purified CYP2K1 (LMC2), CYP2K1 was the first hepatic cytochrome P450 form cloned from a hepatic cDNA library from untreated juvenile rainbow trout (Buhler et al., 1994). The deduced *N*-terminal sequence of CYP2K1 matched 12 of the 15 amino acids derived from purified CYP2K1 by Edman degradation. However, it was not known whether the protein encoded by CYP2K1 cDNA was functionally identical to the purified trout CYP2K1. CYP2K1 has been shown to exhibit lauric acid (LA) ( $\omega$ -1)- and ( $\omega$ -2)-hydroxylation activity (Buhler et al., 1997) and to convert AFB1 to AFB1-8,9-epoxide (Williams and Buhler, 1983). Human and rat liver microsomes produce two forms of AFB1 epoxide, namely, the *endo*- and *exo*- AFB1 epoxides (Raney et al., 1992) and the *exo*-epoxide stereoisomer is considerably more reactive and carcinogenic than the *endo*-epoxide (Raney et al., 1992; Eaton and Gallagher, 1994). The identities of the epoxides produced by trout liver microsomes and purified CYP2K1, however, have not been determined.

In this report, we describe the heterologous expression of CYP2K1 in a baculovirus expression system and the determination of the catalytic activities of the recombinant CYP2K1 protein toward the diagnostic substrates LA and AFB1. The evidence shows that the protein encoded by CYP2K1 cDNA is functionally identical to the purified trout CYP2K1 in hydroxylating LA and in activating AFB1 to its *exo*-epoxide.

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<sup>1</sup> Abbreviations used are: CYP, cytochrome P450; AFB1, aflatoxin B1; LA, lauric acid; AcMNPV, *Autographa californica* nuclear polyhedrosis virus; GST, glutathione S-transferase; PCR, polymerase chain reaction; BV-CYP2K1, recombinant trout CYP2K1 produced in baculovirus-infected insect cells; DLPC, dilaurylphosphatidylcholine; GC/MS, gas chromatography/mass spectrometry; GSH, glutathione.

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### Experimental Procedures

**Experimental Animals.** One-year-old juvenile rainbow trout of the Mt. Shasta strain were obtained from the Oregon State University Marine/Freshwater Biomedical Sciences aquatic facility. Microsomes were prepared by differential centrifugation from the livers of sexually immature trout as described previously by Miranda et al. (1989). Purified trout CYP2K1 also was prepared as described by Miranda et al. (1989). Cytosol was prepared from the livers of male C57B mice as described by Guengerich (1989). Liver homogenates were subjected to centrifugation initially at 10,000g and thereafter at 105,000g and the resulting cytosol stored at  $-80^{\circ}\text{C}$  until used.

**Materials.** [ $^{14}\text{C}$ ]Lauric acid (dodecanoic acid; 40 mCi/mmol) was purchased from ICN Pharmaceuticals Inc. (Costa Mesa, CA) and [ $^3\text{H}$ (G)]AFB1 (28 Ci/mmol) was obtained from Moravak Biochemicals (Brea, CA). Protein A [ $^{125}\text{I}$ ] (>30  $\mu\text{Ci}/\mu\text{g}$ ) was from ICN (Costa Mesa, CA). Insect cell (BTI-TN-5B1-4) microsomes (supersomes) containing cDNA-expressed human cytochromes P450s CYP1A2 and CYP3A4 were purchased from Gentest Corporation (Woburn, MA). Restriction enzymes (*Bam*HI and *Hind*III) were from Stratagene (La Jolla, CA) or New England Biolabs (Beverly, MA). T4 DNA ligase, fetal bovine serum, *Trichoplusia ni* Medium-Formulation Hink and SF-900 II serum-free medium were from Life Technologies (Grand Island, NY). pBlueBacIII and Sf9 cells were kind gifts from Dr. George F. Rohrmann, Department of Microbiology, Oregon State University. Linear *Autographa californica* nuclear polyhedrosis virus (AcMNPV) DNA and liposomes were purchased from Invitrogen (San Diego, CA). Hybond-N+ nylon membrane was from Amersham (Amersham, UK). Acetonitrile (HPLC grade) was from J.T. Baker Chemical (Phillipsburg, NJ). Lauric acid (sodium salt), AFB1, purified rat glutathione transferases (GSTs), BSA, hemin, and NADPH were purchased from Sigma Chemical Co. (St. Louis, MO). Sodium cholate was prepared by recrystallization (2 $\times$ ) of cholic acid (free acid, Sigma Chemical Co.) from hot ethanol containing Celite and charcoal. Crystals obtained were filtered and dried in a desiccator. To prepare a 20% sodium cholate solution, 100 g of recrystallized cholic acid and 10 g of sodium hydroxide were dissolved in 500 ml of water, the pH adjusted to 7.5 with concentrated acetic acid, and the resulting solution filtered through a 0.45- $\mu\text{m}$  filter.

**cDNA-Directed Expression of CYP2K1 in Sf9 Insect Cells.** The baculovirus expression system successfully used in the overexpression of CYP2 M1 (Yang et al., 1998) was also used for the expression of CYP2K1. CYP2K1 cDNA was excised from pSPORT1 vector by *Bam*HI and *Hind*III digestion and ligated to the compatible ends of baculovirus transfer vector pBlueBacIII. The recombinant baculovirus was generated by cotransfection of linear AcMNPV DNA and pBlueBacIII containing CYP2K1 cDNA using AcMNPV linear transfection kit. Sf9 cells were maintained in complete *Trichoplusia ni* Medium-Formulation Hink/10% fetal bovine serum at  $27^{\circ}\text{C}$ . The recombinant baculovirus was isolated by plaque assay with X-gal for color selection and purified as described (O'Reilly et al., 1994). The purity of the plaques and the presence of cDNA insert were confirmed by polymerase chain reaction using recombinant baculovirus PCR forward (5'-TTACTGTTTCGTAA-CAGTTTTCG-3') and reverse (5'-CAACAACGCACAGAATCTAGC-3') primers. The PCR products were further analyzed by diagnostic restriction digestion using *Bam*HI and *Hind*III. Due to the differences in expression level between each recombinant virus, at least three different recombinant viruses were selected from each transfection to examine the protein yield by Western blotting (Buhler et al., 1994) probed with rabbit polyclonal antibodies raised against CYP2K1 (Miranda et al., 1989). The most productive virus was amplified to prepare high titer stock for expression. The presence of cDNA and the absence of contaminating virus in the high-titer stock were checked again using PCR.

To express CYP2K1, Sf9 cells were grown in SF-900 serum-free medium to a density of  $2 \times 10^6$  cells/ml in spinner flasks (Bellco Glass, Vineland, NJ) at room temperature ( $23\text{--}24^{\circ}\text{C}$ ). Cells were infected at a multiplicity of infection of 1 to 5. An equal molar mixture of hemin and BSA stock solution was prepared according to Grogan et al. (1995) and was added at the time of infection to a final concentration of 1  $\mu\text{g}/\text{ml}$  hemin to compensate for the low endogenous level of hemin in the insect cells. P450 production during the infection was monitored by measuring the CO-reduced difference spectra as described by Omura and Sato (1964). Sf9 cells were harvested 84 to 90 h after infection, washed once with PBS, and resuspended in 100 mM potassium

phosphate, pH 7.4, 1 mM EDTA, 1 mM dithiothreitol, 20% glycerol, and 0.2 mM phenylmethylsulfonyl fluoride. Cell lysate was prepared by sonication as described previously (Yang et al., 1998). Total P450 content of lysate was measured using reduced CO-difference spectrometry (Omura and Sato, 1964). Because previous studies (Sigle et al., 1994) have shown that cholate extraction of baculovirus lysate significantly improved P450 catalytic activity, aliquots of cell lysate were treated with 1.4% sodium cholate overnight at  $4^{\circ}\text{C}$  to solubilize the expressed P450 and then centrifuged at 16,000g for 20 min. The P450-containing supernatant and the crude cell lysate were used for Western blotting and enzyme assays. The protein concentration was determined using Coomassie Plus protein assay reagent (Pierce, Rockford, IL).

**Western Blot Analysis.** Western blot analysis of cell lysate was carried out as described previously (Miranda et al., 1989; Buhler et al., 1994). Polyclonal antibodies raised against trout CYP2K1 (Miranda et al., 1989) and monoclonal antibodies were used as probes. Monoclonal antibodies 3 M1 and 3 M3, immunoglobulin type IgG<sub>1</sub> and IgG<sub>2b</sub>, were produced in our lab (unpublished results; Buhler et al., 1994). Two stable hybridoma cell lines were selected from the third fusion of myeloma cells and spleen cells obtained from a mouse immunized with purified rainbow trout CYP2K1 (LMC2). The 3 M1 and 3 M3 monoclonal antibodies were found to react only with trout purified CYP2K1 and have no cross-reactivities with trout CYP1A1, CYP2M1, CYP3A27, LMC3, and LMC4 isozymes (unpublished results). Detection was facilitated by [ $^{125}\text{I}$ ]-protein A (0.2  $\mu\text{Ci}/\text{ml}$ ) for polyclonal antibodies and enhanced chemiluminescence Western blotting detection reagents (Amersham, Arlington Heights, IL) for monoclonal antibodies.

**Enzyme Assays.** Purified CYP2K1 (0.05 nmol) or 0.1 nmol of expressed BV-CYP2K1 (cholate treated or crude lysate) was preincubated with 0.4 nmol of purified rat liver NADPH-cytochrome P450 reductase, 0.2 nmol of rabbit cytochrome *b*<sub>5</sub> and 10  $\mu\text{g}$  of dilaurylphosphatidylcholine (DLPC) at room temperature for 10 min. Buffer (50 mM Tris-HCl, pH 7.4) and substrate (200  $\mu\text{M}$  [ $^{14}\text{C}$ ]LA, or 100  $\mu\text{M}$  [ $^3\text{H}$ ]AFB1) were then added to the mixture followed by 1 mM NADPH to initiate the reaction. All additions yielded the indicated concentrations in a total volume of 0.5 ml. After 1-h incubation at  $25^{\circ}\text{C}$ , the reactions were stopped by the addition of 0.1 ml of 10% sulfuric acid (for LA hydroxylation) or 0.5 ml of methanol (for AFB1 oxidation).

**Analysis of Lauric Acid and Aflatoxin B1 Metabolites.** LA metabolites were extracted and analyzed by HPLC and GC/MS as described by Buhler et al. (1997). Separation of AFB1 metabolites (as Tris-diols or dihydrodiols) was performed on a 5-mm Prodigy ODS-2 column ( $250 \times 4.6 \mu\text{m}$ ; Phenomenex, Belmont, CA) using methanol and 0.1% ammonium phosphate, pH 3.0, as the mobile phase; a flow rate of 1 ml/min, with detection at 362 nm; and by a radioactivity flow monitor (FLO-ONE  $\beta$ eta; Packard, Meriden, CT). The mobile phase concentration was 28% methanol for 15 min followed by a linear gradient to 80% methanol in 5 min. At 25 min, the methanol was returned to 28% in 2 min and the column equilibrated with 28% methanol for 13 min before the next injection.

**Aflatoxin B1 Epoxide-GSH Conjugate Assays.** *Endo*- and *exo*-forms of AFB1 epoxide are conjugated with GSH in the presence of GST and can be separated and identified by HPLC (Raney et al., 1992). Incubation mixtures contained 0.05 nmol of purified CYP2K1, or 0.1 nmol of either BV-CYP2K1 or baculovirus-expressed human CYP1A2 or CYP3A4 as positive controls, 0.4 nmol of rat NADPH-cytochrome P450 reductase, 0.2 nmol of cytochrome *b*<sub>5</sub>, 10  $\mu\text{g}$  of DLPC, 1 mM GSH, 0.3 mg of purified rat GST or 0.5 mg of mouse liver cytosol, 1 mM NADPH, and 100  $\mu\text{M}$  [ $^3\text{H}$ ]AFB1 in a total volume of 0.5 ml. After incubation at  $25^{\circ}\text{C}$  (for BV-CYP2K1 and purified CYP2K1) or  $37^{\circ}\text{C}$  (for CYP3A4) for 1 h, the reactions were stopped by the addition of 100  $\mu\text{l}$  of 2 M acetic acid. When microsomes from liver of juvenile male trout were used, the NADPH-cytochrome P450 reductase, cytochrome *b*<sub>5</sub> and DLPC were omitted from the incubation mixture. The samples were frozen for 2 h, thawed, and then centrifuged at 16,000g for 20 min. The supernatant was analyzed by HPLC using a Pirkle-concept chiral column packed with D-phenylalanine covalently bound to aminopropyl silica ( $4.6 \times 250 \text{ mm}$ ) as described by Stresser et al. (1994). A bimodal peak was seen when a mixture of the *exo*- and *endo*-GSH conjugates was chromatographed, indicating that the resolution was adequate to distinguish between the two stereoisomeric forms.

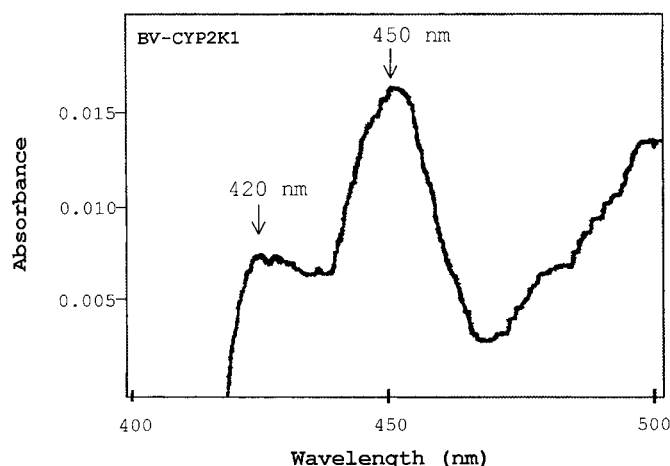


Fig. 1. Identification of BV-CYP2K1 as P450 by CO-reduced difference spectrum.

The spectrum was measured in 0.1 M potassium phosphate (pH 7.4), 1 mM EDTA, 20% glycerol, and 1 mM dithiothreitol using cell homogenate from CYP2K1 recombinant baculovirus-infected Sf9 cells as described under *Experimental Procedures*.

## Results

**Expression of CYP2K1 in Sf9 Cells.** A typical CO-difference spectrum with maximum at 450 nm was detected in the homogenate of Sf9 cells infected with CYP2K1 recombinant baculovirus (Fig. 1). No P450-type CO-difference spectrum was detected in the homogenate from control cells and wild-type virus. The specific content of spectrally active CYP2K1 was estimated to be 30 to 70 nM.

Homogenates from CYP2K1 recombinant baculovirus-infected cells were also analyzed by Western blotting. The virus-infected Sf9 cells contained protein that cross-reacted with polyclonal and monoclonal antibodies (Buhler et al., 1994) raised against CYP2K1. The purified trout CYP2K1 and BV-CYP2K1 both exhibited similar electrophoretic mobilities upon Western blot analysis (Fig. 2). Cell homogenate from Sf9 cells or cells infected with wild-type virus did not show any cross-reactivity with either antibody (data not shown).

**Catalytic Activity of cDNA-Directed CYP2K1 Expression.** CYP2K1 was shown previously to be the major hepatic P450 form catalyzing the ( $\omega$ -1)-hydroxylation of LA (Buhler et al., 1997) and the bioactivation of AFB1 (Williams and Buhler, 1983) in rainbow trout. Therefore, LA and AFB1 were used as substrates to determine the catalytic activities of baculovirus-expressed CYP2K1. BV-CYP2K1-containing insect cell crude lysate showed LA hydroxylation activity with a turnover number of 1.3 nmol/min/nmol P450. Cholate treatment of the cell lysate increased the activity of BV-CYP2K1 to 10.0 nmol/min/nmol P450 (Table 1). In contrast, purified CYP2K1 gave a turnover number of 5.1 nmol/min/nmol P450 (Table 1) with ( $\omega$ -1)-hydroxylauric acid as the major metabolite. A small amount of ( $\omega$ -2)-hydroxylation product also was produced by both purified CYP2K1 and BV-CYP2K1 (Fig. 3). The ratios of LA ( $\omega$ -1)- and ( $\omega$ -2)-hydroxylation was somewhat different between the purified native CYP2K1 enzyme (6:1) and the expressed BV-CYP2K1 (4:1). The reason for this difference in regiospecific hydroxylation may be related to the different lipid environments or other unknown factors but similar differences in catalytic activities between purified and expressed P450s have been previously noted (Gonzalez and Korzekwa, 1995). The identity of LA metabolites produced by BV-CYP2K1 was confirmed by GC/MS analysis (data not shown).

The catalytic activity of BV-CYP2K1 toward AFB1 was also investigated by trapping the AFB1 epoxide metabolites as Tris-diols

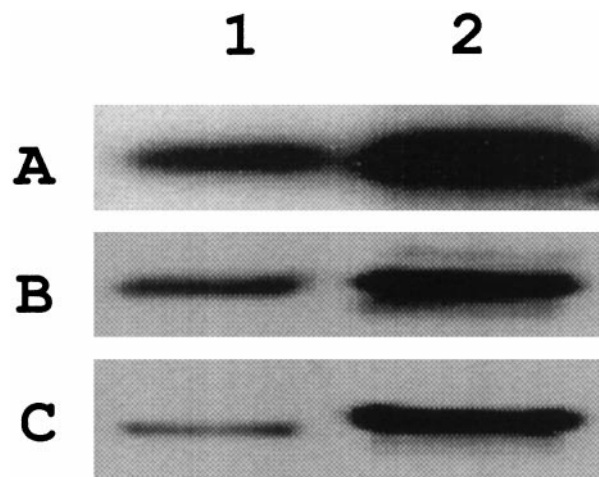


Fig. 2. Western blot analysis of BV-CYP2K1.

Lane 1, 0.25 pmol of purified trout liver CYP2K1. Lane 2, 2.5 mg of Sf9 cell homogenate infected with BV-CYP2K1. The blots were probed with polyclonal anti-CYP2K1 IgG (A), monoclonal anti-CYP2K1 IgG-3 M1 (B), or monoclonal anti-CYP2K1 IgG-3 M3 (C).

TABLE 1

Catalytic activity of BV-CYP2K1 and purified CYP2K1

	( $\omega$ -1)-OH-LA	AFB <sub>1</sub> -diol
	nmol/min/nmol P450	
BV-CYP2K1	1.33	0.39
BV-CYP2K1 (cholate treated)	10.01	2.02
Purified CYP2K1	5.07	6.88

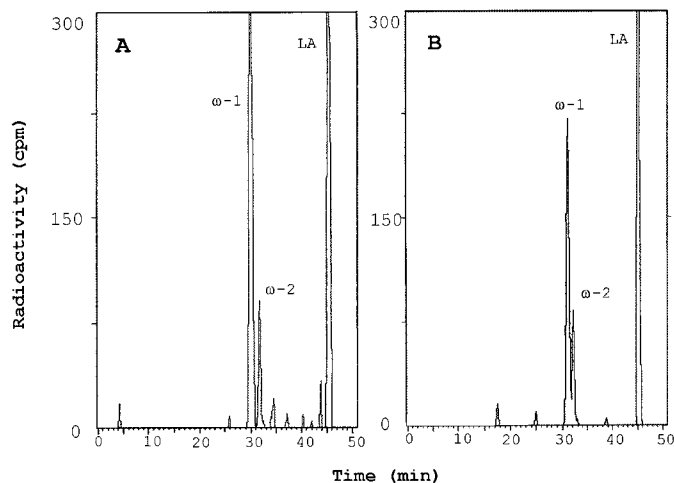


Fig. 3. HPLC chromatogram of [<sup>14</sup>C]LA metabolites produced by purified CYP2K1 (A) and BV-CYP2K1 (B).

(Neal and Colley, 1979), which then were analyzed by HPLC. BV-CYP2K1 in insect cell lysate and in cholate-treated lysate was able to oxidize AFB1 with specific activities of 0.4 and 2.0 nmol/min/nmol P450, respectively (Table 1). Purified trout CYP2K1, however, was more active with a specific activity of 6.9 nmol/min/nmol P450.

**Identification of the AFB1 Epoxide Metabolites of cDNA-Expressed CYP2K1 and Purified CYP2K1 as the *exo*-Form.** To determine whether BV-CYP2K1 or purified CYP2K1 were capable of forming the *exo*- and *endo*-epoxides of AFB1, incubations were carried out using the trout P450s to generate AFB1 epoxides that were then assayed as GSH conjugates after trapping by addition of GSH

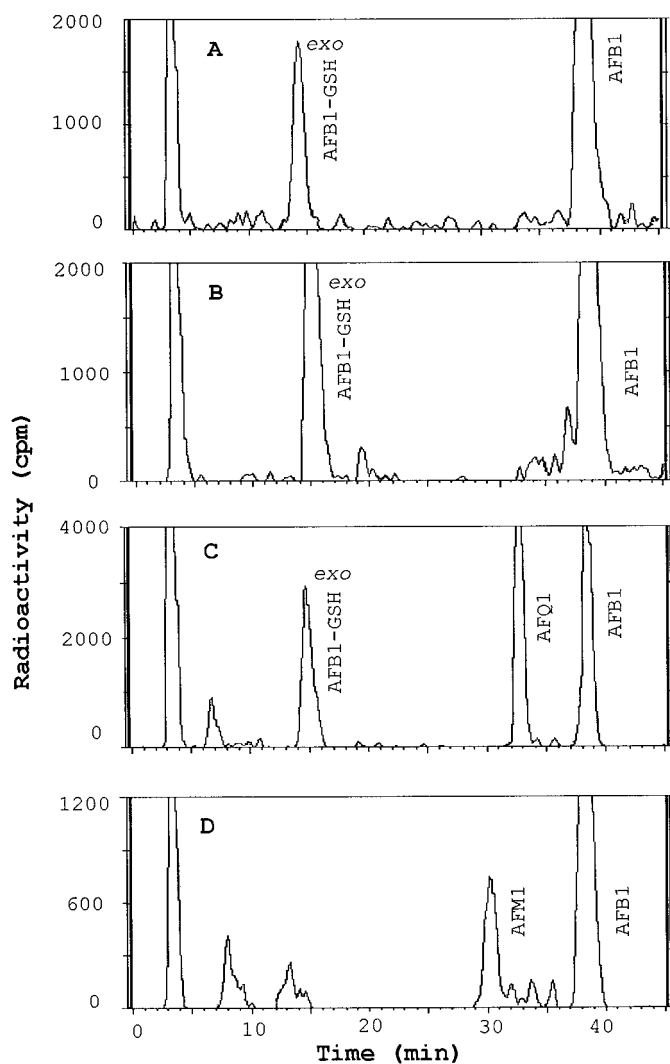


FIG. 4. HPLC chromatogram of AFB1-GSH conjugates produced in the presence of mouse liver cytosol and GSH. BV-CYP2K1 (A), purified CYP2K1 (B), human CYP3A4 (C), and human CYP1A2 (D).

together with purified rat GSTs or mouse liver cytosol containing GSTs. As a positive control, insect cell (BTI-TN-5B1-4) microsomes containing cDNA-expressed human CYP3A4 were used as catalyst for the formation of AFB1 *exo*-epoxide. BV-CYP2K1, purified trout CYP2K1, and human CYP3A4, in the presence of mouse liver cytosol and GSH, all produced a single AFB1 epoxide-GSH peak (Fig. 4, A–C) with a 15.0-min retention time upon HPLC analysis using a chiral column (Stresser et al., 1994). A single major AFB1 epoxide-GSH peak at 15.0-min retention time also was formed when rat GST was used in place of mouse liver cytosol in incubations containing BV-CYP2K1, purified CYP2K1, or CYP3A4 (Fig. 5, A–C). Recombinant human CYP1A2, however, in the presence of rat liver GST gave a single AFB1 *endo*-epoxide-GSH peak (Fig. 5D) with a longer, 16.2-min retention time. When mouse liver cytosol was used instead of rat GST for the incubation of GSH and human CYP1A2, no peak eluted at the retention times of *exo*- or *endo*-epoxide GSH (15.0 or 16.2 min, respectively) (Fig. 4D). The rather broad *exo*-epoxide conjugate peaks and rather high baseline noise level could mask the formation of small quantities of the AFB1 *endo*-epoxide GSH-conjugate (Figs. 4 and 5). It is clear, however, that the major product formed with CYP2K1 and BV-CYP2K1 was the *exo*-AFB1 epoxide-GSH conjugate. Microsomes from the livers of juvenile male trout

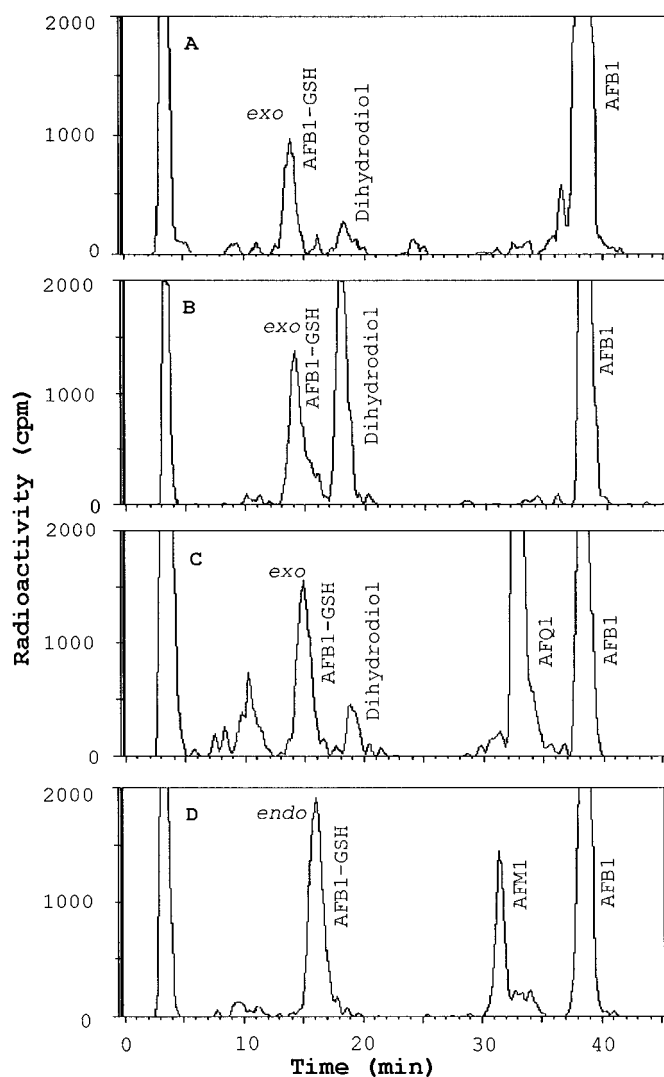


FIG. 5. HPLC chromatogram of AFB1-GSH conjugates produced in the presence of purified rat GSTs ( $\alpha$ - and  $\mu$ -classes) and GSH. BV-CYP2K1 (A), purified trout CYP2K1 (B), human CYP3A4 (C), and human CYP1A2 (D).

incubated with either rat GST or mouse liver cytosol in the presence of GSH also produced a single major AFB1 epoxide-GSH peak with identical retention times as that observed with BV-CYP2K1 and CYP2K1 (data not shown). In addition to formation of the AFB1 epoxide-GSH conjugates, aflatoxin M1 and aflatoxin Q1 (AFQ1) also were formed by human CYP1A2 and CYP3A4, respectively (Fig. 4, C and D; Fig. 5, C and D) but not by trout BV-CYP2K1 or CYP2K1 (Fig. 4, A and B).

## Discussion

Human CYP1A2 has been shown to catalyze the conversion of AFB1 to *exo*- and *endo*-8,9-epoxides, whereas human CYP3A4 only produces the *exo*-epoxide (Ueng et al., 1995), the highly reactive form primarily responsible for AFB1-induced carcinogenesis (Raney et al., 1992; Eaton and Gallagher, 1994). The two AFB1 epoxides are conjugated with GSH by GSTs. In humans and rats, the  $\mu$ -class GSTs have the highest catalytic activity for AFB1 *exo*- and *endo*-epoxides (Raney et al., 1992). In contrast, mouse liver cytosol, which contains mainly  $\alpha$ -class GST, selectively conjugates the *exo*-epoxide almost exclusively (Raney et al., 1992; Eaton and Gallagher, 1994). The resistance of mice to AFB1-induced carcinogenicity is believed to

relate to the high activity of the mouse hepatic GSTs toward the highly reactive AFB1 *exo*-epoxide (Eaton and Gallagher, 1994). Trout liver GSTs, however, have little or no conjugating activity toward AFB1 epoxides (Valsta et al., 1988).

The baculovirus-expressed CYP2K1 (BV-CYP2K1), purified trout CYP2K1, and human CYP3A4, in the presence of mouse liver cytosol and GSH, all showed only a single AFB1 epoxide-GSH peak (Fig. 4, A and C) with a 15.0-min retention time when analyzed by HPLC on a chiral column (Stresser et al., 1994). A single major AFB1 epoxide-GSH peak at 15.0-min retention time also was formed when rat GST was used in place of mouse liver cytosol in incubations containing BV-CYP2K1, purified CYP2K1, or CYP3A4 (Fig. 5, A-C).

When human CYP1A2 was used to oxidize AFB1 in a reconstituted system in the presence of GSH and rat GST, a single AFB1 epoxide-GSH conjugate peak (Fig. 5D) was observed with a longer, 16.2-min retention time. Human CYP1A2 is known to oxidize AFB1 to both the *exo*- and *endo*-epoxides (Ueng et al., 1995) but rat liver GST is much more reactive with the *endo*-epoxide form than with the *exo*-epoxide (Raney et al., 1992). The slower moving 16.2-min peak produced by CYP1A2 in the presence of rat liver GST (Fig. 5D), therefore, is consistent with the formation of the AFB1 *endo*-epoxide-GSH conjugate. If mouse liver cytosol was used as the source of GST for incubation with AFB1, GSH, and human CYP1A2, only a small broad double peak was seen (Fig. 4D), apparently reflecting relatively low CYP1A2 catalyzed formation of AFB1 *exo*-epoxide (Ueng et al., 1995) together with a negligible capacity of the mouse GSTs to conjugate the stereoisomeric AFB1 *endo*-epoxide (Raney et al., 1992).

Based on these considerations, we conclude, therefore, that the trout BV-CYP2K1 and CYP2K1 both catalyze the oxidation of AFB1 primarily to the carcinogenic *exo*-epoxide stereoisomeric form (Fig. 4, A and B; Fig. 5, A and B). Human CYP3A4, which is known to form only AFB1 *exo*-epoxide (Ueng et al., 1995), gave similar results (Figs. 4C and 5C). After incubation with rat GST, a small peak with a 16.2-min retention time also was seen with BV-CYP2K1 and purified CYP2K1 (Fig. 5, A and B). This could reflect the initial formation of small quantities of the AFB1 *endo*-epoxide GSH followed by its conjugation with GSH.

In summary, we have established a successful system for the overexpression of rainbow trout CYP2K1 in insect cells. The expressed CYP2K1 protein was immunologically and enzymatically similar to the purified trout CYP2K1. Both trout BV-CYP2K1 and purified CYP2K1 catalyzed the oxidation of AFB1 mainly to its highly carcinogenic *exo*-epoxide form with little or no formation of other AFB1 metabolites. Thus, this trout enzyme may have potential application in studying mechanisms of chemical carcinogenesis induced by AFB1 *exo*-epoxide without the interference of other metabolites of AFB1 such as aflatoxin M1 and aflatoxin Q1. Because CYP2K1 is the predominant P450 present in the liver of juvenile rainbow trout, our observations may allow a more positive correlation between AFB1 epoxidation and the high sensitivity (Bailey et al., 1996) of this fish species to AFB1-mediated carcinogenesis.

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