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Catalytic Activity and Stereoselectivity of Purified Forms of Rabbit Liver Microsomal Cytochrome P-450 in the Oxygenation of the (−) and (+) Enantiomers of *trans*-7,8-Dihydroxy-7,8-Dihydrobenzo[α]Pyrene

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SUMMARY

DEUTSCH, J., K. P. VATSIS, M. J. COON, J. C. LEUTZ, AND H. V. GELBOIN: Catalytic activity and stereoselectivity of purified forms of rabbit liver microsomal cytochrome P-450 in the oxygenation of the (−) and (+) enantiomers of *trans*-7,8-dihydroxy-7,8-dihydrobenzo[α]pyrene. *Mol. Pharmacol.* **16**, 1011-1018 (1979).

Purified forms of rabbit liver microsomal cytochrome P-450 (phenobarbital-inducible P-450_{LM_2} and β-naphthoflavone-inducible P-450_{LM_4}) were examined for catalytic activity in the conversion of the (−) and (+) enantiomers of *trans*-7,8-dihydroxy-7,8-dihydrobenzo-[α]pyrene to stereoisomeric, highly reactive and mutagenic 7,8-dihydroxy-9,10-oxy-7,8,9,10-tetrahydrobenzo[α]pyrenes. In the reconstituted enzyme system, P-450_{LM_4} from both phenobarbital- and β-naphthoflavone-induced animals has much higher catalytic activity than P-450_{LM_2} with either enantiomer of the *trans*-7,8-diol. Both forms of the cytochrome exhibit greater activity toward the (−) than the (+) isomer of the substrate, but this is more striking with P-450_{LM_4}. The relative amounts of diol-epoxides formed from either enantiomer of the substrate differ markedly with the form of the cytochrome used. P-450_{LM_2} gives somewhat more diol-epoxide I than diol-epoxide II with both substrates. In contrast, P-450_{LM_4} gives almost exclusively diol-epoxide I from the (−)*trans*-7,8-diol and more diol-epoxide II than diol-epoxide I from the (+)*trans*-7,8-diol. Thus, P-450_{LM_4} is highly stereospecific in oxygenating at the 9,10 double bond, regardless of the absolute configuration of the hydroxyl groups at the 7 and 8 positions of the substrate.

INTRODUCTION

The involvement of microsomal mixed-function oxidases and related enzymes in the metabolism of polycyclic aromatic hydrocarbons renders these membrane-bound enzyme systems of special importance in view of man's constant exposure to BP[3] and similar carcinogens (1). It is presently well established that these versatile catalysts

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[3] The abbreviations used are: BP, benzo[α]pyrene; (−)*trans*-7,8-diol, (−)*trans*-7,8-dihydroxy-7,8-dihydrobenzo[α]pyrene; diol-epoxide I, *r*-7,*t*-8-dihydroxy-*t*-9,10-oxy-7,8,9,10-tetrahydrobenzo[α]pyrene; diol-epoxide II, *r*-7,*t*-8-dihydroxy-*c*-9,10-oxy-7,8,9,10-tetrahydrobenzo[α]pyrene; in the latter two abbreviations, *r*-7 indicates that the substituent at the 7-posi-

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can convert polycyclic hydrocarbon carcinogens both to biologically inactive products and to metabolites which are more cytotoxic, carcinogenic and mutagenic than the parent compounds (2).

As reviewed elsewhere (3), numerous studies have provided compelling evidence that the extraordinarily broad substrate specificity of the hepatic microsomal system (4) can be largely attributed to the existence of multiple forms of P-450$_{LM}$. These have recently been purified to varying extents in several laboratories and shown to be distinct proteins with significantly different subunit molecular weights, spectral and immunological properties, content of certain amino acid residues, and catalytic activities toward a variety of substrates (3, 5–14). In this laboratory, six different forms of the cytochrome have been isolated from rabbit liver microsomes and designated according to their relative electrophoretic mobilities (3, 5, 6). P-450$_{LM_2}$ and $_{LM_4}$, which were purified to homogeneity, are inducible by PB and BNF, respectively (3, 5, 6). Both Ouchterlony double diffusion analysis and sodium dodecyl sulfate-polyacrylamide gel electrophoresis indicate that P-450$_{LM_2}$ is present in only trace amounts in microsomes from untreated and BNF-treated rabbits, whereas P-450$_{LM_4}$ is present at significant levels in microsomes from untreated and PB-treated animals (3, 5, 6, 9). The P-450$_{LM_4}$ preparations purified from these three sources (6), as well as from 3-methylcholanthrene-induced (P-448) (7, 10) and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin-induced rabbits (P-450$_c$) (14), are essentially indistinguishable as judged by a number of independent criteria (6, 7, 12–17) (for reviews see Ref. 3, 18, 19).

The major pathway of BP activation in hepatic microsomes is currently believed to involve three enzymatic steps, namely, epoxidation of the 7,8 double bond of BP (20–25) followed by epoxide hydrase-catalyzed hydration of the 7,8-epoxide to the (−)*trans*-7,8-diol (23, 25, 26), and a further oxygenation of the latter, giving rise to the stereoisomeric diol-epoxides I and II (23–25). The three-step activation of BP is mediated by enzymes that are remarkably stereospecific (25), ultimately resulting in the formation of a major diol-epoxide I and a minor diol-epoxide II in a ratio of about 10 to 1 (23–25). Diol-epoxide I has also been shown to be the predominant metabolite of (−)*trans*-7,8-diol incubated with bovine (27) or human (28) bronchial mucosa explants. Early studies had provided evidence that a stereochemically undefined 7,8-diol-9,10-epoxide of BP interacted with DNA *in vitro* (21) and was covalently bound to the DNA of mammalian tissues incubated with BP (21, 22). It was subsequently demonstrated that diol-epoxide I was the major species formed metabolically from BP (23–25, 27, 28) and, consequently, the more active of the two stereoisomeric 7,8-diol-9,10-epoxides in binding to RNA (27) and DNA (28, 29) of the cultured mammalian cells, including those of bronchial mucosa from humans (28). Independent indication of the importance of the diol epoxides was provided by Huberman *et al.* (30), who showed that diol-epoxide I was by far a more potent mutagen in cultured Chinese hamster V79 cells than the isomeric diol-epoxide II, 4,5-epoxide (K-region), and all the other known BP metabolites.

In a previous study (12) we examined the activities of five forms of rabbit P-450$_{LM}$ with BP and (−)*trans*-7,8-diol in the reconstituted enzyme system, and presented evidence for the regiospecificity of these enzymes toward BP and their stereoselectivity in the metabolism of the (−)*trans*-7,8-diol. In this paper we describe the catalytic activities of P-450$_{LM_2}$ and $_{LM_4}$ toward the (−) and (+) enantiomers of *trans*-7,8-diol, as well as the degree of stereospecificity of these catalysts in the oxygenation of the two optical isomers to diol-epoxides I and II.

MATERIALS AND METHODS

Purification of microsomal enzymes. The purification of P-450$_{LM_2}$ from liver microsomes of PB-treated rabbits and of P-

tion is the reference, and *t* and *c* indicate that the substituents are *trans* and *cis*, respectively, to the reference substituent; PB, phenobarbital; BNF, β-naphthoflavone; 3-MC, 3-methylcholanthrene; dilauroyl-GPC, dilauroylglyceryl-3-phosphorylcholine; HPLC, high pressure liquid chromatography; and P-450$_{LM}$, liver microsomal cytochrome P-450.

450_{LM_4} from the same source as well as from microsomes of BNF-treated animals was carried out according to previously published procedures (6, 31). These preparations were homogeneous as judged by sodium dodecyl sulfate-polyacrylamide gel electrophoresis in the presence of β-mercaptoethanol. The concentration of P-450_{LM} was determined from the absolute spectra of the reduced CO complexes using extinction coefficients of 110/mM/cm for P-450_{LM_2} (Amax at 451 nm) and 119/mM/cm for P-450_{LM_4} (Amax at 448 nm) (6). The specific content of the purified P-450_{LM} samples used in these studies was as follows (expressed as nmol of P-450_{LM} per mg of protein): P-450_{LM_2}, 17.5; and P-450_{LM_4} from PB- and BNF-induced rabbits, 14.0 and 12.9, respectively. NADPH-cytochrome P-450 reductase was partially purified from PB-induced rabbit liver microsomes, and had a specific activity of 7.5 to 17.0 μmol cytochrome *c* reduced per min per mg of protein at 30° in 0.3 M potassium phosphate buffer, pH 7.7 (32); the preparations were free of P-450_{LM}. One unit of reductase is defined as the amount which catalyzes the reduction of 1.0 μmol of cytochrome *c* per min under these conditions.

Assay of trans-7,8-diol metabolism. The reaction mixtures, in a final volume of 1.0 ml, contained the following components added in the order indicated: 0.6 nmol of P-450_{LM}, 4.8 units of NADPH-cytochrome P-450 reductase, and 60 μg of dilauroyl-GPC were mixed and equilibrated at room temperature for 3 min. Tris-chloride buffer, pH 7.7 (100 μmol) and NADPH (1.1 μmol) were then added, and the reactions were initiated with (−), (+), or racemic *trans*-7,8-diol (20 nmol added in 0.02 ml of a 4:1 mixture of ethanol:tetrahydrofuran). Incubations were carried out at 30° for 20 min; the reactions were terminated by the addition of 1.0 ml of acetone at 4°, and the mixtures were extracted twice with 2.0 ml of ethyl acetate.

HPLC analysis of *trans*-7,8-diol metabolites was performed with a Spectra-Physics model 3500 liquid chromatograph using a Dupont Zorbax octadecyltrimethoxysilane column (6.2 mm I.D. × 0.25 m) with monitoring at 254 nm, as previously described (23). The column was eluted at a rate of 0.8 ml per min with a linear gradient from 60% aqueous methanol to 100% methanol at a sweep time of 100 min. Twenty-drop fractions were collected. A mixture of four BP tetrols and two triols (23) was used as an internal standard for the analysis of *trans*-7,8-diol metabolites. Activities are expressed as turnover numbers, *i.e.*, pmol of diol-epoxide I and II formed (measured as tetrols and triols) per min per nmol of P-450_{LM}. Each of the diol-epoxides is hydrolyzed and nonenzymatically reduced to two characteristic tetrols and a triol, respectively (23–25, 33).

Materials. [7-^{14}C](±)*trans*-7,8-diol (specific activity, 53.9 mCi/nmol) was obtained through National Cancer Institute Contract NO1-CP-33387 (34, 35), and the (+) and (−) enantiomers were prepared by HPLC as already described (36). Due to decomposition upon storage, the radiolabeled racemic *trans*-7,8-diol was purified by HPLC (23) just prior to use. Synthetic dilauroyl-GPC was obtained in chloroform solution from Serdary Research Laboratories, London, Ontario; prior to use, the solvent was removed under nitrogen and a 1 mg/ml aqueous suspension was prepared by sonication. The source and preparation of other materials are given elsewhere (5, 6).

RESULTS

During the course of these studies we noticed a rather profound effect of magnesium ions and deoxycholate on the activity of P-450_{LM_4} preparations isolated from two different sources toward (−)*trans*-7,8-diol. In the presence of $MgCl_2$ and deoxycholate, P-450_{LM_4} from PB-treated rabbits was 1.5 times more active than the same cytochrome purified from BNF-induced animals (12), whereas exactly the reverse was seen in the present studies when these components were omitted from the reaction mixtures. Despite such differences in total activity, the ratio of diol-epoxide I to diol-epoxide II was the same with the two P-450_{LM} preparations both in the presence and absence of magnesium ions and deoxycholate. The effect of $MgCl_2$ and deoxycholate on the metabolism of (−)*trans*-7,8-diol by P-450_{LM_2} was much less pronounced.

It is not presently clear why the activities of P-450$_{LM_4}$ preparations from different sources are differentially affected by $MgCl_2$ and deoxycholate. Preliminary evidence suggests that these agents affect the relative distribution of water-soluble as compared to ethyl acetate-soluble metabolites formed under these conditions. Suffice it to say that we have observed similar differences in total products formed from the hydroxylation of R or S warfarin catalyzed by P-450$_{LM_4}$ preparations from untreated, PB-treated, or BNF-treated rabbits (13). Moreover, in agreement with results with BP and its 7,8-diol (12), the relative distribution of metabolites from either warfarin enantiomer, and the ratio of individual or total products formed from R relative to S warfarin were essentially identical for the P-450$_{LM_4}$ enzymes isolated from the three different sources. These findings are consistent with the view that these proteins are highly similar, though slight differences among them cannot be ascertained by these or other techniques so far employed. The three preparations are indistinguishable as judged by immunochemical methods (9), electrophoretic and spectral properties, and the identity of the COOH-terminal amino acids (6).

The catalytic activity of P-450$_{LM_4}$ and $_{LM_2}$ toward the (−) and (+) enantiomers of *trans*-7,8-diol and the degree of stereoselectivity of these catalysts in the oxygenation of the two optical isomers are compared in Table 1. The cytochrome preparations exhibit large differences in their ability to metabolize the two enantiomeric forms of *trans*-7,8-diol, as judged by the total metabolites formed. The specificity for *trans*-7,8-diol in the reconstituted enzyme system resides almost exclusively in P-450$_{LM_4}$, since this preparation from PB- or BNF-induced microsomes is 20 to 30 times more active with the (−) enantiomer and 4 to 7 times more active with the (+) enantiomer than P-450$_{LM_2}$. Both P-450$_{LM_4}$ and $_{LM_2}$ exhibit higher activities with the (−) relative to the (+) optical isomer of the substrate, but this difference is most striking with P-450$_{LM_4}$. Thus, P-450$_{LM_4}$ from either source catalyzes the oxygenation of (−)*trans*-7,8-diol approximately ten times faster than that of (+)*trans*-7,8-diol; the activity of P-450$_{LM_2}$ is only twofold higher with the (−) than with the (+) isomer. Inspection of the metabolite ratios obtained with each enantiomer of the substrate reveals that P-450$_{LM_4}$ oxygenates the (−)*trans*-7,8-diol to give predominantly diol-epoxide I. In contrast, about twice as much diol-epoxide II as diol-epoxide I is produced by P-450$_{LM_4}$ when (+)*trans*-7,8-diol serves as the substrate. Thus, there is a marked reversal of the diol-epoxide I:diol-epoxide II ratio when P-450$_{LM_4}$ is incubated with the (+) as compared to (−) diol. On the other hand, P-450$_{LM_2}$ is devoid of stereospecificity with respect to product formation, for it yields more diol-epoxide I than diol-epoxide II with either enantiomer of the *trans*-7,8-diol. The overall activity of P-450$_{LM_2}$ with the enantiomeric diols is so small that the metabolite ratios shown in Table 1, *i.e.*, 1.8 and 3.0 with the (−) and (+) diol, respec-

TABLE 1

Catalytic activity and stereospecificity of rabbit P-450$_{LM_2}$ and P-450$_{LM_4}$ in oxygenation of (−) and (+) enantiomers of BP trans-7,8-diol

Activities were determined by incubating each of the enantiomeric trans-7,8-diols with P-450$_{LM}$ in the reconstituted enzyme system. Reaction mixtures contained P-450$_{LM}$ (0.6 nmol), NADPH-cytochrome P-450 reductase (4.8 units), dilauroyl-GPC (60 μg), Tris-chloride buffer, pH 7.7 (100 μmol), NADPH (1.13 μmol), and (−) or (+)*trans*-7,8-diol (20 nmol) in a total volume of 1.0 ml. Incubations were carried out for 20 min at 30°, and the metabolites formed were extracted into ethyl acetate and estimated by HPLC as described in MATERIALS AND METHODS.

Metabolites of *trans*-7,8-diol	Activity		
	LM$_{4(PB)}$[a]	LM$_{4(BNF)}$[a]	LM$_2$
	(pmol total diol-epoxide formed/min/nmol P-450$_{LM}$)		
(−)*trans*-7,8-Diol			
Diol-epoxide I	166.4	231.8	6.0
Diol-epoxide II	15.1	25.8	3.4
Ratio, I/II	11.0	9.0	1.8
(+)*trans*-7,8-Diol			
Diol-epoxide I	6.8	9.2	3.5
Diol-epoxide II	11.7	23.0	1.2
Ratio, I/II	0.6	0.4	3.0
Activity ratio, (−)/(+)	9.8	8.0	2.0

[a] The P-450$_{LM_4}$ used was isolated from either PB- or BNF-treated animals as indicated.

tively, are not believed to be significantly different.

The metabolism of racemic *trans*-7,8-diol and the relative distribution of its metabolites in the reconstituted enzyme system containing the different forms of P-450$_{LM}$ are shown in Table 2. The apparent lack of substrate specificity of P-450$_{LM_2}$ is again evident, and corresponds to the very low total activity of this cytochrome similar to that seen with the pure enantiomers of the substrate. As expected, the activities of the P-450$_{LM_4}$ preparations toward (±)*trans*-7,8-diol are somewhat intermediate to those seen with the pure (−) and (+) enantiomers of the diol. In addition, (±)*trans*-7,8-diol is converted by P-450$_{LM_4}$ from PB- or BNF-induced microsomes mostly to diol-epoxide I; the resulting values for the diol-epoxide I:diol-epoxide II ratio are closer to those obtained with the (−) rather than with the (+) optical isomer of the substrate. With racemic *trans*-7,8-diol, the magnitude of the value for the diol-epoxide I:diol-epoxide II ratio is determined by several factors: the total activity of a particular form of P-450$_{LM}$ with each of the pure isomers, competition of each isomer for the active site on the enzyme, and the distribution of diol-epoxides I and II formed from this enantiomer. Rabbit P-450$_{LM_4}$ exhibits a very low activity with (+)*trans*-7,8-diol and oxygenates this substrate to give only twofold more diol-epoxide II than diol-epoxide I. As a result of this very low activity, diol-epoxide II, though being the major metabolite of (+)*trans*-7,8-diol, is formed in amounts even lower than those obtained for diol-epoxide II from (−)*trans*-7,8-diol. This explains why the diol-epoxide I:diol-epoxide II ratio with racemic *trans*-7,8-diol has a value which more closely resembles that of the (−) than that of the (+) optical isomer.

TABLE 2

Metabolism of racemic trans-7,8-diol by P-450$_{LM_2}$ and P-450$_{LM_4}$ in the reconstituted enzyme system[a]

Form of P-450$_{LM}$	Activity[b]	Ratio, diol-epoxide I/ diol-epoxide II
	(pmol products formed/min/nmol P-450$_{LM}$)	
LM_2	4.7	2.2
$LM_{4(PB)}$	41.5	5.5
$LM_{4(BNF)}$	67.8	8.4

[a] Experimental conditions were as in Table 1.

[b] Activity represents the sum of diol-epoxides I and II formed under these conditions.

DISCUSSION

The results in this paper clearly demonstrate that the metabolism of BP *trans*-7,8-diol, whether present as the optically pure (−) or (+) enantiomer, or as a racemic mixture, is catalyzed in the reconstituted enzyme system by BNF-inducible P-450$_{LM_4}$ but not to any appreciable extent by PB-inducible P-450$_{LM_2}$. Similarly, 3-methylcholanthrene-induced rat liver microsomes and purified P-448 isolated from this source exhibit considerably higher total activities toward this substrate than corresponding preparations from PB-treated rats (37, 38).

Recent studies have shown that the *trans*-7,8-diol formed upon incubation of benzo[α]pyrene with 3-MC-induced microsomes is the (−) enantiomer as judged by its optical rotation (23, 24), ORD spectrum (24), and elution profile of its menthoxyacetate derivative on HPLC (36). Moreover, the biosynthetically formed *trans*-7,8-diol gives the same metabolite distribution as the pure (−) enantiomer of the diol when incubated with 3-MC-induced rat liver microsomes (37). As might be expected, P-450$_{LM_4}$ has a pronounced preference for the biologically formed (−) enantiomer of the *trans*-7,8-diol. On the other hand, purified cytochrome P-448 from rat liver microsomes exhibits only a twofold higher activity with the pure (−) as compared to the (+) optical isomer (37, 38).

Figure 1 shows the stereochemistry of the BP 7,8-diols used as substrates and of the resulting 9,10-epoxides (39). P-450$_{LM_4}$ shows a preference for insertion of the oxygen atom below the plane of the molecule as shown, regardless of the absolute configuration of the 7,8 hydroxyl groups. Thus, P-450$_{LM_4}$ gives predominantly diol-epoxide I from the (−) enantiomer and more diol-epoxide II from the (+) enantiomer. The

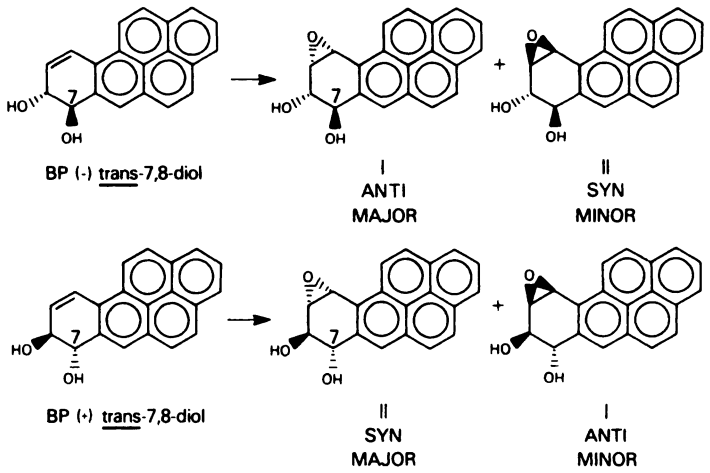

FIG. 1. *Stereospecificity of P-450$_{LM_4}$ in oxygenation of (−) and (+) enantiomers of BP trans-7,8-diol*
Triangles and dashed lines indicate that the substituents are toward and away from the viewer, respectively (24, 25).

remarkable stereospecificity of P-450$_{LM_4}$ is similar to that of 3-MC-inducible cytochrome P-448 purified from rat liver microsomes, though the latter preparation appears to be much more stereoselective in the conversion of the (+)*trans*-7,8-diol, giving diol-epoxide II to diol-epoxide I in a ratio of about 40 (30, 38, 39). In contrast, P-450$_{LM_2}$ shows no selectivity with respect to product formation, oxygenating below the plane with the (−)*trans*-7,8-diol and above the plane with the (+)*trans*-7,8-diol.

It should be noted that, unlike its poor overall activity toward BP-*trans*-7,8-diol, P-450$_{LM_2}$ is the most active of the various forms of rabbit liver microsomal cytochrome P-450 in BP oxygenation (12). The preferential metabolism of BP by P-450$_{LM_2}$ is in accord with the induction of microsomal BP hydroxylase activity by different types of compounds. In this connection, treatment of rabbits with PB leads to a three-fold increase at best in the specific content of P-450$_{LM}$ and a concomitant five-fold enhancement in the specific activity of microsomal BP hydroxylase (41). On the contrary, induction of P-448 in rabbits by polycyclic aromatic hydrocarbons is not associated with an increase in BP hydroxylase activity (7, 40, 41, 42). Conversely, it is well established that BP hydroxylase activity is induced several fold in microsomes from rats treated with polycyclic hydrocarbons. The stereospecificity of the mixed function oxidases may be of paramount importance in understanding their role in polycyclic hydrocarbon activation. It is clear that certain isomers are preferred in mammalian metabolism and binding to DNA and RNA, and a particular isomer ((+)diol-epoxide I) exhibits exceptional tumorigenicity in mice (42) and as an initiating agent for mouse skin tumors (43).

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