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Multiple Forms of Cytochrome P-450: Catalytic Differences Exhibited by Two Homogeneous Forms of Rabbit Cytochrome P-450

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SUMMARY

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Two highly purified forms of hepatic cytochrome P-450 were isolated from rabbits treated with the inducers phenobarbital and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin. Both forms were shown to be metabolically active when reconstituted with lipid and homogeneous NADPH-cytochrome P-450 reductase. Form 2, obtained from rabbits treated with phenobarbital, and form 4, purified from rabbits treated with 2,3,7,8-tetrachlorodibenzo-*p*-dioxin, hydroxylated biphenyl at comparable rates (V_{max}), 4.2 and 3.4 mol min^{-1} mol^{-1} cytochrome P-450, respectively, although with dissimilar apparent K_m values, 31 μM and 9.6 μM, respectively. Form 4 was 60 times more active than form 2 in the deethylation of 7-ethoxyresorufin. Moreover, form 4 was five orders of magnitude more sensitive in the biphenyl reaction to the inhibitory effects of the differential inhibitor α-naphthoflavone than was form 2. Densitometric scans of sodium dodecyl sulfate polyacrylamide gel electrophoretograms revealed that microsomes from rabbits treated with phenobarbital exhibit a relative predominance of form 2. In similar fashion, microsomes from 2,3,7,8-tetrachlorodibenzo-*p*-dioxin-treated animals show a dominance of form 4. The metabolic activities of the induced microsomes reflect the influence of the major form of cytochrome P-450. Similar trends are seen in the activities of phenobarbital-induced microsomes and reconstituted form 2 just as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin-induced microsomes and reconstituted form 4 share like metabolic properties. Thus, the distinct catalytic capacities of multiple forms of cytochrome P-450 represent significant determinants of xenobiotic metabolism by the cytochrome P-450 system.

INTRODUCTION

Multiple forms of cytochrome P-450 are present in the endoplasmic reticulum and metabolize a variety of compounds including drugs, steroids, environmental contaminants, and carcinogens. The age, sex, and pretreatment of the animal from which the microsomes are obtained affect the metabolic profile of activities exhibited by these monooxygenases with a structurally diverse group of substrates (1).

Several forms of cytochrome P-450 have been purified from rabbit, rat, and mouse liver microsomes (2). Two forms of rabbit cytochrome P-450 have been extensively characterized and purified. One cytochrome P-450, designated form 2, is induced in liver microsomes of adult rabbits pretreated with PB[1] and the other, denoted as form 4, is

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[1] The abbreviations used are: PB, phenobarbital; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; ANF, α-naphthoflavone; SDS PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

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induced by treatment of the rabbits with TCDD. These two forms differ by a variety of experimental criteria, including apparent molecular weight (3–5), spectral properties (4, 6), peptide mapping (7), amino acid composition (4), immunological criteria (8), and C-terminal amino acid group (4).

Differences in the occurrence and catalytic function of these two forms of the cytochrome are expected to be significant determinants of the xenobiotic metabolism mediated by the microsomal monooxygenases. We have examined the metabolism of biphenyl and 7-ethoxyresorufin using highly purified preparations of these two cytochromes and of NADPH-cytochrome P-450 reductase. We report here on a comparison of the metabolism of biphenyl by the reconstituted enzymes, on the selective inhibition of this reaction by ANF when catalyzed by form 4 but not form 2, and on the large differences in catalytic activity exhibited by the two forms with 7-ethoxyresorufin as the substrate. These catalytic differences exhibited by forms 2 and 4 comprise two of the most pronounced functional differences yet observed for multiple forms of cytochrome P-450. Moreover, the results presented here suggest that forms 2 and 4 occur naturally, are differentially inducible, and when reconstituted, exhibit properties inherent to each form.

MATERIALS AND METHODS

Isolation of microsomes. Adult male New Zealand white rabbits (3–4 kg) were given either TCDD as a single i.p. injection (30 nmol TCDD in dioxane, 10 mM, per kg body weight) 5 days prior to killing or PB in their drinking water (0.1% as the sodium salt) continuously for 6 days before sacrifice. Untreated rabbits served as controls. All rabbits were housed separately and allowed food and water *ad lib* until 24 hours prior to sacrifice, at which time food was withdrawn.

Liver microsomes were isolated as described by van der Hoeven and Coon (9). Final suspensions of microsomes used in the purification of forms 2 and 4 were made in 0.1 M Tris-acetate, pH 7.4, 0.1 M KCl, 20% glycerol (v/v), 1.0 mM Na_2 EDTA and 1.0 mM dithiothreitol. Microsomes used in the isolation of NADPH-cytochrome P-450 reductase were suspended in 0.01 M Tris-acetate, pH 7.4, 20% glycerol (v/v) and 0.1 mM Na_2 EDTA. Microsomes used in the enzymatic assays of biphenyl and 7-ethoxyresorufin metabolism were suspended in 0.05 M potassium phosphate, pH 7.4, 20% glycerol (v/v), and 0.1 mM Na_2 EDTA.

The average specific contents of cytochrome P-450 in the various microsomal preparations were: TCDD-pretreated, 3.7 ± 0.6 nmol mg^{-1}; PB-pretreated, 3.6 ± 0.5 nmol mg^{-1} and untreated 1.8 ± 0.2 nmol mg^{-1}. The specific content of NADPH-cytochrome P-450 reductase in liver microsomes obtained from PB-pretreated rabbits averaged 0.32 ± 0.03 units mg^{-1} (one unit defined as one μmol cytochrome c reduced per min per mg protein).

Isolation and purification of forms 2 and 4. Form 4 was isolated as described previously (10). All procedures were carried out at 4°. The buffers contained potassium phosphate at the indicated molarity and specified pH, 20% glycerol (v/v), and 0.1 mM Na_2 EDTA, unless noted otherwise.

Form 2 was isolated and purified according to the following methodology (Table 1). Microsomes obtained from PB-treated rabbits were solubilized with sodium cholate and fractionated with polyethylene glycol (9). Protein precipitating between 6 and 10% polyethylene glycol (v/v) was resuspended in a 5 mM buffer, pH 7.7. An aliquot of this material corresponding to 250 mg protein at a concentration of 6 mg ml^{-1} was solubilized by the addition of Nonidet P-40 (a non-ionic detergent) to a concentration of 0.1% (v/v). This solution was applied to a column of DEAE-cellulose (2.5 × 12 cm) equilibrated with 5 mM buffer, pH 7.7, containing 0.1% Nonidet P-40 (v/v). The material which eluted with the equilibration buffer was adsorbed onto 80 mg calcium phosphate gel. After a 15 min incubation, the calcium phosphate gel was separated by centrifugation at 2000 × *g* × 10 min. The pellet with the absorbed protein was resuspended in 20 ml of 0.3 M buffer, pH 6.5, and incubated 15 min. The cytochrome was eluted in the supernatant following centrifugation at 2000 × *g* × 10 min. This material was dialyzed overnight in a 150-fold excess

TABLE 1

Purification of cytochrome P-450 form 2 from PB-induced rabbit liver microsomes

Preparation	Volume	Total protein	Cytochrome P-450 content[a]	Yield[a]
	(ml)	*(mg)*	*(nmol mg protein^{-1})*	*(%)*
Microsomes	36	900	3.6	100
Polyethylene glycol precipitate (6 to 10%)	35	280	6.3	54
DEAE-cellulose column eluate; calcium phosphate gel eluate	40	64	12.0	24
CM-cellulose column eluate; calcium phosphate gel eluate	40	28	14.0	12
Hydroxylapatite-cellulose (1:1) column eluate; calcium phosphate gel eluate	8	3.6	17.5	2

[a] Based on the total amount of cytochrome P-450.

of 5 mM buffer, pH 6.5.

After addition of Nonidet P-40 to the dialysate to a final concentration of 0.1% (v/v), the protein solution was loaded onto a column of CM-cellulose (2 × 5 cm) equilibrated with 5 mM buffer, pH 6.5, containing 0.1% Nonidet P-40 (v/v). The bound material was washed with 1 column volume of the equilibration buffer. The cytochrome(s) was eluted with a linear, 0 to 0.5 M KCl, gradient in the equilibration buffer. The fractions containing form 2, as indicated by SDS PAGE, were pooled and diluted with a two-fold excess of 5 mM buffer, pH 7.4. This material was adsorbed onto 20 mg calcium phosphate gel and treated as before with the exception that the elution buffer used was 0.3 M buffer, pH 7.4. The material which eluted from the calcium phosphate gel was dialyzed against a 150-fold excess of 5 mM buffer, pH 7.4. Nonidet P-40 was added to the clear dialysate to a final concentration of 0.1% (v/v) to ensure complete solubilization. The solution was then applied to a column of hydroxylapatite cellulose (1:1) (1.5 × 6 cm) equilibrated with 5 mM buffer, pH 7.4, containing 0.1% Nonidet P-40 (v/v). The bound material was washed with 4 column volumes of 35 mM buffer, pH 7.4, containing 0.1% Nonidet P-40 (v/v). Form 2 was eluted with 40 mM buffer, pH 7.4, containing 0.1% Nonidet P-40 (v/v). This fraction was dialyzed overnight against a 100-fold excess of 10 mM buffer, pH 7.4. The dialysate was adsorbed onto 20 mg calcium phosphate gel, sedimented by centrifugation at 2,000 × *g* × 10 min, and resuspended in 10 mM buffer, pH 7.4. This wash procedure was repeated two more times. The final pellet was resuspended in 0.3 M buffer, pH 7.4, incubated 15 min, and the cytochrome was separated from the calcium phosphate gel by centrifugation at 2000 × *g* × 10 min.

The specific content of cytochrome P-450 of form 2 averaged 17.5 nmol mg^{-1} with an overall yield of 2%. Form 4 averaged 17.8 nmol mg^{-1} with an overall yield of 3% (10). Both forms were judged homogeneous by SDS PAGE (Fig. 1), with apparent molecular weights corresponding to 48,000 and 55,000 daltons for forms 2 and 4, respectively.

Purification of NADPH-cytochrome P-450 reductase. NADPH-cytochrome P-450 reductase was isolated as described by Yasukochi and Masters (11), with minor modifications. The purification steps are summarized in Table 2. All procedures were carried out at 4°. Microsomes obtained from rabbits treated with PB were solubilized with sodium cholate and Triton N-101. An aliquot of this material corresponding to 2.5 g protein at a concentration of 15 mg/ml was applied to a column of DEAE-cellulose (2.5 × 20 cm) equilibrated with 25 mM Tris-acetate, pH 7.7, 20% glycerol (v/v), 0.05 mM Na_2 EDTA, 0.05 mM dithiothreitol, 0.8% Triton N-101 (v/v), and 0.1% sodium cholate (v/v). The bound material was washed with 4 column volumes of equilibration buffer. The reductase was

eluted as a sharp peak with a linear, 0 to 0.5 M KCl, gradient in the equilibration buffer. The fractions containing reductase (as judged by assaying each fraction for reductase activity) were pooled and applied to a column containing agarose-hexane-(2'-5'-ADP) (1.0 × 10 cm) equilibrated with 10 mM potassium phosphate, pH 7.7, 20% glycerol (v/v) 0.02 mM Na_2 EDTA, 0.2 mM dithiothreitol, and 0.1% Triton N-101 (v/v). The bound material was washed with 6 column volumes of equilibration buffer in which the potassium phosphate and Na_2EDTA concentrations had been increased to 0.2 M and 0.4 mM respectively. This step removed all residual cytochrome b_5 and cytochrome P-450, leaving a well-defined yellow band in the upper fourth of the resin bed. The reductase was eluted with wash buffer containing 1 mM NADPH. This material was dialyzed overnight in a 100-fold excess of 10 mM potassium phosphate buffer, pH 7.7, containing 20% glycerol (v/v). The dialysate was adsorbed onto 20 mg calcium phosphate gel, pelleted by centrifugation at 2000 × g × 10 min and resuspended in 10 mM potassium phosphate, pH 7.7, containing 20% glycerol (v/v). This wash procedure was repeated twice to remove the NADPH and detergent from the reductase. The pellet obtained after the final wash was resuspended in 0.3 M potassium phosphate, pH 7.7, containing 20% glycerol (v/v), incubated 15 min and eluted as above.

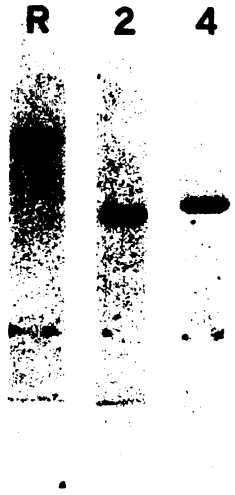

FIG. 1. *Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate of NADPH-cytochrome P-450 reductase (R) and cytochrome P-450 form 2 (2) and form 4 (4)*

Electrophoretic migration is from *top* to *bottom*. The method is that of Laemmli (12). Aliquots of the NADPH-cytochrome P-450 reductase and cytochrome P-450, forms 2 and 4, corresponding to 4 μg of protein were applied to the respective gels.

The average specific activity of the NADPH-cytochrome P-450 reductase was 50 units mg^{-1} with an overall yield of 48%. On SDS PAGE, this material exhibited a single band with an estimated molecular weight of 78,500 daltons (Fig. 1).

Preparation of lipid for reconstitution. Dilauroyl-L-α-lecithin was suspended in 50 mM potassium phosphate, pH 7.4 to a final concentration of 3 mg ml^{-1}. The suspension was sonicated four times of 5s duration at 40% relative output using an Artek Sonic 300 Dismembrator, equipped with microtip.

SDS-Polyacrylamide gel electrophoresis. The individual purified forms of cytochrome P-450 and the various microsomal

TABLE 2

Purification of NADPH-cytochrome P-450 reductase from PB-induced rabbit liver microsomes

Preparation	Volume	Total protein	Total activity[a]	Specific activity[a]	Yield
	(ml)	*(mg)*	*(μmol min^{-1})*	*(μmol min^{-1} mg $protein^{-1}$)*	*(%)*
Microsomes	60.3	1527	485	0.32	100
Solubilized microsomes	93.3	1352	453	0.37	93
DEAE-cellulose column eluate	110.2	58	317	5.47	65
Agarose-hexane 2',5'-ADP column eluate; calcium phosphate gel eluate	10.8	4.7	234	50.0	48

[a] NADPH-cytochrome P-450 reductase activities were assayed as described under METHODS.

preparations were analyzed by SDS PAGE (12). Densitometric scans of the microsomal SDS PAGE stained with Coomassie brilliant blue were recorded at 580 nm using a Gilford Spectrophotometer, Model 2400, equipped with a Gilford Linear Transport model 2410-S.

Miscellaneous assays. Protein concentrations were determined by the method of Lowry *et al.* (13) after precipitation of the proteins in the presence of trichloroacetic acid and deoxycholic acid (14). Crystalline bovine serum albumin served as the standard. Cytochrome P-450 content was estimated by the method of Omura and Sato (15), and NADPH-cytochrome P-450 reductase activity was measured as described previously (16).

Assay of biphenyl hydroxylase and 7-ethoxyresorufin deethylase. The hydroxylation of biphenyl was assayed by the method of Creaven *et al.* (17) as modified by Atlas and Nebert (18), with the following variations. The 1 ml reaction mixture contained 50 μmol potassium phosphate, pH 7.7, 0.7 mg bovine serum albumin. 0.4 μmol biphenyl (prepared by dissolving the biphenyl in a minimal volume of dimethylsulfoxide (2.5 mg μl^{-1}) and bringing the solution to a final volume with methanol (0.6 mg ml^{-1} [18]) and either 0.1 nmol cytochrome P-450, 0.5 units reductase, and 30 μg lipid or 0.05–0.8 mg microsomes. In the reconstitution experiments, the enzymes and lipid were mixed and allowed to incubate 3 min at 4° before any of the remaining assay constituents were added. Following a 2 min incubation at 37°, the reaction was initiated with 1.25 μmol NADPH. The incubation was carried out with continuous shaking at 80 oscillations per minute in a 37° waterbath. After 5 min, the reaction was stopped by the addition of 0.5 ml 2 N HCl and the tubes placed into an ice bath. After addition of 4.0 ml n-heptane, the samples were shaken at 120 oscillations per minute for 20 min at 37°. A 3.0 ml aliquot of the organic phase was extracted with 4.0 ml 0.1 N NaOH by vortexing the mixture 3 times of 5s duration and then aspirating off the organic phase. A 2.0 ml aliquot of the extract was buffered to pH 5.5 by mixing with 0.5 ml of 0.25 M succinic acid. The buffered solutions were allowed to stand 20 min (18), and then the samples were read in a Perkin-Elmer MPF-44A Fluorescence Spectrophotometer using 5 nm spectral slit widths with an excitation wavelength of 278 nm and an emission wavelength of 338 nm (4-hydroxybiphenyl), and an excitation wavelength of 290 nm and an emission wavelength of 415 nm (2-hydroxybiphenyl). The amounts of 2- and 4-isomer produced were estimated from standard curves as described by Creaven *et al.* (17). Complete reaction mixtures containing either boiled microsomes or cytochromes served as blanks and as such were present in the tubes containing the standards.

Resorufin formation was monitored by the direct fluorimetric procedure described by Burke and Mayer (19) with the following modifications. The 2.0 ml reaction mixture contained 100 μmol potassium phosphate, pH 7.7, 1.5 nmol 7-ethoxyresorufin (prepared by dissolving in dimethylsulfoxide) and either 0.025–0.5 mg microsomal protein or the reconstitution mixture described above (which was also preincubated 3 min at 4°). Following a 2 min incubation at 30° the reaction was initiated with 0.5 μmol NADPH, and monitored continuously at 30° in the fluorescence spectrophotometer. An excitation wavelength of 573 nm, an emission wavelength of 586 nm, and 3 nm spectral slit widths were used while monitoring the formation of resorufin (19). After recording the progress of the reaction, the fluorescence intensity was standardized by the addition of 0.5 nmol of resorufin to the reaction mixture.

Studies utilizing the inhibitor ANF necessitated the introduction of additional organic solvent because this compound does not solubilize well in aqueous buffers. The same solvent was employed as was used with the substrate, i.e., methanol was used in the biphenyl assay and dimethylsulfoxide in the 7-ethoxyresorufin assay. In both assays, a constant volume (10 μl) of solvent or solvent plus inhibitor was used to allow for any inhibitory effects of the added solvent. Since ANF in excess of 20 mM crystallized out of methanol, concentrations of ANF in the reaction medium could not exceed 0.2 mM.

Materials. Biphenyl, 4-hydroxybiphenyl, 2-hydroxybiphenyl (zone-refined) and ANF were purchased from the Aldrich Chemical Co., biphenyl and 4-hydroxybiphenyl were further purified by recrystallization from petroleum ether (20); 7-ethoxyresorufin and resorufin were a gift from Russell Prough (Southwestern Medical School, Department of Biochemistry, Dallas, Texas) or were purchased from the Pierce Chemical Co.; bovine serum albumin (Fraction V), NADPH (tetrasodium salt), cytochrome c (horse heart Type III), and PB (tetrasodium salt) were from Sigma Chemical Co., dilauroyl-L-α-lecithin was obtained from Calbiochem; Nonidet P-40 from Accurate Chemical and Scientific Corp., agarose-hexane-(2'-5'-ADP) (Type 2) from PL-Biochemicals, Inc., methanol and *n*-heptane (both glass distilled) were purchased from Burdick and Jackson Labs., Inc., dimethylsulfoxide was from Eastman Kodak Co., and TCDD was a gift from Dow Chemical Co. All other chemicals, solvents, and resins were of the highest commercially available grade.

RESULTS

The activity of both the highly purified forms of the enzyme as well as these enzymes in the microsomal matrix is sensitive to the amount of substrate in the assay medium. As shown in a double reciprocal plot of the rate of reaction versus the substrate concentration, there is a decrease in rate when the substrate concentration exceeds 2.5 μM 7-ethoxyresorufin (Fig. 2) or 0.4 mM biphenyl, indicative of substrate inhibition. We also observe that addition of biphenyl at concentrations greater than 0.4 mM produced crystal formation in the assay medium. Furthermore, the substrate inhibition is dependent on the amount and composition of the solvent used to introduce these water-insoluble substrates into the aqueous assay medium. Of the solvents investigated (methanol, dimethylsulfoxide, and a dimethylsulfoxide-methanol admixture [18]), the most effective was the dimethylsulfoxide-methanol admixture at a final concentration of 1% for biphenyl, and dimethylsulfoxide at a final concentration of 0.5% for 7-ethoxyresorufin.

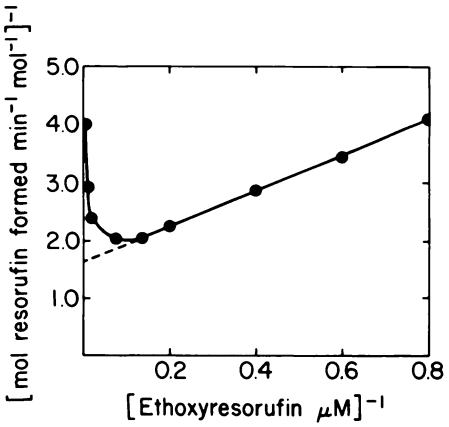

FIG. 2. *Representative Lineweaver-Burk plot of the effect of substrate concentration on the ability of reconstituted form 4 to deethylate 7-ethoxyresorufin*

Various concentrations of 7-ethoxyresorufin in a constant volume of dimethylsulfoxide were incubated with reconstituted form 4, as described in METHODS. Concentrations of 7-ethoxyresorufin in excess of 2.5–4.5 μM initiated substrate inhibition phenomena.

The pH of the incubation mixture affects the relative activities expressed by the reconstituted cytochrome P-450 system and the microsomal ensemble. The hydroxylation of biphenyl is maximal in a pH range from 7.4 to 8.6 for both the reconstituted and microsomal enzyme systems. The deethylation of 7-ethoxyresorufin is maximal from pH 7.5 to 7.9 for both enzyme systems. Accordingly, a pH of 7.7 was chosen for both assays.

In reconstitution experiments, 0.1 nmol of form 2 or 4 was used with saturating amounts of lipid (30 μg) and reductase (0.5 units). Under these conditions, the cytochrome is the rate-limiting protein component of the mixture. Omission of either cytochrome or reductase results in a complete loss of both hydroxylation and deethylation activity. Omission of lipid depresses these activities approximately 50%.

A linear dependence of the rate of product formation on the amount of microsomal protein is observed that extends from 0.05 to 0.8 mg in the biphenyl reaction and 0.025 to 0.5 mg in the 7-ethoxyresorufin assay. The progress of biphenyl hydroxylation is linear with time for the first 8 min for either the microsomal or reconstituted systems, with 5 min routinely used as the incubation

period. The deethylation of 7-ethoxyresorufin also exhibits an initial, constant reaction rate. As such, the initial rate of change in the relative fluorescence with time is used (~2-3 min).

When reconstituted with reductase and lipid, both forms 2 and 4 are active in the 4-hydroxylation of biphenyl (Table 3). Neither catalyzes the formation of detectable levels of 2-hydroxybiphenyl. Double reciprocal plots (1/V vs 1/S) reveal an apparent K_m for form 4 (9.6 μM), which is significantly ($P < 0.001$) less than that manifested by form 2 (31 μM).

The potential use of ANF as a differential inhibitor was explored (21). An ANF I_{50} (the concentration of inhibitor required to evoke a 50% decrease in activity relative to the activity obtained when only the carrier solvent is added) of 27 nM is observed for form 4. In contrast, ANF added to a final concentration 10,000 times greater causes only a 40% decrease in the relative activity of form 2 (Fig. 3). Since ANF was insoluble at higher concentrations, they were not examined.

The deethylation of 7-ethoxyresorufin is supported primarily by form 4 (Table 3). Reconstitution of form 2 results in barely detectible deethylation activities. Form 4 expresses an apparent K_m of 1.8 μM and an ANF I_{50} of 10 nM (Fig. 3). The lack of an easily measured activity in this reaction for form 2 precludes the determination of either an apparent K_m or the ANF I_{50}.

By examining SDS PAGE electrophoretograms of microsomes obtained from PB- and TCDD-treated rabbits, we estimated the relative occurrence of forms 2 and 4 to one another and toward other forms of cytochrome P-450. In order to depict the migration and relative intensity of these forms more graphically, densitometric scans of the SDS PAGE electrophoretograms of microsomes were made. Inspection of Figure 4 shows that both forms 2 and 4 occur naturally in the microsomes of the control rabbit. Furthermore, TCDD-pretreatment effects a predominance of form 4 and pretreatment with PB causes form 2 to predominate. The results of SDS PAGE are corroborated by peptide mapping experiments (7) and immunological studies (8, 16). Upon further examination of this figure, other forms of cytochrome P-450 appear to be positively affected by these compounds, i.e., form 6 by TCDD (22, 23). However, it is the apparent dominance of forms 2 and 4 in microsomes from PB-pretreated and TCDD-pretreated animals, respectively, that is of special interest. In particular, by examining some of the activities of these differentially induced microsomes it may be possible to determine the effect of these principal forms on the metabolic properties of the microsomes.

Biphenyl activity is induced 5- and 3-fold in PB- and TCDD-pretreated rabbits respectively (Table 4). No 2-hydroxylase activity is observed in any of the microsomal preparations. The apparent K_m of the TCDD-microsomes for the hydroxylation

TABLE 3

Apparent K_m and V_{max} of biphenyl 4-hydroxylase and 7-ethoxyresorufin deethylase by reconstituted forms 2 and 4 and control, PB-induced and TCDD-induced microsomes

Assays were conducted as described in METHODS. K_m and V_{max} were obtained by plotting 1/V vs 1/S.

Sample	Biphenyl 4-hydroxylase		7-ethoxyresorufin deethylase	
	V_{max}	K_m	V_{max}	K_m
Cytochromes[a]				
Form 2	4.2 ± 0.4	3.1 ± 0.3 × 10^{-5} M	<0.01	—
Form 4	3.4 ± 0.6	9.6 ± 0.1 × 10^{-6} M	0.62 ± 0.03	1.8 ± 0.1 × 10^{-6} M
Microsomes[b]				
Control	1.8 ± 0.3	3.0 ± 0.4 × 10^{-5} M	0.24 ± 0.07	1.8 ± 0.1 × 10^{-6} M
PB-induced	8.5 ± 0.5	3.1 ± 0.3 × 10^{-5} M	0.14 ± 0.02	—
TCDD-induced	5.8 ± 0.7	1.1 ± 0.3 × 10^{-5} M	2.20 ± 0.21	1.8 ± 0.1 × 10^{-6} M

[a] V_{max} expressed as nmol product formed min^{-1} nmol cytochrome $P\text{-}450^{-1}$.

[b] V_{max} expressed as nmol product formed min^{-1} mg $protein^{-1}$.

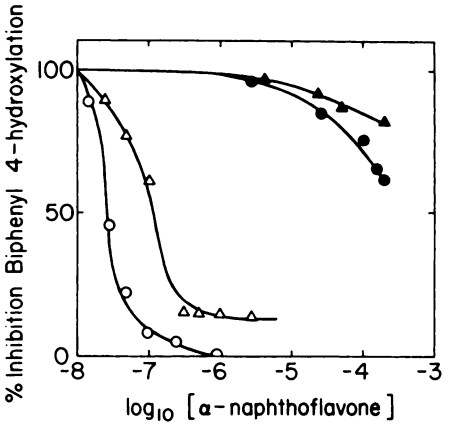

FIG. 3. *Semilog plot of the effect of ANF on biphenyl hydroxylase activity from reconstituted forms 2 and 4 and PB- and TCDD-induced microsomes*

Assays were performed as described in METHODS, with reconstituted forms 2 (●) and 4 (○) and PB-induced (▲) and TCDD-induced (△) microsomes in the presence of various concentrations of ANF in a constant volume of the dimethylsulfoxide-methanol admixture described in METHODS. Activities are expressed as a percentage of the corresponding specific activity in the absence of added ANF, i.e., only added solvent.

reaction is significantly ($P < 0.001$) less than that evidenced by either the PB-induced or control microsomes. The ANF I_{50} is 0.11 μM for the TCDD-induced preparations, whereas PB-induced microsomes are depressed by only 20% when ANF is added at final concentrations 2,000 times greater (Fig. 3).

The deethylation of 7-ethoxyresorufin is supported primarily by TCDD-induced microsomes, and PB-induced microsomes exhibit an activity only half that obtained with control microsomes (Table 3). This again precludes a determination of the ANF I_{50} or apparent K_m for the PB-induced microsomal preparations. Both the TCDD and control microsomes share an apparent K_m of 1.8 μM. The ANF I_{50} is 32 nM for the TCDD-induced microsomes (Fig. 3).

DISCUSSION

Multiple forms of cytochrome P-450 exist in a variety of animals and tissues (2). Differences in the occurrence of these forms, their response to inducers, and catalytic properties are expected to be major determinants of xenobiotic metabolism. The identity of forms 2 and 4 as discrete species of rabbit hepatic cytochrome P-450 was established by virtue of their characteristic physicochemical properties (2–8). Additional evidence, including the results of SDS PAGE presented here, suggests that forms 2 and 4 are the principal cytochromes in the microsomes of adult rabbit liver induced by PB and TCDD, respectively.

Several laboratories (2) have demonstrated differences in the ability of purified forms of cytochrome P-450 to metabolize a given substrate when reconstituted with NADPH-cytochrome P-450 reductase and lipid. The most striking difference reported thus far is the ability of rabbit cytochrome P-450 form 2 to demethylate benzphetamine at rates 20 times higher than that of form 4 (3). In this report, we delineate even

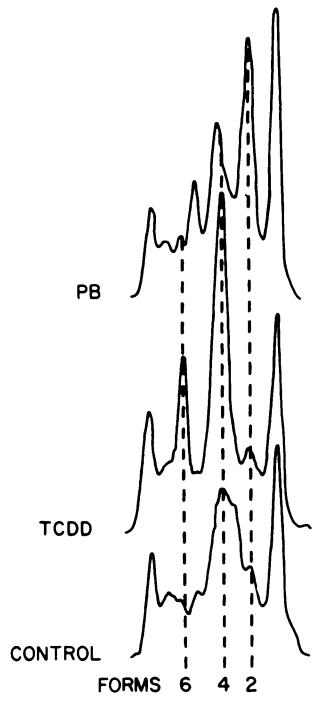

FIG. 4. *Densitometric tracings of rabbit liver microsomes after sodium dodecyl sulfate-polyacrylamide gel electrophoresis obtained from control, PB-treated and TCDD-treated rabbits*

Electrophoretograms and the densitometric tracings were performed as described in METHODS. The origin is at the *left* and the electrophoretic front is to the *right*. The portion of the gel shown corresponds to a molecular weight range of *ca.* 60,000–45,000.

more extreme diversities in the functional properties of reconstituted forms 2 and 4. Evidence is provided that these functional differences among the purified forms also prevail in the microsomes in which these forms exist as the dominant cytochromes.

Biphenyl is hydroxylated at similar rates by forms 2 and 4 in a reconstitution system whereas form 4 is 60 times more active than form 2 in the deethylation of 7-ethoxyresorufin. Although forms 2 and 4 have similar values of V_{max} in the hydroxylation of biphenyl, a closer examination of their kinetic properties reveals that there is a significant divergence in their apparent K_m values for this substrate. Furthermore, form 4 is extremely susceptible to inhibition by ANF in the biphenyl reaction (Fig. 3) whereas form 2 is hardly affected by ANF. The concentration of ANF must be raised 10,000 times above that effective for an I_{50} for form 4, and then ANF causes only a 40% diminution of activity.

These two reactions catalyzed by form 4 are inhibited by less than stoichiometric amounts of ANF. Values of I_{50} for both 7-ethoxyresorufin (10 nM) and biphenyl (27 nM) are less than half of the concentration of the cytochrome (0.1 μM). The origin of this kinetic behavior is unknown, but might arise as a result of the aggregation of the monooxygenase components. Although reductase and cytochrome are present in approximately equimolar concentrations, the system appears to form higher molecular weight aggregates (24). The observed stoichiometry of ANF inhibition may reflect two possibilities: either a portion of the cytochrome may not be active in the aggregate, or ANF bound to one molecule of the cytochrome might inhibit the activity of the other cytochromes which comprise the aggregate. It is unlikely that the biphenyl and 7-ethoxyresorufin reactions are catalyzed by distinct subpopulations of form 4, since 7-ethoxyresorufin (2 μM) inhibits biphenyl hydroxylation by 83%.[2]

The densitometric scans of the SDS PAGE electrophoretogram of microsomes illustrate the induction of forms 2 and 4 in rabbit liver microsomes (Fig. 4). This finding infers that both forms are naturally occurring, and that treatment of the rabbit with TCDD promotes proliferation of form 4 to such an extent that it becomes the dominant species of the cytochrome. The same is true of form 2 in microsomes obtained from rabbits treated with PB. Therefore, the metabolic differences observed with reconstituted forms 2 and 4 may also appear in those microsomes in which these forms predominate. Our evidence indicates that this is the case.

Microsomes from rabbits treated with PB and TCDD each have a similar ability to hydroxylate biphenyl. The same disparity in the apparent K_m's observed in the biphenyl reaction with the cytochromes in the reconstitution experiments is also evident in the microsomes from which they were purified. Likewise, the effects of ANF on microsomal metabolism parallel those seen in reconstitution experiments. The TCDD-derived microsomes are 1,000 times more sensitive to inhibition by ANF than PB-derived microsomes. In the deethylation of 7-ethoxyresorufin, TCDD-induced microsomes are approximately 15 times

TABLE 4

I_{50} ANF of biphenyl 4-hydroxylase and 7-ethoxyresorufin deethylase by reconstituted forms 2 and 4 and control, PB-induced and TCDD-induced microsomes

Assays were conducted as described in METHODS. I_{50} ANF is defined as the concentration of inhibitor that induces a 50% decrease in observed activity relative to that activity obtained when only the added carrier solvent is present.

Sample	Biphenyl 4-hydroxylase I_{50} ANF	7-ethoxyresorufin deethylase I_{50} ANF
Cytochromes		
Form 2	$>2.4 \times 10^{-4}$ M (40%)[a]	——
Form 4	2.7×10^{-8} M	1.0×10^{-8} M
Microsomes		
PB-induced	$>2.4 \times 10^{-4}$ M (20%)[a]	——
TCDD-induced	1.1×10^{-7} M	3.2×10^{-8} M

[a] Numbers in parentheses indicate percent inhibition at indicated ANF concentration.

[2] Unpublished observation.

more active toward this substrate than PB-induced microsomes. It is interesting to note that the activity of PB-induced microsomes in the deethylation reaction is actually less than that of control microsomes. This effect may be the result of several factors, the most probable being that the increased amount of form 2 in PB-treated rabbit microsomes causes a higher demand for the available reductase, thus limiting the amount of reductase available for form 4 and other forms that are capable of deethylating 7-ethoxyresorufin, i.e., form 6 (see footnote in RESULTS). An alternative explanation would be that the induction of one or more specific forms of cytochrome occurs at the expense of other forms, i.e., decreased synthesis of the noninduced forms. This is suggested by studies which indicate a decrease in the amount of electrophoretically distinct microsomal proteins when others are increased following treatment of the animal with inducers of cytochrome P-450 (25, 26).

It is also possible that microsomal metabolism of biphenyl and 7-ethoxyresorufin may reflect the contribution of other forms of cytochrome P-450, such as form 6, which are induced following treatment of the animal with PB or TCDD. Form 6 shares many properties with form 4, including the ability to hydroxylate biphenyl and deethylate 7-ethoxyresorufin, as well as its sensitivity to inhibition by ANF. However, as is illustrated in Fig. 4, form 4 is clearly the dominant of the two forms and the contribution of these forms in the overall microsomal metabolism of these substrates is probably weighted toward form 4.

Throughout the course of investigations dealing with microsomal metabolism, many differences in their functional properties have been observed, which appear to be dependent on a variety of factors such as the age, sex, or pretreatment of the animal from which the microsomes are obtained. It is likely that these differences are actually the result of the differential appearance of certain forms of cytochrome P-450 and have arisen as a result of those conditions, i.e., age or pretreatment of the animal. In this study, the properties inherent to the individual forms of cytochrome P-450 were shown to be characteristic of that form in a reconstitution system and in microsomes. We, therefore, conclude that the properties of forms 2 and 4 are significant determinants of xenobiotic metabolism.

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