Cytochrome P-450 Isozyme 1 from Phenobarbital-Induced Rat Liver: Purification, Characterization, and Interactions with Metyrapone and Cytochrome b_5[†]

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ABSTRACT: Cytochrome P-450 isozyme 1 (PB-1) ($M_r \simeq 53\,000$) was purified to apparent homogeneity from phenobarbital (PB)-induced rat liver microsomes, and its spectral, structural, immunochemical, and catalytic properties were determined. PB-1, present in significant amounts in uninduced rat liver microsomes, is induced ~2–4-fold by phenobarbital, as compared to the >30-fold induction typical of the major PB isozymes characterized previously. PB-1 was distinguished from the major PB-induced isozymes PB-4 and PB-5 [Waxman, D. J., & Walsh, C. (1982) *J. Biol. Chem. 257*, 10446–10457] by the absence of a Fe^{2+}–metyrapone P446 complex, by its unique NH_2-terminal sequence and distinct peptide maps, by the lack of immuno-cross-reactivity to PB-4, and by its characteristic substrate-specificity profile. Metyrapone effected a saturable enhancement of several PB-1-catalyzed reactions in the reconstituted system [K_m(metyrapone) $\simeq$ 200 μM], which varied in magnitude with the substrate, with a maximal stimulation of 5–8-fold in the case of acetanilide 4-hydroxylation. That metyrapone enhanced the corresponding microsomal activities only in cases where the metyrapone-sensitive PB-4 did not catalyze the same reaction at significant rates suggested that PB-1 is probably responsible for the substrate-dependent stimulatory effects of metyrapone on microsomal monooxygenations. In contrast to PB-4 and PB-5, PB-1 was characterized by a marked, but not absolute, dependence on cytochrome b_5 (b_5) for catalytic activity, with 4–7-fold stimulations typically effected by inclusion of stoichiometric b_5 in the reconstituted system. That these b_5-stimulations were lipid dependent and were abolished with specific proteolytic fragments lacking b_5's COOH-terminal membranous segment evidenced the importance of this segment for efficient, b_5-mediated electron transfer to P-450 PB-1 in the reconstituted monooxygenase system.

Hepatic microsomal cytochrome P-450[1] catalyzes the oxidative metabolism of a braod range of lipophilic substrates including drugs, insecticides, and hydrocarbon pollutants, as well as endogenous steroids and fatty acids (Lu & West, 1978; Wislocki et al., 1980). The capacity of an individual organism for these oxidative transformations can be modulated by exposure to any of several hundred monooxygenase inducers (Conney, 1967), several of which have been shown to induce the de novo biosynthesis of specific forms or isozymes of P-450, each of which displays broad and overlapping substrate-specificity profiles (Guengerich, 1979; Lu & West, 1980). The major rat liver P-450 isozymes induced by phenobarbital and by 3-methylcholanthrene and other polycyclics have been purified and are well characterized (Guengerich, 1977, 1978; Masuda-Mikawa et al., 1979; Ryan et al., 1979; West et al., 1979), as are corresponding forms isolated from rabbit liver (Haugen & Coon, 1976). Additional, distinct forms of P-450 have been purified from rat liver microsomes induced with isosafrole or certain chlorinated biphenyls (Ryan et al., 1980; Fisher et al., 1981; Goldstein et al., 1982), as well as from microsomes induced with synthetic steroids (Elshourbagy & Guzelian, 1980). Although several induced forms of P-450 have thus been purified and are well characterized, both biochemical and immunochemical analyses indicate the presence of additional, yet to be characterized P-450 isozymes in PB-induced rat liver [e.g., Thomas et al. (1981), Pickett et al. (1981), and Guengerich et al. (1982a)].

In order to better define the number of distinct forms of P-450 and to assess the contributions of each form to overall microsomal metabolism, purification and characterization of both the major and the minor P-450 isozymes found in PB-induced rat liver have been undertaken in several laboratories. Recent studies have demonstrated that the major PB-induced P-450 consists of two homologous isozymes, one of high activity (termed P-450 PB-4 in our nomenclature) and another of low activity (P-450 PB-5) (Waxman & Walsh, 1982a; Ryan et al., 1982). Other isozymes known to be present after PB induction include P-450 PB-3 (also termed P-450a; Ryan et al., 1979), a minor isozyme that catalyzes androgen B-ring hydroxylations with high regioselectivity (Waxman et al., 1983) and PCN-P-450, a unique form induced by synthetic steroids and recently determined to be present in significant amounts in PB-induced rat liver (Heuman et al., 1982). The present study describes the purification and characterization of a new isozyme, termed P-450 PB-1, from PB-induced rat liver. In addition to its distinct spectral, catalytic, structural, and immunochemical properties, PB-1 exhibits a marked (but not absolute) dependence on cytochrome b_5 and also catalyzes several monooxygenase reactions that are stimulated by metyrapone both in liver microsomes and in a reconstituted

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[1] Abbreviations: P-450, cytochrome P-450; isozymes of P-450, experimentally distinguishable forms of liver microsomal P-450, without regard to whether the individual forms have highly homologous primary structures (the terms "isozyme" and "form" are used interchangeably); PB, phenobarbital; PB-1, PB-2, etc., P-450 isozymes 1, 2, etc. purified from livers of PB-treated rats; b_5, cytochrome b_5; P-450 reductase, NADPH cytochrome P-450 reductase; SDS, sodium dodecyl sulfate; NAD(P)H, reduced nicotinamide adenine dinucleotide (phosphate); HPLC, high-pressure liquid chromatography; metyrapone, 2-methyl-1,2-di(3-pyridyl)-1-propanone; SKF-525A, (diethylamino)ethyl 2,2-diphenylvalerate; EDTA, disodium ethylenediaminetetraacetate; Tris, tris(hydroxymethyl)aminomethane.

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system. A preliminary report of some of these findings has appeared (Waxman & Walsh, 1982b).

Materials and Methods

P-450 PB-1 Purification. Liver microsomes prepared from PB-induced, Sprague-Dawley rats (Charles River, Willmington, MA) were detergent-solubilized and chromatographed on Whatman DE-52 in buffer A [10 mM KP_i (pH 7.4), 0.1 mM EDTA, 20% glycerol (v/v), 0.5% sodium cholate (w/v), and 0.2% emulgen 911 (w/v)] as described (Waxman & Walsh, 1982a). Both the major heme-protein fraction that eluted upon washing this DE-52 column with buffer A (fraction I) and the first fraction subsequently eluted with buffer A plus 20 mM KCl (fraction II) contained P-450 PB-1 as the predominant heme-protein species. Only those P-450 PB-1 fractions with $A_{417}/A_{295} \geq \sim 1.0$ were pooled in order to minimize contamination by epoxide hydrase, which eluted just prior to P-450 PB-1 on DE-52.

P-450 PB-1 (from either fraction I or II)[2] was applied at room temperature to a column (1.4 × 6 cm for 120 nmol of P-450) of hydroxylapatite (Bio-Rad HTP) equilibrated in 0.1 mM EDTA, 20% glycerol (v/v), 0.2% emulgen 911 (w/v), and 10 mM KP_i (pH 7.4) ("10 mM buffer B") and then washed stepwise with equilibration buffer, with 35 mM buffer B, with 90 mM buffer B, and finally with 150 mM buffer B. The major heme-protein fraction eluted with the 90 mM KP_i wash. Fractions exhibiting $A_{417}/A_{295} \geq 1.2$ were pooled, precipitated with 18% poly(ethylene glycol) 6000, dissolved in 2–3 mL of modified buffer A [containing 3 mM KP_i (pH 7.4) rather than 10 mM KP_i (pH 7.4)], and applied to a column of DE-52 (1.5 × 25 cm) equilibrated in the same buffer. After the column was washed with equilibration buffer (~30–40 mL), P-450 PB-1 was eluted with a buffer A wash. In cases where further purification was necessary (as determined by SDS gel analysis), PB-1 was dialyzed against 5 mM KP_i (pH 6.5), 20% glycerol (v/v), and 0.2% emulgen 911 ("CM buffer") and then applied to a column of CM-Sepharose (1.1 × 30 cm for 10–30 nmol of P-450). After the column was washed with CM buffer plus 30 mM KCl, highly purified PB-1 was eluted with CM buffer plus 50 mM KCl, giving an overall yield of ~1–2.5%, on the basis of total microsomal P-450.

Emulgen 911 was removed from purified P-450 isozymes for reconstitution studies by cholate exchange on hydroxylapatite. Samples were dialyzed against 20% glycerol (v/v), 0.1 mM EDTA, and 10 mM KP_i (pH 7.4) ("10 mM buffer C") and then applied to hydroxylapatite equilibrated in the same buffer (20–40 nmol of P-450/mL of resin). Columns were washed with 25 mM buffer C plus 0.5% sodium cholate until the A_{280} of the effluent equaled that of the wash buffer. P-450 isozymes essentially free of unbound emulgen ($A_{407}/A_{280} \geq 0.8$) were eluted with 150 mM buffer C plus 1.5% sodium cholate (yield 70–85%) and then dialyzed extensively (3 days at 4 °C with two changes) against 100 mM buffer C to remove the cholate.

Other Enzymes. Rat liver P-450s PB-4 and PB-5, P-450 reductase, and cytochrome b_5 were purified to apparent homogeneity as described previously (Waxman & Walsh, 1982a). NADH-cytochrome b_5 reductase purified from rabbit liver (Mihara & Sato, 1975) was kindly provided by Dr. Y. Takagaki of this department. Trypsin-cleaved cytochrome b_5 (trypsin-b_5; $M_r \sim 17\,500$) was prepared by incubating purified cytochrome b_5 [$M_r \sim 18\,000$; 30 nmol in 0.35 mL of 20 mM Tris–acetate (pH 8.1), 0.1 M KCl, and 0.1 mM EDTA] with TPCK-trypsin (60 μg; Worthington) for 20 h at room temperature and then purified by chromatography on Bio-Gel A 1.5 M (1.3 × 60 cm) equilibrated in the same buffer to remove undigested cytochrome, which migrates as a high molecular weight aggregate under these conditions (Calabro et al., 1976). Adventitious proteolysis during the final cytochrome b_5 purification steps yielded a fragment (termed protease-b_5; $M_r \sim 15\,500$) that was also purified to apparent homogeneity on Bio-Gel A 1.5 M.

Analytical Procedures. Lowry protein determinations, spectral analyses for P-450 and b_5, and NH_2-terminal sequencing were as described previously (Waxman & Walsh, 1982a). SDS gel electrophoresis used the buffer system of Laemmli (1970) except that the Tris–glycine concentration in both the upper and lower reservoirs was doubled. M_r values were determined from plots of log molecular weight vs. mobility as in Waxman et al. (1983). NADH-cytochrome b_5 reductase activity was determined by ferricyanide reduction and by b_5-dependent cytochrome c reduction (Mihara & Sato, 1975).

Rabbit Anti-P-450 PB-4 Antibody. New Zealand white rabbits were injected subcutaneously at 4–5 sites with 550 μg of purified, detergent-free P-450 PB-4 emulsified with Freund's complete adjuvant (Difco) and then boosted at 4 weeks and again at 6 weeks, each boost with 100 μg of PB-4 emulsified in Freund's incomplete adjuvant (Difco). Rabbits were bled at 3 weekly intervals beginning 1 week after the last boost. Serum was fractionated by precipitation with 40% saturation ammonium sulfate and assayed for specific immunoprecipitation activity by double-diffusion analysis in Ouchterlony plates in the presence of 0.2% emulgen 911, as described previously (Thomas et al., 1981).

Kinetic Analyses. Steady-state kinetic parameters (V_{max} and K_m) and their standard deviations were derived from Lineweaver–Burk double-reciprocal plots by weighted least-squares linear-regression analysis of Roberts (1977) with the velocity squared as the weighting factor. Calculations were performed with a computer program kindly provided by Dr. R. A. Pascal, Jr., of this laboratory.

Monooxygenase Assays. Incubations with PB-induced microsomes were as described (Waxman et al., 1982). Purified P-450s PB-1 and PB-4 (30 nM) were each reconstituted with P-450 reductase (90 nM) and cytochrome b_5 (35 nM) in a dilauroylphosphatidylcholine (Sigma; 4–7 μg/mL) system containing sodium deoxycholate (40 μg/mL). Assays (0.4–1.0 mL) were performed in 100 mM buffer C for 5–30 min at 37 °C as described previously (Waxman & Walsh, 1982a), with the oxidation products of 7-ethoxycoumarin, coumarin (Waxman & Walsh, 1982a), and testosterone (Waxman et al., 1983) determined by modifications of standard methods as described in the references indicated. Assays for (*R*)-warfarin metabolism were kindly performed by Dr. L. S. Kaminsky, N.Y. State Department of Health, as described (Kaminsky et al., 1981). Formaldehyde derived from *N*,*N*-dimethylaniline (Aldrich), benzphetamine (Upjohn), or ethylmorphine (Mallinckrodt) was quantitated by a modification of the Nash assay (Cochrin & Axelrod, 1959) designed to increase sensitivity and reduce backgrounds. Assay mixtures (1.0 mL) were quenched with saturated $Ba(OH)_2$ (0.2 mL), followed by 0.35 M $ZnSO_4$ (0.1 mL), and then centrifuged briefly, and 0.9 mL of the supernatant was added to 0.1 mL of Nash reagent [an aqueous solution of 6 M ammonium

[2] PB-1 isolated from fraction II exhibited the same SDS gel mobility, catalytic specificity, peptide maps (not shown), and NH_2-terminal sequence (see Table I) as that from fraction I. The relative distribution of PB-1 between these two fractions varied somewhat from one preparation to the next.

acetate and 0.15 M acetic acid, with 2,4-pentadione (Aldrich) added to 60 mM just prior to use]. Samples were incubated 30 min at 30 °C and then quantitated by fluorescence [excitation at 425 nm and emission at 515 nm; Perkin-Elmer Model MPF-4 fluorometer; also see Belman (1963)] in comparison to authentic formaldehyde standards.

Oxidation products of toluene and *p*-tolyl ethyl sulfide were analyzed by HPLC on a Waters μporasil column with 1.5% (v/v) 2-propanol in hexane (for toluene oxidation products) or 3.5% (v/v) 2-propanol in hexane (for *p*-tolyl ethyl sulfide products) with two modifications of the methods described previously (Waxman & Walsh, 1982a; Waxman et al., 1982). First, improved sensitivity was obtained on a Waters Model 441 214-nm absorbance detector. Second, reaction mixtures were saturated with NaCl (~0.3 g/mL) prior to the hexane extraction, increasing the extraction yields from the low values obtained previously to 84% *o*-cresol, 77% *p*/*m*-cresol, 48% benzyl alcohol, 90% *p*-(hydroxymethyl)phenyl ethyl sulfide, and 70% *p*-tolyl ethyl sulfoxide.

Acetanilide hydroxylation products were ether extracted (2 × 2 mL) from NaCl-saturated reaction mixtures (1 mL), dried under N_2, dissolved in 13% (v/v) 2-propanol in hexane (1 mL), and analyzed by HPLC on μporasil with 13% (v/v) 2-propanol in hexane (8 mL/min) as the mobile phase with detection by A_{254} and/or A_{214}. Elution times were 1.30, 1.30, 1.65, and 2.45 min for acetanilide and the 2-hydroxy, 3-hydroxy, and 4-hydroxy derivatives, respectively. Although 2-hydroxyacetanilide was not resolved from acetanilide in this system, HPLC analysis on C_{18} (Guenthner et al., 1979) indicated that P-450s PB-1 and PB-4 both catalyzed aromatic ring hydroxylation of acetanilide only at the para position. HPLC on μPorasil gave sharper peaks and was more rapid than analysis on C_{18}, particularly in cases where reaction mixtures contained metyrapone [$t_{elution}$ = 5.4 min on μporasil vs. 39 min on C_{18} in the solvent system of Guenthner et al. (1979)].

Peptide Mapping. Purified isozymes (1.5 nmol of P-450/mL) were dialyzed against 0.125 M Tris-HCl (pH 6.7 at 25 °C) and 15% glycerol (v/v). Aliquots (0.04 mL, each containing ~3 μg of protein) were added to SDS [5 μL of 2.5% (w/v); Bethesda Research Laboratories and boiled for 1 min. Protease [*Staphylococcus aureus* V8 protease (Miles)] or α-chymotrypsin (Worthington) was added in 5 μL of buffer to yield the final concentrations indicated in Figure 3. Samples were incubated for 90 min at 37 °C, 15 μL of denaturant/dye was then added (final concentrations: 3% SDS, 10% 2-mercaptoethanol, and 0.015% bromophenol blue), and the samples were boiled 5 min prior to electrophoresis on 13.5% gels.

P-450 Nomenclature. P-450 forms (isozymes) purified from PB-induced rat liver are numbered in their relative order of elution from Whatman DE-52, i.e., P-450 PB-1, P-450 PB-2, etc. Purification and characterization of P-450 PB-1 are described in the present study. The heterogeneous P-450 PB-2/PB-3 (Waxman & Walsh, 1982b) has now been resolved into four distinct heme proteins including an isozyme equivalent to P-450a (Ryan et al., 1979), termed P-450 PB-3 (Waxman et al., 1983), one equivalent to PCN-P-450 (Elshourbagy & Guzelian, 1980), termed P-450 PB-2a, and a male-specific testosterone 16α-hydroxylase, termed P-450 PB-2c (Waxman et al., 1983). P-450s PB-4 and PB-5, two closely related isozymic forms (Waxman & Walsh, 1982a), are equivalent[3] to P-450bH and P-450e, respectively, of Ryan et al. (1982). P-450 PB-4 probably corresponds to the major phenobarbital-induced isozyme purified by other groups and variously termed PB-B (Guengerich, 1977), fraction C (West et al., 1979), P-450 (Masuda-Mikawa et al., 1979), and PB-P-450 (Elshourbagy & Guzelian, 1980). P-450 PB-6 (Waxman & Walsh, 1982b) has apparently not been studied by others.

[3] F. G. Walz, Jr., A. Rampersand, and D. J. Waxman, unpublished results.

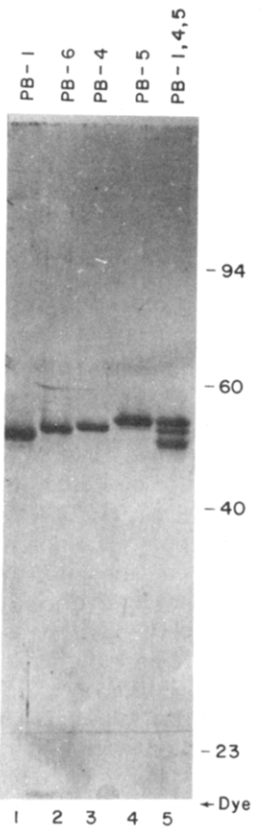

FIGURE 1: SDS gel (10% acrylamide) showing purified P-450 isozymes PB-1, PB-6, PB-4, and PB-5. Apparent molecular weights were 53000 (PB-1), 54500 (PB-6), 55000 (PB-4), and 57000 (PB-5). Molecular weights of protein standards were as indicated.

Results

P-450 PB-1 Purification. DE-52 chromatography of cholate-solubilized PB-induced rat liver microsomes (see Materials and Methods) yielded four heme-protein fractions: fractions I and II, both containing P-450 PB-1 as the major hemeprotein component,[2] and two heterogeneous P-450 fractions (III and IV), both containing "PB-2/PB-3" (Waxman & Walsh, 1982b) from which four additional, distinct P-450 isozymes have been resolved and purified (see P-450 Nomenclature). Two additional heme-protein fractions (V and VI, not shown) were more tightly bound to DE-52 than fractions I–IV and can be eluted with salt gradients after their excision and application to fresh DE-52 columns (Waxman & Walsh, 1982a). Fraction V, bound at the middle of the first DE-52 column, yielded P-450s PB-4, PB-5, and PB-6; Fraction VI, tightly bound at the top of the first DE-52 column, contained cytochrome b_5 as the principle heme-protein component (Waxman & Walsh, 1982a,b). P-450 PB-1 represented 25 ± 4% of the total P-450 resolved in fractions I–VI, as compared to 36 ± 2% for PB-4 and 16 ± 3% for PB-5 plus PB-6 (n = 5). Further chromatography on hydroxylapatite, repeat DE-52, and in some cases CM-Sepharose (see Materials and Methods) yielded preparations of PB-1 >95% pure by SDS gel electrophoresis (M_r ~ 53000; Figure 1) with specific contents ranging from 13.3 to 16.4 nmol/mg.

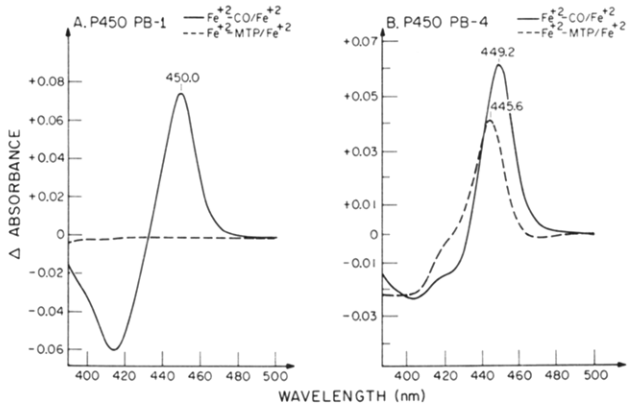

FIGURE 2: Metyrapone-reduced and CO-reduced difference spectra for P-450 PB-1 (A) and P-450 PB-4 (B). Dithionite reduction was in the presence of CO-saturated samples (—) or 1 mM metyrapone (--). Spectra were recorded as ligand/reduced vs. reduced difference spectra at ~0.7–0.8 nmol of P-450/mL in 10 mM buffer C.

DE-52 chromatography of cholate-solubilized, uninduced rat liver microsomes yielded PB-1-containing fractions, in this case corresponding to only 14 ± 4% (n = 3) of the total chromatographed P-450. Hydroxylapatite and repeat DE-52 chromatography yielded partially purified cytochrome (specific content = 8.9), which was equivalent to PB-1 as judged by its peptide maps (see below) and substrate-specificity profile (not shown). These results suggest a 2–4-fold induction of PB-1 by phenobarbital (per milligram of protein), a value that is significantly lower than the ≥30-fold induction seen with the major PB-induced isozyme (P-450 PB-4) (Thomas et al., 1981; Pickett et al., 1981; Guengerich et al., 1982a).

Spectral and Structural Properties. P-450 PB-1 exhibited absolute oxidized, reduced, and CO-reduced spectra similar to those of other low-spin microsomal P-450 isozymes (e.g., Haugen & Coon, 1976). The Soret maximum of oxidized P-450 PB-1 was, however, at ~415–416 nm as compared to ~417–418 nm for other isozymes (not shown). Whereas both PB-1 and PB-4 exhibited similar CO-reduced difference spectra, PB-1 did not form the metyrapone-reduced difference spectrum ("P446") detected with PB-4 and PB-5 (Figure 2; Waxman & Walsh, 1982a). Thus, a P446 metyrapone-reduced difference spectrum is characteristic of only a subset of the PB-induced isozymes in rat liver.

Peptide maps generated by cleavage of PB-1 with V8 protease, α-chymotrypsin (Figure 3), or cyanogen bromide (not shown) were largely unrelated to those of PB-4 or PB-5, demonstrating that PB-1 is not derived by posttranslational modification (e.g., proteolysis) of these other isozymes. PB-1 isolated from uninduced rat liver yielded peptide maps that were closely related to those of PB-1 from PB-induced liver after digestion with α-chymotrypsin (lane 9 vs. 10) or V8 protease (not shown). Small differences in these latter peptide maps probably reflect the inhomogeneity of the isozyme preparation from uninduced animals.

Nucleotide sequence analysis has indicated that at least two and possibly four of the ~15 predicted amino acid differences between two closely related PB-induced isozymes (probably corresponding to P-450 PB-4 and P-450 PB-5) are at aspartate and glutamate residues (Fujii-Kuriyama et al., 1982; Kumar et al., 1983). Consistent with those results, the most significant differences in the peptide maps of the closely related isozymes PB-4 and PB-5 were obtained with the aspartate- and glutamate-specific V8 protease (Figure 3, lanes 3–7).

Antibody raised to PB-4 did not cross-react with purified PB-1 upon Ouchterlony double-diffusion analysis (not shown).

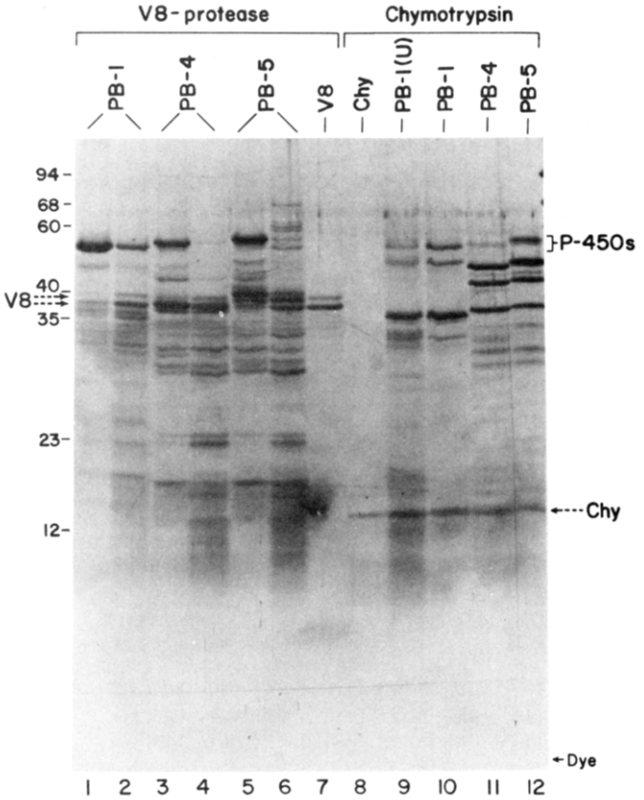

FIGURE 3: Peptide mapping of P-450 isozymes PB-1, PB-4, and PB-5. Purified isozymes (~3 μg per lane; P-450 samples as labeled) were digested with either V8 protease (lanes 1–7) or α-chymotrypsin (lanes 8–12) at protease concentrations of 9 (lanes 1, 3, and 5), 36 (lanes 2, 4, 6, and 7), or 20 μg/mL (lanes 8–12) in a volume of 0.05 mL for 90 min at 37 °C (see Materials and Methods). Shown is a 13.5% SDS gel with molecular weights of standard proteins as indicated. (Lanes 7 and 8) V8 protease and chymotrypsin controls, respectively, incubated in the absence of P-450. Protease-specific bands were as marked. [PB-1 (U)] Peptide map of P-450 PB-1 partially purified from uninduced animals (see text).

Similar analyses indicated that PB-1 is immunochemically distinct from the rat liver isozymes P-450a, P-450b, P-450c, and P-450d purified by the Hoffmann-La Roche group (Ryan et al., 1979, 1980), in addition to being resolved from those isozymes upon SDS gel electrophoresis (not shown). The unique nature of P-450 PB-1 is further supported by its NH_2-terminal amino acid sequence (Table I), which is different from the corresponding sequences reported for seven rat liver and three rabbit liver P-450 isozymes (Figure 4). The sequence obtained for P-450 PB-1 does, however, suggest some homology to rabbit liver P-450 LM 3b (Koop et al., 1981; Ozols et al., 1981), although the data base for comparison (20 residues) is somewhat limited.

Catalytic Activities of P-450 PB-1. The substrate specificity of PB-1 in a reconstituted monoxygenase system was compared to that of PB-4 (Table II). PB-1 catalyzed formation of 4-hydroxyacetanilide, (*R*)-7-hydroxywarfarin, and *p*-(hydroxymethyl)phenyl ethyl sulfide at >20-fold the rates catalyzed by PB-4. By contrast, PB-4 catalyzed formation of 7-hydroxycoumarin, *o*-cresol, *p*/*m*-cresol, and several monohydroxytestosterone derivatives at >10-fold the rates of PB-1. Both isozymes appeared to recognize specific substrates as opposed to exhibiting specificity for a given category of oxidative transformation. Thus, although PB-4 was significantly more efficient than PB-1 as an aromatic hydroxylase with toluene as substrate, PB-1 was a much more active aromatic hydroxylase with either acetanilide or (*R*)-warfarin as substrate.

Table I: Amino-Terminal Sequence Analysis of P-450 PB-1[a]

cycle	residue identified	yield (nmol)[b] I	yield (nmol)[b] II	cycle	residue identified	yield (nmol) I	yield (nmol) II	cycle	residue identified (tentative)[c]
1	Met	7.5	3.6	12	Thr	*d*	0.8	23	Ser
2	Asp	3.6	3.6	13	*e*			24	Ser
3	Leu	2.9	3.0	14	Leu	3.5	2.2	25	Gly
4	Val	4.2	3.3	15	Ile	3.1	1.6	26	*e*
5	Met	3.8	1.9	16	Leu	4.0	2.4	27	Gly
6	Leu	3.9	3.0	17	Leu	3.7	2.3	28	*e*
7	Leu	4.8	2.3	18	Ser[c]			29	Glu
8	Val	3.1	2.2	19	Ile	2.7	1.5	30	Lys
9	Leu	3.7	2.0	20	Trp	1.1	0.6	31	Pro
10	Thr	*d*	0.8	21	*e*				
11	Leu	4.3	2.6	22	Gln[f]	*g*	0.6		

[a] PB-1 from either fraction I or fraction II (see Materials and Methods) and obtained from independent microsomal preparations was subjected to automated Edman degradation. Phenylthiohydantoin derivatives were identified and quantitated as described previously (Waxman & Walsh, 1982a). [b] Starting with 8.5 nmol of PB-1 for the sample purified from fraction I and 5.0 nmol for PB-1 from fraction II. [c] Identifications of residues 18 and of 23–31 must be obtained as *tentative*, as they are based on an increase of only ~40–60% over background levels in each case and, for cycles 23–31, were obtained with a single sample (II) of PB-1. [d] Identified as dehydrothreonine by HPLC and not quantitated. [e] Not identified. Cysteine, arginine, and histidine were not determined in these analyses. [f] Identified as a mixture of Gln and Glu (~1:1). [g] Sequence analysis of sample I halted after 20 cycles.

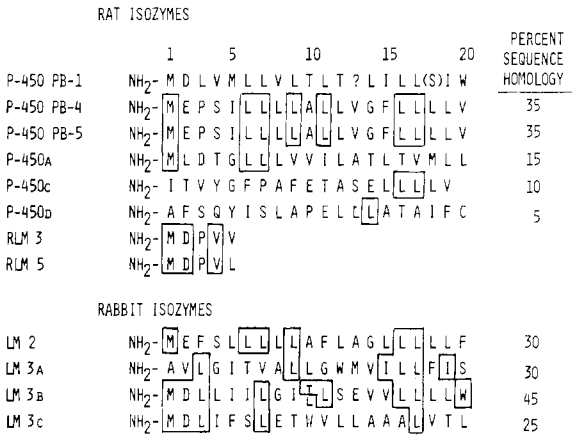

FIGURE 4: Comparison of amino-terminal sequence of P-450 PB-1 to other P-450 isozymes. The amino-terminal sequence obtained for PB-1 (Table I) is compared to those obtained for rat isozymes PB-4 and PB-5 (Waxman & Walsh, 1982a), P-450a and P-450c (Botelho et al., 1979), P-450d (Botelho et al., 1982), RLM3 and RLM5 (Cheng & Schenkman, 1982), and rabbit isozymes LM2, 3a, 3b, and 3c (Koop et al., 1982). Boxed are those residues identical with the corresponding residues of PB-1, with the homology expressed as percent sequence identity. The data base for RLM3 or RLM5 is too small for meaningful comparison to PB-1. Heterogeneity has been observed for LM3b at residue 10.

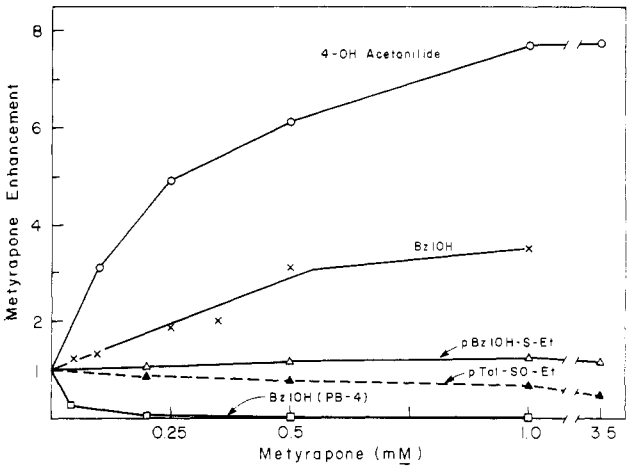

FIGURE 5: Metyrapone enhancement of P-450 PB-1 activities. Metyrapone enhancements were calculated from catalytic activities in the presence of metyrapone (concentrations as indicated) relative to its absence under standard assay conditions. The extent of metyrapone enhancement varied with substrate (see text). Inhibition of the P-450 PB-4 catalyzed conversion of toluene to benzyl alcohol [BzlOH (PB-4)] is included for comparison.

The effects of three P-450 inhibitors on the catalytic activities of reconstituted PB-1 and PB-4 and of PB-induced rat liver microsomes are shown in Table III, along with the microsomal turnover numbers for these reactions. The high sensitivity to metyrapone inhibition displayed by PB-4 contrasts to an *enhancement* of several PB-1-catalyzed reactions by this dipyridyl ligand (Table III, Figure 5, and see below). In comparison to PB-4, PB-1 was less sensitive to inhibition by *n*-octylamine and approximately equally sensitive to SKF-525A inhibition.

With several substrates there was a good correlation between the sensitivity of individual isozymes to particular ligands and the sensitivity of the composite microsomal activity to those same ligands (Table III). Thus, PB microsomes reflected the metyrapone enhancement observed with purified PB-1 in the case of the two activities preferentially catalyzed by PB-1 in reconstituted systems [formation of 4-hydroxyacetanilide and *p*-(hydroxymethyl)phenyl ethyl sulfide]. Conversely, the high sensitivity of microsomal cresol formation to inhibition by metyrapone or *n*-octylamine paralleled the sensitivity of purified PB-4, consistent with PB-4 catalyzing the majority of microsomal cresol formation. In the case of monooxygenations catalyzed in reconstituted systems by both PB-1 and PB-4 (e.g., conversion of toluene to benzyl alcohol), the PB microsomes displayed sensitivities that were intermediate to those of the two purified isozymes. Evaluation of the effects of the three ligands studied on *p*-tolyl ethyl sulfoxide formation is complicated by the participation of microsomal FAD-containing *S*,*N*-monooxygenase in this oxygenation (Light et al., 1982; Waxman et al., 1982).

Metyrapone Enhancement of PB-1 Activities. Although metyrapone inhibits many P-450-catalyzed reactions [e.g., Jonen et al. (1974) and Testa & Jenner (1981)], this dipyridyl heme ligand enhances (up to 2- or 3-fold) the microsomal metabolism of trichloroethylene, acetanilide, and *p*-tolyl ethyl sulfide [yielding *p*-(hydroxymethyl)phenyl ethyl sulfide] (Leibman, 1969; Leibman & Ortiz, 1973; Waxman et al., 1982). Our present results (Table III) confirm these previous observations and suggest that this substrate-dependent metyrapone enhancement reflects the stimulation of P-450 PB-1 or of PB-1-like isozymes present in the microsomal fractions. This metyrapone-enhancement was only observed when the

Table II: Monooxygenase Activities of P-450s PB-1 and PB-4[a]

substrate	concn (mM)	product determined	mol min^{-1} (mol of P-450)$^{-1}$ P-450 PB-1	P-450 PB-4	PB-1/PB-4 (rel rates)
		N,O-Dealkylation			
N,N-dimethylaniline	5.0	formaldehyde	8.2	38	1/4.6
benzphetamine	2.5	formaldehyde	12.6	43	1/3.4
ethylmorphine	7.0	formaldehyde	3.8	2.7	1.4/1
7-ethoxycoumarin	1.0	7-hydroxycoumarin	0.4	4.5	1/11
		Aromatic Hydroxylation			
acetanilide	4.0	4-hydroxyacetanilide[b]	3.4	0.15	23/1
toluene	5.0	*p/m*-cresol[c]	<0.1	3.5	1/>35
		o-cresol	<0.1	2.5	1/>25
(*R*)-warfarin[d]	0.8	(*R*)-4′-hydroxywarfarin	0.06	0.25	1.2/1
		(*R*)-6-hydroxywarfarin	0.11	0.01	11/1
		(*R*)-7-hydroxywarfarin	0.80	<0.01	>80/1
		(*R*)-8-hydroxywarfarin	0.12	<0.01	>12/1
coumarin	1.0	7-hydroxycoumarin	<0.02	<0.02	not applicable
		Aliphatic, Benzylic Hydroxylation			
p-tolyl ethyl sulfide	0.5	*p*-(hydroxymethyl)phenyl ethyl sulfide	5.3	<0.1	>53/1
toluene	5.0	benzyl alcohol	4.1	16.0	1/3.9
testosterone[e]	0.1	15α-hydroxytestosterone	<0.02	0.2	1/>10
		16α-hydroxytestosterone	0.1	3.4	1/34
		16β-hydroxytestosterone	0.1	3.8	1/38
		androstenedione	0.1	3.2	1/32
		Sulfur Oxidation			
p-tolyl ethyl sulfide	0.5	*p*-tolyl ethyl sulfoxide[f]	27	21	1.3/1

[a] Catalytic activities were determined for each of the purified P-450s reconstituted with P-450 reductase, cytochrome b_5, dilauroylphosphatidylcholine, and sodium deoxycholate as described under Materials and Methods. Activities are expressed as turnover numbers per mole of P-450 and are grouped according to reaction type. Ratios of activities for the two isozymes (last column) indicate an upper limit of 1–3% for their cross contamination. [b] 2-Hydroxyacetanilide and 3-hydroxyacetanilide were formed at rates <0.05 min^{-1} for both isozymes tested. In the presence of 1 mM metyrapone, acetanilide 4-hydroxylase activity was ≥5-fold higher than the value listed for P-450 PB-1 while the activity of PB-4 was inhibited by metyrapone (see Figure 5). [c] The two isomeric cresols were not resolved [see Waxman & Walsh (1982a)]. [d] Assays were performed at 0.5 μM P-450, 0.6 μM cytochrome b_5, 0.5 μM P-450 reductase, and 10 μg/mL dilauroylphosphatidylcholine in 50 mM Tris-HCl, pH 7.4. [e] Other products determined to be formed with a turnover of ≤0.02 min^{-1} (mol of P-450)$^{-1}$ included the 2α-, 2β-, 6α-, 6β-, 7α-, 11α-, 11β-, 14α-, 18-, and 19-hydroxytestosterone derivatives [see Waxman et al. (1983)]. [f] The chirality of the sulfoxide product was ~79% *S* for PB-1 and ~84% *S* for PB-4, determined as described in greater detail in Waxman et al. (1982).

Table III: Effects of P-450 Ligands on Monooxygenase Activities[a]

ligand	product determined (relative activity) 4-hydroxyacetanilide	*p*-(hydroxymethyl)phenyl ethyl sulfide	*p*-tolyl ethyl sulfoxide	benzyl alcohol	*o*-cresol	*p/m*-cresol
metyrapone (0.5 mM)						
PB microsomes	1.56	1.70	0.50	0.35	0.08	0.05
P-450-PB-1	3.90	1.32	0.82	1.76	*b*	*b*
P-450 PB-4	*b*	*b*	0.13	0.04	0.05	0.04
SKF-525A (0.2 mM)						
PB microsomes	0.33	0.53	0.59	0.14	ND[c]	ND
P-450 PB-1	0.19	0.10	0.18	0.14	*b*	*b*
P-450 PB-4	*b*	*b*	0.37	0.13	0.15	0.14
n-octylamine (0.5 mM)						
PB microsomes	0.16	0.67	0.51	0.24	0.10	0.07
P-450 PB-1	0.34	0.44	0.40	0.38	*b*	*b*
P-450 PB-4	*b*	*b*	0.11	0.03	0.03	0.04
turnover no. for PB microsomes [min^{-1} (mol of P-450)$^{-1}$]	0.8	0.6	7.5	5.2	0.5	0.5

[a] Catalytic activities were determined for PB microsomes or for purified, reconstituted P-450 PB-1 or P-450 PB-4 as described under Materials and Methods. Activities in the presence of metyrapone, SKF-525A, or *n*-octylamine are expressed relative to the activities in the absence of these ligands such that values <1 indicate inhibitions and values >1 indicate ligand-induced stimulations. [b] The activity was too low to be measured even in the absence of added ligand (see Table II). [c] ND, not determined due to interfering substances coeluting from the HPLC column.

metyrapone-sensitive PB-4 did not contribute significantly to overall microsomal activity, e.g., in formation of 4-hydroxyacetanilide and *p*-(hydroxymethyl)phenyl ethyl sulfide (Tables II and III). By contrast, although metyrapone enhanced the toluene hydroxylase activity of purified PB-1 (Table III, Figure 5), this effect was not observed in microsomes, where the contribution of the more active (Table II) and more abundant PB-4 is dominant.

As the metyrapone enhancement of PB-1 was saturable [K_m = ~200 μM; Figure 5 and Table IVA], it probably reflects specific interactions between PB-1 and metyrapone. Such interactions were not, however, manifest as a P446 complex between the reduced cytochrome and metyrapone as was the case for PB-4 (Figure 2). Metyrapone did not stimulate all PB-1-catalyzed monooxygenations to the same extent (Figure 5). Thus, with *p*-tolyl ethyl sulfide as substrate, PB-1-catalyzed

Table IV: Metyrapone Enhancement of P-450 PB-1 Catalytic Activity[a]

	+metyrapone	−metyrapone
(A) Kinetic Parameters[b]		
V_{max} [min^{-1} (mol of P-450)$^{-1}$]	19 ± 5	5.0 ± 1.4
K_m (mM) (acetanilide)	4.6 ± 1.4	5.9 ± 1.8
V_{max}/K_m	4.1 ± 0.6	0.84 ± 0.10
K_m[c] (μM) (metyrapone)	192 ± 16 (4-hydroxyacetanilide)	
K_m[c] (μM) (metyrapone)	250 ± 75 (benzyl alcohol)	

	nmol of 4-hydroxyacetanilide		metyrapone enhancement[d]
	+metyrapone	−metyrapone	
(B) Influence of Reaction Conditions			
expt I			
(1) standard assay	1.85	0.24	7.7
(2) +10 μg/mL catalase and 10 μg/mL superoxide dismutase	1.85	0.23	8.0
(3) +0.1 M mannitol	1.70	0.27	6.4
expt II			
(4) standard assay	2.0	0.42	4.8
(5) low P-450 reductase reductase[e]	0.63	0.25	5.3
(6) *minus* cytochrome b_5[f]	0.41	0.077	4.2

[a] Metyrapone enhancement is defined as the ratio of monooxygenase activity in the presence of metyrapone to that in its absence. Metyrapone was at a final concentration of 1.0 mM and acetanilide 4-hydroxylase activity determined at 4 mM acetanilide. [b] Values ± SD determined as described under Materials and Methods. [c] Determined from a titration of metyrapone (e.g., as in Figure 5) in either an acetanilide 4-hydroxylase or toluene hydroxylase enhancement assay, as indicated in parentheses. [d] Variability in the extent of metyrapone enhancement largely reflects differences in activity levels in the absence of metyrapone. [e] 18 nM P-450 reductase as compared to 90 nM in the standard assay. [f] In the presence of metyrapone the cytochrome b_5 stimulation (see text and Table 5) was 4.9 vs. 5.5 in its absence (compare samples 4 and 6).

Table V: Cytochrome b_5 Stimulation of Monooxygenase Activity

product determined	relative activity[a] P-450 PB-1	P-450 PB-4[b]
(A) Substrate Specificity		
7-hydroxycoumarin	5.7	1.4
4-hydroxyacetanilide	5.5	ND[c]
benzyl alcohol	4.5	1.3
p-(hydroxymethyl)phenyl ethyl sulfide	6.2[d]	ND[c]
p-tolyl ethyl sulfoxide	4.7[d]	1.2

	relative activity[a] *p*-(hydroxymethyl)phenyl ethyl sulfide	*p*-tolyl ethyl sulfoxide
(B) Influence of Reconstitution Conditions		
(1) standard conditions	4.0[d]	3.1[d]
(2) low reductase[e]	5.0	3.3
(3) *minus* lipid	0.7	1.0
(4) +trypsin-b_5[f]	0.9	1.1
(5) +protease-b_5[f]	0.8	0.9

[a] Turnover numbers in the presence of 1.17 mol of b_5 per mole of P-450 relative to activity in the absence of cytochrome b_5. Assays for the indicated products were performed under standard assay conditions (see Materials and Methods and Table II) excepting that sodium deoxycholate was at 10 μg/mL. [b] Unlike the b_5 stimulation of PB-1 activities, the much smaller stimulations of PB-4 activity were somewhat reduced at 40 μg/mL deoxycholate. [c] ND, not determined due to the low turnover for these products (see Table II). [d] Variability in the degree of cytochrome b_5 stimulation (e.g., compare parts A and B of this table for metabolism of *p*-tolyl ethyl sulfide) can be attributed to variable product formation in the absence of cytochrome b_5. [e] 18 nM reductase, resulting in ~25–30% the activity (per P-450) as with 90 nM reductase. [f] Prepared as described under Materials and Methods and in the text.

p-(hydroxymethyl)phenyl ethyl sulfide formation was stimulated to a small extent (30–50%) while *p*-tolyl ethyl sulfoxide formation was inhibited. The prominent enhancement of acetanilide hydroxylase activity (up to 8-fold with purified PB-1; Figure 5) principally reflected an increase in V_{max}, although a small but reproducible decrease in the K_m for acetanilide also contributed somewhat (Table IVA). Leibman & Ortiz (1973) reported an increase in V_{max} for the corresponding enhancement in rat liver microsomes.

Recent studies indicate that several P-450-dependent reactions proceed via diffusable reduced oxygen species, such as hydroxyl radicals, which directly mediate the oxygenations (Ingelman-Sundberg & Johansson, 1981; Ingelman-Sundberg & Ekstrom, 1982). Metyrapone might therefore enhance a P-450-catalyzed reaction by increasing the uncoupling of substrate oxygenation from NADPH oxidation (Nordblom & Coon, 1977; Ingelman-Sundberg & Johansson, 1980), resulting in formation of H_2O_2 and O_2^- and, ultimately, hydroxyl radicals. This process is, however, not likely to contribute to the metyrapone enhancement of acetanilide 4-hydroxylase activity, the enhancement being unaffected by scavengers for H_2O_2, O_2^-, and ·OH (Table IVB, experiment I).

Other possible mechanisms for the observed metyrapone enhancement include increasing the rates for transfer of either the first or the second electron from NADPH via P-450 reductase to P-450. Metyrapone (1 mM) did not, however, increase but rather decreased by 25–30% the rate of P-450 reductase catalyzed reduction of cytochrome *c*. Another possibility is that the K_D of the P-450–P-450 reductase complex is decreased by metyrapone. In such a case, one would anticipate a greater metyrapone-enhancement under conditions where reductase is limiting, an expectation that was not met (Table IVB). Finally, omission of cytochrome b_5, which most likely stimulates transfer of the second electron to P-450 (see below), had no effect on the magnitude of the metyrapone stimulation (Table IVB).

Interactions with Cytochrome b_5. Inclusion of cytochrome b_5 in the reconstituted system effected a significant increase in the turnover number of P-450 PB-1 for all substrates tested (Table VA). This b_5-stimulation (~4–7-fold) was much more significant than that observed for PB-4 reactions (≤2-fold stimulation) and saturated at ~1 mol of b_5/mol of P-450 (Figure 6A). Monooxygenase activity was stimulated to a similar extent by cytochrome b_5 in the presence of nonsaturating P-450 reductase (Table VB).

In the absence of added lipid, the stimulatory effects of b_5 were abolished (Table VB, Figure 6B), consistent with the previously observed requirement of detergent or lipid for reduction of b_5 by purified P-450 reductase (Enoch & Strittmatter, 1979). This finding suggests the importance of lipid in facilitating productive interactions between b_5 and P-450 and/or P-450 reductase. Since these interactions probably involve the COOH-terminal, membrane-binding region of b_5, two distinct forms of protease-cleaved b_5 were prepared and purified (see Materials and Methods) and tested for their capacity to stimulate PB-1 activities. Both forms, termed

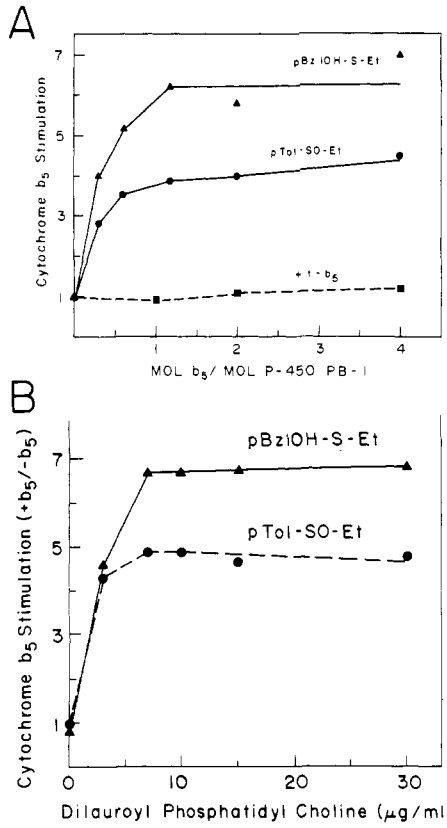

FIGURE 6: Cytochrome b_5 stimulation of P-450 PB-1 catalyzed oxygenation of *p*-tolyl ethyl sulfide. Catalytic activities were determined as a function of concentration of cytochrome b_5 (A) or lipid (B) in the reconstituted system. Formation of *p*-(hydroxymethyl)phenyl ethyl sulfide (pBzlOH-S-Et) was always stimulated by b_5 to a somewhat greater extent than was *p*-tolyl ethyl sulfoxide formation (pTol-SO-Et). These stimulations were not effected when trypsin-b_5 (t-b_5) was used (A) or in the absence of added lipid (B).

trypsin-b_5 (M_r ~17 500) and protease-b_5 (M_r ~15 500), were >95% pure on SDS gels and were readily resolved from uncleaved b_5 (M_r ~18 500) upon Bio-Gel A 1.5 M chromatography (not shown). The small difference in apparent molecular weights of b_5 and trypsin-b_5 (<1000) suggests that trypsin cleavage of only a short peptide segment from rat b_5 is sufficient both to prevent its aggregation upon Bio-Gel A 1.5 M chromatography in detergent-free buffers and also to destroy its competence for several enzymatic reactions (see below). Both of these properties are indicative of the loss of a functional COOH-terminal hydrophobic domain that is effected only by cleavage of a much larger peptide (~40 residues) from b_5 isolated from other species [e.g., Takagaki et al. (1980)].

Although both trypsin-b_5 and protease-b_5 had oxidized visible spectra indistinguishable from uncleaved cytochrome b_5, neither was active in mediating the NADH-dependent reduction of cytochrome *c* by NADH-cytochrome b_5 reductase (≤3% the rate as with uncleaved b_5). Similarly, neither cleaved b_5 derivative stimulated the monooxygenase activity of P-450 PB-1 (Table VB, Figure 6A). This probably reflects the markedly reduced ability of P-450 reductase to transfer electrons to trypsin-b_5 (<1% the rate as with uncleaved b_5; Enoch & Strittmatter, 1979) and is consistent with the involvement of electron-transfer processes in the b_5-stimulation of PB-1-catalyzed reactions.

Discussion

Phenobarbital induces de novo biosynthesis of several distinct isozymes of cytochrome P-450 in mammalian hepatic tissue. In the rat, PB induction yields a major P-450 form now known to be comprised of two closely related isozymes (Waxman & Walsh, 1982a; Ryan et al., 1982; termed P-450 PB-4 and P-450 PB-5 in our nomenclature) differing at less than 15 residues along the 491 amino acid long polypeptide chain (Fujii-Kuriyama et al., 1982; Kumar et al., 1983). These isozymes, together comprising only ~2–4% of total P-450 in uninduced liver, are induced ≥30-fold (per mg of protein) upon PB administration (Thomas et al., 1981; Pickett et al., 1981; Guengerich et al., 1982a). A third isozyme, one induced by synthetic steroids, is increased 13-fold upon PB induction (Heuman et al., 1982). In the present study, we report that a fourth P-450 isozyme, termed P-450 PB-1, is also induced by phenobarbital, but in this case the induction is more modest, estimated at only 2–4-fold. This situation is somewhat analogous to that of P-450a, induced ~3-fold by phenobarbital in immature rats, although not in mature rats (Thomas et al., 1981). In contrast to the low levels of PB-4 and PB-5 in the absence of PB-induction, the basal level of P-450 PB-1 is significant, enabling us to isolate the isozyme from uninduced rat liver. These findings have been extended by the demonstration that PB-1 is indistinguishable from IF-SDS polypeptide PB2, a resolved polypeptide in the two-dimensional SDS–gel isoelectric focusing system of Vlasuk & Walz (1980, 1982), both chromatographically and by ^{125}I-labeled tryptic peptide mapping.[3] Thus P-450 PB-1 is found in both male and female rats, is induced by several inducers of PB-4 and PB-5, and is repressed upon 3-methylcholanthrene or Aroclor 1254 administration (Vlasuk & Walz, 1982; Vlasuk et al., 1982a,b).

P-450 PB-1 was shown to be distinct structurally and immunochemically from P-450s PB-4 and PB-5 in addition to being electrophoretically and immunochemically distinct from isozymes P-450a, P-450b, P-450c, and P-450d prepared by the Hoffmann-La Roche group (Ryan et al., 1979, 1980). In addition, the NH_2-terminal sequence of P-450 PB-1 is different from the seven other sequences reported for rat liver P-450 isozymes. Some homology to the rabbit liver isozyme LM3b (Koop et al., 1981; Ozols et al., 1981) is suggested (Figure 4). LM3b is, however, induced in rabbit liver by triacetyloleandomycin (Bonfils et al., 1982), whereas an isozyme distinct from P-450 PB-1 is induced by this antibiotic in rat liver.[4] It is of interest that all the liver microsomal P-450 isozymes having an amino-terminal methionine (known to be the initiator methionine in the case of PB-4 and/or[5] PB-5; Bar-Nun et al., 1980) have an acidic residue at either the second or third position, a feature not generally found in signal sequences [e.g., Davis & Tai (1980)]. This, in addition to the absence of basic residues typically found in the amino-terminal portion of signal sequences, further supports the suggestion that the amino-terminal hydrophobic region characteristic of hepatic P-450 isozymes is inherently different than that expected for an uncleaved signal sequence and that it may play a role in membrane anchoring (Waxman & Walsh, 1982a).

Significant differences were found when the substrate specificity profile of P-450 PB-1 was compared to that of the major PB-induced isozyme P-450 PB-4 (Table II). Although PB-1 hydroxylates testosterone at the same three sites as does PB-4 (Table II), the observed PB-1 activities do not reflect PB-4 contamination, as shown with PB-4-selective inhibitors (Waxman et al., 1983). PB-1/PB-4 activity ratios for several substrates indicate an upper limit of 1–3% cross-contamination

[4] D. J. Waxman, unpublished results.

[5] PB-4 and PB-5 were not distinguished in that study of the major PB-induced isozyme.

between our purified preparations of PB-1 and PB-4. Substrate specificities determined for these isozymes in a reconstituted monooxygenase system probably reflect their microsomal specificities, as suggested by experiments with isozyme-specific substrates and isozyme-selective inhibitors and activators (Table III). Thus it may eventually be possible to assess the contributions of individual P-450 isozymes to microsomal activities in a more quantitative fashion. The significant influence of cytochrome b_5 on the catalytic activity of PB-1 (but not PB-4) emphasizes the importance of including this heme protein in the reconstituted monooxygenase system when undertaking such analyses.

Metyrapone, a dipyridyl heme ligand generally regarded as an effective inhibitor of cytochrome P-450 (Leibman & Ortiz, 1973; Testa & Jenner, 1981; Netter, 1980), especially that induced by phenobarbital (Jonen et al., 1974; Hultmark et al., 1979; Parkinson et al., 1982), was found to *enhance* several PB-1-catalyzed reactions in the reconstituted system. That the stimulatory effects of metyrapone on microsomal monooxygenations (Leibman, 1969; Leibman & Ortiz, 1973; Waxman et al., 1982) appear to be characteristic of PB-1-preferred substrates indicates that PB-1 probably mediates the observed microsomal enhancements. Metyrapone enhancement of PB-1 activity was principally reflected by an increase in V_{max}, was saturable, and varied in magnitude with the monooxygenase substrate assayed. Maximal stimulation (5–8-fold) was observed for the PB-1-catalyzed 4-hydroxylation of acetanilide. As to the mechanism of this metyrapone enhancement, the following is known: (a) Direct heme ligation is likely not involved, as indicated by the absence of a metyrapone-reduced PB-1-P446 complex (Figure 2). Formation of such complexes probably accounts for the metyrapone sensitivity of P-450 isozymes such as PB-4 and PB-5 (Waxman & Walsh, 1982a). (b) An increase in the uncoupling of NADPH oxidation from substrate oxygenation leading to formation of reactive oxygen species (e.g., O_2^-, H_2O_2, ·OH) is not responsible for the metyrapone enhancement (Table IV). (c) The enhancement is independent of the stimulation effected by cytochrome b_5. (d) Metyrapone does not increase the coupling of P-450 reductase to P-450, nor does it stimulate electron flow from the reductase to cytochrome *c*. It seems most likely that the substrate-dependent metyrapone enhancement reflects a differential facilitation of electron flow from P-450 reductase to different substrate–P-450 PB-1 complexes, a possibility that requires further investigation. Other possibly analogous situations include the isozyme-specific stimulation by flavonoids (Huang et al., 1981a,b) and the SKF-525A enhancement of testosterone 7α-hydroxylase activity observed both with solubilized rat liver microsomes (Shiverick & Neims, 1979) and with purified P-450 PB-3 (Waxman et al., 1983).

Recent studies demonstrate the possibility of transfer of the second electron from either P-450 reductase or NADH-cytochrome b_5 reductase to oxycytochrome P-450 via cytochrome b_5 [e.g., Hildebrant & Estabrook (1971), Noshiro et al. (1980, 1981), and Imai (1981)]. Thus, with P-450 isozymes and for particular substrates for which transfer of the second electron is rate limiting, reconstitution in the presence of b_5 can result in a stimulation of monooxygenase activity (Brunström & Ingelman-Sundberg, 1980; Sugiyama et al., 1980; Vatsis et al., 1982). In the present study, b_5 effected a saturable, 4–7-fold stimulation of several P-450 PB-1 catalyzed reactions, an effect significantly greater than the ≤2-fold stimulation of PB-4 or PB-5 activities (Table V; Waxman & Walsh, 1982a). Previous studies have established the importance of the membranous segment of b_5 for the b_5-mediated reduction of cytochrome *c* and for its reduction by P-450 reductase (Mihara & Sato, 1975; Enoch & Strittmatter, 1979). That the b_5 stimulation of PB-1 activities was dependent on the presence of added lipid and was not observed with specific proteolytic fragments of b_5 that lacked the heme protein's COOH-terminal hydrophobic domain emphasizes the importance of productive interaction of the hydrophobic domain of b_5 with P-450 and/or P-450 reductase for electron transfer to P-450. The availability of a pure P-450 isozyme whose monooxygenase activities are greatly enhanced by cytochrome b_5 should facilitate studies aimed at further elucidating the mechanistic basis for the b_5 stimulation of P-450-catalyzed monooxygenase reactions.

After completion of this paper, Guengerich et al. (1982b) reported the purification to apparent homogeneity of a P-450 isozyme from PB-induced rat liver (termed PB-C) having several properties similar to those of P-450 PB-1. We have recently confirmed the probable identity of P-450 PB-1 and PB-C on the basis of direct catalytic comparisons and electrophoretic analysis in SDS gels.

Acknowledgments

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Registry No. P-450, 9035-51-2; PB, 50-06-6; metyrapone, 54-36-4; cytochrome b_5, 9035-39-6; *N*,*N*-dimethylaniline, 121-69-7; benzphetamine, 156-08-1; ethylmorphine, 76-58-4; 7-ethoxycoumarin, 31005-02-4; acetanilide, 103-84-4; toluene, 108-88-3; (*R*)-warfarin, 5543-58-8; coumarin, 91-64-5; *p*-tolyl ethyl sulfide, 622-63-9; testosterone, 58-22-0; SKF-525A, 62-68-0; *n*-octylamine, 111-86-4; monooxygenase, 9038-14-6.

References

Bar-Nun, S., Kreibich, G., Adebujm, N., Alterman, L., Negishi, M., & Sabatini, D. D. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 965–969.

Belman, S. (1963) *Anal. Chim. Acta 29*, 120–126.

Bonfils, C., Dalet-Beluche, I., & Maurel, P. (1982) *Biochem. Biophys. Res. Commun. 104*, 1011–1017.

Botelho, L. H., Ryan, D. E., & Levin, W. (1979) *J. Biol. Chem. 254*, 5634–5640.

Botelho, L. H., Ryan, D. E., Yuan, P.-M., Kutny, R., Shively, J. E., & Levin, W. (1982) *Biochemistry 21*, 1152–1155.

Brunström, A., & Ingelman-Sundberg, M. (1980) *Biochem. Biophys. Res. Commun. 95*, 431–439.

Calabro, M. A., Katz, J. T., & Holloway, P. W. (1976) *J. Biol. Chem. 251*, 2113–2118.

Cheng, K.-C., & Schenkman, J. B. (1982) *J. Biol. Chem. 257*, 2378–2385.

Cochin, J., & Axelrod, J. (1959) *J. Pharmacol. Exp. Ther. 125*, 105–110.

Conney, A. H. (1967) *Pharmacol. Rev. 19*, 317–366.

Davis, B. D., & Tai, P.-C. (1980) *Nature (London) 283*, 433–438.

Elshourbagy, N. A., & Guzelian, P. S. (1980) *J. Biol. Chem. 255*, 1279–1285.

Enoch, H. G., & Strittmatter, P. (1979) *J. Biol. Chem. 254*, 8976–8981.

Fisher, G. J., Fukushima, H., & Gaylor, J. L. (1981) *J. Biol. Chem. 256*, 4388–4394.

Fujii-Kuriyama, Y., Mizukami, Y., Kawajiri, K., Sogawa, K., & Muramatsu, M. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 2793–2797.

Goldstein, J. A., Linko, P., Luster, M. I., & Sundheimer, D. W. (1982) *J. Biol. Chem. 257*, 2702–2707.

Guengerich, F. P. (1977) *J. Biol. Chem. 252*, 3970–3979.

Guengerich, F. P. (1978) *J. Biol. Chem. 253*, 7931–7939.

Guengerich, F. P. (1979) *Pharmacol. Ther. 6*, 99–121.

Guengerich, F. P., Wang, P., & Davidson, N. K. (1982a) *Biochemistry 21*, 1698–1706.

Guengerich, F. P., Dannan, G. A., Wright, S. T., Martin, M. V., & Kaminsky, L. S. (1982b) *Biochemistry 21*, 6019–6030.

Guenthner, J. M., Negishi, M., & Nebert, D. W. (1979) *Anal. Biochem. 96*, 201–207.

Haugen, D. A., & Coon, M. J. (1976) *J. Biol. Chem. 251*, 7929–7939.

Heuman, D. M., Gallagher, E. J., Barwick, J. L., Elshourbagy, N. A., & Guzelian, P. S. (1982) *Mol. Pharmacol. 21*, 753–760.

Hildebrant, A. G., & Estabrook, R. W. (1971) *Arch. Biochem. Biophys. 143*, 66–79.

Huang, M.-T., Chang, R. L., Fortner, J. G., & Conney, A. H. (1981a) *J. Biol. Chem. 256*, 6829–6836.

Huang, M.-T., Johnson, E. F., Muller-Eberhard, U., Koop, D. R., Coon, M. J., & Conney, A. H. (1981b) *J. Biol. Chem. 256*, 10897–10901.

Hultmark, D., Sundh, K., Johansson, L., & Arrhenius, E. (1979) *Biochem. Pharmacol. 28*, 1587–1590.

Imai, Y. (1981) *J. Biochem. (Tokyo) 89*, 351–362.

Ingelman-Sundberg, M., & Johansson, I. (1980) *Biochemistry 19*, 4004–4011.

Ingelman-Sundberg, M., & Johansson, I. (1981) *J. Biol. Chem. 256*, 6321–6326.

Ingelman-Sundberg, M., & Ekstrom, G. (1982) *Biochem. Biophys. Res. Commun. 106*, 625–631.

Jonen, H. G., Müthwohl, B., Kahl, R., & Kahl, G. F. (1974) *Biochem. Pharmacol. 23*, 1319–1329.

Kaminsky, L. S., Fasco, M. J., & Guengenich, F. P. (1981) *Methods Enzymol. 74*, 262–272.

Koop, D. R., Persson, A. V., & Coon, M. J. (1981) *J. Biol. Chem. 256*, 10704–10711.

Koop, D. R., Morgan, E. T., Tarr, G. E., & Coon, M. J. (1982) *J. Biol. Chem. 257*, 8472–8480.

Kumar, A., Raphael, C., & Adesnick, M. (1983) *J. Biol. Chem.* (in press).

Laemmli, U. K. (1970) *Nature (London) 227*, 680–685.

Leibman, K. (1969) *Mol. Pharmacol. 5*, 1–9.

Leibman, K. C., & Ortiz, E. (1973) *Drug Metab. Dispos. 1*, 184–190.

Light, D. R., Waxman, D. J., & Walsh, C. (1982) *Biochemistry 21*, 2490–2498.

Lu, A. Y. H., & West, S. B. (1978) *Pharmacol. Ther., Part A 2*, 337–358.

Lu, A. Y. H., & West, S. B. (1980) *Pharmacol. Rev. 31*, 277–295.

Masuda-Mikawa, R., Fujii-Kuriyama, Y., Negishi, M., & Tashiro, Y. (1979) *J. Biochem. (Tokyo) 86*, 1383–1394.

Mihara, K., & Sato, R. (1975) *J. Biochem. (Tokyo) 78*, 1057–1073.

Netter, K. J. (1980) *Pharmacol. Ther. 10*, 515–535.

Nordblom, G. D., & Coon, M. J. (1977) *Arch. Biochem. Biophys. 180*, 343–347.

Noshiro, M., Harada, N., & Omura, T. (1980) *J. Biochem. (Tokyo) 88*, 1521–1535.

Noshiro, M., Ullrich, V., & Omura, T. (1981) *Eur. J. Biochem. 116*, 521–526.

Ozols, J., Heinemann, F. S., & Johnson, E. F. (1981) *J. Biol. Chem. 256*, 11405–11408.

Parkinson, A., Robertson, L. W., & Safe, S. (1982) *Biochem. Pharmacol. 31*, 3489–3494.

Pickett, C. B., Jeter, R. L., Morin, J., & Lu, A. Y. H. (1981) *J. Biol. Chem. 256*, 8815–8820.

Roberts, D. V. (1977) in *Enzyme Kinetics*, pp 292–295, Cambridge University Press, Cambridge, UK.

Ryan, D. E., Thomas, P. E., Korzeniowski, D., & Levin, W. (1979) *J. Biol. Chem. 254*, 1326–1374.

Ryan, D. E., Thomas, P. E., & Levin, W. (1980) *J. Biol. Chem. 255*, 7941–7955.

Ryan, D. E., Thomas, P. E., & Levin, W. (1982) *Arch. Biochem. Biophys. 216*, 272–288.

Shiverick, K. T., & Neims, A. H. (1979) *Drug Metab. Dispos. 7*, 290–295.

Sugiyama, T., Miki, N., & Yamano, T. (1980) *J. Biochem. (Tokyo) 87*, 1457–1467.

Takagaki, Y., Gerber, G. E., Nihei, K., & Khorana, H. G. (1980) *J. Biol. Chem. 255*, 1536–1541.

Testa, B., & Jenner, P. (1981) *Drug Metab. Rev. 12*, 1–117.

Thomas, P. E., Reik, L. M., Ryan, D. E., & Levin, W. (1981) *J. Biol. Chem. 256*, 1044–1052.

Vatsis, K. P., Theoharides, A. D., Kupfer, D., & Coon, M. J. (1982) *J. Biol. Chem. 257*, 11221–11229.

Vlasuk, G. P., & Walz, F. G., Jr. (1980) *Anal. Biochem. 105*, 112–120.

Vlasuk, G. P., & Walz, F. G., Jr. (1982) *Arch. Biochem. Biophys. 214*, 248–259.

Vlasuk, G. P., Ghrayeb, J., Ryan, D. E., Reik, L., Thomas, P. E., Levin, W., & Walz, F. G., Jr. (1982a) *Biochemistry 21*, 789–798.

Vlasuk, G. P., Ryan, D. E., Thomas, P. E., Levin, W., & Walz, F. G., Jr. (1982b) *Biochemistry 21*, 6288–6292.

Waxman, D. J., & Walsh, C. (1982a) *J. Biol. Chem. 257*, 10446–10457.

Waxman, D. J., & Walsh, C. (1982b) in *Cytochrome P-450, Biochemistry, Biophysics and Environmental Implications* (Hietanen, E., Laitinen, M., & Hänninen, O., Eds.) pp 311–316, Elsevier Biomedical Press, New York.

Waxman, D. J., Light, D. R., & Walsh, C. (1982) *Biochemistry 21*, 2499–2507.

Waxman, D. J., Ko, A., & Walsh, C. (1983) *J. Biol. Chem.* (in press).

West, S. B., Huang, M.-T., Miwa, G. T., & Lu, A. Y. H. (1979) *Arch. Biochem. Biophys. 193*, 42–50.

Wislocki, P. G., Miwa, G. T., & Lu, A. Y. H. (1980) in *Enzymatic Basis of Detoxification* (Jakoby, W. B., Ed.) Vol. 1, pp 135–182, Academic Press, New York.