Characterization of Testosterone 16α-Hydroxylase (I-P-450$_{16\alpha}$) Induced by Phenobarbital in Mice

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Received January 10, 1985

ABSTRACT: Cytochrome P-450 (I-P-450$_{16\alpha}$), which is associated with phenobarbital-induced testosterone 16α-hydroxylation activity, was purified from livers of phenobarbital-treated female 129/J mice on the basis of the specific hydroxylation activity in fractions eluted from columns of octylamino-Sepharose 4B, hydroxylapatite, DEAE-Bio-Gel A, and isobutyl-Sepharose 4B. The specific cytochrome P-450 content of the purified I-P-450$_{16\alpha}$ fraction was 12.4 nmol/mg of protein, and it had an apparent molecular weight of 54K. The specific activity of reconstituted testosterone 16α-hydroxylation activity with the purified I-P-450$_{16\alpha}$ fraction was 6–8 nmol min^{-1} (nmol of cytochrome P-450)$^{-1}$. Rabbit antibody raised against the purified I-P-450$_{16\alpha}$ fraction inhibited nearly 100% of the 16α-hydroxylation activity in liver microsomes of phenobarbital-treated female 129/J mice but did not affect hepatic microsomal 16α-hydroxylation activity of untreated male and female 129/J mice at all. In hepatic microsomes of phenobarbital-treated male 129/J mice, 70% of the 16α-hydroxylation activity, at most, was catalyzed by I-P-450$_{16\alpha}$, and the residual 30% of the activity was catalyzed by C-P-450$_{16\alpha}$. The increase of I-P-450$_{16\alpha}$ by phenobarbital was due to de novo synthesis of I-P-450$_{16\alpha}$, and this induction was not sexually regulated in 129/J mice. Anti-C-P-450$_{16\alpha}$ [Harada, N., & Negishi, M. (1984) *J. Biol. Chem. 259*, 12285–12290] did not inhibit the 16α-hydroxylation catalyzed by I-P-450$_{16\alpha}$; thus, I-P-450$_{16\alpha}$ and C-P-450$_{16\alpha}$ are immunochemically distinct isozymes of testosterone 16α-hydroxylase.

The liver microsomal cytochrome P-450 system is known to oxidize steroid hormones, and this is believed to be a degradation step of these biologically active compounds. In some steroid hydroxylation reactions, sexual dimorphism is known (Ray & Chatleajee, 1983). In certain inbred strains of mice such as 129/J and AKR/J, liver microsomal testosterone 16α-hydroxylation activity is higher in male than in female mice (Ford et al., 1975, 1979; Harada & Negishi, 1984; Hawke et al., 1983). In other strains such as C57BL/6J, the 16α-hydroxylation activity in microsomes is equal in female and male mice (Ford et al., 1975, 1979). Through purification of hepatic microsomal cytochrome P-450 specific for testosterone 16α-hydroxylation, it was shown that sexual dimorphism of the hydroxylation activity in 129/J mice is due to the presence of a constitutive form of cytochrome P-450 (C-P-450$_{16\alpha}$)[1] that is highly active and regio- and stereospecific for 16α-hydroxylation (Harada & Negishi, 1984a). Nearly 100% of the testosterone 16α-hydroxylation activity in liver microsomes of untreated 129/J mice was accounted for by C-P-450$_{16\alpha}$.

During the course of this investigation, we found that phenobarbital administration increased testosterone 16α-hydroxylation activity in liver microsomes of female 129/J mice although the 16α-hydroxylation activity in microsomes of male 129/J mice was not altered significantly by phenobarbital. In this paper, we describe the purification of an inducible form of cytochrome P-450 (I-P-450$_{16\alpha}$) that is associated with phenobarbital-induced testosterone 16α-hydroxylation activity in female 129/J mice. The immunochemically distinct nature of the two cytochrome P-450 isozymes (C-P-450$_{16\alpha}$ and I-P-450$_{16\alpha}$) is also reported.

Experimental Procedures

Animals and Preparation of Liver Microsomes. Two- to three-month-old male and female 129/J mice were purchased from Jackson Laboratories (Bar Harbor, ME), and female New Zealand white rabbits were bought from Charles River (Wlington, MA). Sodium phenobarbital (50 mg/kg) in saline was injected intraperitoneally for 4 consecutive days. Liver microsomes were prepared by the method previously described (Harada & Negishi, 1984b).

Materials. [^{14}C]Testosterone (50–60 mCi/mmol) was purchased from New England Nuclear. DEAE-Bio-Gel A, Bio-Gel HTP, and protein assay kits were from Bio-Rad. Sepharose 4B was from Pharmacia (Uppsala, Sweden). *d*-Benzphetamine hydrochloride was a generous gift from Upjohn. 7-Ethoxycoumarin, dilauroylglycerol 3-phosphate, serum albumin, catalase, glutamate dehydrogenase, ovalbumin, and aldolase were purchased from Sigma. Sodium cholate and 3-[(cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) were from Calbiochem, isobutylamine was from Aldrich, and X-Omat films were from the Eastman Kodak Co. Silica gels were purchased from Whatman. Emulgen 913 was a kind gift from Kao-Atlas Co. (Tokyo, Japan). 16α-Hydroxytestosterone and 15α-hydroxytestosterone were obtained from the Reference Collection of the British Medical Council (London, England). All other chemicals were of the highest quality commerically available. Rat liver NADPH–cytochrome P-450 reductase was purified by the method of

[1] The term cytochrome P-450 was used to represent total cytochrome P-450 as measured by Omura & Sato (1964). I-P-450$_{16\alpha}$ was defined as the inducible form of testosterone 16α-hydroxylase which was purified from phenobarbital-treated female 129/J mice. Because we now have a second P-450 isozyme catalyzing testosterone 16α-hydroxylation, the previously characterized testosterone 16α-hydroxylase (P-450$_{16\alpha}$) from untreated male 129/J mice (Harada & Negishi, 1984a) was properly redefined as a constitutive form of testosterone 16α-hydroxylase (C-P-450$_{16\alpha}$). In this report, the term isozyme is used only for forms of cytochrome P-450 that can be categorized by the classical isozyme concept (Lehninger, 1982) as in the case of C- and I-P-450$_{16\alpha}$.

Yasukochi & Masters (1976). Isobutyl-Sepharose 4B and octylamino-Sepharose 4B were synthesized according to the procedure previously published (Harada & Negishi, 1984a; Cuatrecases & Anfinsen, 1971) except that 7 mg of CNBr was used to activate 100 g of Sepharose 4B for the preparation of isobutyl-Sepharose 4B.

Purification of I-P-450$_{16\alpha}$. Liver microsomes (1.1 g) prepared from 50 phenobarbital-treated female 129/J mice were solubilized in 350 mL of 100 mM potassium phosphate buffer, pH 7.25, containing 20% glycerol, 1 mM ethylenediaminetetraacetic acid (EDTA), 1 mM dithiothreitol, 0.5% sodium cholate, and 0.05% CHAPS (buffer A) and centrifuged at 105000*g* for 90 min. The resulting supernatant fraction was subjected to column chromatography with a series of packing materials.

Octylamino-Sepharose 4B Step. The supernatant fraction was applied to an octylamino-Sepharose 4B column (2.6 × 30 cm) previously equilibrated with buffer A. After the column was washed with buffer A containing 0.04% Emulgen 913, I-P-450$_{16\alpha}$ was eluted by buffer A containing 0.1% Emulgen in the absence of 1 mM EDTA. First, the protein concentration, total cytochrome P-450 content, and testosterone hydroxylation activities were measured in the fractions eluted from the column, and then the fractions enriched in I-P-450$_{16\alpha}$ which was eluted broadly after the majority of cytochrome P-450 eluted, were pooled and applied to the next column. This basic procedure was followed throughout the purification to the fifth column chromatography step.

Hydroxylapatite Step. A hydroxylapatite column (1.5 × 20 cm) was equiilibrated with 25 mM potassium phosphate buffer, pH 7.25, containing 20% glycerol, 0.4% cholate, 0.1% Emulgen 913, and 1 mM dithiothreitol (buffer B). The pooled fractions eluted from the octylamino-Sepharose 4B column enriched for I-P-450$_{16\alpha}$ were dialyzed to decrease the potassium phosphate buffer concentration to 25 mM and then applied to the equilibrated hydroxylapatite column. The hydroxylapatite column was washed with buffer B and then eluted by stepwise increases of potassium phosphate buffer concentration to 80, 120, 180, and finally 250 mM containing 20% glycerol, 0.4% sodium cholate, 0.1% Emulgen 913, and 1 mM dithiothreitol. I-P-450$_{16\alpha}$ was eluted mainly by the 180 mM potassium phosphate buffer.

DEAE-Bio-Gel A Step. The pooled fractions enriched for I-P-450$_{16\alpha}$ from the hydroxylapatite column were dialyzed against 10 mM potassium phosphate buffer, pH 7.25, containing 20% glycerol, 0.2% cholate, 0.05% Emulgen 913, 1 mM EDTA, and 1 mM dithiothreitol (buffer C) and applied to a column of DEAE-Bio-Gel A (1.0 × 10 cm) previously equilibrated with buffer C. After the column was washed with buffer C, I-P-450$_{16\alpha}$ was eluted by linearly increasing the potassium phosphate concentration in buffer C from 10 to 200 mM. A typical elution pattern for protein, total cytochrome P-450, and I-P-450$_{16\alpha}$ is shown in Figure 1. The fractions containing the highest testosterone 16α-hydroxylation activity (I-P-450$_{16\alpha}$) were pooled and subjected to chromatography on the next column.

Isobutyl-Sepharose 4B Step. The pooled fractions from the DEAE-Bio-Gel A column were diluted with an equal volume of 25 mM potassium phosphate buffer, pH 7.25, containing 20% glycerol, 0.2% cholate, 1 mM EDTA, and 1 mM dithiothreitol and applied to a column of isobutyl-Sepharose 4B (0.5 × 5 cm) equilibrated with 50 mM potassium phosphate buffer, pH 7.25, containing 20% glycerol, 0.2% sodium cholate, 1 mM EDTA, and 1 mM dithiothreitol. After the column was washed with 100 mM potassium phosphate buffer, pH 7.25, containing 20% glycerol, 0.2% cholate, 1 mM EDTA, and 1 mM dithiothreitol (buffer D), it was eluted with buffer D containing 0.05% Emulgen 913. I-P-450$_{16\alpha}$ was primarily recovered in the fractions eluted with buffer D which contained Emulgen 913.

Hydroxylapatite Step To Remove Detergents. The purified I-P-450$_{16\alpha}$ fraction was diluted with equal volumes of 20% glycerol and 1 mM dithiothreitol and applied to a small hydroxylapatite column (0.5 × 3 cm) equilibrated with 50 mM potassium phosphate buffer, pH 7.25, containing 20% glycerol and 1 mM EDTA (buffer E). The column was washed with buffer E to remove detergents, and I-P-450$_{16\alpha}$ was eluted with 250 mM potassium phosphate buffer, pH 7.25, containing 20% glycerol and 1 mM dithiothreitol.

Preparation of Antibody. About 350 μg of the purified I-P-450$_{16\alpha}$ was used to raise antiserum in rabbits according to the method of Harada & Negishi (1984a,b). Purification of the C-P-450$_{16\alpha}$ fraction and its antibody (anti-C-P-450$_{16\alpha}$) was carried out as described previously (Harada & Negishi, 1984a).

Enzyme Assays. Testosterone hydroxylation activity in liver microsomes and reconstistuted systems was measured as previously described (Ford et al., 1975; Harada & Negishi, 1984b). Benzphetamine N-demethylation and 7-ethoxycoumarin O-deethylation activities were also determined by methods previously described (Nash, 1953; Greenlee & Poland, 1978).

Radiolabeling of Microsomal Proteins in Vitro and Immunoprecipitation of Radioactive I-P-450$_{16\alpha}$. Microsomes were incubated with 1 mM pyridoxal phosphate and then sodium [^{3}H]borohydride by the procedure previously described (Negishi & Kreibich, 1978). Immunoprecipitation by anti-I-P-450$_{16\alpha}$ from solubilized radioactive microsomes was carried out by the method of Negishi & Nebert (1979) and analyzed by sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis and fluorography.

Other Analytical Methods. SDS–polyacrylamide gel electrophoresis was performed in 9% acrylamide gels using the procedure described by Maizel (1971). The proteins used for markers were serum albumin (M_r 68 000), catalase (M_r 58 000), glutamate dehydrogenase (M_r 53 000), ovalbumin (M_r 45 000), and carbonic anhydrase (M_r 30 000). The total cytochrome P-450 content was determined by the method of Omura & Sato (1964). Protein concentration was measured from the absorption of the protein–Coomassie Brilliant Blue G-250 complex at 595 nm by the method of Bradford (1976). Bovine serum albumin was used as the standard.

Results

Effect of Anti-C-P-450$_{16\alpha}$ on Testosterone Hydroxylase Activity in Microsomes. Liver microsomes were prepared from untreated and phenobarbital-treated male and female 129/J mice. The 16α-hydroxylase activity in female microsomes was increased an average of 5-fold by phenobarbital and reached the level of activity present in untreated or phenobarbital-treated male microsomes; the 16α-hydroxylase activity in male microsomes was not changed much by phenobarbital treatment (Figure 1). Antibody raised against purified C-P-450$_{16\alpha}$ was tested for inhibition of testosterone 16α-hydroxylase activities in these microsomes. As seen in Figure 1, the antibody effectively inhibited the 16α-hydroxylase activity in untreated male and female microsomes, and this is consistent with the results previously reported (Harada & Negishi, 1984a). On the other hand, the antibody had little effect on phenobarbital-induced female 16α-hydroxylase activity and inhibited only 30% of the activity in pheno-

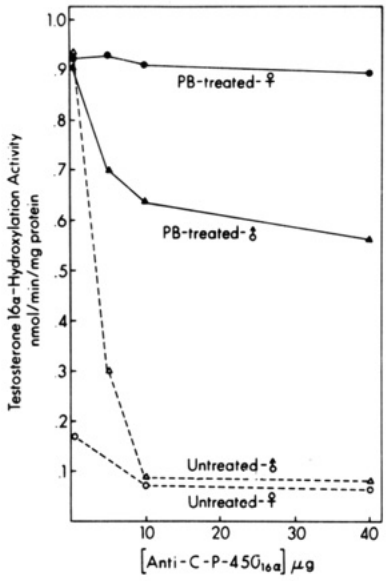

FIGURE 1: Effect of anti-C-P-450$_{16\alpha}$ on testosterone 16α-hydroxylase activity in liver microsomes prepared from 129/J mice. Liver microsomes were prepared from untreated and phenobarbital-treated male and female 129/J mice according to the procedures described under Experimental Procedures. Various amounts of anti-C-P-450$_{16\alpha}$ were incubated with 250 μg of microsomes for 3 min at 4 °C. The incubation was performed in the reaction buffer for the testosterone hydroxylation assay described in Figure 4, in the absence of NADPH. After preincubation for 1 min at 37 °C, the hydroxylation reaction was initiated by adding NADPH. The metabolites formed were subjected to two-dimensional thin-layer chromatography. The radioactive spots representing 16α-hydroxytestosterone were scraped from the thin-layer plate, and the radioactivity was determined.

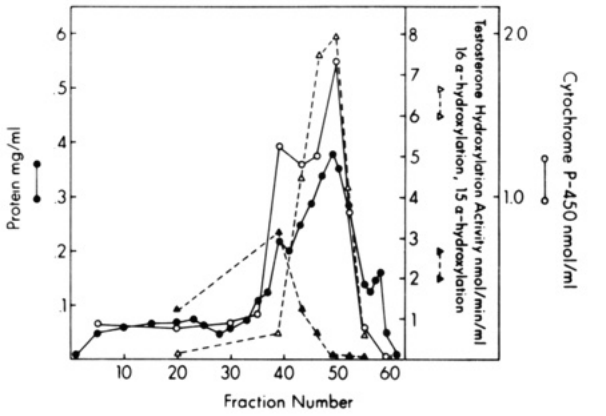

FIGURE 2: Elution profile from the DEAE-Bio-Gel A column. The pooled fraction containing I-P-450$_{16\alpha}$ from the hydroxylapatite column, which was dialyzed accordingly, was chromatographed. Protein concentrations, total cytochrome P-450 contents, and testosterone hydroxylation activities were measured by the methods described under Experimental Procedures. The linear gradient of phosphate buffer was started at fraction 20. Each fraction contained 5 mL of eluate.

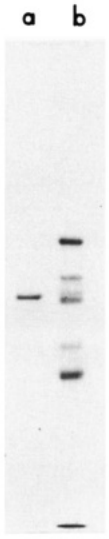

FIGURE 3: SDS–polyacrylamide gel electrophoresis of the purified I-P-450$_{16\alpha}$ fraction. The purified I-P-450$_{16\alpha}$ (1 μg) was analyzed by 9% polyacrylamide gel electrophoresis, and serum albumin, catalase, glutamate dehydrogenase, ovalbumin, and carbonic anhydrase were used as molecular weight markers. Electrophoresis was from top to bottom, and the gel was stained with Coomassie Blue and destained with methanol–acetic acid.

barbital-treated male microsomes. These observations suggested the presence of a form of cytochrome P-450 other than C-P-450$_{16\alpha}$ that catalyzes testosterone 16α-hydroxylase and appears only after induction by phenobarbital.

Purification of I-P-450$_{16\alpha}$. First of all, the sensitivity of phenobarbital-induced 16α-hydroxylase activity to detergents such as Emulgen 913 and CHAPS was determined. Unlike constitutive testosterone 16α-hydroxylase (C-P-450$_{16\alpha}$), phenobarbital-induced 16α-hydroxylase (I-P-450$_{16\alpha}$) activity was not inhibited by up to 0.2% Emulgen 913 (data not shown). At this concentration of Emulgen 913, more than 70% of the activity with C-P-450$_{16\alpha}$ was inhibited (Harada & Negishi, 1984a). Because of the relative insensitivity of I-P-450$_{16\alpha}$ to Emulgen 913, octylamino-Sepharose 4B was chosen as the first column chromatography step. From this column, I-P-450$_{16\alpha}$ was eluted broadly on the shoulder of the major cytochrome P-450 elution peak. Elution profiles from the second DEAE-Bio-Gel A column are shown in Figure 2. I-P-450$_{16\alpha}$ was eluted from the DEAE-Bio-Gel A column at a phosphate buffer concentration of about 100 mM; the cytochrome P-450 (P-450$_{15\alpha}$) associated with testosterone 15α-hydroxylation was eluted earlier in this column. Finally, the majority of I-P-450$_{16\alpha}$ was eluted from a column of isobutyl-Sepharose 4B with buffer D containing Emulgen 913. The specific cytochrome P-450 content of purified I-P-450$_{16\alpha}$ was 12.5 nmol/mg of protein, and the hemoprotein appeared as a single major band on SDS–polyacrylamide gel electrophoresis (Figure 3). Its apparent molecular weight on the gel was 54K; serum albumin, catalase, glutamate dehydrogenase, ovalbumin, and carbonic anhydrase served as molecular weight markers. Recoveries of cytochrome P-450 and protein in the purified I-P-450$_{16\alpha}$ fraction were 2% and 0.1%, respectively. On the basis of the enrichment of specific 16α-hydroxylase activity in the purified fractions from solubilized microsomes, which were approximately 8 and 1 nmol min^{-1} (nmol of P-450)$^{-1}$, respectively, it is estimated that I-P-450$_{16\alpha}$ accounts for about 10–15% of the total cytochrome P-450 in the phenobarbital-treated female microsomes.

Regio- and Stereoselectivity of Testosterone Hydroxylase and Substrate Specificity of Drug Oxidations. Testosterone metabolites formed by the reconstituted system with purified I-P-450$_{16\alpha}$ were analyzed by two-dimensional thin-layer chromatography. It was found that 16α-hydroxytestosterone is a major metabolite (Figure 4). The specific activity of 16α-hydroxylase by the purified I-P-450$_{16\alpha}$ fraction was 6–8 nmol min^{-1} (nmol of cytochrome P-450)$^{-1}$. One unidentified metabolite was less polar and contained about one-fourth of the radioactivity of 16α-hydroxytestosterone. There is no way to test whether formation of this metabolite was catalyzed by I-P-450$_{16\alpha}$ or by some other contaminant cytochrome P-450 isozyme in the purified I-P-450$_{16\alpha}$ fraction at the present time. Purified I-P-450$_{16\alpha}$ exhibited extremely high catalytic activity [193 nmol min^{-1} (nmol of cytochrome P-450)$^{-1}$] for benzphetamine N-demethylation and moderate activity (2 nmol

Table I: Oxidation Activity in a Reconstituted Monooxygenase System Containing Purified I-P-450$_{16\alpha}$ in Comparison with That in Microsomes

	act. in microsomes of 129/J (female) [nmol of product formed min^{-1} (mg of protein)$^{-1}$]		reconstituted act. with purified I-P-450$_{16\alpha}$ [nmol min^{-1} (nmol of P-450)$^{-1}$]
	untreated	phenobarbital treated	
testosterone 16α-hydroxylase	0.2	0.8	8.0
benzphetamine N-demethylation	18.5	40.5	193.0
7-ethoxycoumarin O-deethylation	0.85	1.9	2.1

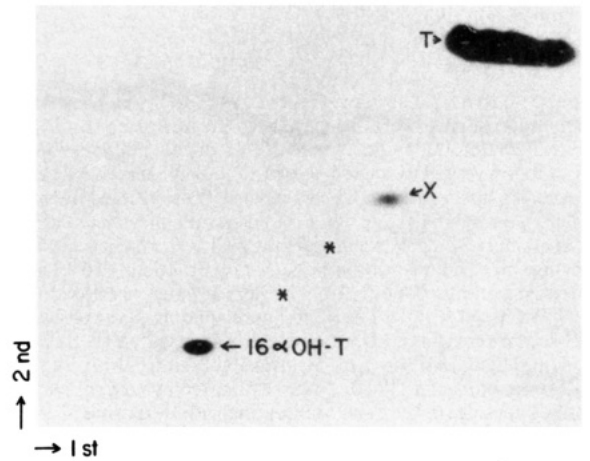

FIGURE 4: Two-dimensional thin-layer chromatography of testosterone metabolites formed by the reconstituted system containing the purified I-P-450$_{16\alpha}$ fraction. Testosterone hydroxylation activity was reconstituted with the purified I-P-450$_{16\alpha}$ fraction. The reconstituted system contained 3 units of NADPH–cytochrome P-450 reductase, 25 μg of dilauroylphosphatidylcholine, and 50 μmol of I-P-450$_{16\alpha}$ in 10 mM tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl), pH 7.5, containing 1 mM $MgCl_2$. The reaction was started by adding 250 nmol of NADPH and continued for 10 min at 37 °C. Metabolites formed were analyzed by two-dimensional thin-layer chromatography by the method described under Experimental Procedures. The two radioactive spots indicated by stars were not metabolites whose formation was catalyzed by I-P-450$_{16\alpha}$; they were either contaminants of the substrate or products formed nonenzymatically. The thin-layer plate was exposed to X-ray film overnight. The radioactive spot indicated by "X" was an unknown testosterone derivative.

min^{-1} nmol^{-1}) for 7-ethoxycoumarin O-deethylation. Together with the oxidation activities in microsomes, these results are summarized in Table I.

Effect of Anti-C-P-450$_{16\alpha}$ on Reconstituted Testosterone Hydroxylase Activity Catalyzed by Purified I- or C-P-450$_{16\alpha}$. Anti-C-P-450$_{16\alpha}$ was tested for inhibitory action on the testosterone 16α-hydroxylase activity of I-P-450$_{16\alpha}$. As shown in Figure 5, anti-C-P450$_{16\alpha}$ inhibited testosterone 16α-hydroxylase activity reconstituted with C-P-450$_{16\alpha}$ very well, and this is consistent with our previous report (Harada & Negishi, 1984a). However, the 16α-hydroxylase activity reconstituted with I-P-450$_{16\alpha}$ was not inhibited at all, consistent with the observation that anti-C-P-450$_{16\alpha}$ did not inhibit phenobarbital-induced testosterone 16α-hydroxylase activity in microsomes of female 129/J mice. These data show that I- and C-P-450$_{16\alpha}$ are immunochemically different. Further evidence for this was obtained by using an antibody against the purified I-P-450$_{16\alpha}$ fraction.

Effect of Anti-I-P-450$_{16\alpha}$ on Testosterone 16α-Hydroxylation in Microsomes. Anti-I-P-450$_{16\alpha}$ almost completely inhibited testosterone 16α-hydroxylase activity in phenobarbital-treated female liver microsomes but did not change the activity in untreated male microsomes, confirming further that I-P-450$_{16\alpha}$ is, in fact, immunochemically different from C-P-450$_{16\alpha}$ (Figure 6). The 16α-hydroxylation reconstituted with purified I-P-450$_{16\alpha}$ fraction was also inhibited completely (data not shown). On the contrary, it was found

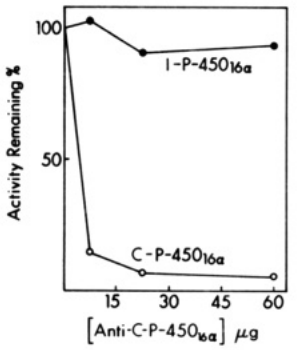

FIGURE 5: Effect of anti-C-P-450$_{16\alpha}$ on 16α-hydroxylase reconstituted with purified I-P-450$_{16\alpha}$ or C-P-450$_{16\alpha}$. Reconstitution of testosterone hydroxylation with the purified I-P-450$_{16\alpha}$ or C-P-450$_{16\alpha}$ was carried out as described under Experimental Procedures. Fifty micromoles of I-P-450$_{16\alpha}$ or 10 μmol of C-P-450$_{16\alpha}$ was used in the reconstituted system. Prior to starting the hydroxylase reaction with NADPH, various amounts of anti-C-P-450$_{16\alpha}$ were added for 3 min at 4 °C. Testosterone metabolites formed by the reconstituted monooxygenase system were separated by one-dimensional thin-layer chromatography, and the amount corresponding to 16α-hydroxytestosterone was measured.

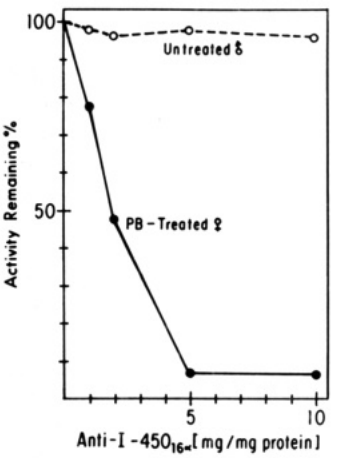

FIGURE 6: Effect of anti-I-P-450$_{16\alpha}$ on testosterone 16α-hydroxylase in hepatic microsomes of untreated or phenobarbital-treated mice. Two hundred micrograms of microsomal protein prepared from untreated or phenobarbital-treated female 129/J mice was incubated with various amounts of anti-I-P-450$_{16\alpha}$ for 3 min at 4 °C. All other reaction conditions were the same as those described in Figure 1.

that anti-I-P-450$_{16\alpha}$ decreased testosterone 16α-hydroxylase activity in phenobarbital-treated male microsomes about 60–70%. Therefore, the following experiment was performed (Figure 7).

Reciprocal Effect of Anti-I- and Anti-C-P-450$_{16\alpha}$ on Testosterone 16α-Hydroxylase Activity of Phenobarbital-Treated Male Microsomes. Liver microsomes were prepared from male 129/J mice treated with phenobarbital for different periods of time as described in Figure 7. Effects of anti-I- or anti-C-P-450$_{16\alpha}$ on microsomal testosterone 16α-hydroxylase activity were studied, and the results are shown in Figure 7. Anti-I-P-450$_{16\alpha}$ had little effect on 16α-hydroxylase activity in untreated male microsomes. This is consistent with the fact that testosterone 16α-hydroxylase (C-P-450$_{16\alpha}$) is the only

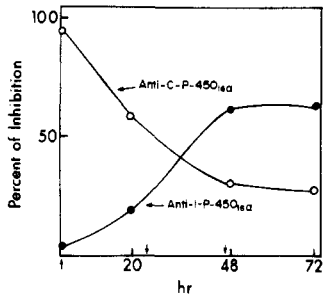

FIGURE 7: Reciprocal effects of anti-I- and anti-C-P-450$_{16\alpha}$ on 16α-hydroxylase activity in phenobarbital-treated male hepatic microsomes. Liver microsomes were isolated from male 129/J mice at 20, 48, and 72 h after the first administration of phenobarbital (50 mg/kg body weight). Five mice were used for each time point. The arrows on the horizontal axis indicate the times when phenobarbital was administered intraperitoneally. Microsomes (250 μg of protein) were preincubated with either anti-C-P-450$_{16\alpha}$ (25 μg) or anti-I-P-450$_{16\alpha}$ (500 μg), and testosterone 16α-hydroxylase activity in the microsomes was measured.

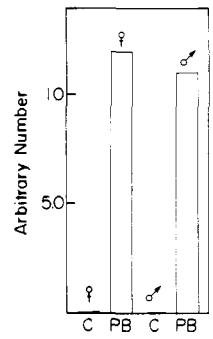

FIGURE 8: Increase of de novo synthesis of I-P-450$_{16\alpha}$ by phenobarbital. Liver microsomes were prepared from untreated and phenobarbital-treated male and female 129/J mice. Phenobarbital (50 mg/kg body weight) was administered on 3 consecutive days. The prepared microsomes were labeled in vitro. The tritiated microsomal proteins were solubilized in 100 mM potassium phosphate buffer, pH 7.5, containing 2% cholate and 150 mM KCl at a protein concentration of 4 mg/mL. The resultant supernatant fractions (50 μL), after centrifugation at 105000g for 1 h, were immunoprecipitated with anti-I-P-450$_{16\alpha}$ (1 mg). The immunoprecipitates were solubilized in SDS sample-treating buffer and electrophoresed on an SDS–polyacrylamide gel, and the dried gel was exposed to X-ray film. The relative amounts of I-P-450$_{16\alpha}$ were estimated by comparison of the relative intensities of the band comigrating with the purified I-P-450$_{16\alpha}$ by laser scanning densitometry.

isozyme catalyzing the 16α-hydroxylation reaction in untreated mice. After administration of phenobarbital, the amount of inhibition by anti-I-P-450$_{16\alpha}$ was increased to 20% and 65–70% at 20 and 48 h, respectively. Reciprocally, the degree of inhibition by anti-C-P-450$_{16\alpha}$ was decreased to about 30% by 48 h after phenobarbital administration. Incubation of phenobarbital-treated female microsomes with both anti-I- and anti-C-P-450$_{16\alpha}$ resulted in total loss of the 16α-hydroxylase activity (data not shown). Phenobarbital treatment did not significantly enhance microsomal testosterone 16α-hydroxylase activity per milligram of protein in male mice, the specific activity being in the range of ±10% of the activity of untreated male microsomes.

Increase of de Novo Synthesis of I-P-450$_{16\alpha}$ by Phenobarbital in Male and Female Mice. The inhibition by anti-I- and anti-C-P-450$_{16\alpha}$ of microsomal testosterone 16α-hydroxylase activity suggested that C-P-450$_{16\alpha}$ in untreated male microsomes is replaced by I-P-450$_{16\alpha}$ after phenobarbital administration. To investigate whether this occurs by an increase of de novo synthesis of I-P-450$_{16\alpha}$ or by some sort of activation of preexisting I-P-450$_{16\alpha}$ in untreated mice, the relative amounts of I-P-450$_{16\alpha}$ protein in microsomes were measured by direct immunoprecipitation. Microsomal protein fractions were tritiated in vitro, and the radioactive microsomes were solubilized by the procedure described under Experimental Procedures. The immunoprecipitate formed by anti-I-P-450$_{16\alpha}$ was collected from the tritiated solubilized microsomes and analyzed by SDS–polyacrylamide gel electrophoresis. The results are shown in Figure 8. Immunoprecipitable I-P-450$_{16\alpha}$ was not detected from untreated male and female microsomes under the experimental conditions used. Instead of I-P-450$_{16\alpha}$, a protein smaller than I-P-450$_{16\alpha}$ was precipitated from untreated male and female microsomes (data not shown). It is not yet understood whether this is due to cross-reactivity of anti-I-P-450$_{16\alpha}$ with the smaller molecular weight protein or to an antibody to a protein contaminant in the purified I-P-450$_{16\alpha}$ fraction. After phenobarbital treatment, the amount of immunoprecipitable I-P-450$_{16\alpha}$ was dramatically increased in male as well as female liver microsomes. Since the level of I-P-450$_{16\alpha}$ in untreated mice is undetectable by this method, the amount of induction of I-P-450$_{16\alpha}$ is indefinite. The smaller protein was also increased by phenobarbital, but induction was only 2–3-fold.

DISCUSSION

In this report, we describe the purification of liver cytochrome P-450 (I-P-450$_{16\alpha}$) which is associated with phenobarbital-induced testosterone 16α-hydroxylase activity. Purification was monitored by measuring the specific testosterone 16α-hydroxylase activity in the fractions eluted from various columns. The specific 16α-hydroxylase activity in a reconstituted system with the purified I-P-450$_{16\alpha}$ fraction was 6–8 nmol min^{-1} (nmol of cytochrome P-450)$^{-1}$. The purified I-P-450$_{16\alpha}$ exhibited a relatively high regio- and stereoselectivity for 16α-hydroxylation of testosterone. Besides 16α-hydroxytestosterone, only one other metabolite was detected under our experimental conditions, and it was formed at a rate of 1–2 nmol min^{-1} (nmol of cytochrome P-450)$^{-1}$.

An attempt to characterize liver microsomal cytochrome P-450's in phenobarbital-treated mice was previously made in Huang et al. (1976). Among the four purified or partially purified cytochrome P-450 fractions obtained, fraction C1 somewhat resembles the purified I-P-450$_{16\alpha}$ with regard to its enzymatic properties. Fraction C1 catalyzed testosterone 16α-hydroxylation a rate of 1.64 nmol) min^{-1} (mol of cytochrome P-450)$^{-1}$ and was effective at benzphetamine N-demethylation [45 nmol min^{-1} (nmol of cytochrome P-450)$^{-1}$] as well, although the rates of both activities catalyzed by fraction C1 were 2–5-fold lower than those catalyzed by I-P-450$_{16\alpha}$. Fraction C1 possessed good 7-ethoxycoumarin O-deethylation activity [6.3 nmol min^{-1} (nmol of cytochrome P-450)$^{-1}$] in comparison with the other three purified fractions. As the study of fraction C1 has not been pursued, further comparison such as the immunochemical characteristics between the two cytochrome P-450 fractions is not possible.

In a previous report, we described the purification of C-P-450$_{16\alpha}$ from untreated male 129/J mice and its high activity and absolute regio- and stereospecificity for testosterone 16α-hydroxylation (Harada & Negishi, 1984a). Although the specific activity of I-P-450$_{16\alpha}$ is 10 times lower than that of C-P-450$_{16\alpha}$, I-P-450$_{16\alpha}$ does catalyze testosterone 16α-hydroxylation. Also, both forms of P-450$_{16\alpha}$ possessed high benzphetamine N-demethylation activities. In spite of the fact that the two cytochrome P-450's exhibited similar catalytic properties, they differed in their apparent molecular weight and hydrophilic and hydrophobic natures, judged from their elution profiles from column chromatography. Furthermore, I- and C-P-450$_{16\alpha}$ are immunochemically distinct. Therefore,

it can be concluded that C- and I-P-$450_{16\alpha}$ are isozymes based on the classic definition (Lehninger, 1982). One isozyme, C-P-$450_{16\alpha}$, exists primarily in untreated male microsomes of 129/J mice; thus, this isozyme shows strong sexual dimorphism in its expression. The other isozyme, I-P-$450_{16\alpha}$, is essentially absent in untreated male and female microsomes of 129/J mice and is induced by compounds such as phenobarbital equally in male and female mice.

While the increase of I-P-$450_{16\alpha}$ by phenobarbital is due to de novo synthesis of apo-I-P-$450_{16\alpha}$, the mechanism of the decrease of the contribution of C-P-$450_{16\alpha}$ to microsomal 16α-hydroxylase activity in male microsomes after treatment by phenobarbital remains to be elucidated. There are several possible mechanisms to explain this phenomenon; for example, phenobarbital may decrease the transcriptional or translational activity of C-P-$450_{16\alpha}$, or the interaction with cytochrome P-450 reductase may be disturbed. These possibilities are now under investigation in our laboratory.

In conclusion, we have demonstrated the existence of two P-450 isozymes (C- and I-P-$450_{16\alpha}$) for testosterone 16α-hydroxylase in mouse liver microsomes. C-P-$450_{16\alpha}$ is a constitutive isozyme and is primarily expressed in male 129/J mice. Its testosterone 16α-hydroxylase activity is 10-fold higher than that of the other isozyme (I-P-$450_{16\alpha}$). I-P-$450_{16\alpha}$ is a phenobarbital-inducible isozyme and does not contribute to the 16α-hydroxylase activity in untreated liver microsomes of 129/J mice. The phenobarbital-inducible de novo synthesis of I-P-$450_{16\alpha}$ is not sex dependent. Finally, the two isozymes are immunochemically distinct.

Registry No. Testosterone 16α-hydroxylase, 37364-16-2; cytochrome P-450, 9035-51-2; phenobarbital, 50-06-6.

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