MOLECULAR PHARMACOLOGY, 27:471–479

Sexual Differentiation of Cytochrome P-450 in Rat Liver

Evidence for a Constitutive Isozyme as the Male-Specific 16α-Hydroxylase

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Received August 7, 1984; Accepted January 14, 1985

SUMMARY

Cytochrome P-450 isozyme RLM_5 [Cheng, K-C., and J. B. Schenkman, *J. Biol. Chem.* **257**:2378–2385 (1982)], possessing a high steroid 16α-hydroxylating activity, was isolated from male rat livers, and the hypothesis that this protein is the sexually differentiated 16α-hydroxylase was tested. Isozyme RLM_5 was not detected when the same purification procedure was applied to female rat liver microsomes. However, a female isozyme named DEa and possessing a similiar M_r was obtained from similar column fractions. The DEa isozyme had insignificant steroid-hydroxylating activity and a substrate specificity different from isozyme RLM_5. The two proteins could also be distinguished in their primary structures and immunochemical properties. Rabbit antibodies were raised to isozyme RLM_5 and were made specific by immunoabsorption with a crude female cytochrome P-450 fraction coupled to Affi-Gel 10. The antibodies were used in a Western blot immunoassay to demonstrate that isozyme RLM_5 can be detected in liver microsomes of male rats at levels at least 20 times higher than those in the female, and that its sexual differentiation is neonatally imprinted by androgen. The antibodies were able to specifically inhibit 70% of the testosterone 16α-hydroxylase activity in male rat liver microsomes but had no effect on the activity in females. It was concluded that isozyme RLM_5 is the major sexually differentiated microsomal 16α-hydroxylase.

INTRODUCTION

The hepatic microsomal drug-metabolizing system catalyzes the oxidative and reductive metabolism of a wide variety of xenobiotics and endogenous compounds (1, 2), and thereby is a major route in the detoxification and excretion of such compounds. Sex differences in the microsomal metabolism of drugs (3, 4) and steroids (4, 5) by rat liver have been well documented. In general, males display higher rates of metabolic conversion of most substrates than do females (3–7), although female microsomes do catalyze certain reactions more readily than those of males (8).

The hormonal basis for this sexual differentiation is on two levels. Circulating androgens are important in maintaining a male pattern of metabolism. Thus, castration of adult males results in a decrease in male-specific activities such as the steroid 16α-hydroxylase (4, 5, 7), although not to female levels. Androgen exposure in the neonatal period is also important, imprinting a basic male pattern of metabolism and the ability to respond to androgens later in life (4, 5, 7). The gonadal hormones do not act direclty on the liver, however, but rather affect the pulsatile secretion of growth hormone by the pituitary, which regulates the liver enzymes (4, 9, 10).

The metabolic reactions of drugs and steroids discussed above are each catalyzed by one or more isozymes of cytochrome P-450 (2), which display distinct substrate preferences and turnover numbers (2, 11, 12). The relative concentrations of different isozymes in the endoplasmic reticulum are major determinants of the metabolic profile of the microsomes (13). Recently, efforts have been made to isolate and characterize sexually differentiated forms of P-450.[1] Kato and co-workers (14) have succeeded in purifying isozymes from both male and female rat liver, which were shown to be sexually differentiated by both chromatographic and immunological criteria. Claims from other laboratories that rat isozymes P-450h (11) and P-450 2c (15) may be sexually differentiated await confirmation by immunological methods. This laboratory has recently succeeded in the purification of a female-specific 15β-hydroxylase iso-

This work was supported by Grants 13X-2819 and 03V-6456 from the Swedish Medical Research Council.

[1] The abbreviations and trivial name used are: P-450, cytochrome P-450; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; ELISA, enzyme-linked immunosorbent assay; androstenedione, 4-androstene-3,17-dione; AH-Sepharose, aminohexyl-Sepharose; GPC, glycerophosphatidylcholine; 37K, fraction of M_r = 37,000 (other forms are analogous); MOPS, 4-morpholine propanesulfonic acid.

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zyme, which is normally present only in female rat liver and is regulated by growth hormone (16).

One of the best characterized, sexually differentiated, P-450-dependent activities in microsomes is the testosterone or androstenedione 16α-hydroxylase discussed above. In order to reach an understanding of the hormonal mechanisms governing the sexual differentiation of this activity, it is clearly desirable to obtain the enzyme in purified form. Chao and Chung (17) have partially purified a male P-450 isozyme with high 16α-hydroxylase activity and demonstrated its absence in female rats or neonatally castrated males. However, the preparation had a major protein contaminant. A P-450 isozyme with a high steroid 16α-hydroxylating activity has been purified from normal male rats by Cheng and Schenkman (18) and designated isozyme RLM_5 by these workers. No evidence that this protein is sexually dimorphic was presented. We have reasoned that such a major constitutive isozyme in the male rat, possessing the 16α-hydroxylase activity, merited research into the possibility that it is the sexually differentiated 16α-hydroxylase.

In this study, we have purified isozyme RLM_5 from male rat liver and raised antibodies to it in rabbits. Chromatographic and immunological evidence shows that isozyme RLM_5 is present in significant amounts in the livers of male rats only, indicating that it is indeed a sexually differentiated isozyme.

EXPERIMENTAL PROCEDURES

Preparation of microsomes. Eight-week-old male or female Sprague-Dawley rats were fasted for 16 hr prior to sacrifice by decapitation. Liver microsomes were prepared immediately according to Haugen and Coon (19), extracted with pyrophosphate and used immediately, or stored at −20° until used.

Purification of cytochrome P-450, NADPH-cytochrome P-450 reductase, and cytochrome b_5. P-450 RLM_5 was isolated from male rat liver microsomes by a modification of the published procedure (18). All steps were at 4°. Microsomes from 24 rats were solubilized and chromatographed on a 3.2 × 28 cm column of lauric acid-AH-Sepharose 4B as described (18). The 1% Emulgen 913 eluate was divided in half and applied to two CM-Sepharose columns, which were developed according to the published method (18). Instead of the final step of hydroxylapatite chromatography, the fraction eluted by 45 mM phosphate buffer from the CM-Sepharose column was treated as follows. The combined, dilute pools from the two columns, containing 45 nmol of P-450 in 250 ml, were dialyzed against 10 volumes of 10 mM Tris-acetate buffer, pH 7.4, 25% (v/v) glycerol, and 0.1 mM EDTA and concentrated to approximately 10 ml by calcium phosphate gel absorption (19). The sample was dialyzed against 2 × 1 liter of 5 mM potassium phosphate buffer, pH 7.4, containing 25% (v/v) glycerol, 0.5% (w/v) sodium cholate, 0.2% (w/v) Emulgen 913, and 0.1 mM EDTA, and then loaded onto a 0.9 × 24 cm DE52 column equilibrated with the same buffer. The column was washed with several volumes of the equilibration buffer, and the electrophoretically homogeneous isozyme eluted in the unbound fraction.

Isozyme DEa was isolated from female rat liver microsomes by the same procedure except that it eluted later in the gradient of the CM-Sepharose columns. Thus, the 60 mM sodium phosphate eluate was taken for further purification on DE52 column chromatography. The male P-450 isozyme 2c was isolated to homogeneity by precipitation with polyethylene glycol, followed by column chromatography at room temperature on DE52 and hydroxylapatite, exactly as described by Waxman *et al.* (15).

Detergent was removed from each P-450 isozyme preparation by absorption onto small hydroxylapatite columns (18). The preparations were finally dialyzed against 100 volumes of 50 mM potassium phosphate buffer, pH 7.4, 25% (v/v) glycerol, and 0.1 mM EDTA, and stored at −20°. The final purified, dialyzed preparations of RLM_5, DEa, and 2c had specific contents of 16.7, 12.5, and 14.1 nmol of P-450/mg of protein, respectively.

Electrophoretically homogeneous NADPH-cytochrome P-450 reductase was isolated from liver microsomes of male phenobarbital-treated rats according to Guengerich and Martin (20). The preparation used had a specific activity of 44 units/mg of protein. One unit of reductase catalyzes the NADPH-dependent reduction of 1 μmol of cytochrome *c*/min.

Preparation of antibodies. Antibodies against highly purified P-450 RLM_5 or 2c were raised in male Little German Lop rabbits weighing 2.8–3.2 kg. Each rabbit was injected with 200 μg of the P-450 intradermally in a 1:1 emulsion of Freund's complete adjuvant at multiple sites along the back of the animal. The rabbits were booster injected with 100 μg of protein in Freund's incomplete adjuvant 3 and 6 weeks later. Blood was collected 4 weeks after the second booster, and serum was prepared. The serum titer of antibody was 1:100,000 as determined using an ELISA method (21).

IgG was prepared by absorption of sera on a column of protein A-Sepharose and subsequent elution with 0.15 M acetic acid. Antibodies cross-reacting with nonspecific antigen were removed by immunoabsorption with a total female liver P-450 fraction coupled to Affi-Gel 10. The 1% (w/v) Emulgen 913 eluate (P-450 fraction) from the lauric acid-AH-Sepharose 4B column (female preparation) was concentrated by calcium phosphate gel absorption and dialyzed for 24 hr against 2 × 600 ml of 50 mM MOPS, pH 7.0, containing 0.2% (w/v) Emulgen 913 and 20% (v/v) glycerol. The solution, containing 30 mg of protein in 6.5 ml, was added to 2 ml of washed Affi-Gel 10 and mixed gently for 4 hr at 4°; 0.2 ml of 1.0 M ethanolamine, pH 8.0, was added, and the mixing continued for 1 hr. The red gel was then harvested by centrifugation, transferred to a 0.9-cm-diameter column, and equilibrated with 10 mM potassium phosphate buffer, pH 7.4, containing 0.2 M KCl and 0.2 mM EDTA. The immunoglobulin samples, dialyzed against the same buffer, were passed twice over the column in 100-mg aliquots at a flow rate of 0.1 ml/min. The efficiency of coupling of the P-450 fraction to the Affi-Gel column was 65%.

Enzyme assays. Catalytic activities of P-450 isozymes were determined at 37° in the reconstituted system in which P-450 was the rate-limiting component. The final concentrations of P-450, NADPH-cytochrome P-450 reductase, dilauroyl-GPC, and cytochrome b_5 were 0.05 μM, 0.5 units/ml, 50 μg/ml, and 0.05 μM respectively, unless otherwise stated. Reactions were performed under conditions where product formation was proportional to the time of incubation and the P-450 concentration.

The *N*-demethylation of benzphetamine, aminopyrine, and ethylmorphine was determined in reaction mixtures containing 50 mM potassium phosphate buffer, pH 7.4, 0.5 mM NADPH, and 1.0 mM benzphetamine, 7.0 mM aminopyrine, or 7.0 mM ethylmorphine in a total volume of 1.0 ml. After incubation of the complete mixture for 10 min, 0.3 ml of cold 50% (w/v) trichloroacetic acid was added, and formaldehyde was assayed (22). Aniline 4-hydroxylation was measured in reaction mixtures like that for benzphetamine, but containing 2.5 mM aniline, and *p*-aminophenol was assayed by the method of Schenkman *et al.* (23). The *O*-deethylations of 7-ethoxycoumarin (0.3 mM) and 7-ethoxyresorufin (4 μM) were measured in similar reaction mixtures, except that the concentrations of P-450 and NADPH-cytochrome P-450 reductase were doubled. 7-Hydroxycoumarin formed was assayed by the method of Greenlee and Poland (24), and 7-hydroxyresorufin according to Prough *et al.* (25).

Testosterone hydroxylation in the reconstituted system was determined by the method of Shiverick and Neims (26) in a reaction mixture like that for benzphetamine, but containing 0.12 mM [4-^{14}C]testosterone (13.3 kBq). The reaction was stopped after 10 min and the metabolites were extracted and quantified as described (26). The 16α- and 6β-hydroxylated metabolites were identified by co-migration with au-

thentic cold standard. The 2- and 7-hydroxylated products were identified by comparison of the R_f values with those in the original method. Androstenedione hydroxylation was measured in identical incubation mixtures, except that 0.25 mM [4-^{14}C]androstenedione replaced testosterone. The metabolites were separated and quantified as described previously (5).

Analytical procedures. Cytochrome P-450 concentrations were determined from the CO difference spectrum of the reduced protein, with an extinction coefficient of 91 mM^{-1} cm^{-1} for the difference between the maximum absorbance of the Soret peak and at 490 nm (27). Protein was determined by the method of Lowry *et al.* (28) as modified by Bensadoun and Weinstein (29). Polyacrylamide slab gel electrophoresis was performed in the presence of sodium dodecyl sulfate as described by Laemmli (30) on a Bio-Rad Protean apparatus. Gels were stained with Coomassie brilliant blue, or silver stained by the method of Wray *et al.* (31) as indicated. Standard proteins used and their M_r values were: phosphorylase *b* (94,000), bovine serum albumin (67,000), ovalbumin (43,000), carbonic anhydrase (30,000), and soybean trypsin inhibitor (20,000).

Peptide maps. Proteolytic maps were obtained by a modification of the method of Cleveland *et al.* (32). To a solution containing 5 μg of P-450 in 24 μl of 50 mM potassium phosphate buffer, pH 7.4, and 25% (v/v) glycerol was added 3 μl each of 1% (w/v) Emulgen 913 and 1% (w/v) sodium dodecyl sulfate. After boiling for 2 min, the protease was added in a volume of 3 μl to give the desired final concentration. The proteolysis proceeded at 37°, until the reaction was stopped by the addition of 30 μl of SDS-PAGE sample solubilization buffer and boiling for 3 min. After cooling, the samples were applied in their entirety to 12.5% polyacrylamide slab gels, 0.75 mm thick, in order to separate the peptides.

Western blot immunoassay. Proteins from SDS-PAGE gels were blotted on nitrocellulose filters and detected immunologically as described previously (33), except that 5% (w/v) nonfat dried milk powder replaced bovine serum albumin in the solutions (Semper brand). Antibody-antigen complexes were visualized by reaction with ^{125}I-protein A and autoradiography.

Materials. Dilauroyl-GPC, aminopyrine, 16α-hydroxytestosterone, and papain were from Sigma. Testosterone and androstenedione were obtained from the Upjohn Co.; the 4-^{14}C-labeled steroids and ^{125}I-protein A were from New England Nuclear. 7-Ethoxycoumarin and aniline were from Aldrich, 7-ethoxyresorufin was from Pierce, ethylmorphine was from Leo Pharmaceuticals, 6β-hydroxytestosterone was from Steraloids Inc., Affi-Gel 10 was from Bio-Rad, sodium cholate was from Merck, protein A-Sepharose and protein standards were from Pharmacia, and α-chymotrypsin was from Worthington. Emulgen 913 was a generous gift from Kao-Atlas, Japan, and benzphetamine was kindly donated by Dr. M. J. Coon, University of Michigan. Lauric acid-AH-Sepharose 4B was synthesized according to Gibson and Schenkman (34). Highly purified rat liver epoxide hydrase and phenobarbital-inducible P-450 were kindly donated by Dr. James Halpert, University of Arizona.

RESULTS

Purification of male and female P-450 isozymes. Solubilization of male rat liver microsomes, followed by column chromatography on lauric acid-AH-Sepharose 4B and CM-Sepharose as described by Cheng and Schenkman (18), yielded a partially purified RLM_5 preparation having a specific content of 12 nmol/mg. However, the 45 mM phosphate eluate contained only RLM_5 as the major protein, whereas the previous authors also obtained a lower molecular weight isozyme termed RLM_3 in this fraction. The major contaminants of our RLM_5 preparation at this stage were of higher molecular weight. Chromatography on hydroxylapatite (18) gave no further purification. Highly purified RLM_5 was obtained by chromatography on DE52 instead. The fractions in the unbound eluate were monitored by SDS-PAGE, and the homogeneous ones were combined.

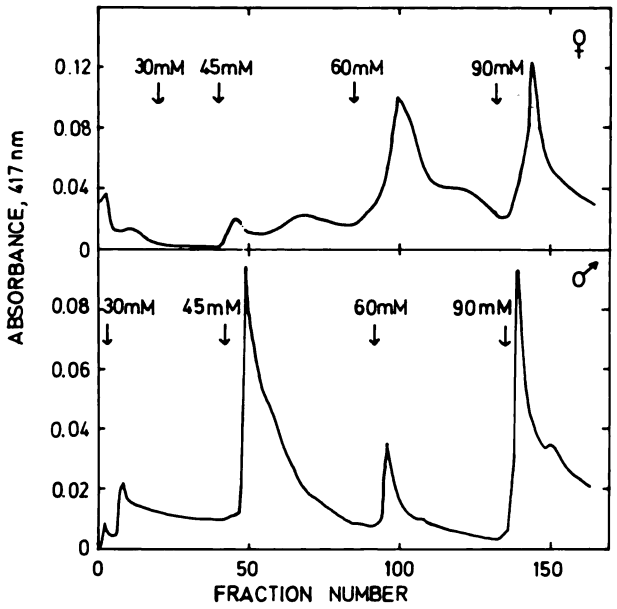

FIG. 1. *CM-Sepharose column profiles of partially purified P-450 fractions from male and female rats*

The P-450 peak eluted from the laurate column by 1% (w/v) Emulgen 913 was applied to a 2.5 × 14 cm CM-Sepharose column (17). The column was eluted with a step gradient of sodium phosphate buffer, pH 6.5, as indicated. Upper chart: P-450 from female rats. Lower chart: P-450 from male rats. The fraction size was 4 ml.

The same purification procedure was applied to female microsomes. In Fig. 1, the CM-Sepharose column profiles from the male and female preparations are compared. Whereas the 45 mM buffer elutes a major peak of male P-450, only a small amount of hemoprotein is usually eluted from the female column. When the buffer strength is increased to 60 mM, however, a major female P-450 peak is eluted. This peak contains essentially two proteins, both P-450s. The lower molecular weight protein is the female-specific steroid sulfate 15β-hydroxylase, and the higher M_r protein, having electrophoretic mobility similar to RLM_5, is termed DEa. Purification of the two female forms is effected on a DE52 column as described in Experimental Procedures, or a DEAE-Sepharose column[2] where form DEa elutes in the unbound fraction and form 15β elutes in a salt gradient. An SDS-PAGE gel of the final purified isozymes DEa and RLM_5, purified from female and male rats, respectively, is shown in Fig. 2. Isozyme RLM_5 is electrophoretically homogeneous, while this preparation of isozyme DEa has two minor contaminants. The minimal molecular weight of both purified isozymes is 52,000.

Isozymes RLM_5 and DEa showed extremely similar chromatographic behavior, differing only in that they eluted at 45 and 60 mM phosphate buffer, respectively, from the CM-Sepharose column. Indeed, occasionally some DEa did elute with the 45 mM buffer in the female purification, and some RLM_5 eluted with the 60 mM buffer in the male purification. In the ensuing experi-

[2] E. T. Morgan and J.-Å. Gustafsson, unpublished observations.

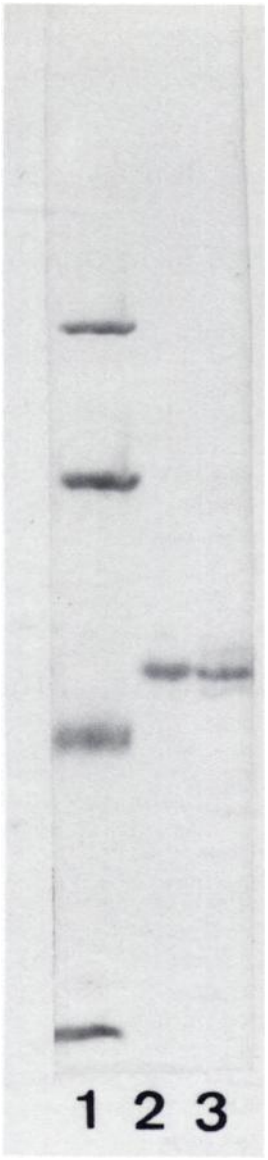

FIG. 2. *SDS-PAGE of purified P-450 preparations*

The samples were analyzed at a protein load of 0.5 μg on slab gels 0.75 mm thick containing 7.5% acrylamide in the separating gel and stained with Coomassie brilliant blue. *1*, molecular weight standards. *2*, P-450 RLM_5. *3*, P-450 DEa.

ments, the possibilities that the two isozymes were identical (i.e., not sexually differentiated) or that the difference between them was due to post-translational modification were carefully tested.

While this work was in progress, Waxman *et al.* (15) reported the purification of P-450 isozyme 2c from male rat liver, possessing high androstenedione and testosterone 16α-hydroxylase activity. These authors could not purify the isozyme from immature male rats and suggested that it might be the sexually differentiated 16α-hydroxylase. We have purified this isozyme to electrophoretic homogeneity by the published method. We have found that isozyme 2c is identical to RLM_5 by all the criteria tested in our laboratory: catalytic activity and regiospecificity, peptide mapping after proteolytic digestion, chromatographic behavior, co-migration on SDS-PAGE, and immunological comparison using antibodies raised separately to both protein preparations together with the Western blot method (results not shown). The following results presented here, however, were all obtained using the isozyme purified by the Cheng and Schenkman method (18), for the purpose of comparison with the female DEa protein.

TABLE 1

Catalytic activities of P-450 isozymes RLM_5 and DEa

Substrate and position hydroxylated	Catalytic activities of isozymes	
	RLM_5	DEa
	nmol product formed/min/nmol P-450	
Aminopyrine	30	2.3
Ethylmorphine	20	9.3
Benzphetamine	24	1.8
Aniline	<0.01[a]	<0.01[a]
7-Ethoxyresorufin	<0.01[a]	0.1
7-Ethoxycoumarin	0.3	<0.01[a]
Testosterone		
16α	4.5	<0.05[a]
7α	0.2	<0.05[a]
6β	0.6	<0.05[a]
2α	3.4	<0.05[a]
Androstenedione		
16α	7.5	0.2
7α	0.5	<0.05[a]
6β	1.0	<0.05[a]

[a] Values at the limits of detection in the assays used.

Catalytic activities and effects of cytochrome b_5. Table 1 shows the catalytic activities of P-450 isozymes RLM_5 and DEa in the reconstituted system. The data for RLM_5 agree well with those of Cheng and Schenkman: the isozyme has high activities in the *N*-demethylation of aminopyrine, ethylmorphine, and benzphetamine and in the 16α- and 2α-hydroxylation of testosterone. We also observed a high activity of RLM_5 in the 16α-hydroxylation of androstenedione. Form DEa, however, has negligible activity towards either of the steroid substrates and relatively low activities with aminopyrine and benzphetamine. Of the substrates studied, this isozyme was best in catalyzing the metabolism of ethylmorphine. The addition of cytochrome b_5 to the incubation medium did not significantly affect the relative abilities of the two isozymes to catalyze testosterone or androstenedione metabolism (results not shown).

The possibility was considered that the differences in catalytic activities of the two isozymes were due to an endogenous inhibitor present in the DEa fraction. This theory was tested as shown in Table 2 by measuring the testosterone-hydroxylating activity of the two forms mixed together in a reconstituted system. The combined 16α- or 2α-hydroxylating activities of the isozymes mixed in a 1:1 ratio was about 70% of the activity expected if the individual turnover numbers are summed. It seems unlikely that such a small decrease in activity could explain the greater than 80-fold difference in, for example, 16α-hydroxylating activities of RLM_5 and DEa by a dissociable inhibitor mechanism. Possibly the deviation from theoretical activities could be caused by unequal competition of the P-450 isozymes for NADPH-cytochrome P-450 reductase.

TABLE 2

Effect of P-450 isozyme DEa on testosterone-hydroxylating activity of isozyme RLM$_5$

The total P-450 concentration in each incubation was held constant at 0.05 μM.

Isozyme	Hydroxylating activity at position			
	16α	7α	6β	2α
	nmol/min/nmol P-450			
RLM$_5$	4.5	0.2	0.6	3.4
DEa	<0.05[a]	<0.05[a]	<0.05[a]	<0.05[a]
RLM$_5$ + DEa[b]	1.8	0.1	0.2	1.3
	(72)[c]	(100)	(67)	(76)

[a] Values at the limit of detection of the assay.

[b] RLM$_5$ and DEa were mixed in a 1:1 molar ratio and incubated for 2 hr at 4° before addition of the other reaction mixture components.

[c] Percentage of calculated theoretical activity.

Peptide map comparison of isozymes RLM$_5$ and DEa. As shown in Fig. 3, the patterns of peptides generated by the proteolytic digestion of isozymes RLM$_5$ and DEa differ distinctly. Digestion of RLM$_5$ with α-chymotrypsin leads to rapid formation of two peptides of M_r = 39,000 and 37,000, which later give way to smaller peptides. With form DEa, the 37K peptide is not formed. In addition, two peptide fragments at M_r = 34,000 and 32,000 are generated from form DEa but not form RLM$_5$. (The gel shown is stained with silver for clarity, but Coomassie-stained gels give the same pattern.) The papain digests of the two isozymes give rather similar peptide patterns, except that form DEa is much less susceptible to digestion than is RLM$_5$. *Staphylococcus aureus* V8 protease digestion of RLM$_5$ also showed differences in the peptide patterns generated from the two isozymes (not shown). These results suggest that the amino acid sequences of the two proteins are different, indicating that they are distinct gene products. However, there may be considerable homology between the two isozymes, since the peptide patterns do show some similarities.

Immunological evidence for sexual differentiation of P-450 RLM$_5$. When rabbit antibody to form RLM$_5$ was used to detect cross-reacting proteins blotted from SDS-PAGE gels onto nitrocellulose filters, cross-reactivity with P-450 isozymes RLM$_5$, DEa, 15β, and PBB$_2$ (phenobarbital-inducible form) was observed when the IgG concentration used was above 50 μg/ml (results not shown).

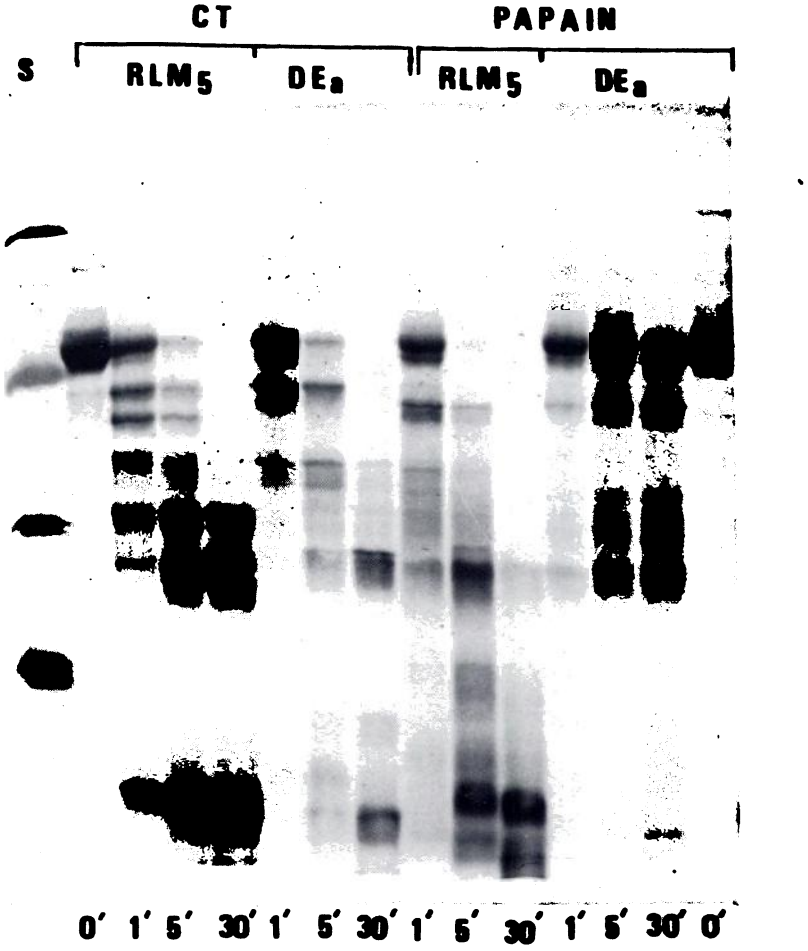

FIG. 3. *SDS-PAGE of peptides generated by proteolytic digestion of isozymes RLM$_5$ and DEa by papain and α-chymotrypsin (CT)*

Electrophoresis of the reaction mixtures was carried out following digestion of the P-450 proteins for 0, 1, 5, or 30 min at 37° as indicated. The protease:P-450 ratios (weight basis) were: α-chymotrypsin, 1:150, and papain, 1:30. Five μg of protein were applied to each lane. The gel was silver-stained. *S*, molecular weight standards.

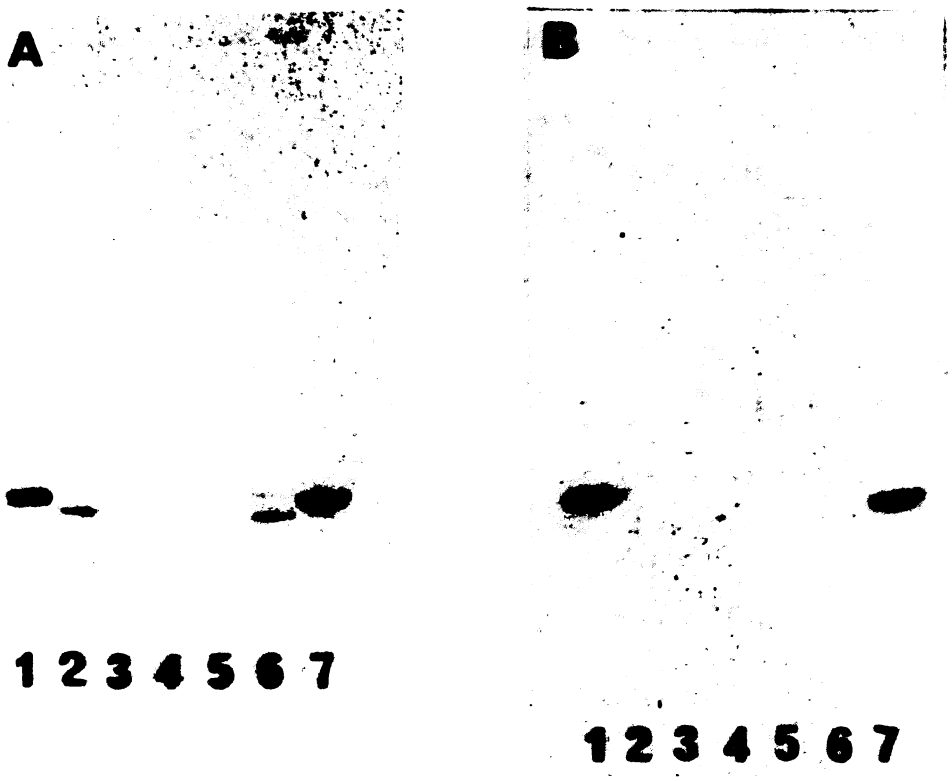

FIG. 4. *Immunoblot of purified P-450 fractions and rat liver microsomes with (A) anti-RLM$_5$ IgG and (B) immunoabsorbed anti-RLM$_5$ IgG*
The protein samples were electrophoresed at the amounts indicated on 0.75-mm-thick, 7.5% polyacrylamide gels and blotted on nitrocellulose filters. The order of proteins in the lanes was identical for the two experiments: *1*, RLM$_5$, 0.5 μg; *2*, purified female isozyme 15β, 0.5 μg; *3*, DEa, 0.5 μg; *4*, purified phenobarbital-inducible P-450, 0.5 μg; *5*, epoxide hydrase, 0.5 μg; *6*, female rat liver microsomes, 10.0 μg; *7*, male rat liver microsomes, 10.0 μg. A, blot incubated with anti-RLM$_5$ IgG, 10 μg/ml. B, blot incubated with immunoabsorbed anti-RLM$_5$ IgG, 20 μg/ml. The procedure for visualization of the blots is described in the text.

Further dilution of the antibody, however, revealed that it was much more specific than indicated by this finding. Fig. 4A shows the blot obtained using an IgG concentration of 10 μg/ml. The antibody recognizes RLM$_5$ under these conditions: only a slight cross-reactivity is observed with form 15β, even less with form DEa, and none with the PBB$_2$ isozyme. A major sex difference is seen in the microsomes. Male microsomes (lane 7) show a single major immunodetectable protein, corresponding to RLM$_5$. Only slight cross-reactivity with forms DEa and 15β in female microsomes is observed. (Identical blots using preimmune serum gave no immunoreactive bands. Radioactivity in the anti-RLM$_5$ blots was proportional to the amount of purified RLM$_5$ or male microsomal protein applied.) We eliminated the residual cross-reactivity by immunoabsorption of the anti-RLM$_5$ IgG with the crude lauric acid column P-450 fraction from female rats. As observed in Fig. 5B, of the purified proteins only isozyme RLM$_5$ is recognized. No immunoreactive proteins were present in female microsomes, while only isozyme RLM$_5$ was detected in male rat liver microsomes. Thus, isozyme RLM$_5$ is a sexually differentiated form of P-450. The specificity of the immunoabsorbed antibody for RLM$_5$ was confirmed using a dot-blot assay in the absence of SDS (not shown).

Inhibition of male microsomal 16α-hydroxylase by anti-RLM$_5$ IgG. Anti-RLM$_5$ IgG prepared by immunoabsorption specifically inhibited the 16α-hydroxylase activity of microsomes from male rats. Fig. 5 shows that microsomes incubated with the immunoabsorbed IgG were significantly inhibited in their testosterone 16α- (Fig. 5A) and 2α-hydroxylating (Fig. 5B) activities, relative to microsomes incubated with preimmune IgG. The maximum observed inhibitions obtained were 79 and 72%, for 16α- and 2α-hydroxylations, respectively. A 26–32% inhibition was seen with preimmune IgG at the same concentrations, so that the activities in the presence of anti-RLM$_5$ IgG were 31 and 38% of those with the control IgG. In contrast, testosterone 6β-hydroxylation, a reaction poorly catalyzed by form RLM$_5$, was unaffected or even stimulated by the anti-RLM$_5$ IgG (Fig. 5C).

The effect of anti-RLM$_5$ on the much lower testosterone 16α-hydroxylating activity of female rat liver microsomes was tested. In contrast to the effects seen with male microsomes, anti-RLM$_5$ IgG at the same concentration did not affect the testosterone 16α-hydroxylase activities of female microsomes (Fig. 6). Indeed, the activities were higher than in the presence of control IgG, which caused a 69% inhibition at the highest concentration. 2α-Hydroxylase activity was not accurately measurable in female microsomes because of the relatively high background of radioactivity in that region.

The anti-RLM$_5$ IgG inhibited the testosterone 16α- and 2α-hydroxylase activities of purified RLM$_5$ by 80%

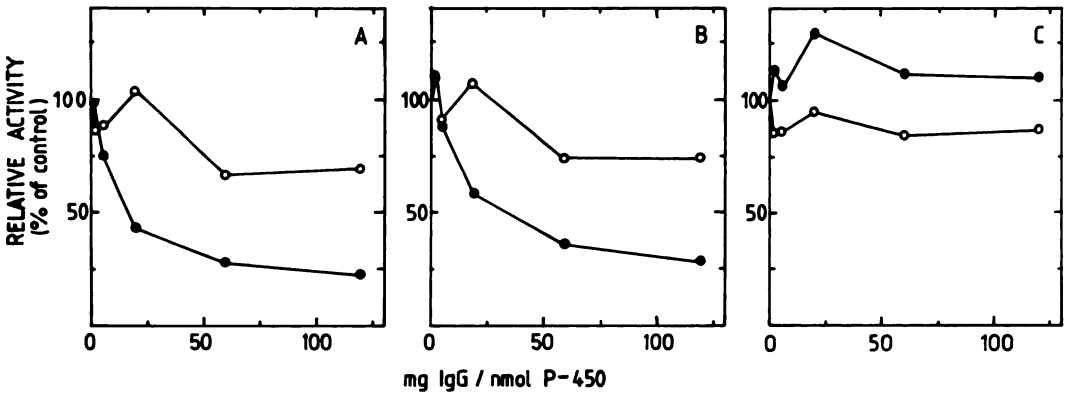

FIG. 5. *Inhibition of testosterone hydroxylase activity of male rat liver microsomes with antibodies to P-450 RLM$_5$*

Pooled microsomes from 10 male rats were preincubated with preimmune IgG (O) or immunoabsorbed anti-RLM$_5$ IgG for 2 hr at 4° (●). The total preincubation volume was held constant by the addition of 50 mM potassium phosphate buffer, pH 7.4, containing 0.2 M KCl. Testosterone-hydroxylating activities were then measured as described in Experimental Procedures. A, 16α-hydroxylation; B, 2α-hydroxylation; C, 6β-hydroxylation. The control activities (100%) were 2.6, 2.3, and 2.0 nmol/nmol P-450/min, respectively.

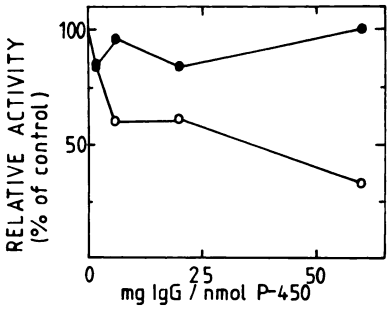

FIG. 6. *Lack of inhibition of testosterone 16α-hydroxylase activities of female rat liver microsomes by antibodies to P-450 RLM$_5$*

Pooled microsomes from 25 female rats were preincubated with preimmune IgG (O) or immunoabsorbed anti-RLM$_5$ IgG (●) for 2 hr at 4°, and testosterone 16α-hydroxylase was determined as described in the text. The control activity (100%) was 32 pmol/nmol P-450/min.

at a concentration of 2 mg of IgG/nmol of P-450, where preimmune IgG had no effect (not shown). Further increases in IgG concentration resulted in significant nonspecific inhibition by the preimmune IgG.

Neonatal imprinting of RLM$_5$ expression by androgen. The sexually differentiated expression of RLM$_5$ was shown to be neonatally imprinted by androgen using the Western blot technique. As seen in Fig. 7, the enzyme is present in male rat liver microsomes at levels 20-fold higher than those observed in the female. Neonatal castration reduces the level in the adult male almost to that of the female. Castration of the male rat after puberty, however, results in only a 40% decrease in RLM$_5$ levels relative to the sham-operated control. This neonatal androgen imprinting is a characteristic property of the microsomal steroid 16α-hydroxylase activity (4, 5, 7), providing strong evidence that isozyme RLM$_5$ is the sexually differentiated 16α-hydroxylase.

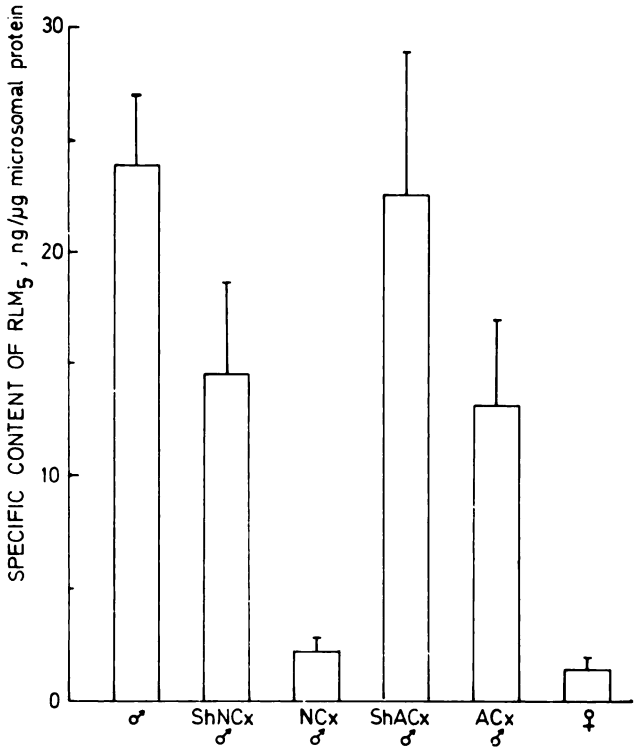

FIG. 7. *Neonatal androgen imprinting of P-450 RLM$_5$ expression determined by immunoquantitation*

Male rats were castrated or sham operated on their first day of life (*NCx*, *ShNCx*), or at the age of 6 weeks (*ACx*, *ShACx*). All animals were killed at 63 days of age, and hepatic microsomes were prepared. The levels of RLM$_5$ protein in the microsomes were determined using the Western immunoblot procedure with a standard curve of the purified protein as described. Values are expressed as the mean ± standard error for each group of three rats.

DISCUSSION

Of all the cytochrome P-450-dependent activities of rat liver microsomes that show sex differences, the steroid 16α-hydroxylase has been the most thoroughly characterized in terms of its hormonal regulation. The dependence of this activity on androgen imprintng in the neonatal period and on circulating androgen later in life has been demonstrated using both androstenedione (4, 5) and testosterone (7) as substrates. The androstenedione 16α-hydroxylase, furthermore, is dependent on the secretory pattern of growth hormone from the pituitary

as a primary mode of control (10). We have recently confirmed that testosterone 16α-hydroxylase in the microsomes is also suppressed by growth hormone infusion.[2] The testosterone and androstenedione 16α-hydroxylases are thus identical in terms of their hormonal regulation.

This paper provides strong evidence that the rat P-450 isozyme RLM_5 is a sexually differentiated form of P-450. Using the Western blot immunoassay and specific antibodies to form RLM_5, we have shown that the isozyme is present in significant quantities in the male rat only, where it may comprise about 40% of the total spectrally measurable P-450 in the microsomes (estimated from the Western blots). It is present in at least 20-fold lower concentrations in female microsomes than in males. Furthermore, RLM_5 cannot be purified from female liver using the same procedure. The corresponding fraction from female rat microsomes gives another purified P-450 isozyme that we have termed DEa, which lacks steroid 16α-hydroxylase activity and which differs from form RLM_5 in other catalytic properties, primary structure (peptide map experiments), and immunological properties. In view of the high activity of the RLM_5 isozyme in the 16α-hydroxylation of testosterone and androstenedione, its sexual differentiation and neonatal imprinting, and the high levels of it in normal male rat microsomes, we propose that P-450 RLM_5 is the sexually differentiated 16α-hydroxylase of rat liver microsomes. This conclusion is supported by our observation of a strong inhibition of 16α-hydroxylase activity by antibodies to RLM_5 in microsomes from male rats but not in those from females. Although anti-RLM_5 IgG inhibited the male 16α-hydroxylase by 80%, a 32% inhibition was observed with preimmune IgG at the same concentration, resulting in a specific inhibition by anti-RLM_5 IgG of 70% relative to the preimmune IgG. The substantial inhibition by preimmune IgG is explained by the high amounts of IgG that had to be used, due to the large decrease in titer of the anti-RLM_5 IgG that occurred during immunoabsorption (not shown). Similar effects of the preimmune IgG were seen with female microsomes, where the specific antibody was not inhibitory. Since the purified RLM_5 was only specifically inhibited by 80% in the reconstituted system, this agrees well with the results observed in the male microsomes. Furthermore, we have observed that growth hormone infusion of normal male rats results in a decrease of RLM_5 levels to that of the female,[2] which also suggests the identity of RLM_5 and the sexually differentiated 16α-hydroxylase. We cannot completely exclude the possibility, however, that other sexually differentiated enzymes may contribute to the microsomal activity.

That the male isozyme we have purified is identical to Cheng and Schenkman's RLM_5 (18) is supported by a comparison of the catalytic and spectral properties (low spin P-450, 451-nm peak in the CO-reduced difference spectrum; results not shown) as well as the minimal M_r values and chromatographic behavior. Ryan *et al.* (11) have proposed that their P-450h is identical to RLM_5, and reported that this protein was purified in only trace amounts from female rats, supporting our data. These authors also speculated that the male-specific P-450 ("P-450 male") purified by Kamataki *et al.* (14) and form UT-A of Guengerich *et al.* (12) are the same protein. P-450 male has been shown to be sexually differentiated, occurring in levels about 30 times higher in the male (14), the differentiation occurring at puberty (35). Form UT-A also appears to be sexually differentiated (36). The steroid-metabolizing capacity of P-450 male has not yet been examined, however, making comparisons difficult. Interestingly, Ryan *et al.* purified another constitutive P-450 isozyme, P-450f, from male rats with an M_r identical to P-450h. It is unlikely, however, that our electrophoretically homogeneous preparation contains P-450f, since the latter is a high spin form of P-450 (11). Our purified preparation has negligible high spin character in the presence or absence of nonionic detergents, as judged by the lack of absorbance maxima at 394 and 640 nm in the UV-visible spectrum (not shown). Form DEa is also a low spin protein.

Waxman *et al.* (15) have purified to homogeneity a P-450 isozyme that they named 2c and also suggested that it might be the sexually differentiated 16α-hydroxylase on the basis of purification data. We have purified this isozyme by the published procedure and found it to be identical to P-450 RLM_5 in its catalytic, physical, and immunochemical properties, supporting these authors' observation. We have found that this procedure is quicker, simpler, and easier to reproduce and gives higher yields than the method of Cheng and Schenkman.

Purification of form DEa from female rat liver microsomes by the described scheme allows simultaneous purification of a female-specific steroid sulfate 15β-hydroxylase. The sexual differentiation of P-450 15β, and the purification of both female proteins are described in more detail in a contemporary publication (16). Form DEa has chromatographic behavior almost identical to isozyme RLM_5. Yet, in the male purification, no protein corresponding to form DEa was ever seen in the equivalent column fractions, only isozyme RLM_5. We believe, therefore, that form DEa may also be a female-specific isozyme of cytochrome P-450. Furthermore, the cross-reactivity of the polyclonal antibodies to form RLM_5 with forms 15β and DEa and the reciprocal cross-reactivity of anti-15β serum (16) suggest a close structural similarity among these three forms. This is supported by the observed similarities in the peptide maps for the RLM_5 and DEa isozymes. Thus, the sexually differentiated isozymes may be products of a single, distinct family of genes.

In view of the known substrate specificity of form RLM_5, it may account for at least two other sexually differentiated activities which are higher in male microsomes. The sex difference in aminopyrine demethylation was discovered by Kato and Takahashi in 1968 (3). P-450 RLM_5 has a high turnover number with this substrate. Cheng and Schenkman (18) have recently reported that this isozyme is also capable of hydroxylating estradiol in the 2-position. Estradiol 2-hydroxylase is also a well characterized male-specific activity (6). Further studies on the substrate specificity of P-450 RLM_5, together with antibody inhibition experiments, should

elucidate how many sex-dependent activities can be accounted for by the male-specific isozyme RLM_5.

ACKNOWLEDGMENT

We wish to thank Ms. Anne-Gerd Nilsson for her excellent technical assistance.

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