Characterization of a Female-Specific Hepatic Mitochondrial Cytochrome P-450 Whose Steady-State Level Is Modulated by Testosterone†

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ABSTRACT: Polyclonal antibody to mitochondrial P-450c27/25 reacted with two proteins of apparent molecular masses of 52 kilodaltons (kDa) and 50 kDa from the female rat liver mitochondrial proteins bound to an ω-octylaminoagarose column. The two proteins were purified to >85% homogeneity by DEAE-Sephacel and hydroxylapatite column chromatography, and both were found to be P-450 as judged by dithionite-reduced CO difference spectra. Both of the P-450 forms required mitochondrial-specific ferredoxin and ferredoxin reductase for in vitro reconstitution of enzyme activities, suggesting that they are mitochondrial forms. The 52-kDa P-450 exhibited the properties of mitochondrial 27/25-hydroxylase with respect to high vitamin D_3 25-hydroxylase activity [1.4 nmol (nmol of P-450)$^{-1}$ min^{-1}] and N-terminal amino acid sequence. The 50-kDa P-450, on the other hand, lacked significant vitamin D_3 25-hydroxylase activity, but showed 17β-reductase [0.380–0.400 nmol (nmol of P-450)$^{-1}$ min^{-1}] and 17β-oxidase [0.1–0.16 nmol (nmol of P-450)$^{-1}$ min^{-1}] activities with both androgens and estrogens as substrates. Immunoblot analysis of proteins using a monoclonal antibody specific for P-450c27/25 showed a 2–3-fold higher level of this enzyme in the female liver mitochondria than in the males. Similarly, use of a polyclonal antibody in the immunoblot analysis showed that the 50-kDa P-450 is female-specific. The relative level of P-450c27/25 was reduced significantly in castrated females, while the level of the female-specific 50-kDa P-450 was increased. However, the levels of both enzymes were increased in castrated males. Testosterone treatment reduced the levels of both enzymes in castrated males as well as in the normal females. Our results suggest that testosterone may directly or indirectly be involved in modulating the levels of these mitochondrial P-450 forms.

Hepatic mitochondria contain some of the enzyme activities needed for the metabolic activation of vitamin D_3 and bile acid synthesis (Jefcoate, 1986; Björkhem, 1989). 27-Hydroxylation of the C-27 sterol side chain, a key step of a major pathway for the conversion of cholesterol to bile acid, is believed to be carried out by a cytochrome P-450 (P-450)[1] localized in the hepatic mitochondria (Björkhem & Gustafsson, 1973; Taniguchi et al., 1973; Björkhem, 1989), while the enzyme activities for the hydroxylation at 3α, 7α, and 12α positions of the sterol ring structure are located in the hepatic microsomal fraction (Danielsson & Sjovall, 1975; Jefcoate, 1986). A mitochondrial P-450 exhibiting the properties of sterol 27-hydroxylase has been purified from rabbit and rat livers (Wikvall, 1984; Okuda et al., 1988) and recently has been characterized by cDNA cloning and expression in monkey COS cells (Andersson et al., 1989). Similarly, conversion of vitamin D_3 to a hormone-like biologically active form involves hydroxylation at C-1 and C-25 positions (DeLuca & Schnoes, 1976; Jefcoate, 1986). It is well documented that vitamin D_3 1α-hydroxylation is carried out by a P-450 located in the kidney mitochondria (DeLuca & Schnoes, 1976; Pedersen et al., 1976) and 25-hydroxylation is carried out in the hepatic tissue. A cytochrome P-450 active in vitamin D_3 25-hydroxylation has recently been purified from rat and rabbit liver mitochondria by a number of groups (Masumoto et al., 1988; Dahlbäck & Wikvall, 1988; Shayiq & Avadhani, 1989; Shayiq et al., 1991). A recent study in our laboratory on the characterization of a cDNA clone by expression in COS cells (Su et al., 1990) demonstrated that the same P-450 enzyme can catalyze both the 25-hydroxylation of vitamin D_3 and the 27-hydroxylation of cholesterol. Thus, the 25-hydroxylase characterized by us (Su et al., 1990) as well as by another group (Usui et al., 1990) is the same P-450 form as the 27-hydroxylase characterized by different investigators (Wikvall, 1984; Okuda et al., 1988; Andersson et al., 1989). This P-450, the gene product of CYP27 (Nebert et al., 1991), will be referred to as P-450c27/25, or 27/25-hydroxylase to adequately describe its functional attributes. An additional P-450 catalyzing the vitamin D_3 25-hydroxylation has also been shown to occur in the microsomal fraction of male rats (Andersson & Jornvall, 1986; Dahlbäck & Wikvall, 1987; Hayashi et al., 1988) and appears to be absent from the female liver (Andersson & Jornvall, 1986). These results suggest an interesting possibility that the physiologically important function of D_3 25-hydroxylation is exclusively delegated to the mitochondrial compartment in the females.

Although mitochondria in animal cells carry out a number of tissue-specific as well as general types of metabolic functions, thus far there is no evidence for the sex-specific differentiation of this organelle with respect to any of the known metabolic functions. In the present study, using a polyclonal antibody to P-450c27/25 as a probe, we have purified and characterized a female-specific mitochondrial P-450 active in 17β-oxidation and reduction of androgens and estrogens. Our results also

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[1] Abbreviations: P-450, cytochrome P-450; SDS, sodium dodecyl sulfate; PEG, poly(ethylene glycol); Fdx, dog hepatic mitochondrial ferredoxin; Fdr, dog hepatic mitochondrial ferredoxin reductase; HPLC, high-pressure liquid chromatography; IgG, immunoglobulin G; P-450 reductase, NADPH–cytochrome P-450 reductase; PB, phenobarbitol; 1α-OH D_3, 1α-hydroxyvitamin D_3; 1α,25-$(OH)_2$ D_3, 1α,25-dihydroxyvitamin D_3.

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show that the level of P-450c27/25 is 2–3-fold higher in the female liver mitochondria than in the male.

Experimental Procedures

Materials. 4-(Dimethylamino)antipyrine, ω-octylamino-agarose, sodium cholate, PEG (8 kDa), $NADP^+$, phosphatidylcholine, *p*-(chloromercuri)benzoic acid, testosterone propionate, and Lubrol PX were purchased from Sigma Chemical Co (St. Louis, MO). DEAE-Sephacel was from Pharmacia Fine Chemicals (Freehold, NJ). Hydroxylapatite (Bio-Gel HTP), Bio-Beads SM2 (20–50 mesh), and chemicals for SDS–polyacrylamide gel electrophoresis were obtained from Bio-Rad Laboratories (Richmond, CA). Nitrocellulose membrane for the Western blot analysis was obtained from Schleicher & Schuell Corp. (Keene, NH). Alkaline phosphatase conjugated secondary antibody and chromogenic substrates for the immunoblot experiments were purchased from Promega Biotech Corp. (Madison, WI). HPLC-grade 2-propanol, hexane, water, and methanol were supplied by Fisher Scientific Corp. (Pittsburg, PA). Bondapak C-18 reverse-phase column (0.39 × 30 cm) and Sep-Pak C-18 cartridges were purchased from Millipore–Waters Corp. (Milford, MA). A Zorbox-Sil column (0.46 × 25 cm) for straight-phase HPLC was purchased from DuPont Co. (Wilmington, DE). [^{3}H]Testosterone (60 Ci/mmol), estrone (60 Ci/mmol), and estradiol (94 Ci/mmol) were purchased from DuPont Co. (Wilmington, DE). [^{14}C]Androstenedione and [^{3}H]-1α,25-$(OH)_2$ D_3 tracer were from Amersham Radiochemical Corp. (Arlington Heights, IL). Unlabeled 1α,25-dihydroxyvitamin D_3 was purchased from Biomol Research Laboratories (Plymouth Meeting, PA). 1α-Hydroxyvitamin D_3 was a kind gift from Professor H. DeLuca, University of Wisconsin, Madison, WI. Female Sprague Dawley rats (150–200-g weight) and surgically treated male as well as female Sprague Dawley rats (castrated at adult stage, 100–120-g weight) were obtained from Harlan Sprague Dawley (Indianapolis, IN).

Purification of Mitochondrial Cytochrome P-450. For each purification, 40–45 overnight-fasted female Sprague Dawley rats (150–200 g) were sacrificed; livers were removed and used for the isolation of mitochondria after washing and perfusing with ice-cold Tris-buffered saline. Livers were homogenized in a sucrose–mannitol–EDTA buffer, and mitochondria were isolated by differential centrifugation as described before (Bhat et al., 1982). Mitochondria were washed 3 times with the isolation buffer, treated with digitonin at a final concentration of 75 μg/mg of protein as described before (Greenawalt, 1974), and washed 3 more times with the isolation buffer to reduce microsomal and peroxisomal contamination. Cytochrome P-450 was solubilized and purified essentially as described for the purification of control P-450 from uninduced male livers (Raza & Avadhani, 1988; Shayiq & Avadhani, 1989; Shayiq et al., 1991), except that higher concentrations of Lubrol PX were used for eluting the P-450 from various columns to improve the yield (Shayiq et al., 1991). Briefly, the PEG fraction solubilized in buffer A (100 mM KH_2PO_4, pH 7.4, 1 mM EDTA, 1 mM DTT, and 20% glycerol) was applied to a preequilibrated ω-octylamino agarose column (2.5 × 50 cm) and washed with 800 mL of equilibration buffer, and the P-450 was eluted with 800 mL of buffer A containing 0.4% Lubrol PX. The P-450-rich fractions were dialyzed against buffer B (10 mM KH_2PO_4, pH 7.7, 0.1 mM EDTA, 0.2% sodium cholate, 0.2% Lubrol PX, and 20% glycerol), concentrated to 30 mL, and applied to a 2.5 × 40 cm DEAE-Sephacel column, preequilibrated with 500 mL of buffer B. The P-450 was eluted with a linear gradient of 0–0.25 M NaCl in buffer B, and fractions of 8 mL each were collected. Fifty microliters each of alternate fractions was subjected to SDS–polyacrylamide gel electrophoresis, and fractions rich in the 50- and 52-kDa P-450 were pooled separately, concentrated, and dialyzed against buffer C (10 mM KH_2PO_4, pH 7.7, 20% glycerol, 0.2% sodium cholate, 0.1 mM EDTA, and 0.05% Lubrol PX). Five to six milliliters of the concentrated and dialyzed sample was loaded onto a 1.4 cm × 12 cm hydroxylapatite column preequilibrated with buffer C. The column was washed with 150 mL of buffer C, and P-450 was eluted with a step gradient of 100 mL each of buffer C containing 40, 60, and 80 mM KH_2PO_4, pH 7.7, respectively, as described before (Shayiq & Avadhani, 1989, 1990). The female-specific isoform of P-450 with an apparent molecular mass of 50 kDa eluted at 40 mM KH_2PO_4, while the isoform with an apparent molecular mass of 52 kDa eluted at 60 mM KH_2PO_4. The purified preparations were freed of detergents by treatment with Bio-Beads SM2 (Shayiq et al., 1991) and stored in aliquots at −80 °C until use. The P-450 content was determined spectrophotometrically as described by Omura and Sato (1964) using an extinction coefficient of 91 cm^{-1} mM^{-1} for sodium dithionite reduced CO-bound P-450.

Purification of Electron-Transfer Proteins. Fdx and Fdr were purified from dog liver mitochondria as described before (Foster & Wilson, 1975; Raza & Avadhani, 1988). Cytochrome P-450 reductase was purified by affinity chromatography on an adenosine diphosphate–agarose column according to Yasukochi & Masters (1976).

Electrophoresis of Proteins and Western Blot Analyses. Proteins were subjected to electrophoresis on 12% SDS–polyacrylamide gels as described by Laemmli (1970) and visualized by staining with Coomassie Blue. The conditions of Western blot analysis were as described by Towbin et al. (1979), and the immunodetection of protein bands on the nitrocellulose membranes was as described before using the appropriate secondary antibodies conjugated to alkaline phosphatase (Raza & Avadhani, 1988; Shayiq & Avadhani, 1989). The relative intensities of bands on the immunoblots were quantitated by scanning the strips through an LKB Ultroscan XL automated system (Pharmacia–LKB Biotechnologies, Piscataway, NJ).

Preparation of Polyclonal and Monoclonal Antibodies. Polyclonal antibodies against purified P-450 were developed in female BALB/c mice by injecting 16 μg of purified 52- or 50-kDa P-450 antigens in Freund's complete adjuvant. The immunization was repeated 2 more times after a 2-week interval each, using Freund's incomplete adjuvant. Following two boosters of 8 μg of antigen each at 2-day intervals, the mice were killed by cervical dislocation, and the blood was taken out directly from the heart. The IgG-rich fraction was precipitated by $(NH_4)_2SO_4$ fractionation (Parham, 1983) and dialyzed against phosphate-buffered saline.

Monoclonal antibodies for P-450c27/25 were developed by fusing the spleenocytes from mice showing the highest antibody titer with the mouse myeloma SP2/O cells using 50% PEG (1000-Da molecular mass) essentially as described by Köhler and Milstein (1975). The antibody-producing hybridoma cells were identified by ELISA screening of culture fluids with purified antigen against a background of myeloma culture fluid used as controls. Finally, the identities of the positive clones were established by Western blot analysis using culture fluids as the source of antibody. Large-scale antibodies were prepared by intraperitioneal injection of 10^5 cells of the positive hybridoma clones. The 2000*g* supernatant of ascites fluid from tumor-bearing animals was subjected to $(NH_4)_2SO_4$ fractionation, and the antibody-rich fraction was dialyzed against

phosphate-buffered saline and stored in 0.1-mL aliquots at −20 °C.

Enzyme Reconstitution. Reconstitution of enzyme activity with purified P-450 preparations was carried out in 0.5-mL final volumes using 0.2 nmol of dog liver Fdx + 0.025 nmol of Fdr or 0.1 nmol of P-450 reductase essentially as described before (Niranjan et al., 1984; Raza & Avadhani, 1988). 1α-OH D_3 (25 nmol/mL) was used as the substrate for assaying the 25-hydroxylase activity. After 30-min incubation at 37 °C, the reaction was terminated by adding 0.5 mL of acetonitrile. The membranous precipitate was removed by centrifugation at 4000g for 10 min, and the clear supernatant was applied to a Sep-Pak C18 cartridge, prewashed with 10 mL of acetonitrile and 10 mL of H_2O. The cartridge was washed successively with 5 mL of water and 3 mL of water–methanol (3:7 v/v) before the vitamin D_3 metabolites were eluted with 3 mL of acetonitrile as described by Gray and Ghazarian (1989). The eluate was evaporated to dryness under N_2, and 1α,25-$(OH)_2$ D_3 was resolved and quantitated by using the straight-phase and reverse-phase HPLC as described before (Raza & Avadhani, 1988; Shayiq & Avadhani, 1990) with some modifications. The dried Sep-Pak C-18 eluate was dissolved in 100 μL of mobile phase (15% 2-propanol in hexane) and injected onto a Zorbox-Sil column. The column was eluted with 15% 2-propanol in hexane at a flow rate of 0.8 mL/min, and the elution pattern was monitored at 254 nm. The retention time for 1α-OH D_3 was 12.1 min, and that for 1α,25-$(OH)_2$ D_3 was 19.9 min. The fractions corresponding to the 1α,25-$(OH)_2$ D_3 peak were collected, dried under N_2, and subjected to reverse-phase HPLC on a Bondapak C18 column. The samples were injected onto the column in 100 μL of 90% methanol and eluted with the same organic phase at a flow rate of 0.8 mL/min. The elution pattern was monitored at 265 nm. Under these conditions 1α,25-$(OH)_2$ D_3 was eluted with a retention time of 8.2 min, while 1α-OH D_3 eluted at 20.5 min. The integration of peaks at 265 nm was carried out by using a Hitachi D-2000 Chromato-Integrator (EM Science, Gibbstown, NJ). The total recovery of metabolite was 69% based on the recovery of [^{3}H]-1α,25-$(OH)_2$ D_3. The 1α,25-$(OH)_2$ D_3 peak was identified and quantitated by comparing with known amounts of authentic metabolite resolved on the reverse-phase column, and the value was corrected for the recovery rate stated above. The 25-hydroxylase activities of intact mitochondria were assayed by using the incubation conditions described by Saarem and Pedersen (1987) at 37 °C for 30 min. The reaction was terminated by adding an equal volume (0.5 mL) of acetonitrile, and the metabolites were extracted and quantitated by the HPLC method as described above.

The 17β-oxidase and reductase activities were assayed by using 27 nmol each of testosterone and androstenedione, and 29 nmol each of 17β-estradiol and estrone as substrates. In each case, the reaction mixture was spiked with 150 000 cpm of respective ^{3}H- or ^{14}C-labeled compounds. After 30-min incubation at 37 °C, the reactions with androstenedione and testosterone were stopped by adding 6.0 mL of ethyl acetate, and those with estrone and estradiol were stopped by adding 0.5 mL of methanol. The steroids were extracted in the organic phase (Waxman et al., 1983), resolved by thin-layer chromatography, and quantitated by using published procedures (Waxman et al., 1983; Barbieri et al., 1986; Wood et al., 1983). Dimethylaminoantipyrine *N*-demethylase and benzphetamine demethylase activities were measured spectrophotometrically as described by Nash (1953) and Cochin and Axelrod (1959).

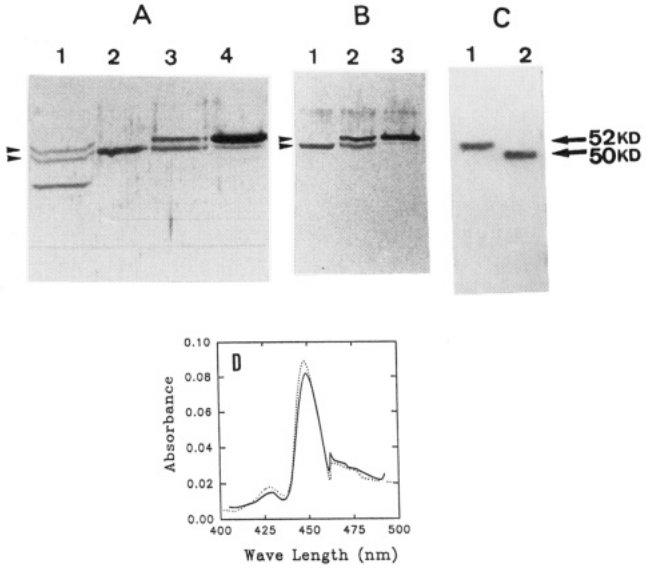

FIGURE 1: Purification of two constitutive forms of mitochondrial P-450 from female rat liver. P-450 was solubilized from female rat liver mitoplasts by sonic disruption and cholate treatment, and purified by chromatography on ω-octylaminoagarose, DEAE-Sephacel, and hydroxylapatite columns as described under Experimental Procedures. Proteins were resolved by electrophoresis through SDS–polyacrylamide gels and either stained with Coomassie Blue or transblotted onto a nitrocellulose membrane for the Western blot analysis also as described in the text. Panel A shows the immunoblot analysis of proteins eluted from the ω-octylaminoagarose column (lane 1) and DEAE-Sephacel column fractions 22–26 (lane 2), 27–30 (lane 3), and 31–38 (lane 4). Twenty micrograms of protein in lane 1 and 8–15 μg in lanes 2–4 were run. Panel B shows the immunoblot analysis of 3 μg each of P-450 from DEAE fractions 22–26 (lane 1) and fractions 31–38 (lane 3) further purified by hydroxylapatite column chromatography. In lane 2, a mixture of the two proteins was loaded. A polyclonal antibody to rat P-450c27/25 (Su et al., 1990) was used to develop the immunoblots in both panels A and B; 4 μg/mL $(NH_4)_2SO_4$-fractionated IgG was used. Panel C shows the Coomassie Blue stained patterns of 3 μg each of the 52-kDa (lane 1) and 50-kDa (lane 2) P-450s (3 μg of each) subjected to electrophoresis. Proteins of known molecular weight were run as markers. Panel D shows the dithionite-reduced CO difference spectra of the 52-kDa P-450c27/25 (—) and the 50-kDa P-450mtf (···).

N-Terminal Sequence Analysis. About 70–90 pmol of P-450 protein electrophoretically transferred to a PVDF Immobilon membrane was sequenced in an Applied Biosystems 475A gas-phase sequencer by the phenylthiohydantoin procedure. The conditions for SDS–polyacrylamide gel electrophoresis and electrophoretic transfer were as described by Matsudaira (1987) and Moos et al. (1988). The sequencing was carried out in Dr. Audree Fowler's facility at UCLA School of Medicine, Los Angeles, CA.

RESULTS

Identification of Two Distinct P-450 Forms in the Female Liver Mitochondria. In the initial experiments, Western blot analysis using the polyclonal antibody to P-450c27/25 revealed the presence of two antibody cross-reacting protein species of 52 and 50 kDa in the octylaminoagarose fractions of female liver mitochondria (Figure 1A, lane 1). The antibody also reacted with a 30-kDa protein which might either be a proteolytic product of the larger species or be an independent protein. A similar fraction from the male liver mitochondria, on the other hand, exhibits only the 52-kDa species characteristic of the 27/25-hydroxylase (Shayiq & Avadhani, 1989; Shayiq et al., 1991), with no detectable 50-kDa species. This apparent sex difference prompted further purification of the 50-kDa protein by DEAE-Sephacel and hydroxylapatite column chromatography. The separation of the 52- and 50-

Table I: In Vitro Reconstitution of Purified P-450 with Different Substrates and Electron-Transfer Systems[a]

P-450 species	electron-transfer systems and additions	activity [nmol (nmol of P-450)$^{-1}$ min^{-1}] aminoantipyrine demethylase	benzphetamine demethylase	D_3 25-hydroxylase
52 kDa (P-450c27/25)	none	0.0	0.0	0.0
	Fdx/Fdr[b]	0.0	0.0	1.38
	Fdx/Fdr + PCMB[c]	0.0	0.0	0.16
	P-450 reductase[d]	0.0	0.0	0.03
50 kDa (P-450mtf)	none	0.0	0.0	0.0
	Fdx/Fdr	9.9	8.2	0.03
	Fdx/Fdr + PCMB	1.02	0.8	0.0
	P-450 reductase	0.0	0.0	0.0

[a] In vitro reconstitution of P-450 and measurements of products were carried out as described under Experimental Procedures. [b] Fdx/Fdr = hepatic mitochondrial ferredoxin and ferredoxin reductase. [c] PCMB = *p*-(chloromercuri)benzoate added at 40 μM concentration. [d] P-450 reductase = rat liver microsomal NADPH–cytochrome P-450 reductase.

Table II: N-Terminal Sequence Analysis of Mitochondrial P-450[a]

P-450 species	amino acid residues 1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
52 kDa (P-450c27/25)	Ala	Ile	Pro	Ala	Ala	Leu	Arg	Asp	His	Glu						
50 kDa (P-450mtf)	Met	Asp	*b*	Phe	Val	Val	Leu	Val (Phe)	Leu	Thr	Leu	*b*	*b*	Leu	Phe	Pro
P-4502C12 (15β)	Met	Asp	Pro	Phe	Val	Val	Leu	Val	Leu	Ser	Leu	Ser	Phe	Leu	Leu	Leu

[a] About 80–90 pmol of P-450 transferred to a PVDF Immobilon membrane was sequenced by the phenylthiohydantoin derivation method as described under Experimental Procedures. The yield of amino acid residues ranged from 56 pmol in early cycles to 20 pmol in the late cycles. The residues shown in parentheses represent minor residues detected during the sequencing. [b] The yield of residues in cycles 3, 12, and 13 of P-450mtf sequencing was less than 4 pmol.

kDa species on the DEAE-Sephacel column was monitored by electrophoretic analysis and Western blotting of column fractions. As shown in the Western blot in Figure 1A (lane 2), DEAE fractions 22–26 (eluting at 40–50 mM NaCl) showed predominantly the 50-kDa antibody-reactive species, while fractions 27–30 (eluting at 50–60 mM NaCl) showed a mixture of both 52- and 50-kDa species (see lane 3). Fractions 31–38 (eluting at 60–75 mM NaCl), however, contained mainly the 52-kDa species as shown in lane 4. The DEAE fractions 22–26 and 31–38 were therefore subjected to further purification by hydroxylapatite column chromatography. Figure 1B shows the immunoblot patterns of 50- (lane 1) and 52-kDa (lane 3) proteins and a mixture of the two (lane 2) proteins purified by hydroxylapatite column chromatography. The electrophoretic patterns of purified 52-kDa protein (lane 1) and purified 50-kDa protein (lane 2) are presented in Figure 1C. The scanning of the Coomassie Blue stained gels using a Pharmacia-LKB Ultroscan indicates that the 52-kDa and the 50-kDa preparations are over 85% homogeneous. As shown in Figure 1D, both of these purified proteins exhibit a CO-reduced absorption maximum in the range of 448–450 nm, characteristic of cytochrome P-450 heme proteins. The P-450 contents of the purified fractions ranged from 13.5 to 15.3 nmol/mg of protein. For the sake of presentation, the 50-kDa P-450 purified in this study is referred to as P-450mtf (for the mitochondrial female-specific form).

Substrate Specificities of the Two P-450s. The P-450s cross-reacting with the polyclonal antibody were studied by reconstitution of enzyme activities using different substrates. As shown in Table I, the 52-kDa species exhibits high 25-hydroxylase activity in the range of 1.3–1.4 nmol (nmol of P-450)$^{-1}$ min^{-1} using 1α-OH D_3 as the substrate. This enzyme also exhibited cholesterol 27-hydroxylase activity in the range of 515–525 pmol (nmol of P-450)$^{-1}$ min^{-1} as reported previously for the 27/25-hydroxylase (Su et al., 1990). It is also seen that the reconstitution of enzyme activity is dependent on the addition of Fdx and Fdr as electron-transport proteins. The P-450mtf, on the other hand, exhibits no detectable 25-hydroxylase activity (Table I), although it has significant aminoantipyrine demethylase [9.9 nmol (nmol of P-450)$^{-1}$ min^{-1}] and benzphetamine demethylase [8.2 nmol (nmol of P-450)$^{-1}$ min^{-1}] activities (Table I). It should be noted that P-450mtf is reconstituted only with the mitochondrial-specific flavoprotein and flavoprotein reductases but not with P-450 reductase, demonstrating that it is truly a mitochondrial-specific form. *p*-(Chloromercuri)benzoate, a sulfhydryl blocking agent, inhibits the activities of both of the P-450s, further supporting that the observed hydroxylations are indeed enzyme-catalyzed reactions.

Female Predominance of Mitochondrial P-450c27/25. Previous studies from this laboratory showed that the PB-induced form P-450mt3 shares immunochemical homology with a P-450 of similar size (52 kDa) isolated from the male hepatic mitochondria as well as with the 27/25-hydroxylase (Shayiq & Avadhani, 1989; Shayiq et al., 1991). Although, both of these forms exhibit identical electrophoretic migration compared to P-450c27/25, they have different catalytic properties (Shayiq & Avadhani, 1989, 1990; Shayiq et al., 1991). Further, as shown in Table II, the 52-kDa P-450 exhibiting high D_3 25-hydroxylase activity has an N-terminal sequence of Ala-Ile-Pro-Ala-Ala-Leu-Arg-Asp-His-Glu, which is identical with the sequence of P-450c27/25 deduced by cDNA sequencing (Su et al., 1990; Usui et al., 1990). The first six residues are also similar to the N-terminal sequence of PB-inducible P-450mt3 reported from this laboratory (Shayiq & Avadhani, 1989). These results suggest a significant level of sequence conservation among different forms of hepatic mitochondrial P-450s characterized thus far.

The observed cross-reactivity of the polyclonal antibody with two other enzymes of similar size, in addition to a 50-kDa P-450mtf, raised further questions on their molecular relationship. It was decided to address these questions by developing monoclonal antibodies which could be used as discriminating probes. Although we obtained an array of hybridoma clones showing different levels of cross-reactivities to different enzymes, a clone designated as 1A9 showed a unique specificity to P-450c27/25. It is seen from Figure 2A that antibody 1A9 does not cross-react with either the male hepatic mitochondrial P-450 or the PB-induced P-450mt3

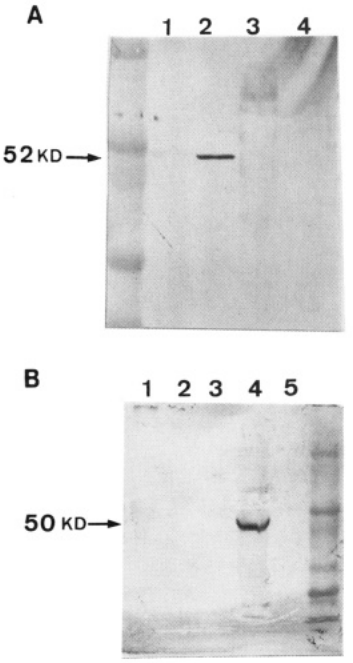

FIGURE 2: Specificity of monoclonal antibody to P-450c27/25 and polyclonal antibody to P-450mtf. Purified mitochondrial P-450 (3 μg of protein each) was subjected to Western blot analysis using either 2.5 μg/mL clone 1A9 monoclonal antibody (panel A) or 4 μg/mL polyclonal antibody to P-450mtf (panel B) as the primary antibodies. (Panel A) Lane 1, constitutive P-450 from male liver mitochondria; lane 2, 52-kDa P-450c27/25 from Figure 1; lane 3, PB-induced P-450mt3; lane 4, 50-kDa P-450mtf from Figure 1. (Panel B) Lane 1, P-450mt3; lane 2, P-450mt4; lane 3, P-450mt2; lane 4, P-450mtf; lane 5, P-450c27/25. Details of electrophoresis and Western blot analysis were as described under Experimental Procedures.

(lanes 1 and 3) though it cross-reacts well with purified P-450c27/25 (lane 2). Additionally, this monoclonal antibody fails to react with the 50-kDa P-450mtf (lane 4). These results firmly establish that P-450c27/25 is distinct from the other similarly sized P-450 isoforms purified from the induced and uninduced mitochondria.

The relative levels of P-450c27/25 in male and female mitochondria under different physiological conditions were studied by the Western blot method using the highly specific 1A9 antibody as the probe, and compared with the 25-hydroxylase activities of intact mitochondrial particles. As shown in Figure 3 (lanes 1 and 6), mitoplasts from female liver contain 2–3-fold higher levels of the antibody-reactive 52-kDa protein than that detectable in the male liver mitoplasts. Castration of male rats results in 50–60% higher levels of the enzyme (lane 2) whereas ovariectomized female rats contain about a 70% reduced level of the enzyme (lane 3). Testosterone treatment results in 40–50% reduced levels of the enzyme in castrated males and also in normal females (see lanes 4 and 5). It is also seen from Figure 3 that the 25-hydroxylase activities of mitochondria from various control and treated rats closely correlate with the levels of antibody-reactive P-450 in these preparations. These results together suggest the female predominance of the hepatic mitochondrial P-450c27/25 and that the level of this enzyme is modulated by sex hormones.

The monoclonal antibody was also used as a unique probe to determine the relative levels of P-450c27/25 in a number of extrahepatic tissues. The Western blot analysis of protein in Figure 4 shows that the level of P-450c27/25 in brain mitochondria is nearly 60–70% of that detected in male liver mitochondria (lanes 1 and 5). Mitochondria from rat lung (lane 3) contain significant levels, while those from kidney

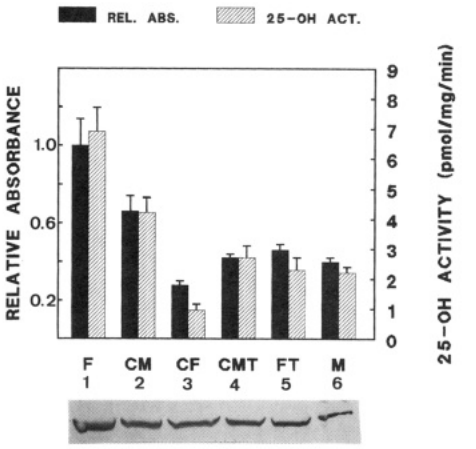

FIGURE 3: Relative levels of P-450c27/25 in the male and female liver mitochondria. Testosterone propionate in 100 μL of corn oil was administered intraperitoneally at the rate of 1 mg/rat for 8 days. The untreated controls received the vehicle alone. Mitochondria were isolated from control, castrated, or testosterone-treated rat livers, and protein samples of 25 μg each were subjected to Western blot analysis as described under Experimental Procedures, using the monoclonal antibody to P-450c27/25 (2.5 μg/mL) as the probe. The mitochondrial preparations were also assayed for 25-hydroxylase activity using 1α-OH D_3 as the substrate. The solid bars represent the relative absorbance of immunoreactive bands scanned through an LKB Ultroscan scanner, and the hatched bars represent the 25-hydroxylase activities (picomoles per milligram of protein per minute) of mitochondrial preparations. Lane 1, mitochondria from female liver (marked F); lane 2, castrated male (marked CM); lane 3, castrated female (marked CF); lane 4, castrated males treated with testosterone (CMT); lane 5, females treated with testosterone (marked FT); lane 6, male (marked M). The mean and SD were calculated on the basis of three independent experiments.

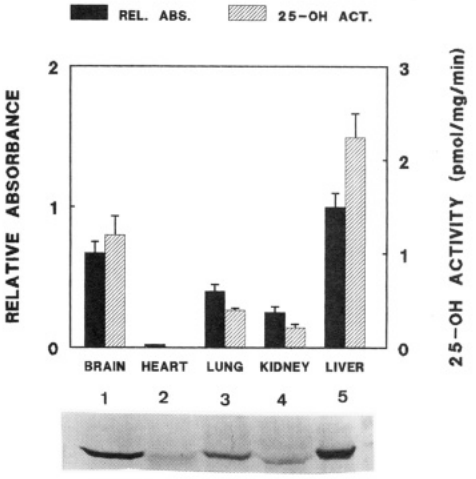

FIGURE 4: Relative levels of P-450c27/25 in mitochondria from different tissues. Mitochondria were prepared from male liver, brain, kidney, heart, and lung and compared by Western blot analysis using the monoclonal antibody to P-450c27/25 (2.5 μg/mL IgG fraction) as described in Figure 3. The 25-hydroxylase activities of mitochondrial preparations were assayed as described under Experimental Procedures. The solid bars indicate the relative intensities of the immunoreactive bands on the Western blot, and the hatched bars represent the 25-hydroxylase activities of various mitochondrial preparations. Lane 1, mitochondria from brain; lane 2, heart; lane 3, lung; lane 4, kidney; lane 5, liver. The mean value and SD were calculated on the basis of three independent experiments.

(lane 4) and heart (lane 2) contain very low to negligible levels. The results of Western blot analysis are consistent with the 25-hydroxylase activities of the respective mitochondrial preparations presented alongside of optical density scans.

Characteristics of P-450mtf. The molecular characteristics of P-450mtf were studied by using a polyclonal antibody raised

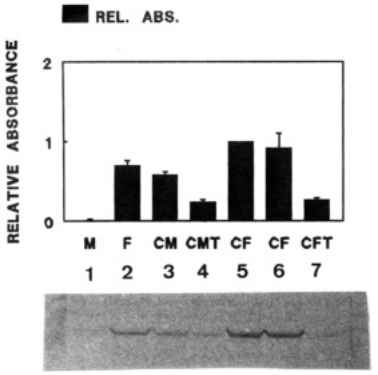

FIGURE 5: Female specificity of hepatic mitochondrial P-450mtf. Mitochondrial proteins (50 μg of each) from various control, castrated, and testosterone-treated rat livers were analyzed by the Western blot procedure using a polyclonal antibody to P-450mtf (4 μg of IgG fraction/mL) as described in Figure 3 and under Experimental Procedures. Lane 1, male (marked M); lane 2, female (marked F); lane 3, castrated male (marked CM); lane 4, castrated males treated with testosterone (marked CMT); lanes 5 and 6, samples from two different castrated females (marked CF); and lane 7, castrated female treated with testosterone (marked CFT). The mean value and the SD were calculated on the basis of three separate experiments.

against the purified enzyme. As shown in Figure 2B, the antibody cross-reacts strongly with P-450mtf (lane 4) but not with any of the other mitochondrial forms tested such as the PB-induced P-450mt3 (lane 1) and mt4 (lane 2), β-naphthoflavone-induced P-450mt2 (lane 3), and P-450c27/25 (lane 5). The observed cross-reactivity of the polyclonal antibody to P-450c27/25 with the 50-kDa P-450mtf may therefore be due to a minor epitope homology between the two enzymes or possibly to a minor contaminating P-450mtf in the P-450c27/25 preparation used for raising the antibody.

The Western blot analysis using the antibody to P-450mtf (see Figure 5) shows a negligible amount of P-450mtf apoprotein in the male liver mitoplasts (lane 1), as against significant levels in mitoplasts from female livers (lane 2). Mitoplasts from castrated male livers show a relatively high level of the enzyme corresponding to about 70% of the level detected in the female liver mitoplasts (lane 3). It is also seen that the level of the enzyme was increased by 40–50% in mitoplasts from castrated female liver (lanes 5 and 6). Administration of testosterone to castrated animals resulted in a 60–70% reduction in the level of immunodetectable protein (Figure 5, lanes 4 and 7). These results suggest the possibility that testosterone may have a repressive effect on this female-specific mitochondrial enzyme.

As shown in Table II, P-450mtf shows an N-terminal sequence 10 out of 16 residues similar to the sequence of female-specific microsomal P-4502C11 with 15β-hydroxylase activity (MacGeoch et al., 1984; Zaphiropoulos et al., 1988). The ability of P-450mtf to metabolize diverse substrates, such as aminoantipyrine, benzphetamine, and structurally diverse steroids, was studied to understand the possible physiological role of this enzyme. As shown in Table III, P-450mtf can reduce androstenedione to testosterone and estrone to estradiol at comparable turnover rates of 0.380–0.400 min^{-1}. Further, this enzyme can also carry out the oxidation of testosterone and estradiol to respective keto forms, though at considerably lower efficiencies of 0.100–0.160 nmol (nmol of P-450)$^{-1}$ min^{-1}. Although not presented, the enzyme exhibited very low to negligible 2α-, 7α-, and 16α-hydroxylase activities. Furthermore, as shown in Table III, CO treatment results in a near 90% inhibition of the activity, confirming that the observed steroid oxidation and reduction are indeed due to a P-450-catalyzed reaction.

Table III: 17β-Oxidation and -Reduction of Steroid Hormones by P-450mtf[a]

substrate	product assayed	treatment	activity [pmol (nmol of P-450)$^{-1}$ min^{-1}] 17β-oxidase	17β-reductase
androstenedione	testosterone	none		400
		CO		18.7 (95)
testosterone	androstenedione	none	100	
estrone	estradiol	none		380
		CO		16.0 (96)
estradiol	estrone	none	160	

[a] P-450mtf was reconstituted with the mitochondrial Fdx/Fdr electron-transfer system using ^{14}C-labeled androstenedione and ^{3}H-labeled testosterone, estrone, and estradiol as substrates. The extraction of steroids in the organic phase and analysis by TLC were as described under Experimental Procedures. Values in parentheses indicate the percent inhibition by CO treatment.

Discussion

Previous studies from this laboratory showed that P-450 inducers, β-naphthoflavone and PB, induced four different hepatic mitochondrial forms suggesting the occurrence of multiple species of P-450 in hepatic mitochondria (Shayiq & Avadhani, 1989; Raza & Avadhani, 1988; Niranjan et al., 1984). The results also showed that the mitochondrial P-450mt4, resembling the major microsomal P-4502B1, was induced by PB predominantly in the male livers, suggesting a sex-dependent induction (Shayiq & Avadhani, 1990). The present study describes two different constitutive forms of mitochondrial P-450 expressed in a sex-predominant or sex-specific fashion in the female rat livers as follows: (1) Using a highly specific monoclonal antibody, we report that mitochondrial P-450c27/25 occurs at 2–3-fold higher levels in the female livers than in the males. (2) Using the polyclonal antibody to P-450c27/25 as a probe, we have purified and characterized a female-specific mitochondrial P-450 which can catalyze the 17β-oxidation and reduction of androgens and estrogens.

It is well established that mitochondria from steroidogenic tissues, such as adrenal cortex, contain P-450 enzymes involved in cholesterol side-chain cleavage (P-45011A1) and 11β-hydroxylation (P-45011B1) as well as 18α-hydroxylation steps of the steroid hormone biosynthetic pathways (Shikita & Hall, 1973; Mitani et al., 1982; Jefcoate, 1986). A recent report indicates the presence of a yet another P-450 exhibiting the properties of aldosterone synthase in the glomerulosa zona of rat adrenal cortex (Ogishima et al., 1989). It has also been demonstrated that oligodendroglial cells contain P-45011A1 (P-450ssc), which might be involved in the synthesis of "neuro steroids" (Goascogne et al., 1987; Yi-Hu et al., 1987). A constitutive form of P-450c27/25 exhibiting vitamin D_3 25-hydroxylase activity has been purified by several groups including our own and characterized to varying degrees (Masumoto et al., 1988; Dahlbäck & Wikvall, 1988; Shayiq & Avadhani, 1989; Su et al., 1990; Usui et al., 1990; Shayiq et al., 1991). It has also been shown that a polyclonal antibody to P-450c27/25 cross-reacts with a constitutive form of mitochondrial P-450 from male liver of yet unknown function (Shayiq & Avadhani, 1989), and also the PB-induced P-450mt3 (Shayiq & Avadhani, 1989; Shayiq et al., 1991). The results of N-terminal sequencing presented here along with those reported previously suggest that there may be at least three different P-450 forms in rat liver mitochondria having close structural and immunochemical similarities but different catalytic properties. Similarly, Northern blot analysis of our

own and others (Su et al., 1990; Andersson et al., 1989) indicated the presence of multiple RNA species in some of the rat and rabbit tissues hybridizing with the P-450c27/25 cDNA probe. For these reasons, the use of polyclonal antibody or the cDNA probes to quantitate the level of P-450c27/25 can be misleading. We have circumvented the problem of quantitating the levels of P-450c27/25 by developing a monoclonal antibody highly specific for this enzyme. The results of quantitation using the monoclonal antibody probe clearly show the female predominance of P-450c27/25 in the adult rats (see Figure 3). These results confirm and extend the observations of Saarem and Pedersen (1987), which showed significantly higher levels of D_3 25-hydroxylation activity in isolated intact mitochondria from the female rats in comparison to mitochondria from adult males. Further, the results of Andersson and Jornvall (1986) showed the apparent lack of the microsomal form of P-450 with vitamin D_3 25-hydroxylase activity in the female liver which was shown to be present in livers of male rats (Andersson & Jornvall, 1986; Dahlbäck & Wikvall, 1987; Hayashi et al., 1988). It is therefore possible that the relative abundance of the mitochondrial P-450c27/25 in the female liver observed in the present study might be to compensate for the lack of the microsomal form of the enzyme from the female liver.

The monoclonal antibody was also used as a selective probe to quantitate the levels of P-450c27/25 in mitochondria from different extrahepatic tissues. The results of immunoblot analysis and enzyme activity measurements in Figure 4 show a surprisingly high level of P-450c27/25 in brain, and very low to negligible levels in kidney and heart mitochondria. Recent studies have shown that brain mitochondria contain enzyme systems for the conversion of cholesterol to progesterone (Goascogne et al., 1987; Yi-Hu et al., 1987). It is likely that P-450c27/25 may be involved in the synthesis of regulatory oxysterols which regulate cholesterol homeostasis and thus modulate the levels of cholesterol available for the synthesis of neurosteroids. The present results on the occurrence of P-450c27/25 in the extrahepatic tissues are in general agreement with the results of Northern blot analysis (Andersson et al., 1989; Su et al., 1990) using P-450c27/25 cDNA, and in vitro studies on the detection of 27-hydroxylase activity in brain and kidney mitochondria (Pedersen et al., 1989; Postlind & Wikvall, 1989).

It has been shown that castration of male rats results in reduced levels of some male-specific P-450 isoforms (Dannan et al., 1986; Wong et al., 1987) while similar treatments result in the induction of the female-specific P-4502C12 with 15β-hydroxylase activity (Morgan et al., 1985; Wong et al., 1987). The results of this study show that the levels of both the female-predominant P-45027/25 and female-specific P-450mtf are elevated markedly in castrated males. However, the levels of these enzymes in ovariectomized females show contrasting patterns in that P-450c27/25 is reduced by nearly 60%, while P-450mtf is increased. The reasons for these divergent effects remain unknown at the present time. Further, as shown for the mouse and rat P-4502C12 (Kamataki et al., 1983; Squires & Negishi, 1988), administration of testosterone significantly reduces the levels of the enzymes in both castrated males and normal females, suggesting that the androgens might be directly or indirectly involved in modulating the levels of their expression. In contrast to the induction of male-specific P-4502C11 (Dannan et al., 1986; Wong et al., 1987), testosterone appears to negatively modulate the level of the female-specific mitochondrial enzyme. It remains to be seen if this observed effect is due to the direct action of androgens at the level of gene expression or if it involves the pituitary pathway as shown for two different female-specific P-450 forms (Noshiro & Negishi, 1988; Guzelian et al., 1988).

The immunoblot analysis of octylaminoagarose column fractions from the female liver mitochondria, using the polyclonal antibody to P-450c27/25, led to the identification and eventual purification of a female-specific 50-kDa P-450mtf. The results of N-terminal sequence analysis together with the immunoblot analysis using a highly specific monoclonal antibody suggested that P-450mtf is a distinct P-450 different from P-450c27/25 and two related P-450 isoforms purified from uninduced and PB-induced male rats. This view is further supported by the fact that P-450mtf has no detectable 25-hydroxylase activity as opposed to a turnover number of 1.3–1.4 min^{-1} observed with the 52-kDa P-450c27/25 using 1α-OH D_3 as the substrate. P-450mtf, on the other hand, exhibits high benzphetamine demethylase and aminoantipyrine demethylase activities (Table I), which are essentially absent from the 52-kDa P-450c27/25 (Shayiq & Avadhani, 1990). It should also be noted that the 50-kDa female-specific mitochondrial P-450mtf exhibits partial resemblance to the female-specific microsomal P-4502C12 (also referred to as P-450i or P-45015β) with respect to N-terminal sequence. The mitochondrial isoform, however, represents an independent species as shown by its distinct catalytic properties and its requirement for Fdx and Fdr as carriers of electrons from NADPH. In this respect, the present P-450 isoform falls in the category of two other mitochondrial P-450 forms, P-450mt2 and P-450mt4, which show immunological similarity to some of the major microsomal forms (Raza & Avadhani, 1988; Shayiq & Avadhani, 1990), further suggesting the possibility that a number of mitochondrial forms might have evolved from genes coding for different microsomal counterparts.

A key observation of this study is the ability of P-450mtf to carry out oxidation and reduction of androgens and estrogens. As shown in Table III, this enzyme can reversibly reduce androstenedione to active testosterone and also carry out a similar reduction of estrone to 17β-estradiol. Both of these activities are dependent on the presence of NADPH and require both Fdx and Fdr, suggesting that they are indeed P-450-dependent activities. It is widely believed that distinct forms of non-P-450 types of dehydrogenases are involved in the interconversion of androstenedione to testosterone and of estrone to 17β-estradiol, respectively (Engel & Gorman, 1974; Pittaway, 1978; Barbieri, 1988), though a male-specific microsomal P-4502C11 (P-450h) and a major microsomal PB-induced form of P-4502B1 (Waxman et al., 1983; Sonderfan et al., 1987) have been shown to contain some activity for the interconversion of androgens. To our knowledge, the female-specific hepatic mitochondrial form characterized in this study is the only P-450 which can carry out the 17β-oxidation of both estrogens and androgens. Although the physiological significance of the P-450 exhibiting the 17β-oxidoreductase activity remains to be elucidated, the present study describes the first mitochondrial enzyme expressed in a sexually dimorphic form.

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Registry No. Ferredoxin reductase, 9029-33-8; vitamin D_3 25-hydroxylase, 65589-62-0; steroid 17β-reductase, 9028-63-1; testo-

sterone, 58-22-0; cytochrome P450, 9035-51-2.

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