Participation of two structurally related enzymes in rat hepatic microsomal androstenedione 7 α -hydroxylation

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Rat hepatic cytochrome *P*-450 form 3 (testosterone 7 α -hydroxylase; *P*-450 gene *IIA1*) and *P*-450 form RLM2 (testosterone 15 α -hydroxylase; *P*-450 gene *IIA2*) are 88% identical in primary structure, yet they hydroxylate testosterone with distinct and apparently unrelated regioselectivities. In this study, androstenedione and progesterone were used to assess the regioselectivity and stereospecificity of these two *P*-450 enzymes towards other steroid substrates. Although *P*-450 RLM2 exhibited low 7 α -hydroxylase activity with testosterone or progesterone as substrate (turnover number ≤ 1 –2 nmol of metabolite/min per nmol of *P*-450), it did catalyse androstenedione 7 α -hydroxylation at a high rate (21 min⁻¹) which exceeded that of *P*-450 3 (7 min⁻¹). However, whereas *P*-450 3 exhibited a high specificity for hydroxylation of these steroids at the 7 α position (95–97% of total activity), *P*-450 RLM2 actively metabolized these compounds at four or more major sites including the nearby C-15 position, which dominated in the case of testosterone and progesterone. The observation that androstenedione is actively 7 α -hydroxylated by purified *P*-450 RLM2 suggested that this *P*-450 enzyme might make significant contributions to microsomal androstenedione 7 α -hydroxylation, an activity that was previously reported to be associated with immunoreactive *P*-450 3. Antibody inhibition experiments were therefore carried out in liver microsomes using polyclonal anti-(*P*-450 3) antibodies which cross-react with *P*-450 RLM2, and using a monoclonal antibody that is reactive with and inhibitory towards *P*-450 3 but not *P*-450 RLM2. *P*-450 3 was thus shown to catalyse only around 35% of the total androstenedione 7 α -hydroxylase activity in uninduced adult male rat liver microsomes, with the balance attributed to *P*-450 RLM2. The *P*-450-3-dependent 7 α -hydroxylase activity was increased to ~65% of the total in phenobarbital-induced adult male microsomes, and to > 90% of the total in untreated adult female rat liver microsomes. These observations are consistent with the inducibility of *P*-450 3 by phenobarbital and with the absence of *P*-450 RLM2 from adult female rat liver respectively. These findings establish that *P*-450 RLM2 and *P*-450 3 can both contribute significantly to microsomal androstenedione 7 α -hydroxylation, thus demonstrating that the 7 α -hydroxylation of this androgen does not serve as a specific catalytic monitor for microsomal *P*-450 3.

INTRODUCTION

Steroid hormones are actively metabolized in mammalian liver through pathways involving oxidation, reduction and conjugation reactions. Steroid hydroxylation in this tissue is catalysed by members of the cytochrome *P*-450 superfamily, which exhibit diverse activities for the metabolism of these endogenous substrates at multiple sites. Rat hepatic *P*-450 form 3 (*P*-450 gene *IIA1*) is a female-predominant cytochrome *P*-450 enzyme that displays a high stereoselectivity for hydroxylation of steroid hormones at the 7 α -position [1,2]. This hydroxylation reaction appears to be specifically catalysed by *P*-450 3, and has been useful for monitoring changes in *P*-450 3 protein levels in liver microsomal fractions isolated from rats differing in their developmental or hormonal status or in their history of exposure to xenobiotics (for review, see [3]). Recent studies have identified a distinct *P*-450 form, designated RLM2 (gene *IIA2*) [4], that is immunochemically related to *P*-450 3 but which is

subject to markedly different regulation by developmental and hormonal factors [5]. Although these two *P*-450s are 88% identical in primary structure [6], their testosterone hydroxylase profiles reveal no apparent similarities [6,7]. This situation contrasts with that of several other pairs of structurally related *P*-450s, such as the rat *P*-450 forms PB-4 (gene *IIB1*) and PB-5 (gene *IIB2*) [1,2], as well as the human *P*-450s PCN1 (gene *IIIA4*) and PCN3 (gene *IIIA5*) [8], which yield rather similar patterns of steroid metabolites. This suggests that the site-specificity of steroid hydroxylation catalysed by these microsomal *P*-450s may be determined in part by a segment of the polypeptide chain that corresponds to one of the local regions of dissimilarity [6] between the two otherwise closely related cytochromes *P*-450 3 and *P*-450 RLM2.

In addition to testosterone, two other steroid hormones, androstenedione and progesterone, have also been used as catalytic probes for *P*-450 enzyme specificity [4,9–11]. It was therefore of interest to compare the

Abbreviation used: *P*-450, cytochrome *P*-450. Rat liver *P*-450 3 (gene *IIA1*) (originally designated *P*-450 PB-3 [1]) is also termed *P*-450a [2] and *P*-450 UT-F [29]. *P*-450 RLM2 (gene *IIA2*) is designated according to Jansson *et al.* [4]. See [30] for an updated listing of *P*-450 gene designations and [3,5] for listings of alternative *P*-450 protein nomenclatures.

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activities of *P*-450 3 and *P*-450 RLM2 with these other steroids to probe for any catalytic similarities or overlapping hydroxylation specificities that might become apparent. The results presented in this study indicate that purified *P*-450 RLM2 is an active catalyst of 7α -hydroxylation of androstenedione but not of progesterone, and that in contrast with *P*-450 3, *P*-450 RLM2 hydroxylates these endogenous substrates at several other positions as well. Moreover, microsomal androstenedione 7α -hydroxylation in adult male rat liver is shown to be catalysed by both *P*-450 3 and *P*-450 RLM2, demonstrating that this microsomal hydroxylation reaction cannot be used as a specific monitor for any single hepatic *P*-450 form.

METHODS

P-450 enzymes and antibodies

P-450 forms 3 [1] and RLM2 [6] and NADPH *P*-450 reductase [12] were purified to apparent homogeneity from Sprague-Dawley rat liver as described previously. Inhibitory polyclonal antibody to *P*-450 3 [5,10], and a monoclonal antibody raised to *P*-450 PB-4 (IIB1) that is cross-reactive with *P*-450 3 (antibody B3; clone 4-7-1 [13,14]) but does not recognize *P*-450 RLM2 (see the Results section), were as described previously. Monoclonal antibodies were kindly provided by Dr. S. S. Park and Dr. H. V. Gelboin, NCI, Bethesda, MD, U.S.A. Purified IgG fractions prepared as described by McKinney & Parkinson [15] were used for antibody inhibition experiments. *P*-450 form specificities of these antibodies were assessed using a dot immunoblotting assay [14].

Steroid hydroxylase assays

Purified and detergent-free *P*-450 enzymes (10 pmol of haemoprotein) were reconstituted for 20 min at 20–22 °C with rat liver NADPH *P*-450 reductase (1 unit) and freshly sonicated dilauroylphosphatidylcholine (2 µg) in 0.05 ml of 0.1 M-KP_i, pH 7.4/20% (w/v) glycerol/0.1 mM-EDTA containing 30 nmol of the steroid substrate (i.e. [4 - 14 C]androstenedione, [4 - 14 C]progesterone or [4 - 14 C]testosterone, 3.5–8.7 mCi/mmol; Amersham). Samples were then diluted to 0.375 ml with 0.1 M-KP_i (pH 7.4)/0.1 mM-EDTA and reactions were initiated by the addition of 120 nmol of NADPH in 0.025 ml. Reactions were incubated for 10 min at 37 °C and then extracted and analysed on silica gel t.l.c. plates developed sequentially in solvents AA (androstenedione), NRR (progesterone) and AB (testosterone) using methods described elsewhere [1,9] (see Table 1 for t.l.c. solvent compositions).

Identification of hydroxysteroid metabolites: t.l.c. analysis

Radiolabelled steroid metabolites were purified from enzyme incubations on silica gel plates and then compared with monohydroxy steroid standards by co-chromatography on silica gel plates developed in three or four independent solvent systems [1]. Androstenedione metabolites were analysed in solvents AA, PQ and I, and progesterone metabolites were analysed in solvents M, N, OI and NRR (Table 1). Each hydroxysteroid metabolite was thus shown to co-chromatograph with its corresponding authentic standard and to be distinct from all other available hydroxylated derivatives of the parent steroid (listed below). Similarly, metabolites

Table 1. Solvent systems used for silica gel analysis of hydroxysteroid metabolites

Hydroxysteroid metabolites and/or their acetylated derivatives were chromatographed on silica gel t.l.c. plates developed in these mixtures of solvents as specified in the text. In some cases plates were sequentially developed in multiple solvent systems to optimally resolve the metabolites of interest (e.g. solvent system N followed by two developments in solvent R is solvent NRR for progesterone metabolites of *P*-450 RLM2). See [1] for further methodological details.

Solvent system	Solvent components	Ratios by vol.
A	Methylene chloride/acetone	4:1
B	Chloroform/ethyl acetate/100% ethanol	4:1:0.7
F	Benzene/acetone	4:1
G	Chloroform/ethyl acetate	20:1
H	Chloroform/ethyl acetate	1:2
I	Ethyl acetate/n-hexane/acetic acid	19:5:1
M	Diethyl ether/acetone	4:1
N	Ethyl acetate/n-hexane/acetic acid	16:8:1
O	Benzene/ethyl acetate	3:1
P	Methylene chloride/100% ethanol	97:3
Q	Chloroform/ethyl acetate	1:1
R	Benzene/ethyl acetate/acetone	10:1:1
X	Chloroform/ethyl acetate/100% ethanol	4:1:0.2

designated as unidentified or tentatively identified were clearly distinguished from all of the available product standards. Finally, the identities of the three major androstenedione metabolites produced by *P*-450 RLM2 (7α OH, 6β OH, 16α OH) were confirmed by acetylation of the purified metabolites using the methods of Swinney *et al.* [11] followed by analysis of t.l.c. plates developed in solvents F and GG [1] in comparison with acetylated standards prepared in parallel.

Gas chromatography and h.p.l.c. analysis

The identities of the 7α OH- and 6β OH-androstenedione metabolites produced by *P*-450 RLM2 were confirmed by gas chromatography on an HP-17 series 530 µ column following derivatization (Hewlett-Packard instrument HP5890A; oven temperature, 250 °C; detector temperature, 270 °C; He flow, 12 ml/min) [R_t of 7α OH-androstenedione (relative to 5α -cholestane), 1.48; R_t of 6β OH-androstenedione, 1.27]. Briefly, enzyme incubations were extracted and metabolites were resolved by t.l.c. (solvents AA or PQ). Individual metabolites were eluted from the plate with ethyl acetate, dried under N₂, and then derivatized with 0.01 ml of Tri-Sil (Pierce) (30 min at 20–22 °C). Samples were N₂-dried and then extracted twice with hexane (0.05 ml each; solvent-dried over MgSO₄). The combined hexane extracts were concentrated under N₂ and then analysed by gas chromatography.

The 15α OH- and 6β OH-progesterone metabolites produced by *P*-450 RLM2 were confirmed to co-migrate with authentic standards by h.p.l.c. in solvent system I of Swinney *et al.* [11]; these latter analyses were kindly carried out by Dr. Wayne Levin, Hoffmann-La Roche, Nutley, NJ, U.S.A.

Metabolite standards

Available androstenedione metabolite standards were those hydroxylated at the 2 α , 2 β , 4, 6 α , 6 β , 7 α , 7 β , 11 α , 11 β , 14 α , 15 α , 16 α and 16 β positions, and progesterone metabolites were those with hydroxyl groups at the 2 α , 6 α , 6 β , 7 α , 11 α , 11 β , 12 α , 14 α , 15 α , 16 α , 17 α , 18, 19, 20 α , 20 β and 21 positions. Available testosterone metabolites were as listed previously [6]. Steroid standards were obtained from Professor D. N. Kirk, M.R.C. Steroid Reference Collection, Queen Mary College, University of London, U.K. and from other sources identified previously [1,9,16]. ^{14}C -labelled 16 β OH-androstenedione and 7 α OH-progesterone were isolated from incubations with purified *P*-450 PB-4 [12] and *P*-450 3 [1] respectively.

RESULTS

Site-specific hydroxylation of testosterone and androstenedione catalysed by *P*-450 RLM2

As reported previously and confirmed in the present study, *P*-450 3 and *P*-450 RLM2 hydroxylate testosterone at similar rates, but with markedly different regioselectivities and stereospecificities (Table 2). *P*-450 3 hydroxylated testosterone (as well as androstenedione and progesterone) at the 7 α position (turnover 13.4 min $^{-1}$, corresponding to > 95% of the observed metabolism) and at the 6 α position (turnover 0.6 min $^{-1}$, ~5% of

the total). In addition, Δ^6 -testosterone (i.e. 17 β OH-4,6-androstadiene-3-one) [17], which co-migrates with unmetabolized testosterone on t.l.c., could be detected on h.p.l.c. by its characteristic absorbance at 280 nm [18]. In contrast, *P*-450 RLM2 hydroxylated testosterone at the 7 α position to only a minor extent (~6% of total metabolism), with the major hydroxylation occurring at the 15 α position (49%) and at an unidentified site (30%). This hydroxylated metabolite was originally thought to be 7 β OH-testosterone [4] but was subsequently shown to be distinct from an authentic 7 β OH standard [6]. Although it was recently concluded on the basis of h.p.l.c. elution position that this unknown RLM2 metabolite is probably 18-OH-testosterone [19], the unknown was clearly distinguished from 18-OH-testosterone in the present study by chromatography on t.l.c. plates developed in solvents AB [R_F of unknown, 0.30; R_F of 18-OH-testosterone, 0.45]; it thus remains unidentified.

Although *P*-450 RLM2 exhibited low 7 α -hydroxylase activity with testosterone, it catalysed androstenedione 7 α -hydroxylation at a high-rate (20.8 min $^{-1}$) which exceeded that determined for *P*-450 3 (7 min $^{-1}$) in a parallel incubation. This 7 α -hydroxylation reaction corresponded to the most prominent activity of *P*-450 RLM2 with androstenedione as substrate (51% of total activity). Other prominent metabolites of *P*-450 RLM2 included 6 β OH-androstenedione and 16 α OH-androstenedione (Fig. 1; Table 2).

Table 2. Site specificity of androgen hydroxylation catalysed by *P*-450 RLM2 and *P*-450 3

Purified *P*-450 RLM2 and *P*-450 3 (10 pmol) were assayed in parallel for hydroxylation of [^{14}C]androstenedione and [^{14}C]testosterone (75 μM) after reconstitution with NADPH *P*-450 reductase. Metabolites were resolved on t.l.c. plates developed in solvents AA and AB respectively and then quantified by liquid scintillation counting (e.g. Fig. 1). Rates of formation of the individual hydroxylated metabolites are expressed as apparent turnover numbers (min $^{-1}$ $\cdot$ *P*-450 $^{-1}$) and as a percentage of total hydroxylation activity (numbers in parentheses). Formation of dehydro metabolites [17] (which generally co-migrated with unmetabolized steroid on these t.l.c. plates) was not monitored. Other monohydroxylated metabolites for which authentic standards are available (see the Methods section) were not detected (≤ 0.1 – 0.2 nmol of metabolite/min per nmol of *P*-450).

Hydroxylation site	Isoenzyme ...	Activity (nmol of metabolite/min per nmol of <i>P</i> -450)			
		<i>P</i> -450 RLM2 (IIA2)		<i>P</i> -450 3 (IIA1)	
	Substrate...	Androstenedione	Testosterone	Androstenedione	Testosterone
6 α		—*	—†	0.23 (3)	0.6 (4)
6 β		10.7 (26)	2.1 (12)	—	—
7 α		20.8 (51)	1.1 (6)	7.0 (97)	13.4 (96)
7 β		—	—	—	—
14 α		0.3 (1)	—	—	—
15 α		1.5* (4)	8.4 (49)	—	—
15 β		—‡	0.5 (3)	—	—
16 α		5.5 (13)	—	—	—
Unidentified		2.2§ (5)	5.2 (30)	—	—
Total activity		41	17.3	7.2	14.0

* 6 α OH-Androstenedione was not sufficiently resolved from 15 α OH-androstenedione in any of the t.l.c. solvent systems examined. The minor *P*-450 RLM2 metabolite that chromatographs in this region of the t.l.c. plate is tentatively assigned as 15 α OH-androstenedione (rather than 6 α OH-androstenedione) on the basis of the high testosterone 15 α -hydroxylase activity of this *P*-450 and the absence of 6 α -hydroxylase activity using testosterone (this Table) and progesterone (Table 3) as substrates.

† < 0.1 – 0.2 nmol/min per nmol of *P*-450.

‡ The precise lower limit of this activity could not be specified since standard 15 β OH-androstenedione was not available.

§ T.l.c. mobility similar to that of [^{14}C]16 β OH-androstenedione formed by purified *P*-450 PB-4.

|| This compound corresponds to the major RLM2 metabolite originally designated 7 β OH-testosterone [4] and more recently thought to be 18OH-testosterone [19]. It was clearly distinguished from both of these compounds by t.l.c. analysis in the present study (see also [6]).

Fig. 1. Androstenedione hydroxylation catalysed by *P*-450 forms 3 and RLM2

^{14}C -labelled androstenedione metabolites formed by purified reconstituted *P*-450 3 (lane 2) and *P*-450 RLM2 (lane 3) were extracted into ethyl acetate then resolved on a silica gel t.l.c. plate developed twice in solvent A (see Table 1). Shown is an autoradiogram of the t.l.c. plate, with the three major sites of hydroxylation by *P*-450 RLM2 marked on the right and minor sites of hydroxylation (not visible at this exposure) marked on the left (16β identification is tentative; see also Table 2). Standard 11β -hydroxy and 2α -hydroxy androstenedione migrated to the positions marked by circles drawn on the autoradiograph. Lane 1, control minus *P*-450. The dark spot present in all three samples corresponds to unmetabolized androstenedione.

Progesterone hydroxylation catalysed by *P*-450 RLM2

P-450 RLM2 actively metabolized progesterone at multiple sites (Table 3). This broad profile of metabolites contrasts with the metabolism of progesterone by *P*-450 3, which proceeds with high selectivity for the 7α (95%) and 6α (5%) positions (Table 3) [9,11]. In previous studies, the progesterone metabolites of *P*-450 RLM2 were separated following chromatography on silica gel t.l.c. plates developed once in solvent X [4,7]. However, we found that solvent ineffective at resolving even the

major metabolites; sequential development in solvents NRR was required to adequately separate these metabolites, designated a–d (Table 3). In addition, two minor RLM2 metabolites (designated e and f) could be resolved when a two-dimensional t.l.c. analysis was performed (Fig. 2). [In solvent X, metabolite b precisely co-migrates with metabolite d ($6\beta\text{OH}$ -progesterone, see below), and this appears to account for the significantly higher $6\beta\text{OH}/15\alpha\text{OH}$ -progesterone product ratio reported by Thummel *et al.* [7] ($6\beta/15\alpha = 2.4$) compared with the present study ($6\beta/15\alpha = 1.2$).]

Metabolites a and d were identified as $15\alpha\text{OH}$ -progesterone and $6\beta\text{OH}$ -progesterone respectively by their co-migration with authentic standards on t.l.c. and h.p.l.c. (see the Methods section). Metabolites b and f were distinguished by t.l.c. from each of the 16 individual monohydroxy progesterone available to us (see the Methods section) and thus remain unidentified. Metabolite c, which was also distinguished from each of these monohydroxy progesterone, corresponds to the major RLM2 metabolite characterized by Jansson *et al.* [4] as $15\beta\text{OH}$ -progesterone by comparison of its mass spectrum with a literature spectrum of authentic standard. Metabolite e, corresponding to only 3% of the total metabolites, exhibited a chromatographic mobility similar to that of $7\alpha\text{OH}$ -progesterone. Thus *P*-450 RLM2 catalyses 7α -hydroxylation of progesterone to only a minor extent at most. All six progesterone metabolites were readily distinguished from authentic $16\alpha\text{OH}$ -progesterone and $6\alpha\text{OH}$ -progesterone by t.l.c. in solvents NRR, in disagreement with the earlier identification of these two compounds among the major progesterone metabolites of *P*-450 RLM2 [4,7].

Role of *P*-450 RLM2 in microsomal androstenedione 7α -hydroxylation

The above observation that purified *P*-450 RLM2 catalyses androstenedione 7α -hydroxylation at a high rate suggested that this *P*-450 might make a significant contribution to microsomal androstenedione 7α -hydroxylation, an activity that was previously reported to be specifically associated with immunoreactive *P*-450 3 [10]. This contribution could be particularly important in adult male rat liver microsomes, in which the levels of these two *P*-450 forms are similar [5]. This question was examined in antibody inhibition experiments using two distinct anti-*P*-450 antibodies. The first, a rabbit polyclonal antibody raised to purified *P*-450 3, is cross-reactive with *P*-450 RLM2 (Fig. 3, lane 3) but not with 10 other rat hepatic *P*-450 forms [5]. The other, a monoclonal antibody raised to the phenobarbital-inducible *P*-450 PB-4 (IIB1), is cross-reactive with *P*-450 3 [as well as with *P*-450 PB-5 (IIB2)] [14] but not with *P*-450 RLM2 (Fig. 3, lane 2). Both antibodies are inherently inhibitory, as shown by their inhibition ($\geq 80\%$) of testosterone 7α -hydroxylation catalysed by purified *P*-450 3 and by liver microsomes, where *P*-450 3 appears to be the predominant catalyst ($\geq 80\%$) of this hydroxylase activity [10,14,20]. These antibodies were therefore used as inhibitory probes for the contributions made by *P*-450 3 plus *P*-450 RLM2 (polyclonal antibody) and *P*-450 3 alone (monoclonal antibody) to androstenedione 7α -hydroxylation catalysed by rat liver microsomes. Comparison of the inhibitory activity of this monoclonal antibody with that of another, inhibitory, monoclonal antibody raised to *P*-450 PB-4, but

Table 3. Progesterone hydroxylation catalysed by *P*-450 RLM2

Progesterone metabolism catalysed by *P*-450 RLM2 was assayed as described in the legend to Table 2 (75 μ M steroid substrate). Metabolites a–f (designated according to Fig. 2) were resolved on t.l.c. plates developed sequentially in solvents NRR; adequate resolution of the minor metabolites e and f required two-dimensional t.l.c. analysis (Fig. 2). *P*-450 3 assayed in parallel yielded 7 α OH-progesterone and 6 α OH-progesterone (turnover = 5.8 and 0.4 min⁻¹·*P*-450⁻¹ respectively) as the only resolved metabolites (not shown). Metabolite c is tentatively identified as 15 β OH-progesterone (see text), and metabolite e may correspond to 7 α -OH-progesterone (see footnote^a). Values in parentheses refer to percentages of total hydroxylation activity.

Metabolite	Identification*	T.l.c. mobility (R_F)	Activity (nmol/min per nmol of <i>P</i> -450)
a	15 α OH-P	0.24	9.9 (13)
b	–†	0.31	14.9 (20)
c	15 β OH-P (tentative)	0.36	32.3 (43)
d	6 β OH-P	0.46	12.3 (16)
e	–	0.28	1.9 (3)
f	–	0.47	3.3 (4)
Total activity			74.6

*P; progesterone. 15 α and 6 β metabolites were identified by t.l.c. and confirmed by h.p.l.c. (see the Methods section). Tentative assignment of the 15 β metabolite is based on conclusions of Jansson *et al.* [4] (see the text). Metabolites b and f were shown to be distinct from all 17 available standard progesterone metabolites. Metabolite e exhibited a mobility similar to that of a 7 α OH-[¹⁴C]progesterone standard formed enzymically by purified *P*-450 3.

† This major metabolite was not resolved from metabolite d (6 β OH-progesterone) under the chromatographic conditions used by Jansson *et al.* [4] and Thummel *et al.* [7] to separate progesterone metabolites produced by *P*-450 RLM2 (see the text).

Fig. 2. Resolution of progesterone metabolites produced by *P*-450 RLM2 by two-dimensional silica gel t.l.c.

¹⁴C-labelled progesterone metabolites formed by *P*-450 RLM2 were extracted into ethyl acetate and then resolved on a t.l.c. plate developed in solvent N in the first dimension and then sequentially in solvents RRRX in the second dimension. Metabolites, detected by autoradiography, are designated a–f (see Table 3). P, unmetabolized progesterone. Sample was applied at the lower left hand corner of the plate.

not cross-reactive with *P*-450 3, confirmed earlier conclusions that *P*-450 PB-4 does not participate in microsomal 7 α -hydroxylation reactions [14].

Polyclonal anti-(*P*-450 3/*P*-450 RLM2) IgG inhibited androstenedione 7 α -hydroxylation catalysed by

Fig. 3. Antibody specificity for *P*-450 3 and *P*-450 RLM2: dot immunoblot analysis

Purified *P*-450 antigens (0, 12.5 or 25 ng, as indicated) were spotted onto a nitrocellulose sheet across each row and individual antibodies incubated down each lane using a Deca-Probe apparatus (Hoefer Scientific) as described elsewhere [14]. Shown is the coloured product deposited on the nitrocellulose after detection with peroxidase-linked second antibody. Incubations were with monoclonal antibodies B2 (clone 2-66-3) and B3 (clone 4-7-1) (lanes 1, 2; 20 μ g of ascites protein/ml) or with polyclonal antibody raised to *P*-450 3 (lane 3; 1:1000 dilution of antiserum). Monoclonal antibodies B2 and B3 were raised to *P*-450 PB-4 and both are cross-reactive with *P*-450 PB-5; in addition, antibody B3 is cross-reactive with *P*-450 3 (see also [14]) but not with *P*-450 RLM2 (lane 2). In contrast, the polyclonal anti-(*P*-450 3) is also cross-reactive with *P*-450 RLM2 (lane 3).

Fig. 4. Contribution of *P*-450 forms 3 and RLM2 to microsomal androstenedione 7 α -hydroxylation as determined by antibody inhibition

Liver microsomes isolated from untreated male (*a,b*) and female rats (*d*) as well as phenobarbital-induced males (*c*) were preincubated with increasing amounts of polyclonal anti-(*P*450 3/RLM2) IgG (*a*) or monoclonal antibody (MAb) that reacts with *P*-450 3 but not with *P*-450 RLM2 (*b-d*). Microsomes were then assayed for hydroxylation of androstenedione at the 7 α and 6 β positions, and in the case of the phenobarbital microsomes, for 16 β -hydroxylation as well. The *P*-450-3-dependent component of the microsomal 7 α -hydroxylase activity is seen to vary with the relative microsomal content of *P*-450 forms 3 and RLM2 (see the text). Inhibition by the MAb of microsomal 16 β -hydroxylation (*c*) is due to the reactivity of this antibody with *P*-450 forms PB-4 and PB-5 (see Fig. 3). Microsomal incubations were carried out with 50 μ M-androstenedione in 0.1 M-Hepes buffer (pH 7.4) containing the steroid 5 α -reductase inhibitor 4-MA [17 β -(*NN*-diethylcarbamoyl)-4-aza-methyl-5 α -androstan-3-one]. [Hepes was used in place of KP_i buffer, since it selectively stimulates microsomal 7 α -hydroxylase activity by about 2-fold. It did not, however, affect the fractional inhibition by these antibodies, suggesting that this buffer stimulates the *P*-450 3- and RLM2-dependent microsomal reactions to a similar extent.] Uninhibited activities were as follows: 7 α = 0.33, 0.33, 0.48, 0.78 nmol/min per mg, and 6 β = 1.2, 1.2, 3.2, 0.09 nmol/min per mg for (*a*)–(*d*) respectively. 16 β = 15.2 nmol/min per mg (*c*). The antibodies had only a minimal effect on the 6 β -hydroxylation reaction, which serves as an internal control. NS, effect of non-specific antibody on 7 α -hydroxylation reaction.

uninduced adult male rat liver microsomes in a selective and near quantitative (> 95%) fashion (Fig. 4*a*), in agreement with our earlier observations [10]. In contrast, the monoclonal anti-(*P*-450 3) IgG inhibited androstenedione 7 α -hydroxylation catalysed by these same liver microsomes by a maximum of only 35% (Fig. 4*b*). A comparison of these results with those of Fig. 4(*a*) suggests that *P*-450 RLM2 catalyses the balance of this microsomal activity, i.e. around 65% of the total. This conclusion is consistent with the high turnover exhibited by purified *P*-450 RLM2 for 7 α -hydroxylation of this substrate (Table 2) coupled with the observation that *P*-450 3 and *P*-450 RLM2 are expressed at similar levels in uninduced adult male rat livers [5].

The participation of *P*-450 RLM2 in microsomal androstenedione 7 α -hydroxylation was further studied by antibody inhibition experiments carried out in liver microsomes that exhibit an altered *P*-450 3/*P*-450 RLM2 ratio. These include (*a*) phenobarbital-induced adult male rat liver microsomes, which have a 2–3-fold elevated level of *P*-450 3 and a somewhat lower level (up to ~30% decrease [5]) in *P*-450 RLM2 compared with the uninduced adult male liver microsomes, and (*b*) uninduced adult female rat liver microsomes, which express *P*-450 3 at approx. 3-fold higher levels than uninduced adult male microsomes but which do not contain detectable amounts of *P*-450 RLM2 [5]. The results of these immunoinhibition experiments indicate that *P*-450 3 mediates ~65% of androstenedione 7 α -hydroxylation catalysed by the phenobarbital-induced male liver microsomes (Fig. 4*c*) and > 90% of the activity catalysed by the uninduced female liver microsomes (Fig. 4*d*). These findings are consistent with the relative levels of *P*-450 3 and *P*-450 RLM2 expressed in these microsomal samples.

DISCUSSION

The present report establishes that *P*-450 RLM2 exhibits a much broader regioselectivity than *P*-450 3 for the hydroxylation of androstenedione and progesterone, and thus extends our previous conclusions in a study using testosterone as substrate [6]. A similar conclusion was recently drawn from studies of progesterone hydroxylation catalysed by *P*-450 RLM2 [7]. Steroid 7 α -hydroxylation, the major reaction catalysed by *P*-450 3 with all three steroids examined, was catalysed by *P*-450 RLM2 to an extent that was highly dependent on the steroid's D-ring substituent at C-17 [C-17 = 17 β -OH group (testosterone), 17-oxo group (androstenedione) or 17 β -acetyl group (progesterone)]; only with androstenedione did *P*-450 RLM2 yield significant amounts of 7 α -hydroxylated metabolite. Hydroxylation at the nearby C-15 position appeared to dominate the metabolism of testosterone and progesterone, but was only a minor pathway for androstenedione metabolism. Moreover, the stereospecificity of hydroxylation at C-15 was markedly affected by the steroid's D-ring substituents. In the case of testosterone, hydroxylation at the 15 α position was much greater than that at 15 β (15 α /15 β = 16.8), whereas in the case of progesterone the stereoselectivity was apparently reversed, with a 15 α /15 β ratio of 0.3. Interestingly, 6 β -hydroxylation catalysed by *P*-450 RLM2 was less sensitive to the C-17 substituent (12–26% of total metabolism for all three steroids) than were the 7 α -hydroxylation and the 15 α - and 15 β -hydroxylation

reactions. Overall, these studies establish that, in spite of their high degree of amino acid sequence conservation (88 % sequence identity; [6]), *P*-450 3 and *P*-450 RLM2 exhibit markedly different hydroxylation profiles. This conclusion is substantiated by analysis of the hydroxysteroid metabolites formed by vaccinia-virus-encoded *P*-450 RLM2 (IIA2). After expression in human HepG2 cells, the recombinant *P*-450 yields metabolites that are qualitatively and quantitatively similar to those produced by purified *P*-450 RLM2 (D. J. Waxman, D. P. Lapenson, T. Aoyama & F. J. Gonzalez, unpublished work). Therefore the broad specificity exhibited by the purified enzyme is inherent to this *P*-450 form, and does not reflect contamination by other *P*-450 enzymes.

Antibody inhibition experiments carried out in this laboratory several years ago established that microsomal formation of 7α OH-androstenedione is specifically associated with immunoreactive *P*-450 3 [10]. The subsequent discovery that polyclonal antibodies to *P*-450 3 are highly cross-reactive with *P*-450 RLM2 [5,7], and the present finding that androstenedione 7α -hydroxylation is actively catalysed by *P*-450 RLM2 in addition to *P*-450 3, prompted us to investigate whether *P*-450 RLM2 also contributes to microsomal 7α -hydroxylation of this androgen. Indeed, *P*-450 RLM2 and *P*-450 3 were both found to participate in this microsomal hydroxylation reaction, with the individual contributions of these two *P*-450 forms dependent on their relative microsomal levels. Therefore, androstenedione 7α -hydroxylation cannot be used as a specific catalytic monitor for microsomal *P*-450 3, except perhaps in immature rats and adult females, which do not express *P*-450 RLM2 at detectable levels [5]. With testosterone as substrate, however, 7α -hydroxylation is catalysed by *P*-450 3 at least 10-fold more actively than by *P*-450 RLM2, and indeed no significant contribution of *P*-450 RLM2 to the corresponding microsomal activity is apparent [5,21]. Therefore microsomal testosterone 7α -hydroxylation can be used as a reliable catalytic monitor for *P*-450 3 expression, as has now been shown by several laboratories [10,20,21].

Although *P*-450 forms 3 and RLM2 are highly similar in primary structure, they are subject to markedly different regulation by gonadal and pituitary hormones [5,22]. The present finding that *P*-450 3 and *P*-450 RLM2 both contribute to microsomal androstenedione 7α -hydroxylation may therefore help to explain why total microsomal androstenedione 7α -hydroxylase activity does not appear to be regulated by gonadal factors [23,24], even though expression of the androstenedione 7α -hydroxylase *P*-450 3 is positively regulated by oestrogen and negatively regulated by androgen [22]. Studies that monitor changes in adult male microsomal androstenedione 7α -hydroxylase activity with hormone or drug treatments [e.g. 24–26] cannot be interpreted in terms of *P*-450 3 alone, insofar as the contributions of *P*-450 RLM2 must also be taken into account.

The precise structural features that determine the narrow, well-defined site selectivity of *P*-450 3 versus the much broader hydroxylation specificity of *P*-450 RLM2 are not yet known. Although these two *P*-450s exhibit a total of 57 amino acid differences, these differences are not randomly distributed but are primarily localized to a limited number of short segments of the polypeptide chain. We have previously observed that one of these segments (amino acid residues 103–130; 50 % dissimi-

larity) also marks a region of local dissimilarity between *P*-450 RLM2 and two other rat hepatic steroid hydroxylase *P*-450s [6]. The corresponding region of *P*-450cam makes several substrate contacts and includes tyrosine-96, which hydrogen bonds to the carbonyl oxygen of its camphor substrate [27]. Mutation of this tyrosine to a phenylalanine results in a loss of that enzyme's absolute regioselectivity for hydroxylation of camphor at the 5-exo position [28]. Interestingly, the highly regioselective *P*-450 3 contains a tyrosine in this region (Tyr-106), whereas the more broadly hydroxylating *P*-450 RLM2 contains a phenylalanine at the corresponding position. It is conceivable, therefore, that Tyr-106 may stabilize substrate bound to *P*-450 3 by hydrogen bonding to the steroid carbonyl, and that the absence of this contact contributes to the much broader site specificity of steroid hydroxylation catalysed by *P*-450 RLM2. Further studies, including *P*-450 3/RLM2 chimaera construction and site-directed mutagenesis, will be required to identify definitively which amino acid differences are the most critical determinants of the divergent substrate specificities of these two hepatic *P*-450 forms.

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