Distinct forms of cytochrome *P*-450 are responsible for 6 β -hydroxylation of bile acids and of neutral steroids

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Cytochrome *P*-450-dependent 6 β -hydroxylation of bile acids in rat liver contributes to the synthesis of the quantitatively important pool of 6-hydroxylated bile acids, as well as to the detoxification of hydrophobic bile acids. The lithocholic acid 6 β -hydroxylation reaction was investigated and compared with androstenedione 6 β -hydroxylation. Differential responses of these two activities to inducers and inhibitors of microsomal *P*-450 enzymes, lack of mutual inhibition by the two substrates and differential inhibition by antibodies raised against several purified hepatic cytochromes *P*-450 were observed. From these results it was concluded that 6 β -hydroxylation of lithocholic acid is catalysed by *P*-450 form(s) different from the subfamily IIIA cytochromes *P*-450 which are responsible for the bulk of microsomal androstenedione 6 β -hydroxylation. Similar, but more tentative, results revealed that the 7 α -hydroxylation of lithocholic acid and of androstenedione may be also catalysed by distinct *P*-450 enzymes. The results indicate that cytochromes *P*-450 hydroxylating bile acids are distinct from analogous enzymes that carry out reactions of the same regio- and stereo-specificity on neutral steroids (steroid hormones). A comparison of pairs of cytochromes *P*-450 that catalyse the same reaction on closely related steroid molecules will help to define those structural elements in the proteins that determine the recognition of their respective substrates.

INTRODUCTION

The conversion of cholesterol into bile acids involves several key cytochrome *P*-450-dependent hydroxylation reactions, including the rate-limiting step of the process, namely cholesterol 7 α -hydroxylation. These reactions play an important role in both cholesterol homeostasis and bile acid biosynthesis, and have been studied in some detail [1]. The bile acids formed in this manner are not metabolically inert terminal cholesterol catabolites, but are subject to numerous interconversions, some of which again involve hydroxylation reactions [2]. These hydroxylations can be distinguished from those required for bile acid biosynthesis in so far as the latter reactions do not proceed with fully formed bile acid molecules as substrates [1].

Hydroxylation of a bile acid molecule decreases its hydrophobicity. Since bile acids are, in the course of the enterohepatic circulation, constantly exposed to the action of intestinal microflora which can cause the loss of hydroxyl groups [3], rehydroxylation is essential in restoring the hydrophilic–hydrophobic balance required for the physiological functions of bile acids. Rehydroxylation also prevents the toxicity that is characteristic of very hydrophobic bile acids, such as the monohydroxylated lithocholic acid (3 α -OH). More generally, hydroxylation of bile acids becomes important in any situation, physiological or pathological, in which the hydrophilic–hydro-

phobic balance of bile acids as a group (loosely translatable into the ratio of trihydroxylated to dihydroxylated bile acids) is disturbed.

In the rat, considerable amounts of 6 β -hydroxylated bile acids (approx. 20% of the total bile acid pool [4]) are found. The predominant of these compounds, β -muricholic acid (3 α ,6 β ,7 β -triOH), is thought to be formed by 6 β -hydroxylation of chenodeoxycholic acid (3 α ,7 α -diOH) to α -muricholic acid (3 α ,6 β ,7 α -triOH), followed by epimerization of the 7 α -hydroxy group [5–12] (Fig. 1). This biosynthetic reaction, which takes place under normal physiological conditions, probably evolved as a means of maintaining a ratio of tri- to di-hydroxylated bile acids that is compatible with the functions of the bile acid pool. The 6 β -hydroxylation of bile acids has, however, an additional role. Certain monohydroxylated bile acids are too hydrophobic to contribute to the physiological functions of bile acids; on the other hand, these compounds are hepatotoxic through an only partially understood mechanism. Lithocholic acid (3 α -OH), formed mainly by bacterial 7 α -dehydroxylation of chenodeoxycholic acid (3 α ,7 α -diOH), and 3 β -hydroxychol-5-enoic acid, probably a product of a minor pathway of direct bile acid biosynthesis from cholesterol [13], are the two major examples of such compounds. It is fairly well established, at least for lithocholic acid, that one of the main detoxification reactions is 6-hydroxylation (see Discussion in [14,15]). In the rat, 6 β -hydroxyl-

Abbreviations used: lithocholic acid, 3 α -hydroxy-5 β -cholanoic acid; chenodeoxycholic acid, 3 α ,7 α -dihydroxy-5 β -cholanoic acid; cholic acid, 3 α ,7 α ,12 α -trihydroxy-5 β -cholanoic acid; murideoxycholic acid, 3 α ,6 β -dihydroxy-5 β -cholanoic acid; α -muricholic acid, 3 α ,6 β ,7 α -trihydroxy-5 β -cholanoic acid; β -muricholic acid, 3 α ,6 β ,7 β -trihydroxy-5 β -cholanoic acid; for clarity, the positions of the hydroxyl groups are occasionally given in the text after the trivial name as derivatives of 5 β -cholanoic acid, e.g. murideoxycholic acid (3 α ,6 β -diOH); androstenedione, androst-4-ene-3,17-dione; 4-MA, 17 β -(*N,N*-diethylcarbamoyl)-4-aza-4-methyl-5 α -androstan-3-one; metyrapone, 2-methyl-1,2-bis(3-pyridyl)-1-propanone; *trans*-tamoxifen, *trans*-2-[4-(1,2-diphenyl-1-butenyl)phenoxy]-*N,N*-dimethylethylamine; clotrimazole, 1-[(2-chlorophenyl)diphenylmethyl]imidazole; ketoconazole, 1-acetyl-4-[4-[(2-(2,4-dichlorophenyl)-2-(1H-imidazol-1-ylmethyl)-1,3-dioxolan-4-yl)methoxy]phenyl]piperazine.

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Fig. 1. Selected steps of bile acid metabolism in the rat

Cholesterol is converted in a multi-step pathway to the two primary bile acids, cholic and chenodeoxycholic acids. A part of chenodeoxycholic acid is 6β -hydroxylated to α -muricholic acid, which in turn is converted by epimerization to β -muricholic acid [5–12]. Bacterial 7α -dehydroxylation of chenodeoxycholic acid leads to the formation of lithocholic acid. The latter is efficiently 6β -hydroxylated [18], and the intermediate murideoxycholic acid can be further transformed into β -muricholic acid, either through 7α -hydroxylation followed by epimerization, or through a hypothetical 7β -hydroxylation [6]. The 6β -hydroxylation reactions of interest in the context of the present work are shown as bold arrows. The bile acids on the scheme are arranged, from left to right, in an approximate order of decreasing hydrophobicity.

ation predominates [7,16–18] (Fig. 1). The murideoxycholic acid ($3\alpha,6\beta$ -diOH) formed from lithocholic acid by 6β -hydroxylation can re-enter the main bile acid pool through subsequent further hydroxylation to the muricholic acids [18]. This series of reactions has therefore both a detoxification and a salvage function.

In spite of the physiological importance of bile acid 6β -hydroxylation in the rat, the enzymology of the process has not been studied in detail. Early work demonstrated that the reaction is catalysed by cytochrome *P*-450 [7,19,20]. Since the multiplicity of cytochromes *P*-450 was not fully appreciated at that time, the specific *P*-450 enzyme(s) involved were not identified. The formally analogous 6β -hydroxylation reaction of a different steroidal substrate, androstenedione, known to be catalysed in rat liver microsomes primarily by the constitutive cytochrome *P*-450III_{A2} and the inducible cytochrome *P*-450III_{A1} [21], initially suggested that lithocholic acid may be 6β -hydroxylated by the same enzyme(s). The present work proves that this is not the case; distinct forms of *P*-450 are involved in the hydroxylation of each substrate.

MATERIALS AND METHODS

Animals

Male and female Fischer 344 rats, approx. 10 weeks of age, were treated with phenobarbital (1 g/litre in drinking water for 5 days), dexamethasone (four daily intraperitoneal injections of 100 mg/kg body wt., dissolved in corn oil), clofibrate (three daily intraperitoneal injections of 400 mg/kg body wt., dissolved in corn oil), or remained untreated (controls). At the end of the treatment period, the animals were killed and the livers were excised and frozen. For inhibition experiments involving metyrapone, *trans*-tamoxifen, ketoconazole and clotrimazole, microsomes from untreated male Sprague–Dawley rats were used. All procedures involving animals have been reviewed and approved by the institutional Animal Care and Use Committees.

Preparation of microsomes

Livers were frozen and stored at -80°C . All subsequent steps were carried out at 4°C . After thawing, the tissue was minced with scissors in 0.25 M-sucrose/10 mM-Tris/HCl (pH 7.8)/1 mM-EDTA/0.1 mM-phenylmethanesulphonyl fluoride. After decanting, a fresh portion (3–4 ml/g of original liver wt.) of the above buffer without phenylmethanesulphonyl fluoride was added, and the tissue was disrupted using a motorized glass-Teflon homogenizer. The suspension was centrifuged for 20 min at 13 000 *g*, and the supernatant was centrifuged for 45 min at 105 000 *g*. The pellet was suspended in 0.1 M-potassium phosphate/20% (v/v) glycerol/0.1 mM-EDTA (pH 7.4) and centrifuged for 90 min at 105 000 *g*. The final pellet was suspended in the above buffer and stored in small portions at -80°C . Protein was determined using the Bio-Rad (Richmond, CA, U.S.A.) kit, based on the method of Bradford [22], with BSA as standard.

Enzyme assays

Lithocholic acid 6β -hydroxylase activity was measured using 100 μg of microsomal protein/assay as described previously [18], except that the concentration of Hepes/NaOH was 50 mM and that of EDTA was 0.05 mM. The androstenedione 6β -hydroxylase assay was similar to that described by Waxman *et al.* [23]. Briefly, 12 μg of microsomal protein was incubated in 50 mM-Hepes/NaOH (pH 7.4)/5 μM of the 5α -reductase inhibitor 4-MA/0.05 mM-EDTA/2 mM-dithiothreitol/50 μM -[^{14}C]androstenedione (6 nCi/nmol)/4.0 mM-NADPH in a final volume of 80 μl . The radiolabelled androstenedione (4 nmol) in benzene was dried with a stream of N_2 in individual assay tubes prior to the addition of other assay components. Reactions were started with NADPH, and were stopped after 10 min at 37°C by addition of 20 μl of ice-cold ethanol. A sample of 60 μl was directly applied to the pre-adsorbent layer of a thin layer plate [type Si250-PA(19C); J. T. Baker, Phillipsburg, NJ, U.S.A.]. The plates were developed in dichloromethane/ethanol (95:5, v/v), followed by a second development in chloroform/ethyl acetate (1:1, v/v). Microsomal testosterone 6β -hydroxylation was assayed according to LeBlanc & Waxman [24].

Labelled products were detected by autoradiography (48–96 h exposure on Kodak X-OMAT AR film at -80°C) and quantified by scraping of zones containing the compounds of interest followed by scintillation counting. Products were identified by comparison with authentic standards.

Antibody and inhibition studies

Inhibition of microsomal androstenedione, testosterone and lithocholic acid 6β -hydroxylation by anti-*P*-450 antibodies was measured essentially as described above, with the following modifications. Unless stated otherwise, assays were carried out

Table 1. Inducibility of rat liver microsomal androstenedione and lithocholic acid 6 β - and 7 α -hydroxylation

The specific activities of the above reactions in Fischer rat liver microsomes are shown for untreated control animals (Con) and for phenobarbital (PB)-, dexamethasone (Dex)-, and clofibrate (Clof)-treated male (σ) and female (φ) animals. In parentheses, normalized specific activities, expressed as percentages of the corresponding control value, are listed. The ratio of the normalized specific activities for lithocholic acid and for androstenedione is also shown. A ratio close to unity signifies that the induction of hydroxylation activity by a given inducer is similar for both substrates. Cases in which the induction of lithocholic acid hydroxylation differs significantly from the induction of androstenedione hydroxylation, as shown by Student's *t* test, are marked by * ($P < 0.001$) or ** ($P < 0.0000001$). For each inducing agent, microsomes were prepared from four individual animals, and each microsomal preparation was assayed in duplicate. Results are shown as means $\pm$ S.D.

	Specific activity (nmol/min per mg) (%)		
	Lithocholic acid	Androstenedione	Ratio
6 β -Hydroxylation			
♂ Con	0.60 $\pm$ 0.07 (100 $\pm$ 11)	0.51 $\pm$ 0.11 (100 $\pm$ 21)	1.0
PB	1.15 $\pm$ 0.14 (198 $\pm$ 24)	1.47 $\pm$ 0.14 (289 $\pm$ 27)	0.7*
Dex	0.61 $\pm$ 0.04 (103 $\pm$ 7)	1.56 $\pm$ 0.15 (307 $\pm$ 29)	0.3**
Clof	0.98 $\pm$ 0.16 (164 $\pm$ 27)	1.22 $\pm$ 0.21 (240 $\pm$ 42)	0.7*
♀ Con	0.29 $\pm$ 0.04 (100 $\pm$ 13)	0.23 $\pm$ 0.04 (100 $\pm$ 17)	1.0
PB	0.82 $\pm$ 0.14 (286 $\pm$ 50)	0.62 $\pm$ 0.04 (266 $\pm$ 16)	1.1
Dex	1.19 $\pm$ 0.09 (413 $\pm$ 31)	0.66 $\pm$ 0.18 (285 $\pm$ 77)	1.5
Clof	0.93 $\pm$ 0.06 (322 $\pm$ 20)	0.22 $\pm$ 0.03 (95 $\pm$ 11)	3.4**
7 α -Hydroxylation			
♂ Con	0.020 $\pm$ 0.003 (100 $\pm$ 15)	0.41 $\pm$ 0.05 (100 $\pm$ 12)	1.0
PB	0.039 $\pm$ 0.005 (195 $\pm$ 25)	1.05 $\pm$ 0.15 (257 $\pm$ 38)	0.8
Dex	0.036 $\pm$ 0.003 (180 $\pm$ 15)	0.37 $\pm$ 0.04 (91 $\pm$ 9)	2.0**
Clof	0.027 $\pm$ 0.003 (135 $\pm$ 15)	0.68 $\pm$ 0.09 (168 $\pm$ 21)	0.8
♀ Con	0.026 $\pm$ 0.004 (100 $\pm$ 15)	1.27 $\pm$ 0.07 (100 $\pm$ 5)	1.0
PB	0.060 $\pm$ 0.006 (231 $\pm$ 23)	2.65 $\pm$ 0.35 (209 $\pm$ 28)	1.1
Dex	0.058 $\pm$ 0.013 (233 $\pm$ 50)	1.00 $\pm$ 0.27 (79 $\pm$ 21)	2.8*
Clof	0.035 $\pm$ 0.003 (135 $\pm$ 12)	0.99 $\pm$ 0.14 (78 $\pm$ 11)	1.7**

with 20 μ g of microsomal protein. The assay mixtures contained, in addition to the components listed above, 75 mM-NaCl and 10 mM-potassium phosphate, pH 7.4. All components of the assay except NADPH, but including the antibody or normal rabbit IgG, were combined, and the mixtures were incubated for 40 min at 22 $^{\circ}$ C. The tubes were then transferred to a 37 $^{\circ}$ C water bath, and the assays were started by the addition of NADPH. In the inhibition studies, metyrapone, *trans*-tamoxifen, ketoconazole and clotrimazole, dissolved in ethanol, were added directly to standard assays carried out as described in the previous section. The final concentration of ethanol in the assay was kept below 0.5 %, a concentration which had no effect on the activities measured.

Materials

Polyclonal anti-*P*-450 antibody IgG fractions were prepared from rabbits immunized with purified rat liver *P*-450 forms designated p (*P*-450IIIA1) [25], PCNb (*P*-450IIIA2) [26,27], PB1 (*P*-450IIC6) [28], and RLM2 (*P*-450IIA2) [29] using standard methods. Antibodies to *P*-450p and *P*-450PCNb used in this study were kindly provided by Dr. P. S. Guzelian (Medical College of Virginia, Richmond, VA, U.S.A.) and Dr. J. Halpert (University of Arizona, Tucson, AZ, U.S.A.) respectively. [24- 14 C]Lithocholic acid, [4- 14 C]androst-4-ene-3,17-dione and [4- 14 C]testosterone (specific radioactivity 55–60 Ci/mol) were purchased from Amersham Corp., Arlington Heights, IL, U.S.A. The 5 α -reductase inhibitor 4-MA was generously provided by Dr. G. H. Rasmusson, Merck Sharp and Dohme, Rahway, NJ, U.S.A. NADPH, metyrapone, ketoconazole, clotrimazole, phenobarbital, dexamethasone, clofibrate and normal rabbit IgG were from Sigma Chemical Co. (St. Louis, MO, U.S.A.). *trans*-Tamoxifen was purchased from Aldrich Chemical Co. (Milwaukee, WI, U.S.A.).

RESULTS

Androstenedione [21] and lithocholic acid [18] are both hydroxylated at multiple sites by isolated rat liver microsomes, with the 6 β -hydroxy derivatives formed as major metabolites with each compound. The influence of several mono-oxygenase-inducing agents, administered to adult male and female rats, on the rates of hydroxylation of these compounds at the 6 β and 7 α positions is shown in Table 1. Androstenedione 6 β -hydroxylation was induced in male rats by phenobarbital, dexamethasone and clofibrate, whereas only the first two compounds induced this activity in female rats. In contrast, lithocholic acid 6 β -hydroxylation was enhanced in male rats by phenobarbital and clofibrate, while in females all three inducers used were effective. When both androstenedione and lithocholic acid 6 β -hydroxylation was increased by an inducer, the degree of induction was in most cases significantly different for the two substrates (Table 1). Examination of the microsomal 7 α -hydroxylation rates for both substrates also revealed differences in their response to mono-oxygenase inducers, in particular in the case of dexamethasone, which stimulated lithocholic acid 7 α -hydroxylase but not androstenedione 7 α -hydroxylase in both male and female rats. Since the 6 β -hydroxylation of lithocholic acid is the quantitatively predominant [18] and physiologically most interesting hydroxylation reaction involving this substrate, further work focused on this process.

The inducibility patterns presented in Table 1 suggested that hydroxylations of the same regio- and stereo-specificity on androstenedione and on lithocholic acid are catalysed by distinct cytochrome *P*-450 forms. This prompted a more direct examination of the possible identity of the enzyme(s) involved in lithocholic acid 6 β -hydroxylation. This was accomplished using anti-*P*-450 antibodies that are preferentially inhibitory to select

Fig. 2. Effect of antibodies on microsomal androstenedione and lithocholic acid 6 β -hydroxylase activities

Microsomes were obtained from livers of four untreated (a,c,e) and four dexamethasone-treated (b,d,f) male Fischer rats. Corresponding microsomal samples were pooled and were preincubated with antibody IgG fractions (●, ▲) or with normal non-immune rabbit IgG (○, △) for 40 min. The microsomes were subsequently assayed for androstenedione (●, ○) or lithocholic acid (▲, △) 6 β -hydroxylase activity. Measurements were carried out at least twice, and representative values are shown. The activities are expressed relative to the control activity measured in the absence of added antibodies; these activities were 1.60 nmol/min per mg for lithocholic acid and 1.45 nmol/min per mg for androstenedione 6 β -hydroxylase for microsomes from untreated animals, and 1.51 nmol/min per mg and 5.45 nmol/min per mg respectively for microsomes from dexamethasone-treated rats. Each panel corresponds to an individual antibody, as follows: (a, b) anti-*P*-450III A1; (c, d) anti-*P*-450III A2; (e, f) anti-*P*-450IIC6.

Fig. 3. Effect of anti-*P*450IIA2 antibody on microsomal testosterone and lithocholic acid 6 β -hydroxylase activities

Liver microsomes from untreated male Fischer rats were assayed as described in the legend to Fig. 2. Microsomes (50 μ g) were used per assay. The symbols used are as in Fig. 2, except that ●, ○ denote testosterone 6 β -hydroxylase activity. Enzyme activities measured in the absence of added antibody were 0.62 nmol/min per mg for testosterone 6 β -hydroxylase and 0.36 nmol/min per mg for lithocholic acid 6 β -hydroxylase.

individual *P*-450 forms. Previous studies have established that rat hepatic microsomal androstenedione 6 β -hydroxylation is catalysed primarily by *P*-450III A2, a constitutively expressed male-specific enzyme, and by *P*-450III A1, a closely related (89 % similar) cytochrome that is expressed constitutively at very low levels in both sexes, but can be induced to high levels by dexamethasone and other drugs [30,31]. In agreement with the above findings, microsomal androstenedione 6 β -hydroxylation was inhibited by anti-*P*-450III A2 antibodies and (to a lesser degree) by anti-*P*-450III A1 antibodies. The inhibition patterns were similar for liver microsomes obtained from untreated (control) and dexamethasone-treated male rats (Figs. 2c and 2d). Although *P*-450III A2 and *P*-450III A1 exhibit a high degree of similarity in primary structure (89 % amino acid sequence identity [31]), the antibody preparations used in the present study are only partially cross-reactive. For the interpretation of the above results it should be kept in mind that antibodies may recognize epitopes on an enzyme without blocking the active site and thus without interfering with catalytic activity [32,33]; partial blocking of the active site could result in incomplete enzyme inhibition. Therefore partial inhibition is not necessarily due to the presence of additional, immunologically unrelated, enzymes catalysing the same reaction.

In contrast with its strong inhibitory effect on androstenedione 6 β -hydroxylation in dexamethasone-induced microsomes and its moderate inhibitory effect in uninduced microsomes, anti-*P*-450III A2 did not inhibit the 6 β -hydroxylation of lithocholic acid catalysed by either microsome preparation (Fig. 2). Conversely, anti-*P*-450III A1, which was a comparatively weak inhibitor of androstenedione 6 β -hydroxylation, completely eliminated lithocholic acid 6 β -hydroxylation (Figs. 2a and 2b). In contrast, a second anti-*P*-450III A1 antibody used in this study was partially inhibitory toward androstenedione 6 β -hydroxylation but was ineffective against lithocholic acid 6 β -hydroxylation (results not shown).

An antibody raised against *P*-450IIC6, a cytochrome belonging to a different *P*-450 family than the two androstenedione 6 β -hydroxylases, did not significantly inhibit the latter activity in either untreated or dexamethasone-induced liver microsomes (Figs. 2e and 2f). However, the same antibody was quite effective in inhibiting lithocholic acid 6 β -hydroxylase (Fig. 2). Similarly (Fig. 3), anti-*P*-450IIA2 inhibited lithocholic acid 6 β -hydroxylase without affecting steroid hormone 6 β -hydroxylation, which in this experiment was assayed using testosterone as an alternative substrate [21]. This immunochemical cross-reactivity of the lithocholic acid 6 β -hydroxylase with members of cytochrome *P*-450 family II further sets it apart from the androstenedione 6 β -hydroxylase(s). No inhibition of either 6 β -hydroxylase activity was effected by antibodies reactive with the 3-methylcholanthrene-inducible cytochrome *P*-450IA1 or the phenobarbital-inducible cytochrome *P*-450IIB1 (results not shown), or by non-immune rabbit IgG (Fig. 2).

The results presented above show that microsomal 6 β -hydroxylations of androstenedione and lithocholic acid are catalysed by distinct *P*-450 enzymes. Ancillary experiments were carried out in support of this hypothesis. Results of direct competition assays were consistent with the presence of distinct hydroxylases specific for each substrate: at an androstenedione concentration of 40 μ M, an equal concentration of lithocholic acid inhibited androstenedione 6 β -hydroxylation by less than 5%, whereas at 100 μ M-lithocholic acid the inhibition was approx. 25%. A similarly slight inhibition of lithocholic acid 6 β -hydroxylation by androstenedione was observed (results not shown). Finally, lithocholic acid and androstenedione 6 β -hydroxylase activities could be differentiated by the use of cytochrome *P*-450 inhibitors. Fig. 4 shows the effects of

Fig. 4. Effect of cytochrome *P*-450 inhibitors on microsomal androstenedione and lithocholic acid 6 β -hydroxylase activities

Androstenedione 6 β -hydroxylase (●) and lithocholic acid 6 β -hydroxylase (▲) activities were measured in microsomes obtained from male Sprague–Dawley rats in the presence of metyrapone (a), *trans*-tamoxifen (b), ketoconazole (c) and clotrimazole (d). Measurements were carried out at least twice, and representative values are shown. Control activity for androstenedione 6 β -hydroxylase activity was 0.89 ± 0.10 nmol/min per mg and for lithocholic acid 6 β -hydroxylase it was 0.18 ± 0.02 nmol/min per mg.

metyrapone (a), *trans*-tamoxifen (b), ketoconazole (c) and clotrimazole (d) on the two enzymes. All four compounds are known to inhibit androstenedione 6 β -hydroxylase [34–36], a finding that is confirmed by our present results. In all four cases, lithocholic acid 6 β -hydroxylase activity was more resistant to inhibition than was androstenedione 6 β -hydroxylase, even though major differences in the effectiveness of the four inhibitors were evident. Within the concentration range studied, the imidazole derivatives ketoconazole, and especially clotrimazole, were the only compounds that fully inhibited lithocholic acid 6 β -hydroxylation, but this only occurred at concentrations considerably higher than those needed to inhibit androstenedione 6 β -hydroxylation. Metyrapone and *trans*-tamoxifen had no significant effect on lithocholic acid 6 β -hydroxylation at concentrations up to 100 μ M, which strongly inhibited androstenedione 6 β -hydroxylation. The above results with cytochrome *P*-450 inhibitors were obtained with microsomes from Sprague–Dawley rat livers, and for selected inhibitor concentrations were confirmed in Fischer rat microsomes. For example, 60 μ M-metyrapone did not inhibit lithocholic acid 6 β -hydroxylase (109 % remaining activity), whereas androstenedione 6 β -hydroxylase was markedly inhibited (58 % remaining activity). For 1 μ M-ketoconazole, 93 % and 75 % remaining activity was measured for the two enzymes, and for 1 μ M-clotrimazole these values were 91 % and 13 %. The similar response to chemical inhibitors in both strains indicates that the existence of separate 6 β -hydroxylases for lithocholic acid and for androstenedione is a general phenomenon, rather than being restricted to a specific strain of rats.

DISCUSSION

Several independent approaches were used to demonstrate that the cytochrome(s) *P*-450 that catalyse microsomal lithocholic acid 6 β -hydroxylation, and perhaps 7 α -hydroxylation, are dis-

tinct from those that catalyse the corresponding 6 β - and 7 α -hydroxylations of androstenedione. This conclusion is supported by the differential response of the two activities to *P*-450 inducers, the lack of significant mutual inhibition of hydroxylation by the two compounds, and differential sensitivity to inhibition by compounds such as metyrapone and ketoconazole, as well as to anti-*P*-450 antibodies.

Induction of microsomal detoxifying enzymes, including the cytochromes *P*-450, by xenobiotics is common. Inducibility by a given compound is often highly characteristic for a *P*-450 enzyme, and can aid in its identification [37], especially when no structural and genetic information is available. Since the expression of a number of rat hepatic cytochrome *P*-450s is subject to sex-specific hormonal control [21], induction was studied in both male and female rats. The clear differences in the inducibility patterns of hepatic lithocholic acid 6 β -hydroxylase and androstenedione 6 β -hydroxylase activities indicate that distinct *P*-450 enzymes catalyse these two microsomal reactions. A similar conclusion was reached with regard to the 7 α -hydroxylation reactions (Table 1).

The distinctiveness of cytochromes *P*-450 responsible for the 6 β -hydroxylations of lithocholic acid and androstenedione was confirmed by the differential responses of these activities to chemical inhibitors. While the results obtained in these experiments point to the likelihood of a multiplicity of these 6 β -hydroxylase enzymes, more specific information on their identity was provided by immunological cross-reactivity experiments (Figs. 2 and 3). Due to a pronounced physical similarity of microsomal cytochromes *P*-450 that includes sequence homology [38,39], the presence of highly conserved regions [40–42] and identical overall patterns of folding and membrane attachment [41,43–45], antibodies obtained against these proteins are rarely monospecific, even if homogeneous preparations of single *P*-450 forms are used for immunization. This presents an obvious experimental complication if the objective is to quantify a single

P-450 form in a mixture of similar proteins. However, overlapping specificity of anti-*P*450 antibodies can become a valuable asset if the goal is to probe for similarities between a hitherto unknown *P*-450 and previously identified enzymes. In the present work, antibodies were used to this effect; beyond the simple elimination of certain known cytochromes *P*-450 as the lithocholic acid 6 β -hydroxylase, they were employed to identify similarities between various *P*-450 enzymes.

Several purified cytochromes *P*-450 exhibit high androstenedione 6 β -hydroxylase activity when assayed in reconstituted systems [21,37]. However, in intact liver microsomes the constitutive form *P*-450III A2 and the drug-inducible form *P*-450III A1 are largely, if not solely, responsible for this reaction [21]. While some antibodies prepared against these two cytochromes also inhibited lithocholic acid 6 β -hydroxylation, there were striking differences in the behaviour of the latter activity as compared with androstenedione 6 β -hydroxylation (Fig. 2). On one hand, this finding corroborates the conclusion that separate enzymes catalyse the two reactions. On the other hand, it suggests that the lithocholic acid 6 β -hydroxylase *P*-450(s) share some, but not all, epitopes with the androstenedione 6 β -hydroxylases of *P*-450 subfamily IIIA. This observation suggests that the lithocholic acid 6 β -hydroxylase may belong to family III. However, the partial inhibition of lithocholic acid 6 β -hydroxylation by anti-*P*-450IIC6 (Fig. 2) and the more complete inhibition by anti-*P*-450IIA2 (Fig. 3) are suggestive of some structural similarity with family II cytochromes *P*-450 as well. A more definitive determination of whether the lithocholic acid 6 β -hydroxylase *P*-450 actually belongs to *P*-450 family II or III requires the determination of its primary structure.

Although *P*-450 forms IIIA1 and IIIA2 are the major catalysts of microsomal androstenedione 6 β -hydroxylation in rat liver, several other cytochromes *P*-450 exhibit the same activity in purified reconstituted enzyme systems. The most prominent of these are cytochromes *P*-450IA1 and *P*-450IIC13 [21,37]. These two enzymes can, however, be ruled out as candidates for the lithocholic acid 6 β -hydroxylase. Inhibitory antibodies against cytochrome *P*-450IA1 did not inhibit the latter activity, and β -naphthoflavone, a prototypic inducer of cytochrome *P*-450IA1, did not increase the level of microsomal lithocholic acid 6 β -hydroxylase activity (results not shown). The other steroid 6 β -hydroxylase, cytochrome *P*-450IIC13, is strain-specific and absent in Fischer rats [46], in which the present experiments were carried out. Therefore it appears most likely that lithocholic acid 6 β -hydroxylation is catalysed by a hitherto undescribed cytochrome *P*-450.

The fact that the 6 β -hydroxylations of lithocholic acid and androstenedione are catalysed by distinct enzymes should be seen in a physiological as well as a mechanistic context. The functions of the two reactions are very different. Whereas the 6 β -hydroxylation of bile acids has, as indicated previously, a dual biosynthetic and detoxification/salvage role, 6 β -hydroxylation of androstenedione and other related androgens, e.g. testosterone, is likely to contribute to the inactivation and degradation of these hormones. The physiological regulatory requirements for the two reactions are obviously very different. A catabolic pathway for androgens is necessary only in the post-pubertal male. In fact, a complex hormonal regulatory system present in the rat blocks the expression of androstenedione 6 β -hydroxylase in adult females [21,34,47,48]. In contrast, bile acid 6 β -hydroxylation is probably functional in both sexes throughout life [49], and may be especially important in the perinatal period. During this period of development, the hydrophobic-hydrophilic balance of bile acids is shifted towards a relative deficiency of trihydroxylated bile acids [50]. The situation is remedied in the rat by increased 6 β -hydroxylation, as evidenced by higher levels

Fig. 5. Structures of androstenedione and lithocholic acid

The 6 β positions are marked by arrows.

of 6 β -hydroxylated bile acids at birth as compared with adult levels [49]. Thus, in light of the disparate physiological requirements, the separation of lithocholic acid and androstenedione 6 β -hydroxylations becomes understandable. Since our preliminary data indicate that 7 α -hydroxylation of the two substrates is also catalysed by independent enzymes, it becomes likely that a group of bile acid hydroxylase *P*-450s exists that is distinct from the known cytochromes *P*-450 that hydroxylate neutral steroids, even if the positional specificity of the catalysed reactions is the same.

The discrimination between the two steroidal molecules, androstenedione and lithocholic acid, by cytochromes *P*-450 has interesting mechanistic implications. Despite their overall similarity, the two compounds differ in several respects (Fig. 5). The most striking are (i) their distinctly different shapes, with androstenedione being essentially planar, while ring A of lithocholic acid is bent at an almost right angle relative to the plane of the molecule; (ii) the presence, in androstenedione but not in lithocholic acid, of a conjugated double bond system that increases the inherent chemical reactivity of position 6 β ; (iii) a different substituent in position 17, i.e. an oxo group in androstenedione, and a charged and more bulky side chain in lithocholic acid. Further studies will be required to determine which of these characteristics of the two molecules are crucial for their selective recognition by the *P*-450 enzymes that catalyse their hydroxylation.

The two principal rat liver androstenedione 6 β -hydroxylases, cytochromes *P*-450III A1 and *P*-450III A2, have been purified [30,34,51–53] and reconstituted without loss of activity [34,53,54], and their sequences are known [31,55]. The identification of another cytochrome *P*-450, the lithocholic acid 6 β -hydroxylase, which catalyses a reaction of the same regio- and stereo-specificity on a generally similar substrate, will help to focus on the differences between the enzymes that are important for their function. In a complementary development, it has been recently determined that the human hepatic cytochrome *P*-450III A4 (*P*-450hPCN1) is able to carry out the 6 α -hydroxylation of lithocholic acid [56]. A comparison of this protein, the sequence of which is known [57–59], with the lithocholic acid 6 β -hydroxylase will add another dimension to the correlation of structure with function, as these two enzymes catalyse reactions of identical regio- but opposite stereo-specificity on the same substrate. The two-way comparison of the lithocholic acid 6 β -hydroxylase with the rat androstenedione 6 β -hydroxylases on one hand, and with the human lithocholic acid 6 α -hydroxylase on the other, should facilitate a more detailed understanding of

those structural variables of the proteins that are critical to the recognition and orientation within the active site of their respective steroidal substrates.

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