Biochemical Differences between Rat and Human Cytochrome P450c17 Support the Different Steroidogenic Needs of These Two Species[†]

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ABSTRACT: Microsomal 17α-hydroxylase/17,20-lyase cytochrome P450 (P450c17) catalyzes both the 17α-hydroxylase reaction required to produce cortisol, the major glucocorticoid in many animals, and the 17,20-lyase activity required for the production of androgens in all animals. In rodents such as rat, which utilize corticosterone as the major glucocorticoid, P450c17 is expressed predominantly in the gonads, and is absent in the adrenal. In other species including humans, P450c17 is expressed in both adrenal and gonads and participates in both glucocorticoid and androgen production. Rat and human forms of P450c17 are 69% identical at the amino acid level. Based on the differences in physiological roles between P450c17 in these two species, it could be predicted that major differences would be observed in their hydroxylase activities. Contrary to this hypothesis, using partially purified, recombinant human and rat P450c17, we found that the most significant differences lie in their lyase activities. Lyase activities demonstrate that the rat enzyme favors Δ^4 (progesterone) substrates while the human enzyme favors Δ^5 (pregnenolone) substrates. This substrate preference is also observed in the ability of steroids to decrease uncoupled H_2O_2 production and to increase stability during turnover. Cytochrome b_5, a microsomal electron-transfer protein, enhances lyase activities of rat and human P450c17. However, the most dramatic stimulatory effect is on the human HO-PROG lyase activity. This enhancement of activities is not associated with electron transfer. These differences in biochemical properties between the two forms of P450c17 indicate that human P450c17 has evolved as an enzyme system that limits androgen production to the gonads where a favorable b_5:P450c17 ratio exists. Even though orthologous forms of P450c17 are capable of catalyzing the same enzymatic activities, specific physiological requirements of each species ensure biochemical differences between these enzymes.

17α-Hydroxylase/17,20-lyase cytochrome P450 (P450c17),[1] a steroidogenic, microsomal cytochrome P450 mixed-function oxidase, catalyzes the 17α-hydroxylation of Δ^4- and Δ^5-steroids necessary for glucocorticoid production, and the 17,20-lyase reactions required to produce the 19-carbon androgens, androstenedione (AD) and dehydroepiandrosterone (DHEA). It thus controls an important branch point in steroidogenesis between glucocorticoid and sex hormone biosynthesis. Glucocorticoids play important roles in the maintenance of stress responses, carbohydrate metabolism, and general metabolite homeostasis. Androgens play essential roles in reproductive biology including the development and maintenance of the male phenotype. Mutations that result in decreased activity of P450c17 generally lead to combined 17α-hydroxylase, 17,20-lyase deficiency and the development of abnormal secondary sex characteristics because of the absence of or greatly reduced production of androgens (*1, 2*). On the other hand, excess androgen biosynthesis in men has been associated with prostate cancer, and the P450c17 lyase activity has been proposed as a potential target for treatment of this disease (*3*−*5*). Although the physiological role of DHEA beyond testosterone production is unclear, several therapeutic uses for this steroid have been proposed (*6*−*9*). Detailed analysis of the structure/function relationships of P450c17 will be important for a complete understanding at the biochemical level of both reproductive capacity and metabolic homeostasis.

Steroidogenesis is quite variable between species, and differences in P450c17 expression and activity are major factors in such differences. P450c17 has been purified from steroidogenic organs (adrenal, testis, or ovary) of various animals including pig (*10*), guinea pig (*11*), bovine (*12*), and rat (*13*). Although the P450c17 enzymes from these animals share 60−75% amino acid sequence homology, they exhibit important differences in their expression and activity. In most species, P450c17 is expressed in adrenal glands and gonads. However, adrenal expression is absent in the rat, mouse, and rabbit (*14*−*17*), although pharmacological doses of adrenocorticotropin (ACTH) induce expression of rabbit adrenal P450c17 (*18*). Porcine, rat, trout, and hamster P450c17

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[1] Abbreviations: P450c17, cytochrome P450 17α-hydroxylase/17,-20-lyase; PROG, progesterone; PREG, pregnenolone; HO-PROG, 17α-hydroxyprogesterone; AD, androstenedione; HO-PREG, 17α-hydroxypregnenolone; DHEA, dehydroepiandrosterone; DLPC, L-α-phosphatidylcholine, dilauryl; b_5, cytochrome b_5; IPTG, isopropyl β-D-thiogalactopyranoside; ALA, δ-aminolevulinic acid; PMSF, phenylmethylsulfonyl fluoride.

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catalyze Δ^4 and Δ^5 hydroxylase and lyase reactions (*19*−*22*). The hydroxylase and lyase activities of the guinea pig enzyme appear to have a preference for Δ^4-steroids (*23*). Bovine and human P450c17 also catalyze the 17α-hydroxylation of Δ^4- and Δ^5-steroids. However, the 17,20-lyase activity of bovine and human P450c17 is observed almost exclusively with the Δ^5-steroid HO-PREG, the 17,20-lyase activity with the Δ^4-steroid HO-PROG being negligible (*1, 20, 24*). Thus, comparison of the biochemical properties of P450c17 from different species provides an excellent opportunity to understand the molecular basis of unique steroidogenic pathways in different species. For example, the high ratio of 17α-hydroxylase to 17,20-lyase activity in the human adrenal gland avoids a condition termed "endocrine ambiguity" (*25*). If extensive androgen synthesis occurred in human adrenal glands, this might give rise to the phenotypes similar to those described for 21-hydroxylase deficiency including virulization and hirsutism in females and precocious development of the male phenotype. Rodents avoid the possibility of endocrine ambiguity by not expressing P450c17 in the adrenal gland.

Efforts to elucidate details of the structure/function relationships that contribute to these species-specific properties have combined modeling experiments with properties of site-directed and naturally occurring P450c17 mutants (*26*−*29*). These studies on the rat and human enzymes have predicted residues in P450c17 that may differentially regulate the 17,20-lyase activity of this bifunctional enzyme. Interaction with cytochrome b_5 (b_5), phosphorylation, membrane environment, and the ratio of P450 reductase to P450 have all been proposed to differentially regulate human P450c17 lyase activity with little or no effect on the hydroxylase activity (*24*, *29*−*31*). Utilizing an *E. coli* expression system for mammalian steroidogenic cytochromes P450 (*32*), we now provide a direct comparison of activity, substrate binding, O_2 reduction, and protein stability between recombinant rat and human P450c17s, as well as interaction with b_5. Although the differences in steroidogenesis between these species are primarily reflected in their 17α-hydroxylase capacities, the results demonstrate that the greatest difference in properties between rat and human P450c17 is most evident in lyase activities and not hydroxylase activities. Additional P450c17 species-specific properties identified in this study are useful in explaining the differences in steroidogenesis between rats and humans.

EXPERIMENTAL PROCEDURES

Materials. [^{3}H]PREG, [^{3}H]PROG, [^{3}H]17α-HO-PREG, and [^{3}H]17α-HO-PROG were purchased from ICN Biochemicals (Costa Mesa, CA). The recombinant expression plasmids pCWR17*mod* encoding rat P450c17 and pCWH17-*mod*(His)$_4$ encoding human P450c17 were constructed as described by Barnes et al. (*33*) and Imai et al. (*34*), respectively. Recombinant rat P450 reductase and bovine b_5 were generously provided by Dr. R. W. Estabrook, University of Texas Southwestern Medical Center, Dallas.

Culture Conditions and Enzyme Purification. Single-colony isolates of *E. coli* strain JM109, freshly transformed with either pCWR17*mod* or pCWH17*mod*(His)$_4$, were used to start 5 mL overnight Luria−Bertani broth cultures. These cultures then were seeded into 1 L of terrific broth and grown at 37 °C, 240 rpm. When the cell density reached an absorbance of 0.4 at 600 nm, isopropyl β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 1.0 mM, and the temperature and shaking speed were reduced to 25 °C and 125 rpm, respectively. δ-Aminolevulinic acid (ALA) was added simultaneously with IPTG at a final concentration of 1.0 mM to cultures expressing human P450c17. Cells were harvested 48 h (rat P450c17) and 24 h (human P450c17) after the addition of IPTG. Spheroplast preparation and human P450c17 purification were performed as previously described (*34*). For purification of the rat P450c17, spheroplasts were prepared as above and subjected to Triton X-114 induced phase separation (*35, 36*). Spheroplasts (50 g) were suspended with 200 mL of phase separation buffer (50 mM potassium phosphate, pH 7.4, 35% glycerol, 0.1 mM DTT, 0.1 mM PMSF, 1 μg/mL DNase, 40 μM PROG) in a Teflon homogenizer. Triton X-114, purified by the method of Bordier (*35*), was added dropwise [final concentration = 0.7% (v/v)]. After stirring 30 min on ice, the sample was centrifuged at 100000*g* for 45 min, and the dark, reddish-brown, detergent-rich phase was carefully collected with a plastic pipet. The detergent-rich phase was then diluted with 10 volumes of 10 mM potassium phosphate, pH 7.4, containing 20% glycerol, 0.1 mM PMSF, 40 μM PROG, 0.1% Triton X-100, and 0.1% sodium cholate. The diluted sample was bound to a DEAE Sepharose CL-6B column equilibrated in 10 mM potassium phosphate, pH 7.4, containing 20% glycerol, 0.1 mM PMSF, 40 μM PROG, and 0.2% Triton X-100. The column was washed with 3 column volumes of equilibration buffer and eluted with a linear gradient of NaCl (0−250 mM in 200 mL). Fractions were pooled based on P450 content which was determined from the reduced CO-difference spectrum (*37*). The pooled fractions from the DEAE column were diluted in 5 volumes of 20% glycerol, 40 μM PROG and loaded onto a hydroxylapatite column which had been equilibrated in 1.0 mM potassium phosphate, 20% glycerol, 0.02% Emulgen 913. The column was washed with 5 column volumes of 10 mM potassium phosphate, 20% glycerol, 0.02% Emulgen 913, and the sample was eluted with the same buffer containing 300 mM potassium phosphate. The partially purified enzyme was dialyzed against 50 mM sodium phosphate, pH 7.2, 20% glycerol, 0.02% Emulgen 913, and 50 μM PROG and stored at −80 °C until further use. Hydroxylapatite chromatography was used to remove the majority of PROG from samples used in stability and substrate binding experiments.

Reconstitution of Rat and Human 17α-Hydroxylase and 17,20-Lyase Activities. Activity assays were based on those described in Imai et al. (*34*). Recombinant P450c17, P450-reductase, and 75 000 cpm of ^{3}H-labeled substrate were incubated in reaction buffer (50 mM sodium phosphate, pH 7.2, 20% glycerol, 1 mM magnesium acetate, 100 μg/mL DLPC) for 3 min while shaking at 37 °C. After 3 min, NADPH was added to a final concentration of 1.0 mM. The final volume of each reaction was 0.2−1 mL depending on the experiment. Concentrations of P450c17, P450-reductase, and substrates and reaction times are described in the figure legends. Reactions were extracted in 2 mL of CH_2Cl_2, evaporated to dryness, resuspended in acetone, and subjected to thin-layer chromatography. Δ^4-Steroids (PROG, HO-PROG, and AD) were developed in chloroform/ethyl acetate (80:20) and detected by UV irradiation. Δ^5-Steroids (PREG,

HO-PREG, and DHEA) were developed using the same mobile phase and detected by iodine vapor. Using authentic standards, TLC spots were cut out and immersed in 5 mL of Econo-Safe scintillation cocktail (RPI Corp.) and counted on a Wallac 1409 scintillation counter (Pharmacia).

Determination of Spectral Binding Constants, K_s, for Steroid Substrates. A 1 mL solution of rat or human P450c17 (1 μM) in 50 mM sodium phosphate, pH 7.2, plus 20% glycerol was placed in a pair of optically matched tandem cuvettes. Titrations were performed with ethanol solutions of PROG, PREG, HO-PROG, HO-PREG, AD, and DHEA. Concentration ranges for each steroid are given in the table and figure legends. After each titration, the samples were allowed to equilibrate 2−3 min prior to recording the substrate-induced difference spectrum on a Beckman DU 640 spectrophotometer. K_s values were extrapolated from double reciprocal plots of substrate concentration versus change in absorbance.

P450 Stability. The stability of the two enzymes during turnover of PROG and PREG was measured by recording the reduced CO-difference spectra of reconstituted enzyme reactions at the indicated times after addition of NADPH. The reaction conditions were identical to those noted above for activity assays.

Other Methods. Protein concentrations were determined by the bicinchoninic acid method (Pierce Chemical Co.) (*38*) with bovine serum albumin as standard. Protein purity was assessed by SDS−PAGE (*39*). H_2O_2 formation was measured by the ferrithiocyanate method (*40*).

RESULTS

Expression and Purification of Recombinant Rat P450c17. Because partial purification of recombinant rat P450c17 from *E. coli* has not previously been reported, a brief description is provided. Relatively high levels of rat P450c17 expression are observed compared to expression of the recombinant human enzyme. Twenty hours after addition of IPTG, the rat enzyme was expressed at 600−700 nmol of P450/L compared to 100−300 nmol/L of the human enzyme (not shown). Expression of the rat enzyme is not dependent on the presence of ALA, while maximal levels of expression of the human enzyme required addition of this precursor of heme biosynthesis. The recombinant human enzyme was purified to a specific content of 7.5 nmol of P450/mg of protein (Figure 1). As shown in Table 1, the purification of the recombinant rat enzyme using Triton X-114 phase separation followed by a combination of DEAE Sepharose and hydroxylapatite chromatography resulted in about 10% recovery of a partially purified enzyme (specific P450 content of 7.1 nmol of P450/mg). It was found that lower yields of the recombinant human enzyme were obtained when Triton X-114 induced phase separation was used compared to the sonication protocol (*34*).

Comparison of the Activity and Substrate Binding Properties of Rat and Human P450c17. The activities of the two purified enzymes in reconstituted systems compared in Table 2, demonstrate differences between these enzymes. The recombinant rat and human enzymes catalyzed the 17α-hydroxylation of PROG at initial rates of 2.4 and 5.6 nmol min^{-1} (nmol of P450)$^{-1}$, respectively. 17α-hydroxylation of PREG was catalyzed at rates of 2.6 and 0.80 nmol min^{-1} (nmol of P450)$^{-1}$ by the rat and human enzymes, respectively. HO-PROG and HO-PREG lyase activities were readily observed with the rat enzyme, while the most dramatic difference between these two enzymes is that virtually no HO-PROG lyase activity was observed with the human enzyme. When PROG was used as the starting substrate at a concentration of 50 μM, AD production was detectable with the rat enzyme yet undetectable using the human enzyme. However, very low human lyase activity was observed when the PROG concentration was reduced to 10 μM (data not shown).

To assess the contributions of substrate binding and product release to the enzymatic differences between rat and human P450c17 seen in Table 2, micromolar binding

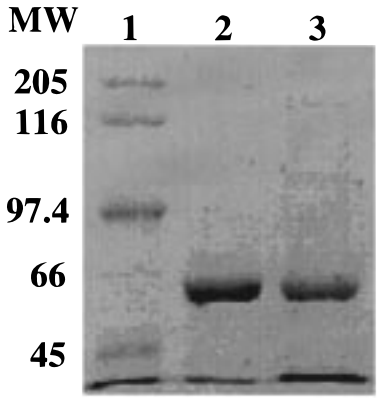

FIGURE 1: SDS−PAGE analysis of purified rat and human P450c17. Molecular weight markers (lane 1); 1 μg of human P450c17 (specific content 7.5 nmol of P450/mg of protein) after Ni^{2+} chromatography (lane 2); 1 μg of rat P450c17 (specific content 7.1 nmol of P450/mg of protein) after hydroxylapatite chromatography (lane 3). The gel was stained with Coomassie blue.

Table 1: Purification of Recombinant Rat P450c17 from *E. coli*[a]

preparation	protein (mg)	P450 (nmol)	yield (%)	specific content (nmol of P450/ mg of protein)
whole cells	nd[b]	2160	100	nd
detergent-rich phase	225	595	60	2.6
DEAE	87	330	15	3.8
hydroxylapatite	39	278	13	7.1

[a] Results of purification from 4 L of culture following expression of pCWratc17 in JM109. [b] nd, not determined.

Table 2: Catalytic Activities of Partially Purified Rat and Human P450c17[a]

substrate	activity [nmol of product min^{-1} (nmol of P450)$^{-1}$]	
	rat P450c17	human P450c17
PROG	2.4 ± 0.12	5.6 ± 0.30
HO-PROG	0.37 ± 0.008	≤0.005[b]
PREG	2.6 ± 0.12	0.80 ± 0.88
HO-PREG	0.25 ± 0.03	0.025 ± 0.002

[a] Reactions were performed as described under Experimental Procedures. Each 0.2 mL reaction contained 50 mM sodium phosphate (pH 7.2), 20% glycerol, 1 mM magnesium acetate, 75 000 cpm of ^{3}H-labeled substrate, 100 μg/mL DLPC, 0.5 μM P450c17, 0.5 μM P450-reductase, and 1 mM NADPH. Hydroxylase and lyase assays were performed for 5 and 20 min at 37 °C, respectively, after addition of NADPH. Values are the average of at least three determinations for each substrate and presented as mean ± SD. [b] This value is at the limit of detection and not amenable to statistical analysis.

Table 3: Binding Properties of Rat and Human P450c17 for Different Substrates and Products[a]

substrate	rat P450c17	human P450c17
progesterone	2.9, 1.4	2.7, 1.0
17α-OH-Prog	52, 40	9.4, 8.3
pregnenolone	1.8, 2.5	1.6, 0.71
17α-OH-Preg	6.7, 3.5	3.6, 0.56
androstenedione	*b*	*b*
DHEA	*c*	22

[a] Spectrally determined binding constants (K_s) were measured in tandem cuvettes using 1 mL solutions of 1 μM P450 in 50 mM sodium phosphate (pH 7.2) plus 20% glycerol and titrating with ethanol solutions of steroids in the following concentration (μM) ranges: PROG and PREG, 1−25; HO-PROG and HO-PREG, 2−150; androstenedione and DHEA, 2−150. Duplicate values are reported for binding constants obtained from two separate preparations of rat and human P450c17. [b] Undetermined value. [c] No spectral perturbation was observed.

constants (K_s) (Table 3) were calculated from absorbance changes observed in substrate-induced difference spectra (*41*). In all cases, binding of substrates to the human P450c17 is either similar or stronger than binding to the rat enzyme. The most striking result in Table 3 is the 5-fold tighter binding for HO-PROG to the human enzyme which poorly catalyzes the lyase reaction. Quantification of lyase product binding is complicated by the fact that different types of binding spectra are observed with the two enzymes (Figure 2). With the exception of DHEA binding to human P450c17, binding constants for AD and DHEA are not reported. PROG, PREG, HO-PROG, and HO-PREG gave the expected type I, high-spin spectra. The spectral perturbation observed upon interaction of human P450c17 with 1−100 μM AD is a broad trough at 425−430 nm, and a broad maximum at 390−400 nm (Figure 2A). Although this resembles type I spectra, it is clearly different from the classical type I observed with steroids that serve as enzymatic substrates. At very high concentrations (>100 μM), AD induces a spectrum more closely resembling a classical type I spectrum (data not shown). AD binding to the rat enzyme produced a reverse type I spectrum (*42*) with minimum and maximum absorbance values at 390 and 420 nm, respectively. In contrast to the results observed with AD, DHEA induced a classical type I spectrum with the human enzyme, while even the highest concentrations (10−200 μM) did not induce a spectral perturbation with the rat enzyme (Figure 2B). The variability in difference spectra induced by androgens indicates unique heme environments within the active sites of these two enzymes which catalyze the same set of reactions.

Substrate-Specific Protection from Heme Loss during Turnover. The stability of P450c17 during turnover in the reconstituted assay system also exhibits species-specific and substrate-specific properties. The enzymes were incubated using the conditions described for activity assays in Table 2. After a 30 min incubation in the absence of NADPH and substrate, a pronounced loss of both rat and human P450c17 450 nm absorbance peaks was observed with the corresponding appearance of 420 nm peaks (Figure 3A,B, −substrate, −NADPH). In the presence of NADPH and the absence of substrate (Figure 3A,B; $t = 30$ − substrate), the loss of the 450 nm peaks occurs without the formation of the 420 nm peaks. Figure 3 also demonstrates substrate-dependent protection from loss of the 450 nm peaks during turnover of

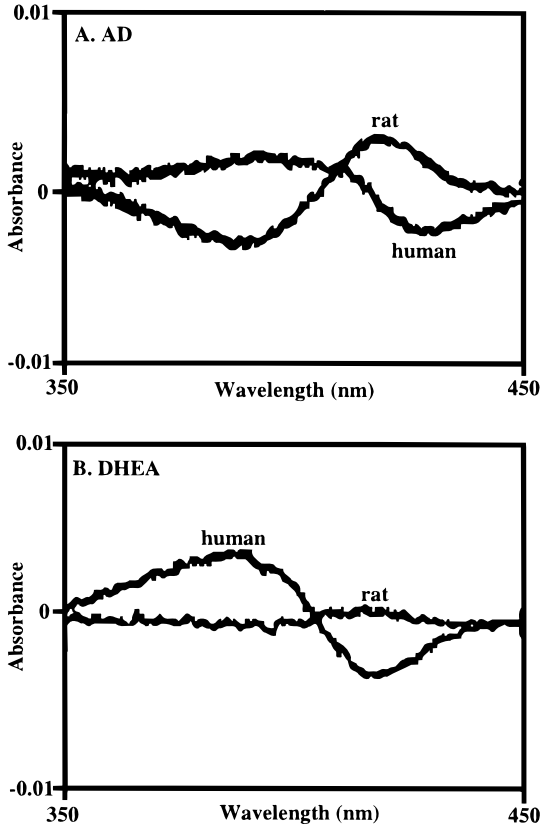

FIGURE 2: Rat and human P450c17-specific AD- and DHEA-induced difference spectra. Ethanol stock solutions of androstenedione (A) or DHEA (B) were added to 1 mL solutions of 1 μM P450c17 in 50 mM sodium phosphate, pH 7.2, 20% glycerol. (A) Rat P450c17 + 20 μM androstenedione, human P450c17 + 40 μM androstenedione. (B) Rat P450c17 + 200 μM DHEA, human P450c17 + 30 μM DHEA.

the rat and human enzymes. As seen in Figure 3A, PROG is more effective in maintaining the rat enzyme heme environment than PREG. Conversely, PREG is more effective in protecting the human enzyme than PROG (Figure 3B).

Substrate-Specific Effects on H_2O_2 Production during Turnover. To better understand the mechanism of substrate-dependent protection from heme loss during turnover, we examined the coupling efficiency of rat and human P450c17 during turnover of Δ^4- and Δ^5-steroids. The substrate-dependent decrease in H_2O_2 production during turnover in vitro is used both as an indicator of substrate specificity and to demonstrate possible intrinsic differences in oxygen activation between the two enzymes. As seen in Figure 4, in the absence of substrate the rat enzyme produces nearly 4 times more H_2O_2 than the human enzyme. Activity assays using both rat and human P450 reductase suggest that the higher H_2O_2 production with the rat enzyme is not due to a more favorable interaction with the rat reductase (not shown). In the presence of PROG, H_2O_2 produced by the rat enzyme is decreased 3−4-fold while that produced by the human enzyme is unchanged. A similar decrease in H_2O_2 production by the rat enzyme is observed in the presence of PREG (Figure 4B), and in contrast to PROG, PREG also causes a noticeable decrease in H_2O_2 production by the human enzyme.

As shown in Figure 5, substrate specificities in H_2O_2 production are also observed with the hydroxysteroids HO-

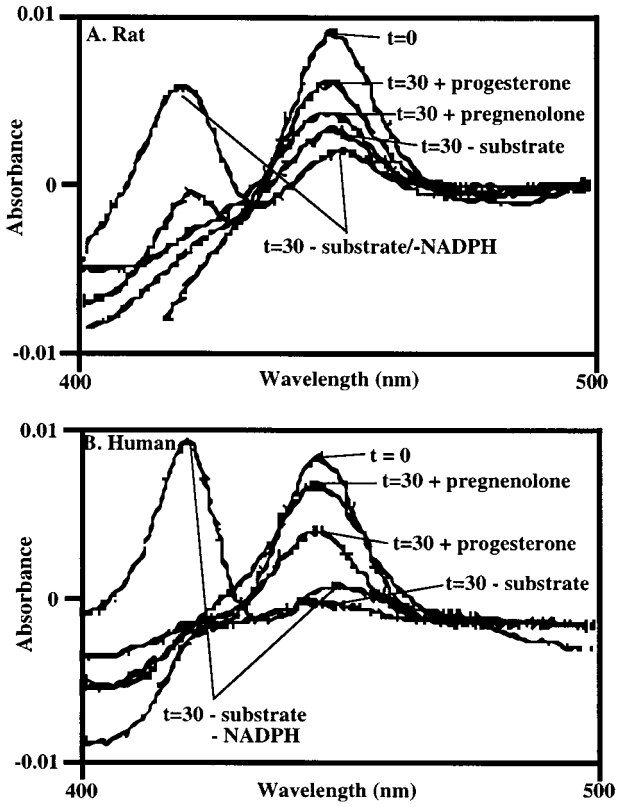

FIGURE 3: Substrate-specific protection from 450 nm loss in rat and human P450c17 during turnover. Reaction compositions were identical to those described for activity measurements in Table 2. 1 mL reactions consisting of 0.5 μM rat (A) or human (B) P450c17 in 50 mM sodium phosphate (pH 7.2), 20% glycerol, 1 mM magnesium acetate, 100 μg/mL DLPC, 0.5 μM P450-reductase, 50 μM PROG or PREG, and 1 mM NADPH were incubated at 37 °C and monitored for loss of the 450 nm peak. A 0.5 mL aliquot of each reconstituted reaction was removed at 0 and 30 min after addition of NADPH, and the reduced CO-difference spectra of each reaction were recorded. The results shown are representative of duplicate experiments.

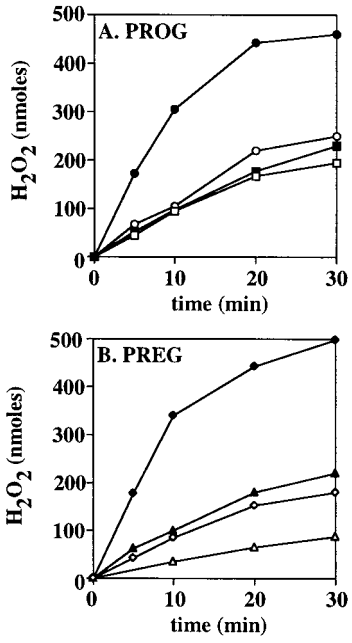

FIGURE 4: H_2O_2 production during turnover of rat and human P450c17 with PROG and PREG. The reaction conditions were identical to those described for Table 2. 1 mL reactions consisting of 0.5 μM rat or human P450c17 in 50 mM sodium phosphate (pH 7.2), 20% glycerol, 1 mM magnesium acetate, 100 μg/mL DLPC, 0.5 μM P450-reductase, 50 μM PROG or PREG, and 1 mM NADPH were incubated at 37 °C. At the indicated time intervals, 150 μL aliquots from each reaction were combined with 0.25 mL of 3% TCA on ice. Following centrifugation at 5000 rpm for 10 min, a 0.25 mL aliquot of the supernatant was added to 0.05 mL of 10 mM ferroammonium sulfate and 0.02 mL of 2.5 M potassium ferricyanate. H_2O_2 concentrations were measured by the absorbance at 480 nm. (A) Effects of PROG on H_2O_2 production: rat P450c17 − substrate (●), rat P450c17 + PROG (○), human P450c17 − substrate (■), human P450c17 + PROG (□). (B) Effects of PREG on H_2O_2 production: rat P450c17 − substrate (◆), rat P450c17 + PREG (◇), human P450c17 − substrate (▲), human P450c17 + PREG (△). The results shown are representative of duplicate experiments.

PROG and HO-PREG. HO-PROG decreases H_2O_2 production by the rat enzyme to a similar extent as seen in Figure 4 with PROG (Figure 5A). Figure 5B shows that HO-PREG decreases H_2O_2 production by the rat enzyme less efficiently than does HO-PROG.

Comparison of the Interaction of Cytochrome b_5 with Rat and Human P450c17. An additional component of the microsomal electron transport system, cytochrome b_5, has been shown to modulate the activity of human P450c17 in vitro (*24, 43*−*46*). The effects of b_5 on the reconstituted Δ^4 activities of rat and human P450c17 are shown in Figure 6. As previously reported (*24*), a profound stimulation of the human Δ^4 lyase reaction is observed in the presence of b_5. The lyase activity of the rat enzyme is also stimulated by b_5, and this reaction approaches quantitative conversion of PROG to AD over a period of 20 min. The b_5-dependent stimulation of activity was measured using either PROG or HO-PROG as starting substrate (Table 4). A small stimulation of rat lyase activity by b_5 is observed when PROG and HO-PROG are used as starting substrates. However, a more pronounced stimulation by b_5 is observed with the human enzyme. The actual degree of b_5-dependent stimulation of human lyase activity is difficult to assess due to the extremely low activity in the absence of b_5. One possible mechanism of b_5-dependent stimulation of P450 activity is facilitation of the second electron transfer to the activated ferro-oxygen intermediate (*47, 48*). To test for the facilitation of electron transfer by b_5 in our reconstituted system, we measured the effect of b_5 on the production of H_2O_2 during PROG metabolism by the rat and human P450c17 (Figure 7). Although b_5 clearly had a stimulatory effect on PROG metabolism with both enzymes (Figure 6, Table 4), it did not further alter H_2O_2 production during PROG metabolism by either the rat or the human P450c17, indicating no role in electron transfer.

DISCUSSION

Different animals utilize distinct steroidogenic pathways to satisfy their own unique physiological requirements. Characterization of individual steroid biosynthetic enzymes among different species can lead to identification of key regulatory steps in these diverse pathways. Understanding the species-specific biochemical properties of rat and human P450c17 provides insight into the molecular basis by which these animals control the important branch point between glucocorticoid and androgen biosynthetic pathways. In rats, this branch point is controlled by not expressing P450c17 in the adrenal, therefore producing corticosterone as the major glucocorticoid and not producing androgens in this organ.

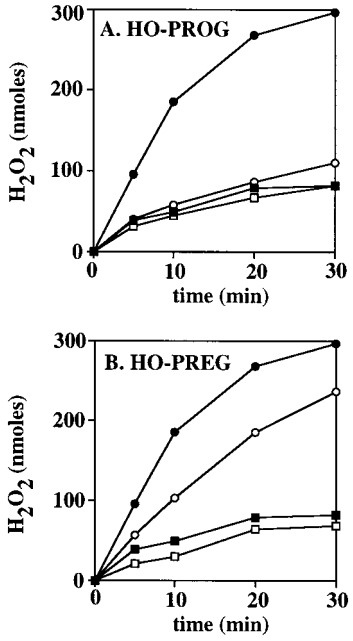

FIGURE 5: H_2O_2 production during turnover of rat and human P450c17 with HO-PROG and HO-PREG. Reaction conditions were as described in Figure 4. (A) Effects of HO-PROG on H_2O_2 production: rat P450c17 − substrate (●), rat P450c17 + HO-PROG (○), human P450c17 − substrate (■), human P450c17 + HO-PROG (□). (B) Effects of HO-PREG on H_2O_2 production: rat P450c17- substrate (●), rat P450c17 + HO-PREG (○), human P450c17 − substrate (■), human P450c17 + HO-PREG (□). The results shown are representative of duplicate experiments.

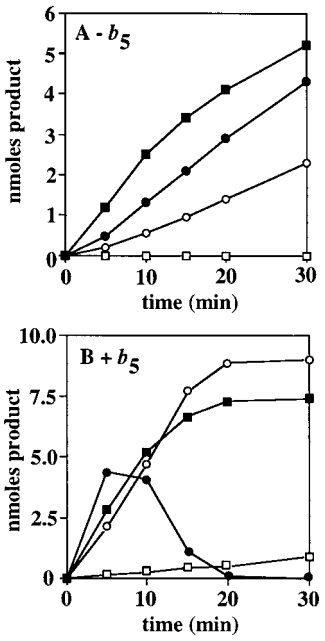

FIGURE 6: Stimulation of PROG metabolism by rat and human P450c17 in the presence of cytochrome b_5. Time course of PROG metabolism by rat and human P450c17 in the absence (A) and presence (B) of b_5. Reactions conditions were identical to those described in Table 2. 1 mL reactions consisting of 0.5 μM rat or human P450c17 and 0.5 μM b_5 in 50 mM sodium phosphate (pH 7.2), 20% glycerol, 1 mM magnesium acetate, 100 μg/mL DLPC, 0.5 μM P450-reductase, 50 μM PROG, and 1 mM NADPH were incubated at 37 °C. At the indicated time points, 0.2 mL aliquots were removed and extracted with 2 mL of CH_2Cl_2. Product analysis was performed as described under Experimental Procedures. Rat HO-PROG production (●), rat androstenedione production (○), human HO-PROG production (■), human androstenedione production (□). The results shown are representitive of duplicate experiments.

Alternatively, humans have evolved a more elaborate steroidogenic system, composed of P450c17 in the adrenals and P450c17 plus b_5 in the gonads, that regulates glucocorticoid and androgen biosynthesis.

Based on studies by our laboratory and by others, it has been predicted that the rat enzyme catalyzes the 17,20-lyase reactions at a more rapid rate than the human enzyme (*20*, *26*, *29*, *34*). This is confirmed here by direct comparison of the partially purified enzymes (Table 2) which indicates little difference in the 17α-hydroxylase activities of rat and human P450c17, while profound differences exist between the lyase activities. This is quite interesting when considering that the physiological difference in steroidogenesis between rats and humans is that rats have no requirement for 17α-hydroxylation as an independent reaction. Stated another way, the capacity for 17α-hydroxylation is very similar in rats and humans even though rats only use this reaction as an intermediate in sex hormone production. Surprisingly, the lyase activities are very different between these enzymes even though both species share an absolute reproductive requirement for androgens. The rat enzyme has evolved into an essentially independent androgen synthesizer while the human enzyme requires the accessory protein cytochrome b_5 to meet physiological androgen requirements. In the testis, where synthesis of androgens is the sole function for P450c17, the presence of a high concentration of b_5 will promote the lyase activity at the expense of HO-PROG accumulation. The b_5:P450 ratio of 10:1 in human testis is the highest reported (*49*), being 30 times higher than that found in rat testis (0.29:1). The high intrinsic Δ^4 lyase activity of the rat enzyme negates the need for high b_5 levels in rat testes. As demonstrated here, the rat P450c17 Δ^4 lyase activity is also stimulated in the presence of b_5, but the fold stimulation is modest relative to that observed with the human enzyme (Figure 6, Table 4). The absence of any effect on H_2O_2 production during turnover argues against a role of b_5 in facilitating electron transport of either enzyme, in agreement with a recent report by Auchus et al. (*50*). The dramatic stimulation of Δ^4 lyase activity when PROG is used as starting substrate in the presence of b_5 suggests that it relieves the inhibition caused by PROG, perhaps by slightly changing the protein conformation, thereby promoting the lyase activity.

Table 4: Cytochrome b_5-Dependent Stimulation of PROG Metabolism by the Rat and Human P450c17 Using PROG or HO-PROG as Starting Substrate[a]

	activity [nmol min^{-1} (nmol of P450)$^{-1}$]			
	hydroxylase activity		lyase activity	
starting substrate	rat	human	rat	human
progesterone	0.95	3.5	0.64	0.005[b]
progesterone + b_5	1.5	3.3	1.6	0.31
HO-Prog	nd	nd	1.5	0.12
HO-Prog + b_5	nd	nd	2.2	0.64

[a] All assays were performed as described under Experimental Procedures with the addition of 0.5 μM cytochrome b_5. Hydroxylase and lyase reactions were carried out for 5 and 30 min, respectively after addition of 1 mM NADPH. These values are representitive of duplicate experiments. [b] Value at the limits of detection. [c] nd, not determined.

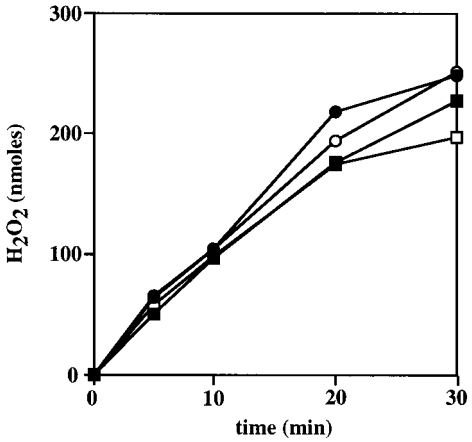

FIGURE 7: Effect of cytochrome b_5 on the coupling efficiency of PROG metabolism by rat and human P450c17. Reaction conditions were as described in Figure 6. 1 mL reactions consisting of 0.5 μM rat or human P450c17 and 0.5 μM b_5 in 50 mM sodium phosphate (pH 7.2), 20% glycerol, 1 mM magnesium acetate, 100 μg/mL DLPC, 0.5 μM P450-reductase, 50 μM PROG, and 1 mM NADPH were incubated at 37 °C. At the indicated time points, H_2O_2 assays were performed as described in Figure 4. Rat P450c17 + PROG (●), rat P450c17 + PROG/b_5 (○), human P450c17 + PROG (■), human P450c17 + PROG/b_5 (□).

Binding studies show quantitative and qualitative differences in steroid binding to rat and human P450c17. The very similar binding constants for PROG and PREG indicate that the large difference between rat and human P450c17 lyase activities seen in Table 2 is not due to large differences in binding of these substrates. It has been previously suggested that the lyase activity in guinea pig P450c17 may require a change in orientation of HO-PROG in the active site (*51*), and that tight binding may inhibit this reorientation. Our results suggest that HO-PROG does indeed bind to the human enzyme more tightly than to the rat enzyme. Although reorientation of the hydroxysteroid is an interesting model to explain the low lyase activity of the human P450c17, it is difficult to understand how the 5-fold difference in binding constants observed in this study could so profoundly affect the rate of catalysis. There are other factors, most notably the oxidation state of the oxygen at the 3-position, that contribute to the Δ^4/Δ^5 preference. It may be that reorientation of the hydroxylated intermediates is more critical for the Δ^4 lyase reactions. This may explain why both enzymes can catalyze the HO-PREG lyase reaction even though HO-PREG has a higher apparent affinity than HO-PROG. A possible mechanism of b_5 stimulation may be to slightly "relax" the enzyme−hydroxysteroid complex, promoting the proposed reorientation and subsequent lyase reaction. The disparity in lyase activities may also result from a combination of differences in substrate binding and product release. It has been suggested that product dissociation from P4501A1 and P4502E1 is the rate-limiting step in catalysis (*52*, *53*). In this study, product binding constants could not be determined in most cases, although the different binding spectra observed with AD and DHEA are indicative of differences in the heme environments of the two enzymes. The degree to which these different active site environments contribute to the rate of product release will require further study.

We carried out a series of substrate-specific and species-specific in vitro enzyme turnover studies designed to identify mechanistic differences between these enzymes. Under the conditions of the reconstituted assays with no turnover (−NADPH, −substrate), both enzymes demonstrated similar stabilities, being converted to P420 during the course of incubation. Similar stabilities between the enzymes were also observed in the absence of substrate (+NADPH, −substrate). Under these conditions of P450-dependent oxygen activation, no P420 formation was observed for either enzyme. Thus, in addition to the similar relative stabilities exhibited under these conditions, the absence of P420 formation by both enzymes suggests that they share a similar NADPH-dependent mechanism of denaturation. Although these relative stabilities of rat and human P450c17 in the absence of substrate are similar, important species-specific differences in stability are observed during turnover of substrates. In the presence of PROG and PREG, both enzymes are protected from loss of the 450 nm peak, and this protective effect exhibits substrate specificity for each enzyme. The rat enzyme is clearly protected more efficiently during turnover of PROG, while the human enzyme is protected more efficiently during turnover of PREG. As described above, DHEA induces a spin shift upon binding to the human enzyme and has no effect on the rat enzyme, providing additional evidence for the Δ^5 preference in the active site of the human enzyme. The substrate-dependent protection from heme loss during turnover can be explained, at least in part, by a substrate-dependent increase in electron coupling. It has been demonstrated for other P450s that uncoupling of electrons from NADPH to product formation occurs when poor substrates are used (*54*−*56*). Such uncoupling contributes to the generation of reactive oxygen species such as H_2O_2 at the active site, and such nonproductive activation of oxygen can result in the oxidative modification of heme that prevents formation of the reduced CO-difference spectrum. The catalytic cycle of rat P450c17 is clearly more tightly coupled during turnover of PROG and HO-PROG than the human enzyme. These substrates decrease the H_2O_2 production with the rat enzyme, yet have no effect on H_2O_2 production by the human enzyme, showing that the rat enzyme is coupled more efficiently by Δ^4-steroids than the human enzyme, and consequently protected from heme loss by Δ^4-steroids. PREG also decreases H_2O_2 production by the rat enzyme, but in contrast to PROG, also decreases H_2O_2 production from the human enzyme. Furthermore, HO-PREG couples the rat catalytic cycle at approximately half the efficiency of HO-PROG. The ability of the Δ^5-steroids to couple the human catalytic cycle, and to be less efficient than Δ^4-steroids in coupling the rat catalytic cycle, correlates with the ability of the human P450c17 to catalyze the Δ^5 lyase reaction and the protection provided by Δ^5-steroids during turnover. Thus, the results from Figures 3, 4, and 5 suggest that rat and human P450c17 demonstrate substrate-specific protection from loss of heme during catalysis, and that substrate-specific coupling effects contribute to this protection. Inactivation of P450c17 in vivo by a turnover-dependent mechanism has previously been reported. Oxygen-dependent loss of P450c17 activity has been observed for P450c17 in rat Leydig cells when testosterone was used as a "pseudosubstrate" (*57*, *58*). This suggested that testosterone binding to P450c17 increased the susceptibility of the enzyme to oxidative modification and subsequent proteolytic degradation. It is possible that testosterone behaved as a poor

substrate, uncoupling the catalytic cycle of P450c17, and promoting the formation of deleterious reactive oxygen intermediates. PREG binding to the human enzyme or PROG binding to the rat enzyme may prevent this type of inactivation.

The higher rat lyase activity might be related to the relatively high amounts of H_2O_2 produced by the rat enzyme in the absence of substrate. The ability of the rat P450c17 to produce 3−4 times more H_2O_2 than the human enzyme points to active site differences as suggested from lyase product binding studies. The lyase reaction has been reported to proceed via a peroxo intermediate as opposed to the oxene intermediate implicated in hydroxylation reactions (*59*, *60*). If the active site of the rat enzyme favors formation of the peroxo intermediate over the oxene intermediate, and if this intermediate were somewhat labile in the absence of substrate, then it would be expected that the rat enzyme would produce more H_2O_2 than the human enzyme. The preferential formation of the peroxo intermediate would be expected for a rat enzyme whose sole function is to catalyze the lyase reaction and produce androgens. Alternatively, the active site of the human enzyme, which must also catalyze the hydroxylase reaction, may preferentially form the oxene intermediate upon reduction.

In summary, differences in activity, substrate binding, stability, and coupling efficiency have been revealed upon comparison of rat and human P450c17. A major difference in the rat and human steroidogenic pathways is the absence of adrenal P450c17 expression in the rat (*14*). The results from the present study provide some insight into the molecular basis of differences in rat and human P450c17, and indicate that these differences are associated with Δ^4/Δ^5 biosynthetic pathway selection. The catalytic efficiencies of rat and human P450c17 appear to be more critical for determining Δ^4/Δ^5 pathway selection than does the binding of PROG or PREG. However, tighter binding of HO-PROG to the human enzyme indicates that binding properties may partially contribute to the negligible human Δ^4 lyase activity. Even though the general reactions catalyzed by P450c17 in all species are the same, important biochemical differences exist between the forms from different species, and these differences can be related to the steroidogenic needs of the species. Certain P450 subfamily members in one species have quite different catalytic specificities from the orthologous subfamily member in another species (*61*). For P450c17 which has no subfamily members, and for other P450s such as P450scc, P450c27, P450aldo, P450c11, P450arom, and P450c21, the general catalytic properties are similar, but the biochemical details differ from species to species, meeting metabolic needs. For example, in the cow, P450c11 catalyzes both cortisol and aldosterone synthesis (*62*) while in human and rats P450c11 does not catalyze aldosterone synthesis and a different enzyme, P450aldo, plays this role (*63*). In the case of P450c17, it is evident that extrapolation of P450 activities from animal models to the corresponding human activity should consider the physiological role of the P450s in both species.

Each of the species-specific differences reported in this study represents a marker to study structure/function relationships in these two P450s. Because these enzymes share 69% amino acid identity yet clearly exhibit different biochemical properties, they are excellent candidates for detailed structure/function analysis. Comparison of amino acids that contribute to any of the properties described in this study will advance our understanding of P450c17 and aid in the elucidation of the molecular basis of steroidogenic disorders.

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REFERENCES

1. Yanase, T., Simpson, E. R., and Waterman, M. R. (1991) *Endrocr. Rev. 12,* 91−108.
2. Ahlgren, R., Yanase, T., Simpson, E. R., Winter, J. S., and Waterman, M. R. (1992) *J. Clin. Endocrinol. Metab. 74,* 667−672.
3. Lowe, F. C., and Bamberger, M. H. (1990) *Urology 36,* 541−545.
4. McCague, R., Rowlands, M. G., Barrie, S. E., and Houghton, J. (1990) *J. Med. Chem. 33,* 3050−3055.
5. Vanden Bossche, H., Koymans, L., and Moereels, H. (1995) *Parmacol. Ther. 67,* 79−100.
6. Boccuzzi, G., Aragna, M., Seccia, M., Brignardello, E., Tamagna, E., Albano, E., Danni, O., and Bellomo, G. (1997) *Free Radical Biol. Med. 22,* 1289−1294.
7. Eberling, P., and Koivisto, V. A. (1994) *Lancet 343,* 1479−1481.
8. Barrett-Connor, E., Khaw, K. T., and Yen, S. S. C. (1986) *N. Engl. J. Med. 315,* 1519−1524.
9. Coleman, D. L., Leiter, E. H., and Schwizer, R. W. (1982) *Diabetes 31,* 830−833.
10. Nakajin, S., and Hall, P. F. (1981) *J. Biol. Chem. 256,* 3871−3876.
11. Kominami, S., Shinzawa, K., and Takemori, S. (1982) *Biochem. Biophys. Res. Commun. 111,* 916−921.
12. Perrin, A., Defaye, G., Guidicelli, C., and Chambaz, E. M. (1991) *J. Steroid Biochem. Mol. Biol. 40,* 431−435.
13. Betz, G., Tsai, P., and Hales, D. (1980) *Endocrinology 7,* 1055−1060.
14. Hofman, F. G. (1962) *Biochim. Biophys. Acta 58,* 343−348.
15. Perkins, I. M., and Payne, A. H. (1988) *Endocrinology 123,* 2675−2682.
16. Nishihara, M., Winters, C. A., Buzko, E., Waterman, M. R., and Dufau, M. L. (1988) *Biochem. Biophys. Res. Commun. 154,* 151−158.
17. Voutilainen, R., Taganainen, J., Chung, B.-C., Matteson, K. J., and Miller, W. L. (1986) *J. Clin. Endocrinol. Metab. 63,* 202−207.
18. Fevold, H. R., Wilson, P. I., and Salanina, S. M. (1978) *J. Steroid Biochem. 9,* 1033−1041.
19. Nakajin, S., Shinoda, M., Haniu, M., Shively, J. E., and Hall, P. F. (1984) *J. Biol. Chem. 259,* 3971−3976.
20. Fevold, H. R., Lorence, M. C., McCarthy, J. L., Trant, J. M., Kagimoto, M., Waterman, M. R., and Mason, J. I. (1989) *Mol. Endrocrinol. 3,* 968−975.
21. Sakai, N., Tanaka, M., Adachi, S., Miller, W. L., and Wagahana, Y. (1992) *FEBS Lett. 301,* 606−664.
22. Cloutier, M., Fleury, A., Courtemanche, J., Ducharme, L., Mason, J. I., and Lehoux, J.-G. (1997) *DNA Cell Biol. 16,* 357−368.
23. Tremblay, Y., Fleury, A., Beaudoin, C., Vallee, M., and Belanger, A. (1994) *DNA Cell Biol. 13,* 1199−1212.
24. Katagiri, M., Kagawa, N., and Waterman, M. R. (1995) *Arch. Biochem. Biophys. 317,* 343−347.
25. Conley, A. J., and Bird, I. M. (1997) *Biol. Reprod. 56,* 789−799.
26. Kitamura, M., Buczko, E., and Dufau, M. L. (1991) *Mol. Endrocrinol. 5,* 1373−1380.
27. Koh, Y., Buczko, E., and Dufau, M. L. (1993) *J. Biol. Chem. 268,* 18267−18271.

28. Geller, D. H., Auchus, R. J., Mendonca, B. B., and Miller, W. L. (1997) *Nat. Genet. 17*, 201−205.
29. Miller, W. L., Auchus, R. J., and Geller, D. H. (1997) *Steroids 62*, 133−142.
30. Zhang, L.-H., Rodriguiez, H., Ohno, S., and Miller, W. L. (1995) *Proc. Natl. Acad. Sci. U.S.A. 92*, 10619−10623.
31. Perrin, A., Chambaz, E. M., and Defaye, G. (1995) *J. Steroid Biochem. 54*, 121−129.
32. Barnes, H. J., Arlotto, M. P., and Waterman, M. R. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 5597−5601.
33. Barnes, H. J. (1992), Ph.D. Dissertation, University of Texas Southwestern Medical Center.
34. Imai, T., Globerman, H., Gertner, J. M., Kagawa, N., and Waterman, M. R. (1993) *J. Biol. Chem. 268*, 19681−19689.
35. Bordier, C. (1981) *J. Biol. Chem. 256*, 1604−1607.
36. Werck-Reichhart, D., Benveniste, I., Teutsch, H., Durst, F., and Gabriac, B. (1991) *Anal. Biochem. 197*, 125−131.
37. Omura, T., and Sato, R. (1964) *J. Biol. Chem. 239*, 2370−2378.
38. Smith, P. K., Krohn, R. I., Hermanson, G. T., Mallia, A. K., Gartner, F. H., Provenzano, M. D., Fujimoto, E. K., Goeke, N. M., Olson, B. J., and Klenk, D. C. (1985) *Anal. Biochem 150*, 76−85.
39. Laemmli, U. K. (1970) *Nature 227*, 680−685.
40. Thurman, R. G., Georg Ley, H., and Scholz, R. (1972) *Eur. J. Biochem. 25*, 420−430.
41. Narasimhulu, S., Cooper, D. Y., and Rosenthal, D. (1965) *Life Sci. 4*, 2101−2107.
42. Jefcoat, C. R. (1978) *Methods Enzymol. 52*, 258−279.
43. Kominami, S., Ogawa, N., Morimune, R., De-Ying, H., and Takemori, S. (1992) *J. Steroid Biochem. Mol. Biol. 42*, 57−64.
44. Sakai, Y., Yanase, T., Hara, T., Takayanagi, R., Haji, M., and Nawata, H. (1994) *Clin. Endocrinol. 40*, 205−209.
45. Lee-Robichaud, P., Kaderbhai, M. A., Kaderbhai, N., Wright, J. N., and Akhtar, M. (1997) *Biochem. J. 321*, 857−863.
46. Lee-Robichaud, P., Wright, J. N., Akhtar, M. E., and Akhtar, M. (1995) *Biochem. J. 308*, 901−908.
47. Hildebrandt, A., and Estabrook, R. W. (1971) *Arch. Biochem. Biophys. 143*, 66−79.
48. Noshiro, M., Ullrich, V., and Omura, T. (1981) *Eur. J. Biochem. 116*, 521−526.
49. Mason, J. I., Estabrook, R. W., and Purvis, J. L. (1973) *Ann. N.Y. Acad. Sci. 212*, 406−419.
50. Auchus, R. J., Lee, T. C., and Miller, W. L. (1998) *J. Biol. Chem. 273*, 3158−3165.
51. Tagashira, H., Kominami, S., and Takemori, S. (1995) *Biochemistry 34*, 10939−10945.
52. Sakaki, T., Kominami, S., Takemori, S., Ohkawa, H., Akiyoshi-Shibata, M., and Yabusaki, Y. (1994) *Biochemistry 33*, 4933−4939.
53. Bell, L. C., and Guengerich, F. P. (1997) *J. Biol. Chem. 272*, 29643−29651.
54. Karuzina, I. I., and Archakov, A. I. (1994) *Free Radical Biol. Med. 17*, 557−567.
55. Guengerich, F. P. (1978) *Biochemistry 17*, 3633−3639.
56. Pompon, D. (1987) *Biochemistry 26*, 6429−6435.
57. Perkins, L. M., Hall, F. J., and Payne, A. H. (1988) *Endrocrinology 122*, 2257−2264.
58. Geogeiou, M., Perkins, L. M., and Payne, A. H. (1987) *Endocrinology 121*, 1390−1399.
59. Swinney, D. C., and Mak, A. Y. (1994) *Biochemistry 33*, 2185−2190.
60. Lee-Robichaud, P., Akhtar, M. E., and Akhtar, M. (1998) *Biochem. J. 330*, 967−974.
61. Guengerich, F. P. (1997) *Chem.-Biol. Interact. 106*, 161−182.
62. Mathew, P. A., Mason, J. I., Trant, J. M., and Waterman, M. R. (1990) *Mol. Cell. Endocrinol. 73*, 73−80.
63. Mornet, E., Dupont, J., Vitek, A., and White, P. C. (1989) *J. Biol. Chem. 264*, 20961−20967.

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