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Hydroxylation and Lyase Reactions of Steroids Catalyzed by Mouse Cytochrome P450 17A1 (Cyp17a1)

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Abstract

Cytochrome P450 17A1 (CYP17A1) catalyzes 17α-hydroxylation and 17,20-lyase reactions with steroid hormones. Mice contain an orthologous Cyp17a1 enzyme in the genome, and its amino acid sequence has high similarity with human CYP17A1. We purified recombinant mouse Cyp17a1 and characterized its oxidation reactions with progesterone and pregnenolone. The open reading frame of the mouse *Cyp17a1* gene was inserted and successfully expressed in *Escherichia coli* and then purified using Ni^{2+}-NTA affinity column chromatography. Purified mouse Cyp17a1 displayed typical Type I binding titration spectral changes upon the addition of progesterone, 17α-OH progesterone, pregnenolone, and 17α-OH pregnenolone, with similar binding affinities to those of human CYP17A1. Catalytic activities for 17α-hydroxylation and 17,20-lyase reactions were studied using UPLC-mass spectrometry analysis. Mouse Cyp17a1 showed cytochrome b_5 stimulation in catalysis. In comparison to human enzyme, much higher specificity constants (k_{cat}/K_m) were observed with mouse Cyp17a1. In the reactions of Δ4-steroids (progesterone and 17α-OH progesterone), the specificity constants were 2100 times higher than the human enzyme. The addition of cytochrome b_5 produced significant stimulation of 17,20-lyase activities of mouse Cyp17a1. Two Arg mutants of mouse Cyp17a1 (R347H and R358Q) displayed a larger decrease in 17,20-lyase reaction (from 17α-OH pregnenolone to DHEA) than 17α-hydroxylation, indicating that –as in human CYP17A1–these basic residues in mouse Cyp17a1 are important in interacting with the cytochrome b_5 protein in the lyase reactions.

Keywords

cytochrome P450; CYP17A1; cytochrome b_5; progesterone; pregnenolone; enzyme kinetics; enzyme mechanism; enzyme inhibitor; enzyme catalysis

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Author Contributions

DK and FPG conceived and supervised the study; DK and SGL designed experiments; SGL, VK, GHL, CK and EJ performed experiments; SGL performed visualization; SGL, VK and DK analyzed data; SGL, FPG, and DK wrote and edited the manuscript.

Conflict of Interest

The authors declare that they have no conflict of interest with the contents of this article.

Supporting Information

Supporting information for this article can be found online at http://xxxx.

1. Introduction

Cytochrome P450 (CYP) is a superfamily of microsomal membrane-bound hemoproteins that exhibit a unique 450 nm spectral absorbance peak in the ferrous-carbon monoxide-bound form [1]. P450s catalyze mixed-function monooxygenase reactions to deliver a single atom of molecular oxygen to a substrate, along with the introduction of electrons from NAD(P)H [2]. CYP17A1 is expressed in the adrenal glands and gonads of various mammals. This enzyme is involved in two consecutive reactions. The first reaction is 17α-hydroxylation, converting pregnenolone and progesterone to 17α-hydroxy (OH) pregnenolone and 17α-OH progesterone, respectively (Fig. 1). The second reaction is a 17,20-lyase reaction, catalyzing the conversion of 17α-OH pregnenolone to dehydroepiandrosterone (DHEA) and 17α-OH progesterone to androstenedione (Fig. 1) [3]. Because CYP17A1 possesses both 17α-hydroxylation and 17,20-lyase activities, it is a key branch point in steroid hormone synthesis [4]. The presence of another hemoprotein, cytochrome b_5 (b_5), has been shown to greatly stimulate 17,20-lyase activity, at least in some species [5]. CYP17A1 is an important drug target for the treatment of breast and prostate cancers, in that proliferation is dependent on estrogens and androgens. Inhibition of the 17,20-lyase activity of CYP17A1 by the drug abiraterone acetate decreases the concentration of circulating testosterone and improves survival in relapsed patients [6]. Therefore, there is recent pharmaceutical interest focused on the development of selective inhibitors of 17,20-lyase activity of CYP17A1 that can improve safety profiles and simplify treatment. Since both the 17α-hydroxylation and 17,20-lyase activities of CYP17A1 originate from a single active site with a limited steroid-binding pocket, it has been very difficult to develop an active site-directed inhibitor that selectively inhibits only the 17,20-lyase reactions [7, 8].

The mouse (*Mus musculus*) is the most commonly used model organism in many fields of research involving human diseases because of the phylogenetic relatedness and physiological similarity to humans, the ease of maintaining and breeding the animals in the laboratory, and the availability of many inbred strains {Morse HCI. Building a better mouse: one hundred years of genetics and biology In: Fox JG. (ed.). The Mouse in Biomedical Research. Amsterdam: Elsevier, 2007, 1–11}. Mice have 102 cytochrome P450 genes, and CYP17A1 is one of the more conserved P450 enzymes in various animal species (https://drnelson.uthsc.edu/). An *in vivo* study of mice with the *Cyp17a1* gene knocked out was reported [25]. The XY KO mice had a female appearance with external genital phenotype and were infertile, like humans, with severe 17α-hydroxylase/17,20-lyase deficiency (17OHD) [25]. Male *Cyp17a1* KO mice have small testes, lacking Wolffian derivates, and female *Cyp17a1* KO mice display underdeveloped uteri [25]. Therefore, this mouse model reflects many aspects of the disorders of sex development phenotype of the human CYP17A1deficiency [25]. Amino acid sequence alignment analysis of mouse Cyp17a1 with human CYP17A1 displayed high similarity with a T-coffee score of 999 and indicates an orthologue Cyp17a1 enzyme in mice (Supplementary Fig. S1). Good sequence alignment with human CYP17A1 suggests that mouse Cyp17a1 is likely to have 17α-hydroxylation and 17,20-lyase reaction catalytic activity.

In this study, we characterized the enzymatic activity of mouse Cyp17a1 (Cyp17a1) using a purified enzyme. The substrate binding titration analysis and the steady-state kinetic analysis

for 17α-hydroxylation and 17,20-lyase reactions were performed in order to compare the mouse to the human enzyme and validate its relevance in understanding human diseases. In particular, the stimulatory effect of b_5 on catalytic activity was studied and compared with human CYP17A1. Two human clinical mutation b_5 binding sites (Arg-347 and Arg-358) were analyzed.

2. Results

2.1. Construction, heterologous expression, and purification

Recombinant mouse Cyp17a1 wild-type and two mutants were successfully expressed in *Escherichia coli* JM109 cells. The mouse Cyp17a1 wild-type protein showed an expression level of ~450 nmol P450 enzyme per liter culture, and the expression levels of the R347H and R358Q mutants were 380 and 400 nmol per liter, respectively. Mouse Cyp17a1 wild-type and mutants were purified using Ni^{2+}-NTA affinity column chromatography (Fig. 2). The CO-binding spectra of purified mouse Cyp17a1 wild-type protein and the mutants displayed no detectable cytochrome P420 peak (Fig. 2).

2.2. Enzymatic activity of mouse Cyp17a1

Mouse Cyp17a1 catalyzes the two-step oxidation of progesterone to 17α-OH progesterone to androstenedione and of pregnenolone to 17α-OH pregnenolone to DHEA (Fig. 1). UPLC-tandem mass spectrometry of Δ4-steroids determined that the retention times of progesterone, 17α-OH progesterone, and androstenedione were 5.20, 4.00, and 3.58 min, respectively (Supplementary Fig. S2), and Δ5-steroids were eluted with retention times of 4.88, 4.18, and 4.27 min for pregnenolone, 17α-OH pregnenolone, and DHEA, respectively (Supplementary Fig. S3). The optimal ratios of Cyp17a1 and b_5 ratio for *in vitro* catalytic activity were analyzed under our experimental condiions (Fig. 3). The catalytic activities of Cyp17a1 were stimulated by b_5 in a concentration-dependent manner up to a 1:20 ratio in both the 17α-hydroxylation and lyase reactions (Fig. 3). Therefore, the molar ratio of 1:20 was chosen for subsequent experiments. Steady-state kinetic parameters for the catalytic activity of Cyp17a1 were determined both with b_5 and without b_5 (Supplementary Figs. 4 and 5, Tables 1 and 2). The presence of b_5 stimulated the 17α-hydroxylation reaction of progesterone (k_{cat}) about 3-fold but the specificity constant (k_{cat}/K_m) was similar to the value in the reaction without b_5 (Supplementary Fig. 4A and 4B and Table 1). The stimulatory effect of b_5 for the 17α-hydroxylation reaction with pregnenolone showed a 4-fold increase in k_{cat} value and 2.4-fold increase in k_{cat}/K_m value (Supplementary Fig 4C and 4D and Table 2). On the other hand, the stimulatory effect of b_5 observed in the lyase reactions was much greater (Supplementary Fig 5 and Table 1). In the lyase reaction with 17α-OH progesterone, b_5 enhanced the specificity constant (k_{cat}/K_m) of Cyp17a1 10-fold, mainly due to the increased k_{cat} value (Supplementary Fig. 5A and 5B and Table 1). In the 17α-OH pregnenolone lyase reaction of Cyp17a1, the product DHEA was observed only when b_5 was added (Supplementary Fig 5C and Table 2). In the reaction without b_5, DHEA was not detected in the mass chromatogram using the multiple reaction monitoring (mrm) conditions determined for DHEA (*m/z* 271.2 > 105.0) with the highest concentration of 17α-OH pregnenolone (used at 30 μM).

2.3. Comparison of catalytic activities of mouse Cyp17a1 and human CYP17A1

In order to compare the catalytic activities of mouse Cyp17a1 and human CYP17A1, the analysis of human CYP17A1 was performed under the same conditions. Steady-state kinetic parameters for hydroxylation and lyase reaction of mouse and human cytochrome P450 17A1 enzymes were compared (Tables 1 and 2). In reactions of Δ4-steroids (progesterone and 17α-OH progesterone), the catalytic efficiencies of Cyp17a1 were significantly higher than those of human CYP17A1; 28-fold higher for hydroxylation and 770-fold higher for the lyase reaction with b_5 (Table 1). The higher catalytic efficiency for hydroxylation was mainly due to the decrease in the K_m value while the combination of the increased k_{cat} (54-fold) and the decreased K_m (13-fold) resulted in the enhanced catalytic efficiency in the lyase reaction (Table 1). However, the combined effect of the increased k_{cat} and the decreased K_m was observed in both hydroxylation and lyase reactions without b_5 (Table 1). In reactions of Δ5-steroids (pregnenolone and 17α-OH pregnenolone), the catalytic efficiencies of Cyp17a1 were also higher than those of human CYP17A1: 5.3-fold higher for the hydroxylation of pregnenolone and 1.5-fold higher in the lyase reaction with 17α-OH pregnenolone (Table 2). Increased catalytic efficiency in the 17α-hydroxylation reaction was mainly due to increased k_{cat} value but the effects of k_{cat} and K_m canceled out in the lyase reaction to result in less change of catalytic efficiency (Table 2). In addition, the enhancement of catalytic efficiencies in Cyp17a1 (compared to human CYP17A1) was observed in the reactions without b_5 (2,100 for progesterone and 47 for pregnenolone). These results indicated that all reactions of mouse Cyp17a1 were much faster than with human CYP17A1, markedly enhanced in the catalysis of reactions of Δ4-steroids (progesterone and 17α-OH progesterone).

2.4. Substrate binding affinities of mouse Cyp17a1

Binding spectral titration analyses of mouse Cyp17a1 were performed with progesterone, 17α-OH progesterone, pregnenolone, and 17α-OH pregnenolone (Fig. 4). Binding titrations of all substrates produced a typical Type I spectral shift, with an increase at 388 nm and a decrease at 423 nm, i.e., a shift from a low- to high-iron spin state (Fig. 4). Cyp17a1 showed K_d values of 0.34 to 3.4 μM, indicative of tight substrate binding affinities (Table 3). These binding affinities of Cyp17a1 are similar to those of human CYP17A1 for all substrates (Table 3). These results suggest that the higher rates of enzyme catalysis in Cyp17a1 compared to human CYP17A1 are not due to the altered substrate binding affinity.

2.5. Comparison of catalytic activity of mouse Cyp17a1 mutants

Previous studies indicated that some Arg residues of human CYP17A1 are involved in binding interactions with b_5 and for lyase reaction of steroids [9], consistent with clinical observations with variants. In order to analyze the roles of Arg residues of mouse Cyp17a1, the R347H and R358Q mutants (of Cyp17a1) were constructed, and the proteins were purified (Fig. 2). The catalytic activities of these two mutant enzymes were analyzed (Supplementary Fig 4 and 5, Table 4). The rates of the 17α-hydroxylation reactions with progesterone and pregnenolone were reduced 49 and 41% in the R347H enzyme and 68 and 45% in the R358Q enzyme, respectively (Table 4). The mutant enzymes displayed more significantly reductions in rates of the lyase reactions (Table 4), indicating that the lyase

activity of Cyp17a1 requires the interaction of b_5. In particular, the k_{cat} value for the R358Q mutant was < 10% that of wild-type Cyp17a1 and no conversion of 17α-OH pregnenolone to DHEA was detected, even with the highest concentration of 17α-OH pregnenolone used at 30 μM. (Table 4). This result indicated that these Arg residues in mouse Cyp17a1 are important in interacting with the b_5 protein, especially in the lyase reactions, as with the human CYP17A1 enzyme.

3. Discussion

CYP17A1 functions at an early stage in the classic steroidogenesis pathway. After cleavage of the cholesterol side chain, the resulting pregnenolone can be converted into sex hormones, glucocorticoids, and mineralocorticoids [10]. However, androgens also lead to the development of prostate cancer, the most common cancer in men in the United States and the second leading cause of cancer death, while estrogens are recognized as a cause of hormone-responsive breast cancer. Therefore, this enzyme has gained considerable interest as a relatively new drug target, validated by the successful use of the CYP17A1 inhibitor abiraterone [11, 12]. Abiraterone acetate, the FDA-approved prodrug form of this CYP17A1 inhibitor, inhibits both 17α-hydroxylation and lyase activities (Fig. 1), with the inhibition of 17α-hydroxylation resulting in side effects because of mineralocorticoid and glucocorticoid imbalances. As such, interest in novel 17,20-lyase selective inhibitors has increased. Understanding the mechanism of the b_5-driven 17,20-lyase stimulation of CYP17A1 may contribute to the design of a novel type of CYP17A1 inhibitor [9, 13, 14].

b_5 is a small hemoprotein that plays an important role in regulating the activity of CYP17A1. Cytochrome b_5 plays an essential role in regulating the catalytic activity of CYP17A1 during 17,20-lyase reactions, although not via direct electron transfer [15]. Nonetheless, it remains unknown exactly how b_5 regulates the enzymatic activity of CYP17A1. Recent studies have benefited significantly from the crystal structures of human CYP17A1 [16], but despite this structural information, questions about specific protein-protein interactions and regulation of androgen synthesis by CYP17A1 remain unresolved [17].

In this study, the enzymatic characterization of mouse Cyp17a1 was carried out *in vitro*. Previous studies of CYP17A1 in rats, guinea pigs, and cattle have been conducted, and these studies involved several approaches and designs, many of which did not include b_5 [18–20]. The catalytic activity was confirmed using the purified mouse Cyp17a1 and b_5. Both 17α-hydroxylation and lyase activities with the four major substrates were significantly stimulated by the presence of b_5 (Supplementary Fig. 5, Tables 1 and 2). Mouse Cyp17a1 had much higher catalytic activity than human CYP17A1 in all reactions (Tables 1 and 2). This catalytic enhancement of mouse Cyp17a1 was seen more dramatically in the reactions of the Δ4-steroids (progesterone series). One speculation for different catalysis can be attributed to the residue 105 (Supplementary Fig. S1). A previous study by Swart *et al.* confirmed that the residue 105 of CYP17A1 is located in the predicted B′ helix and conserved in various species with Leu, but only the CYP17A1 enzymes of humans and chimpanzees are substituted with an Ala residue [21]. The substitution of Ala-105 of human CYP17A1 with Leu produced less 16α-hydroxylation of progesterone, and therefore

17α-hydroxylation occurs more predominantly [22]. Human and mouse CYP17A1 enzymes showed the same Type I binding spectra for major substrates and similar K_d values (Fig. 4 and Table 3). The difference in the catalytic activity of the mouse and human enzymes does not seem to be due to the difference in overall binding affinity to the substrate. The analysis of b_5 dependence was performed using two mutant enzymes of mouse Cyp17a1 with substitution of Arg residues (R347, R358), which are located in the b_5-interacting domain of human CYP17A1 [9, 13, 14]. The structural analysis indicated that these two basic residues Arg-347 and Arg-358 are located in the J´ helix and K helix, respectively, and are exposed to solvent [13, 14]. The significant reduction was observed in production of androstenedione and DHEA in the presence of b_5 (Table 4). In order to confirm the role of b_5, the lyase reaction by these two mutants was performed but the catalytic turnover numbers were not significantly decreased (for androstenedione production 2.86 ± 0.16 min^{-1} for R347H, 1.56 ± 0.08 min^{-1} for R358Q). These results suggested that these basic residues of mouse Cyp17a1 also play an important role in interaction with b_5 for lyase reaction. A previous study by Sondhi *et al.* reported that the b_5 protein in Leydig cells of mouse testis is required for maximal androgen synthesis by preventing 17α-OH progesterone accumulation [3]. However, the b_5-independent 17,20-lyase activity of mouse Cyp17a1 is sufficient for normal male genital development and fertility [3]. Unlike the human and bovine orthologues, rodent CYP17A1 enzymes are expressed in gonads but not in adrenal glands [23]. Previously, Katagiri *et al.* reported the *in vivo* expression of CYP17A1 from immature rat liver and the increasing levels of 17α-hydroxylation and 17,20-lyase activity for pregnenolone and progesterone by three weeks of age [24].

4. Conclusion

We overexpressed and purified mouse Cyp17a1 and analyzed its catalytic activity with relevant steroids. Mouse Cyp17a1 had much higher catalytic activity than human CYP17A1, and its enzyme activity was also significantly stimulated by b_5 protein. The role of b_5 in the biosynthesis of androgens have been previously shown by human and rat CYP17A1 enzymes[23, 26]. This study also indicates that the stimulatory effect of b_5 can be applied not only in human Cyp17a1 enzyme but in various species including mouse. However, the significant difference of the observed enzyme kinetics should be considered in the interpretation of physiological relevance for the biosynthesis of human androgens, although mouse Cyp17a1 is a good model to investigate the human enzyme and related diseases such as prostate cancer.

5. Experimental procedures

5.1. Chemicals and enzymes

Progesterone, pregnenolone, 17α-OH progesterone, and 4-androstene-3,17-dione were purchased from Sigma-Aldrich. 17α-OH pregnenolone was purchased from Cayman Chemical (Ann Arbor, MI, USA). Dehydroepiandrosterone (DHEA) was purchased from Avanti Polar Lipids (Alabaster, AL, USA). 3-[(3-Cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS) was purchased from GoldBio (St. Louis, MO, USA). HisPurTM Ni-NTA resin was purchased from Thermo Fisher (Waltham, MA, USA). A HiTrap® DEAE

Fast Flow column was purchased from Cytiva (Marlborough, MA). *E. coli* JM109 cells were purchased from Enzynomics (Daejeon, Korea). Rat NADPH-cytochrome P450 reductase (POR) was heterologously expressed in *E. coli* HMS174 (DE3) and purified as described elsewhere [27]. Recombinant human b_5 was expressed in *E. coli* JM109 cells from a plasmid [pSE420 (Amp)] and the protein was solubilized and purified by DEAE-Sepharose ion exchange chromatography [28].

5.2. Construction of CYP17A1 expression plasmid

A pBHA plasmid including the *Cyp17a1* gene (cDNA) of *Mus musculus* was purchased from Bionics (Seoul, Korea). The open reading frame for mouse Cyp17a1 was modified to truncate residues 2–19, substitute the hydrophilic sequence 20–23 (Arg-Arg-Cys-Pro to Ala-Lys-Lys-Thr), and add a carboxy-terminal four-histidine tag [16]. The mouse *Cyp17a1* cDNA was cloned into the pCW (Ori+) bicistronic vector (including a human POR cDNA) using *Nde*I and *Xba*I restriction sites. Site-directed mutagenesis (SDM) was carried out using an Agilent QuickChange® II site-directed mutagenesis kit following the procedures as described in the manufacturer's user manual, with the following primers: 5´- CGTCTTTCAATGACCACACTCACCTCCTC −3´ (R347H) and 5´- GGAGGCCACTATCCAAGAAG −3´ (R358Q) [29]. The sequences of the constructed wild-type and mutant CYP17A1 cDNAs were confirmed by nucleotide sequence analysis.

5.3. Expression and purification of mouse Cyp17a1 wild-type and mutants

The expression and purification of the mouse Cyp17a1 enzyme were performed as previously described, with some modifications [14]. Recombinant mouse Cyp17a1 wild-type and mutants were transformed in *E. coli* JM109 cells and inoculated into Luria-Bertani (LB) media containing 50 μg/ml ampicillin and incubated at 37 °C for 16 h. The pre-culture was grown at 37 °C and 230 rpm, and the additive containing 1.0 mM IPTG, 1.0 mM thiamine, 0.5 mM 5-ALA, and trace elements was added to induce the expression of CYP enzymes [30]. The expression cultures were incubated at 30 °C and 200 rpm for 16 h and followed by harvesting by centrifugation. The membranes were prepared after sonicating the lysed cells. Then, the membranes were solubilized overnight at 4°C in 100 mM potassium phosphate buffer (pH 7.4) containing 20% (w/v) glycerol, 0.5 M NaCl, 10 mM β-mercaptoethanol, and 1.5% (w/v) CHAPS. The soluble fractions containing Cyp17a1 (wild-type or mutants) were prepared after ultracentrifugation at 10^5 g for 1 h. The soluble fraction was loaded onto a Ni^{2+}-nitrilotriacetic acid (NTA) column. The purified protein was eluted with 100 mM potassium phosphate buffer (pH 7.4) containing 0.5 M NaCl, 0.5% (w/v) CHAPS, 20% (v/v) glycerol, and 250 mM imidazole. The eluted fraction, containing highly purified Cyp17a1, was dialyzed at 4 °C against 100 mM potassium phosphate buffer (pH 7.4) containing 20% (v/v) glycerol and 0.1 mM EDTA to remove CHAPS and NaCl.

5.4. Enzyme catalytic activity analysis

The catalytic activity of mouse Cyp17a1 enzymes was analyzed by 17α-hydroxylation of progesterone and lyase reaction of 17α -OH progesterone and 17α-hydroxylation of pregnenolone and lyase reaction of 17α-OH pregnenolone using ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS). The reaction mixtures contained 10 pmol purified mouse Cyp17a1 (or human CYP17A1), 40 pmol purified

rat NADPH-P450 reductase (NPR), 200 pmol human b_5, and DLPC, with various concentrations of progesterone and 17α-OH progesterone in 500 μl of 100 mM potassium phosphate buffer (pH 7.4). The reactions were initiated by adding an NADPH-regenerating system (final concentrations of 15 mM glucose 6- phosphate, 1.5 mM $NADP^+$, and 1 IU ml^{-1} glucose 6-phosphate dehydrogenase) after pre-incubation at 37 °C for 3 min. The reactions were incubated at 37 °C for 5 min and added 1.0 ml of CH_2Cl_2 to terminate the reactions. After mixing with a vortex device, the reaction samples were centrifuged at 1260 g for 15 min. The organic phase of each sample was dispensed into a new test tube and dried under an N_2 gas stream. The dried products were dissolved in 200 μl CH_3CN. Samples were injected onto an ACQUITY UPLC™ BEH C18 column (50 × 2.1 mm, 1.7 μm) linked to a Waters ACQUITY UPLC™ (Waters, Milford, MA) and Waters Quattro Premier™ (Waters). The reaction products were resolved using a mobile phase composed of solvents A (60% CH_3OH, 40% H_2O, with 0.1% formic acid, v/v) and B (100% CH_3CN, with 0.1% formic acid), at a flow rate of 0.2 ml min^{-1}. The analytes were observed using positive electrospray ionization and multiple reaction mode. The source temperature was 150 °C and the desolvation temperature was 500 °C, the desolvation gas flow rate was 550 l/h, and the cone gas flow rate was 50 l/h. The column temperature was maintained at 40 °C. The positive ionization transitions of progesterone (*m/z* 315.2 > 97.1), 17α-OH progesterone (*m/z* 331.2 > 96.9), and androstenedione (*m/z* 287.2 > 96.9) were monitored at collision energies of 22, 24, and 22 eV, respectively. The column temperature was maintained at 40 °C. The positive ionization transitions of pregnenolone (*m/z* 317.2 > 90.9), 17α-OH pregnenolone (m/z 297.1 > 105), and DHEA (*m/z* 271.2 > 105.0) were monitored at collision energies of 60, 34, and 32 eV, respectively. The peak areas were calculated using QuanLynx software (Waters).

5.5. Substrate binding analysis

Binding titration analysis was performed using purified mouse Cyp17a1 wild-type and human CYP17A1. Purified Cyp17a1/CYP17A1 enzymes were diluted to 2 μM in 100 mM potassium phosphate buffer (pH 7.4) and then divided between two glass cuvettes. The spectroscopic changes (350 to 500 nm) were recorded using a CARY 100 spectrophotometer (Varian, Palo Alto, CA, USA) with subsequent additions of progesterone, 17α-OH progesterone, pregnenolone, or 17α-OH pregnenolone. Substrate binding affinities (K_d) were estimated by plotting the difference in absorbance between the maximum wavelength (420 nm) and minimum wavelength (380 nm) versus substrate concentration using nonlinear regression analysis in Graph-Pad Prism software (Graph-Pad, San Diego, CA) and the quadratic equation [31].

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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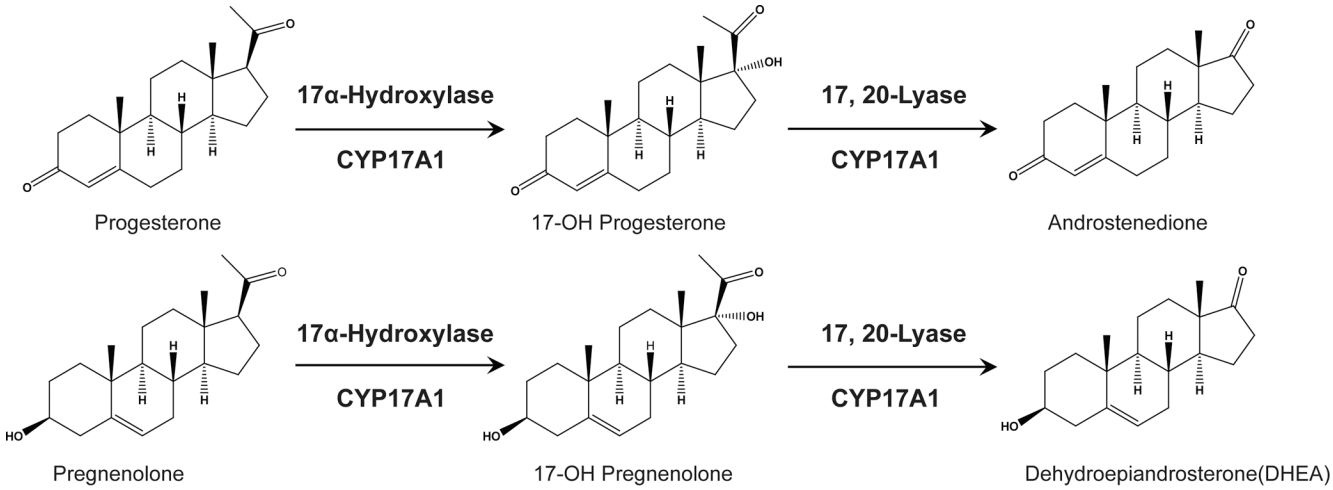

Fig 1.

Oxidation of steroids by 17α-hydroxylation and lyase reaction via CYP17A1.

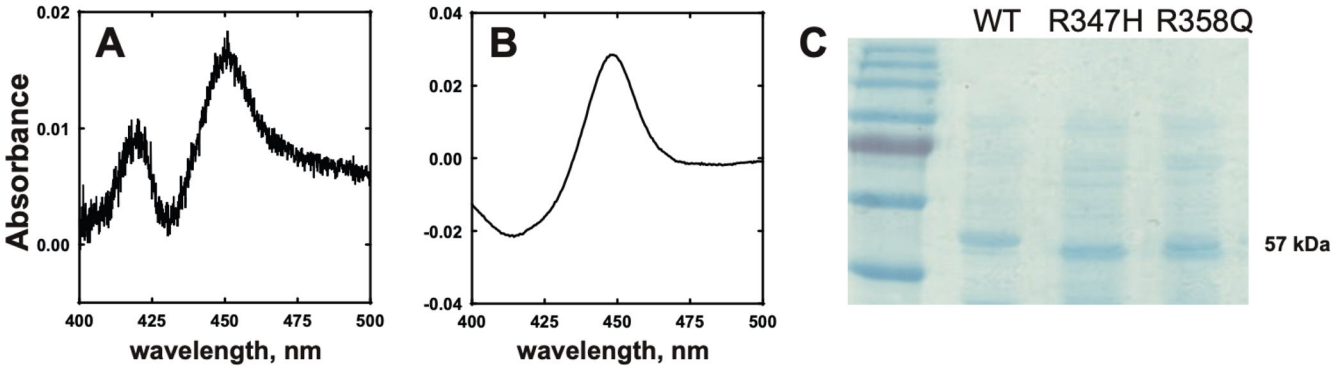

Fig 2. Expression and purification of mouse Cyp17a1.

(A) CO-binding spectra of mouse Cyp17a1 in *E. coli* whole cells. The expression level was 450 nmol/liter. (B) CO-binding spectra of purified mouse Cyp17a1. (C) Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) gels of wild-type mouse Cyp17a1 and mutants. The bands show purified proteins at 57 kDa, as expected for the open reading frame of mouse Cyp17a1 enzyme.

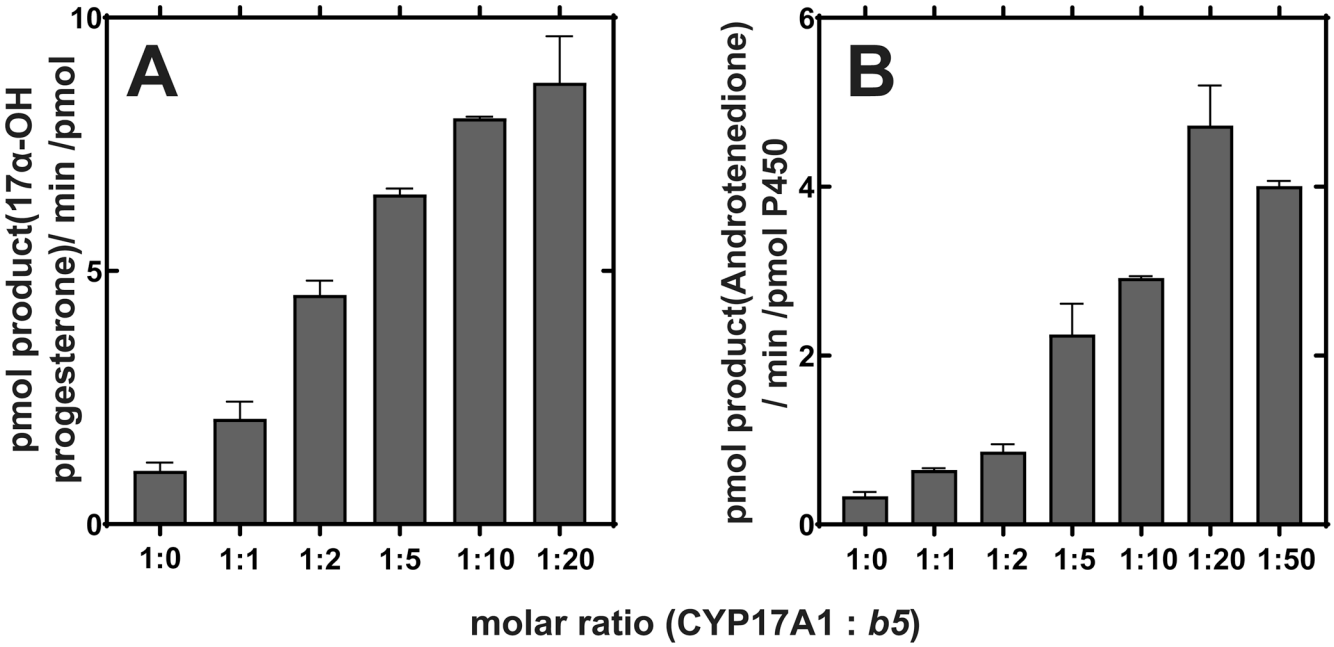

Fig 3. Stimulatory effect of cytochrome b_5 on the catalytic activity of mouse Cyp17a1.
(A) Progesterone hydroxylation of mouse Cyp17a1; (B) 17α-OH progesterone lyase reaction of mouse Cyp17a1.

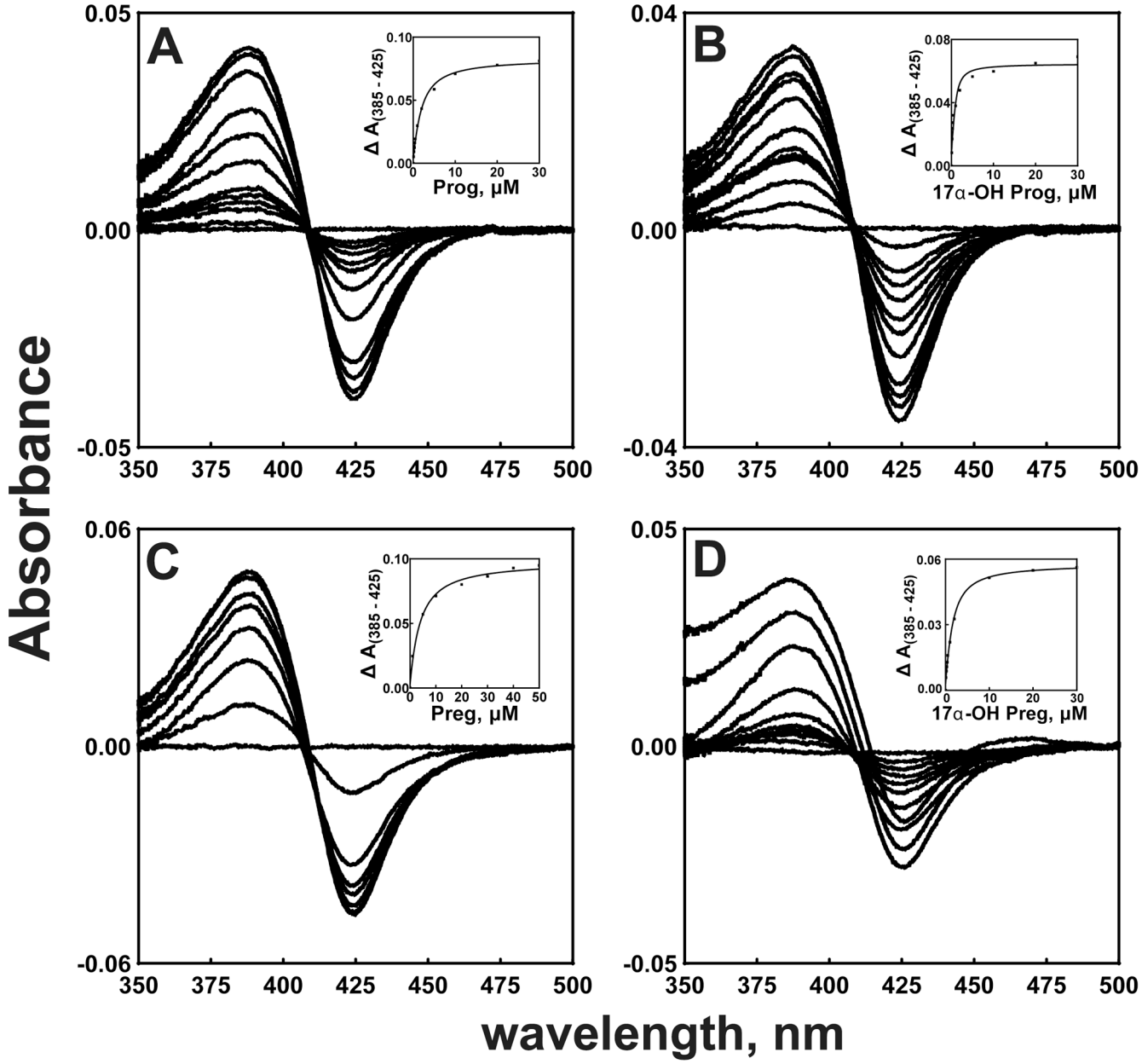

Fig 4. Binding of progesterone, 17α-OH progesterone, pregnenolone, and 17α-OH pregnenolone to mouse Cyp17a1.

(A) Progesterone binding titration. (B) 17α-OH progesterone binding titration. (C) Pregnenolone binding titration. (D) 17α-OH pregnenolone titration. The insets show plots of $\Delta A_{385–425}$ versus the concentrations of substrates. The binding parameters are listed in Table 3.

Table 1.

Steady-state kinetic parameters for Δ4-steroids reactions (progesterone 17α-hydroxylation and 17α-OH progesterone lyase reaction) of mouse and human CYP17A1 enzymes.

CYP17A1	b_5	Progesterone 17α-hydroxylation[a]				17α-OH progesterone conversion to androstenedione			
		k_{cat}, min^{-1}	K_m, μM	k_{cat}/K_m	Ratio to human (k_{cat}/K_m)	k_{cat}, min^{-1}	K_m, μM	k_{cat}/K_m	Ratio to human (k_{cat}/K_m)
Mouse	–	1.63 ± 0.06	0.09 ± 0.02	19.2 ± 4.9	2,100	1.62 ± 0.14	0.43 ± 0.11	4.10 ± 1.34	1,000
Human	–	0.05 ± 0.01	5.87 ± 0.71	0.009 ± 0.003	1	0.08 ± 0.01	21.9 ± 4.70	0.004 ± 0.001	1
Mouse	+	5.42 ± 0.30	0.25 ± 0.05	22.9 ± 5.7	28	15.7 ± 1.2	0.42 ± 0.09	39.8 ± 11.4	770
Human[b]	+	6.4 ± 0.5	7.8 ± 1.4	0.82 ± 0.16	1	0.29 ± 0.02	5.5 ± 0.8	0.052 ± 0.008	1

[a] The kinetic analysis of 17α-hydroxylation of progesterone includes the androstenedione produced as well as 17α-OH progesterone.

[b] These values were obtained in our previous study [9].

Table 2.

Steady-state kinetic parameters for Δ5-steroids reactions (pregnenolone 17α-hydroxylation and 17α-OH pregnenolone lyase reaction) of mouse and human CYP17A1 enzyme.

CYP17A1	b_5	Pregnenolone 17α-hydroxylation[a]				17α-OH pregnenolone conversion to DHEA			
		k_{cat}, min^{-1}	K_m, μM	k_{cat}/K_m	Ratio to human (k_{cat}/K_m)	k_{cat}, min^{-1}	K_m, μM	k_{cat}/K_m	Ratio to human (k_{cat}/K_m)
Mouse	–	1.04 ± 0.05	0.26 ± 0.05	4.2 ± 1.0	47	ND[b]	ND	ND	ND
Human	–	0.10 ± 0.01	1.19 ± 0.26	0.09 ± 0.03	1	ND	ND	ND	ND
Mouse	+	4.35 ± 0.23	0.44 ± 0.07	10.2 ± 2.2	5.3	0.47 ± 0.02	0.38 ± 0.06	1.28 ± 0.25	1.5
Human[c]	+	0.88 ± 0.05	0.47 ± 0.20	1.9 ± 0.8	1	5.5 ± 0.7	6.7 ± 2.1	0.83 ± 0.28	1

[a] The kinetic analysis of 17α-hydroxylation of pregnenolone includes DHEA produced as well as 17α-OH pregnenolone.

[b] Not determined. In the absence of cytochrome b_5, the 17α-OH lyase reaction did not detect any product (DHEA) even with the maximum substrate concentration used.

[c] These values were obtained in our previous study [9].

Table 3.

Substrate binding affinities of mouse and human CYP17A1 enzymes.

CYP17A1	K_d			
	Progesterone, μM	**17-OH progesterone, μM**	**Pregnenolone, μM**	**17-OH pregnenolone, μM**
Mouse	1.5 ± 0.2	0.3 ± 0.2	3.4 ± 0.6	1.1 ± 0.2
Human	1.4 ± 0.2	4.9 ± 0.4	0.3 ± 0.2	0.7 ± 0.1

Table 4.

Comparison of catalytic activities of mouse Cyp17a1 wild-type and mutants.

mouse Cyp17a1	b_5	Progesterone 17α-hydroxylation				17α-OH progesterone conversion to androstenedione			
		k_{cat}, min^{-1}	K_m, μM	k_{cat}/K_m	% WT (k_{cat}/K_m)	k_{cat}, min^{-1}	K_m, μM	k_{cat}/K_m	% WT (k_{cat}/K_m)
WT		5.42 ± 0.30	0.25 ± 0.05	23 ± 6	100	15.7 ± 1.19	0.42 ± 0.09	40 ± 11	100
R347H	+	1.58 ± 0.16	0.15 ± 0.03	11 ± 3	49	2.86 ± 0.16	0.21 ± 0.05	15 ± 4	36
R358Q		1.56 ± 0.08	0.11 ± 0.03	16 ± 5	68	1.56 ± 0.08	0.09 ± 0.05	26 ± 15	64
mouse Cyp17a1	**b_5**	**Pregnenolone 17α-hydroxylation**				**17α-OH pregnenolone conversion to DHEA**			
		k_{cat}, min^{-1}	K_m, μM	k_{cat}/K_m	% WT (k_{cat}/K_m)	k_{cat}, min^{-1}	K_m, μM	k_{cat}/K_m	% WT (k_{cat}/K_m)
WT		4.35 ± 0.23	0.44 ± 0.07	10 ± 2	100	0.47 ± 0.02	0.38 ± 0.06	1.28 ± 0.25	100
R347H	+	1.38 ± 0.07	0.34 ± 0.06	4.2 ± 1.0	41	0.14 ± 0.01	0.47 ± 0.07	0.35 ± 0.03	27
R358Q		2.41 ± 0.18	0.56 ± 0.13	4.6 ± 1.4	45	ND	ND	ND	ND

[a]Not determined. In the presence of cytochrome b_5, the 17α-OH lyase reaction did not detect any product (DHEA) until the maximum substrate concentration.