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ORIGINAL RESEARCH PAPER

Regioselective hydroxylation of 17β-estradiol by mutants of CYP102A1 from *Bacillus megaterium*

Gun Su Cha · Sang Hoon Ryu · Taeho Ahn · Chul-Ho Yun

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Abstract A large set of mutants of CYP102A1 from *Bacillus megaterium* have human cytochrome P450-like activities and the ability to metabolize a number of marketed drugs and steroids. Here, we tested whether the CYP102A1 mutants could be used to produce hydroxylated human metabolites of 17β-estradiol (E_2). A set of the mutants, which were generated by site-directed and random mutagenesis, was used to produce hydroxylated human metabolites of E_2 in this study. Some of the tested mutants could regioselectively generate 2-OH E_2 as a major metabolite but not other hydroxylated products. These results suggest that CYP102A1 mutants would be useful for the bioconversion of steroid hormones to hydroxylated products, which can be used for industrial applications.

Keywords CYP102A1 mutant · 17β-Estradiol · Human metabolite · Hydroxylation · Regioselectivity

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G. S. Cha · S. H. Ryu · C.-H. Yun (✉)
School of Biological Sciences and Technology, Chonnam National University, Gwangju 500-757, Republic of Korea
e-mail: chyun@jnu.ac.kr

T. Ahn
College of Veterinary Medicine, Chonnam National University, Gwangju 500-757, Republic of Korea

Introduction

Steroids play an important role as hormones in mammals, and a variety of steroids are widely used as anti-inflammatory, antiandrogenic, progestational, and anticancer agents, as well as in other applications (Holland 1999). Because it is difficult to conduct selective and efficient synthesis of hydroxylated derivatives of steroids by traditional chemical methods, transformation using enzymes is a useful alternative. A large number of bacterial strains that hydroxylate steroids have been isolated and characterized, and most positions of the steroid can be hydroxylated (Mahato and Gari 1997). CYP106A1 (Lee et al. 2014) and CYP106A2 (Virus and Bernhardt 2008) from *Bacillus megaterium* can catalyze the hydroxylation reaction of a set of steroids that generates novel hydroxylated derivatives. The hydroxylated steroids themselves can be drug leads, and they can also be used to make drug candidates after chemical modifications at the hydroxylated position.

17β-Estradiol (E_2) is metabolized to multiple hydroxylated metabolites, including the catechol estrogens 2-OH E_2, 4-OH E_2, 16α-OH E_2, and 4-hydroxyestrone, by human cytochromes P450 (P450) 1A1, 1A2, and 1B1 (Fig. 1). The carcinogenic effect of the E_2 metabolites, such as 4-OH E_2, 2-OH E_2 and 16α-OH E_2, are still controversial (Turan et al. 2004). The high cost of the hydroxylated E_2 metabolites makes it difficult to study the effects of the metabolites on in vivo and in vitro systems.

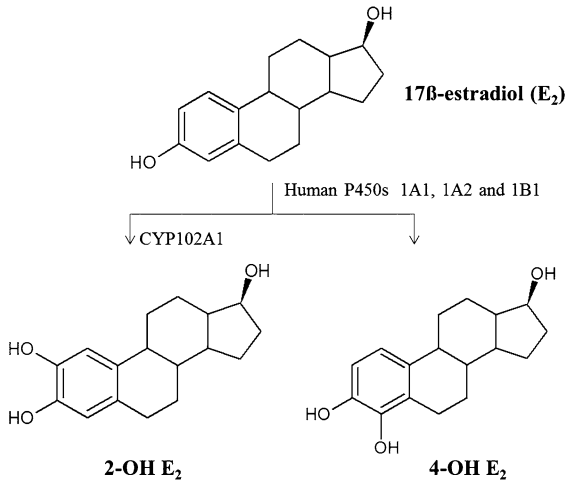

Fig. 1 The chemical structures of 17β-estradiol (E_2) and its human metabolites. E_2 is primarily metabolized by human P450 s 1A1, 1A2, and 1B1 to 2-OH E_2 and 4-OH E_2. Mutants of CYP102A1 from *B. megaterium* ATCC 14581 produced 2-OH E_2 but not 4-OH E_2

P450 BM3 (CYP102A1) from *B. megaterium* is catalytically self-sufficient, containing both a heme domain responsible for substrate oxidation and a diflavin reductase domain responsible for electron transport. At present, a large set of CYP102A1 mutants are known to have human P450-like activities and the ability to metabolize a number of marketed drugs and steroids (Yun et al. 2007; Whitehouse et al. 2012; Caswell et al. 2013; Kang et al. 2014).

In this study, we tested whether the CYP102A1 mutants could be used to produce hydroxylated human metabolites of E_2. We found that some of the tested mutants could generate 2-OH E_2 regioselectively as a major metabolite but not other hydroxylated products.

Materials and methods

Chemicals

17β-Estradiol (E_2), 2-hydroxyestradiol (2-OH E_2), and 4-hydroxyestradiol (4-OH E_2) were purchased from Sigma-Aldrich (St. Louis, MO). All of the other chemicals used were of the highest grade commercially available.

Construction of the CYP102A1 mutants by site-directed and random mutagenesis

The wild type CYP102A1 from *B. megaterium* ATCC 14,581 (Kang et al. 2011) and its 46 different mutants used in this study were prepared as described (Kim et al. 2008; Park et al. 2010; Kang et al. 2014). The mutants were selected based on their activity on a variety of human P450 substrates and drugs. Mutants #1–17 have mutations in the substrate channel and active site (Kim et al. 2008), and mutants #18–26 have mutations outside of the active site and substrate channel (Park et al. 2010). Chimera M16V2 and its 19 mutants (A1 ~ H1), which were obtained by random mutagenesis of the heme domain, were created for a previous work (Kang et al. 2014). Each mutant bears amino acid substitution(s), relative to wild type CYP102A1, as summarized in Supplementary Tables 1 and 2.

Heterologous expression in *Escherichia coli* and purification of the wild type and mutated enzymes of CYP102A1

The wild type and 46 mutants of CYP102A1 were expressed in the *E. coli* strain DH5αF′-IQ and purified as previously described (Kim et al. 2008; Park et al. 2010; Kang et al. 2014). The CYP102A1 concentrations were determined from the CO-difference spectra using $\varepsilon = 91\ mM^{-1}\ cm^{-1}$. For all of the wild type and mutated enzymes, a typical culture yielded 200–700 nM P450.

Activity assay of 17β-estradiol (E_2) hydroxylation catalyzed by the CYP102A1 mutants

The E_2 hydroxylation activity of CYP102A1 was determined as previously described (Shimada et al. 1998), with slight modifications. Briefly, purified CYP102A1 (200 nM) was incubated with 200 μM substrate in 100 mM potassium phosphate buffer (pH 7.4) in 0.25 ml in the presence of a NADPH-generating system (final concentrations: 10 mM glucose 6-phosphate, 0.5 mM $NADP^+$, and 1 IU/ml yeast glucose-6-phosphate dehydrogenase). Ascorbic acid (1 mM) was added to the reaction mixture to protect the E_2 metabolites from oxidative degradation. After incubation for 20 min at 37 °C, the reactions were terminated with 0.5 ml ice-cold ethyl acetate and

centrifuged at $\sim$3,000×g for 10 min. The organic layers were combined, and the ethyl acetate was removed under N_2. The reaction products were analyzed by HPLC using a Gemini C18 column (4.6 × 150 mm, 5 μm; Phenomenex, Torrance, CA) with a mobile phase of 33 % acetonitrile containing 0.5 % acetic acid with detection at 280 nm. The flow rate was 1 ml/min. The retention times for E_2 and its metabolites were: 2-OH E_2, 10.02 min; 4-OH E_2, 16.5 min; and E_2, 19.15 min.

LC/MS analysis

To identify the E_2 metabolite produced by the CYP102A1 mutants, LC–MS analysis was carried out. The hydroxylation reaction of E_2 by the CYP102A1 mutants was performed as described above. For the activity assays of human CYP1A2, a control experiment containing 50 pmol P450, 100 pmol rat NADPH-P450 reductase, and 50 μM L-α-dilauroyl-*sn*-glycero-3-phosphocholine was used. After extraction and centrifugation, the organic phases were evaporated under N_2. Five microlitres of the reconstituted residue was injected onto the LC column. LC–MS analysis was performed on a Shimadzu LCMS-2010 EV system (Shimadzu, Kyoto, Japan), which included the LC–MS solution software. The separation was performed on a Shim-pack VP-ODS column (2 × 250 mm; Shimadzu) with a mobile phase of 50 % acetonitrile containing 0.5 % acetic acid at 0.15 ml/min. The column was maintained at 40 °C. To identify the metabolites, mass spectra were recorded by electrospray ionization in negative mode. The interface and detector voltages were 4.4 and 1.5 kV, respectively. The nebulization gas was at 1.5 l/min. The interface, curve desolvation line, and heat block temperatures were 250, 230, and 200 °C, respectively. The retention time and fragmentation patterns of the E_2 metabolites and authentic compounds were compared to identify the chemical structure of the metabolites.

Stability of the CYP102A1 mutants during the reaction

To estimate the stability of the CYP102A1 mutants during the E_2 oxidation reaction, we measured the CO-difference spectra of the reaction aliquots. After 100 nM of enzyme was incubated with 1 mM of substrate (E_2) for 0.5, 1, 2, 3, and 4 h in the presence of the NADPH-generating system, the remaining concentration of P450 was measured.

Results and discussion

Hydroxylation of E_2 by wild type CYP102A1 and its mutants

Initially, the catalytic activity of the wild type and a set of CYP102A1 mutants toward E_2 was investigated at a fixed substrate concentration of 200 μM using HPLC (Fig. 2a). Although the wild type and some mutants did not show any detectable metabolites, 30 of 46 mutants tested here produced one metabolite hydroxylated at the 2-position (2-OH E_2) with an apparent activity of >1 min^{-1} for E_2 (Fig. 3). However, 4-OH E_2 and 16α-OH E_2, other human metabolites, were not detectable. The metabolite (2-OH E_2) and substrate (E_2) were identified by HPLC and LC–MS by comparison to authentic compounds. The retention time and fragmentation pattern of the major metabolite from E_2 were matched to the 2-OH E_2 standard (Fig. 2b). The turnover numbers from the 34 mutants for 2-OH E_2 formation varied over a wide range. The wild type enzyme, as well as mutants #1, #2, #4 $\sim$#7, #9, #11, B3, B10, E7, and G2, did not show activity for 2-OH E_2 formation (<0.1 min^{-1}). Nine mutants showed much higher catalytic activity (>10 min^{-1}) for 2-OH E_2 formation than those of human CYP1A1 (5.78 min^{-1}) and CYP1A2 (7.68 min^{-1}).

Kinetic parameters and total turnover number (TTN) of E_2 2-hydroxylation by the CYP102A1 mutants

Four mutants (#8, #13, #17, and G1) were selected to study the kinetics of the 2-hydroxylation of E_2 (Table 1 and Supplementary Fig. 1). The k_{cat} values of the mutants were in the range of 13–47 min^{-1}, and the K_m values were in the range of 34–125 μM. Although mutant #17 showed the highest k_{cat} value, mutants #8 and G1 showed very high catalytic efficiency (k_{cat}/K_m) because of their low K_m values.

The same mutants were used to measure the TTN (mol product/mol catalyst) for the 2-hydroxylation reaction of E_2 (Supplementary Fig. 2). The overall TTN values were between 1,040 and 1,210 for the

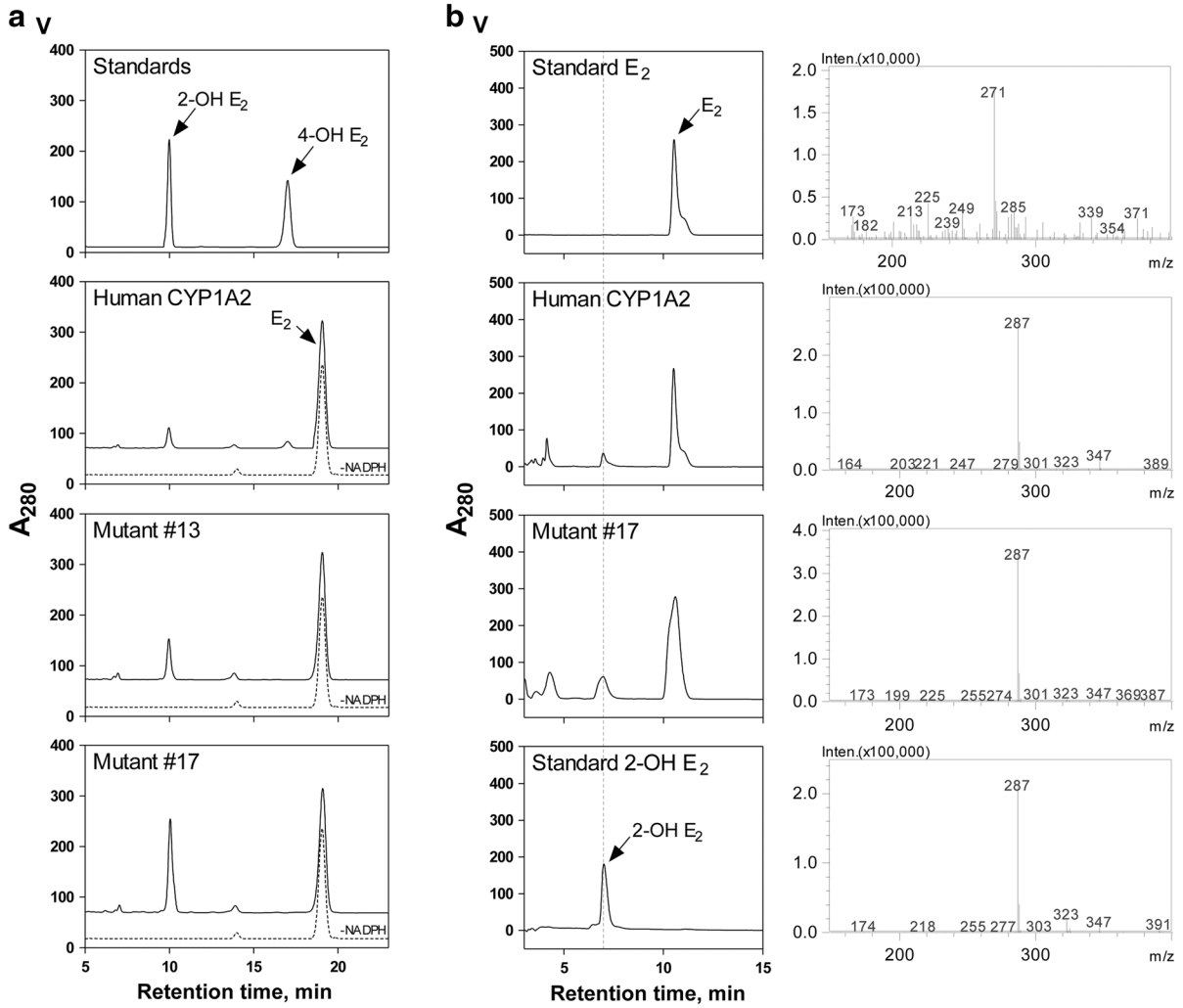

Fig. 2 HPLC and LC–MS analyses of the hydroxylation of E_2 catalyzed by human CYP1A2 and mutants of CYP102A1 from *B. megaterium* ATCC 14581. **a** The HPLC elution profile of E_2 and its major metabolite produced by the CYP102A1 mutants and human CYP1A2. The peaks of the substrate (E_2) and its metabolites (2-OH E_2 and 4-OH E_2) are indicated. Absorbance was monitored at 280 nm. **b** The LC–MS elution and TIC (total ion current) profiles of the metabolites generated by the CYP102A1 mutant and human CYP1A2. The mass spectra of the reaction samples showed two major peaks at 6.96 min (2-OH E_2) and 10.5 min (E_2). The mass spectra of the 2-OH E_2 produced by the CYP102A1 mutant and CYP1A2 and E_2 are also shown. The calculated mass for $[M]^-$ was 271 and 287 for E_2 and 2-OH E_2, respectively

2-OH E_2 formation of mutants #8, #17, and G1 when the reaction mixture was incubated for 4 h in the presence of 1 mM substrate. Mutant #13 showed a TTN of only 122.

Stability of the CYP102A1 mutants as measured by CO-difference spectra

During the hydroxylation reaction of E_2 by P450 in the presence of NADPH, the stability of the P450 enzymes was examined by measuring the CO-difference spectra of reaction aliquots at the indicated time. The stability of the mutants was quite different. Mutant #17, with the highest activity among the tested mutants, also showed the highest stability during the 4 h incubation. After 2 h incubation, <40 % of the intact P450 of mutants #8 and #13 remained under the tested condition (Supplementary Fig. 3). This result might be related to that of the TTN experiments (Supplementary Fig. 2).

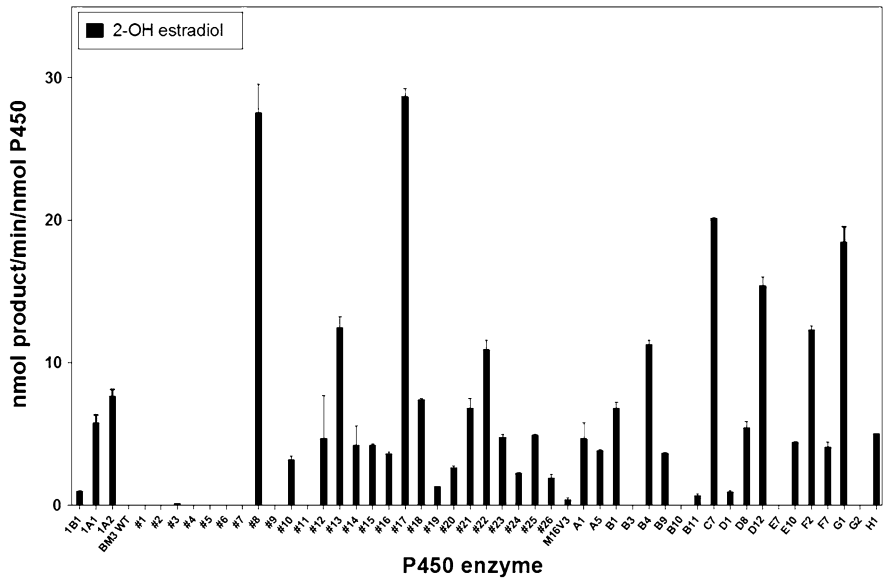

Fig. 3 The rates of 17β-estradiol metabolite production by the 34 mutants of CYP102A1 from *B. megaterium* strain ATCC 14581. Assays were performed for 20 min using 200 μM of E_2. The formation rate of the 2-OH E_2 was determined by HPLC. The values are presented as the mean ± S.E.M. of duplicate measurements

Table 1 Kinetic parameters for the formation of the 2-OH E_2 by mutants of CYP102A1 from *B. megaterium* strain ATCC 14581. The statistical analysis and kinetic parameters (K_m and k_{cat}) were determined using GraphPad Prism software (San Diego, CA)

CYP102A1 mutants	17ß-estradiol (E_2)		
	k_{cat} (min^{-1})	K_m (μM)	k_{cat}/K_m
#8[a]	39 ± 2	63 ± 8	0.62 ± 0.08
#13[b]	13 ± 1	57 ± 9	0.24 ± 0.04
#17[c]	47 ± 2	125 ± 13	0.37 ± 0.4
G1[d]	22 ± 1	34 ± 6	0.63 ± 0.1

Amino acid mutations in the CYP102A1 mutants were as follows

[a] A74G/F87 V/L188Q

[b] R47L/L86I/F87 V/L188Q

[c] R47L/E64G/F81I/F87 V/E143G/L188Q/E267 V

[d] R47L/F81I/F87 V/E143G/T152A/L188Q/E267 V/Q403R/V413A/A474 V/E558D/T664A/P675L/A678E/E687A/A741G/K813E/R825S/R836H/E870 N/I881 V/E887G/P894S/S954 N/M967 V/Q981R/A1008D/H1021Y/Q1022E

According to the revised FDA guideline for the Safety Testing of Drug Metabolites (http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/ucm079266.pdf), any human drug metabolite "formed at >10 % of parent drug systemic exposure at steady state" should be subject to separate safety testing, which requires large quantities of the drug metabolites (MIST issue). Some drug metabolites of concern can be prepared by chemical methods, but others should be prepared by several enzyme sources, including purified human P450 enzymes. Some bacterial P450 s are competitive biocatalysts for the production of commercial drugs and steroids, due to their high activities and stabilities (Julsing et al. 2008; Whitehouse et al. 2012; Caswell et al. 2013; Kang et al. 2014). Therefore, CYP102A1 is now generally accepted to be a prototype monooxygenase for the development of versatile biocatalysts for use in drug discovery and synthesis (Urlacher and Girhard 2012; Kang et al. 2014).

In summary, using a set of CYP102A1 mutants and E_2, a human P450 substrate, this work reveals that bacterial CYP102A1 enzymes catalyze the same reaction as human P450 s. The hydroxylation of E_2 is catalyzed by a subset of the CYP102A1 mutants. One major hydroxylated product, 2-OH E_2, was produced as a result of a hydroxylation reaction. Other hydroxylated products were not produced. 2-OH E_2 formation was confirmed by HPLC and LC–MS by comparing the metabolite to the authentic 2-OH E_2 compound. Thus, the CYP102A1 mutants efficiently produce 2-OH E_2, an authentic human metabolite of E_2.

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Conflict of interest The authors declare no conflict of interests.

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