Short Communication

Regioselective Hydroxylation of Omeprazole Enantiomers by Bacterial CYP102A1 Mutants

Sang Hoon Ryu, Bo-Yeon Park, So-Young Kim, Sun-Ha Park, Hyun-Jin Jung, Min Park, Ki Deok Park, Taeho Ahn, Hyung-Sik Kang, and Chul-Ho Yun

Gwangju Center, Korea Basic Science Institute, Gwangju 500-757, Republic of Korea (K.D.P.);
Department of Biochemistry, College of Veterinary Medicine, Chonnam National University, Gwangju 500-757, Republic of Korea (T.A.).
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Corresponding author: Professor Chul-Ho Yun

School of Biological Sciences and Technology, Chonnam National University, Gwangju 500-757, Republic of Korea.
Tel: +82-62-530-2194; Fax: +82-62-530-2199; E-mail: chyun@jnu.ac.kr

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Abbreviations: CYP or P450, cytochrome P450; CPR, NADPH-cytochrome P450 reductase; LC-MS, liquid chromatography-mass spectrometry; OMP, omeprazole; PPI, proton pump inhibitor; TTN, total turnover number
ABSTRACT: A large set of *Bacillus megaterium* CYP102A1 mutants are known to metabolize various drugs to form human metabolites. Omeprazole (OMP), a proton pump inhibitor, has been widely used as an acid inhibitory agent for the treatment of gastric acid hypersecretion disorders. It is primarily metabolized by human CYP2C19 and CYP3A4 to 5'-OH OMP and a sulfone product, respectively. Recently, it was reported that several CYP102A1 mutants can oxidize racemic and S-OMP to 5'-OH OMP and that these mutants can further oxidize 5'-OH racemic OMP to 5'-COOH OMP. Here, we report that the S- and R-enantiomers of OMP are hydroxylated by 26 mutants of CYP102A1 to produce one major metabolite (5'-OH OMP) regardless of the chirality of the parent substrates. Although the binding of R-OMP to the CYP102A1 active site caused a more apparent change of heme environment compared to binding of S-OMP, there was no correlation between the spectral change upon substrate binding and catalytic activity of either enantiomer. The 5'-OH OMP produced from racemic, S-, and R-OMP could be obtained with a high conversion rate and high selectivity when the triple R47L/F87V/L188Q mutant was used. These results suggest that bacterial CYP102A1 mutants can be used to produce the human metabolite 5'-OH OMP from both the S- and R-enantiomers of OMP.
Introduction

Omeprazole (OMP), a proton pump inhibitor (PPI), acts to regulate acid production in the stomach by irreversible binding to the proton pump (H⁺-K⁺) ATPase in the gastric parietal cell. It is used for the treatment of gastric acid hypersecretion disorders including dyspepsia, peptic ulcer disease, gastroesophageal reflux disease, laryngopharyngeal reflux, and Zollinger–Ellison syndrome. It is administered as a racemic mixture of the R- and S-enantiomers. OMP is primarily metabolized by the hepatic enzymes CYP2C19 and CYP3A4. Both CYP2C19 and CYP3A4 exhibit a stereoselective metabolism of OMP. CYP2C19 favors 5’-hydroxylation of R-OMP, whereas sulfoxidation mediated by CYP3A4 highly favors the S-form (Li et al., 2005; Abelö et al., 2000; Yamazaki et al., 1997) (Figure 1). S-OMP (esomeprazole; Nexium®) has been developed as a new drug with the goal of improving the pharmacokinetic and pharmacodynamic profiles of racemic omeprazole (McKeage et al., 2008). It is known that esomeprazole is significantly more active against Helicobacter pylori than omeprazole. Esomeprazole was not associated with either gastric dysplasia or neoplasia after long-term treatment in patients with healed reflux esophagitis.

CYP2C19 is a highly polymorphic enzyme, with variations in both mRNA and protein expression as well as differences in the protein coding region, which give rise to differing rates of catalysis (Goldstein, 2001). The proportion of the poor metabolizer phenotype varies widely between populations, from ~2% for Caucasians to nearly ~20% in Asians. Several pharmacogenomic studies have suggested that PPI treatment should be tailored according to the patient’s CYP2C19 metabolism status (Furuta et al., 2005). A large set of CYP2C19 alleles from ethnically different populations has been reported (http://www.cypalleles.ki.se/cyp2c19.htm). The PPI omeprazole and related ulcer drugs are primarily oxidized by CYP2C19, and poor metabolizers show a better response to these drugs (Kawabata et al., 2003).
The importance of drug metabolites has been recognized in several International
Committees on Harmonization (ICH) and safety guidelines such as the ICH S7A, the ICH
metabolites of concern can be prepared by chemical methods, but others are generally
prepared by several enzyme sources, including recombinant human P450 enzymes (Yun et al.,
2006). Some bacterial P450s are already known to be competitive biocatalysts for metabolite
production due to their high activities and stabilities (Julsing et al., 2008; Whitehouse et al.,
2012; Kang et al., 2014). Currently, a large set of CYP102A1 mutants are known to have
similar activities to human P450 as well as the ability to metabolize a number of marketed
drugs and steroids (Yun et al., 2007; Whitehouse et al., 2012; Kang et al., 2014). Therefore,
CYP102A1 is generally accepted as a prototype monooxygenase for the development of
versatile biocatalysts for use in drug discovery and synthesis (Urlacher and Girhard, 2012;
Kang et al., 2014). Recently, the 5'-hydroxylation activity of CYP102A1 mutants on racemic
OMP (Yun et al., 2013; Butler et al., 2013) and S-OMP (Butler et al., 2014) has been reported.
It was also shown that 5'-OH racemic OMP can be further oxidized to a carboxylated product
(Butler et al., 2013).

The aim of this study was to investigate the enzymatic properties of CYP102A1
mutants to generate the 5'-OH product from S- and R-OMP enantiomers. It was found that a
set of CYP102A1 mutants could catalyze the formation of 5'-OH OMP regardless of the
chirality of the substrate. Although human CYP2C19 and CYP3A4 form 5'-OH and sulfone
products, respectively, CYP102A1 mutants could produce 5'-OH OMP (but not other
products) as the major metabolite. Some of the CYP102A1 mutants tested here were able to
catalyze the oxidation of OMP to generate its primary metabolite, 5'-OH OMP, from S- and
R- enantiomers with a high conversion rate and high selectivity.
Materials and Methods

Chemicals. OMP (racemic mixture), S-OMP and NADPH were purchased from Sigma-Aldrich (St. Louis, MO). R-OMP was obtained from Santa Cruz Biotechnology (Dallas, Texas, USA). Other chemicals were of the highest grade commercially available.

Construction of CYP102A1 mutants by site-directed mutagenesis and random mutagenesis. Wild type and 26 different mutants of CYP102A1 used in this study were prepared as previously described (Kim et al., 2008; Park et al., 2010). The mutants were selected based on their activities toward 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). Each mutant bears amino acid substitution(s) relative to wild type CYP102A1, as summarized in Supplemental Table S1.

Expression and purification of wild type and mutant enzymes of CYP102A1. Wild type and 26 mutants of CYP102A1 were expressed in the *Escherichia coli* strain DH5α-F′-IQ and purified as previously described (Kim et al., 2008; Park et al., 2010). The CYP102A1 concentrations were determined by CO difference spectra using $\varepsilon = 91 \text{ mM}^{-1}\text{cm}^{-1}$. For all of the wild type and mutated enzymes, a typical culture yielded 200 to 700 nM P450.

Hydroxylation of OMP. Typical steady-state reactions for the hydroxylation of each OMP enantiomer included 50 pmol of CYP102A1 in 0.25 ml of 100 mM potassium phosphate buffer (pH 7.4) containing a final concentration of 100 μM OMP (racemic mixture, *S*, or *R*-enantiomer). To determine the kinetic parameters of several CYP102A1 mutants, a range of concentrations of OMP (2 to 1000 μM) was used. An aliquot of a NADPH-generating system was used to initiate reactions (final concentrations: 10 mM glucose 6-phosphate, 0.5 mM NADP+, and 1 IU/ml yeast glucose 6-phosphate dehydrogenase). A stock solution of OMP (200 mM) was prepared in dimethyl sulfoxide and diluted into the enzyme.
reactions with a final organic solvent concentration of <1% (v/v). OMP is soluble at the
diluted solution of 2 mM concentration (1%, v/v). Reactions were incubated for 30 min at
37°C and terminated with 0.50 ml of ice-cold dichloromethane. After centrifugation of the
reaction mixture, organic phases were evaporated under nitrogen gas. The product was
analyzed by HPLC using a Gemini C18 column (4.6 × 150 mm, 5 μm; Phenomenex,
Torrance, CA) with an acetonitrile-water (30:70, v/v) mobile phase. Elutes were detected by
UV at 302 nm. Phenacetin was used as an internal standard as previously described
(Kobayashi et al., 1994). The kinetic parameters (kcat and Km) were determined using
nonlinear regression analysis based on the Michaelis-Menten kinetics model with GraphPad
Prism software (GraphPad, Software Inc., San Diego, CA).

To determine the total turnover numbers (TTNs) of each CYP102A1 mutant, 1.0 mM
OMP (racemic mixture, S-, or R-enantiomer) and 200 pmol of enzyme were used in 0.50 ml
of 100 mM potassium phosphate buffer (pH 7.4). The reaction was initiated by the addition of
the NADPH-generating system and incubated for 10 min to 4 h at 37°C. The formation rate
of the OMP metabolite was determined by HPLC as described above.

Isolation of 5′-OH OMP. To prepare the metabolites of racemic and S-OMP, we used
a scale of 20 ml reaction volume in 20 test tubes (each 1 ml, under air). Each reaction mixture
contained 400 pmol of CYP102A1 mutant #10, a NADPH-generating system, and 1 mM
racemic OMP or S-OMP in 1 ml of 100 mM potassium phosphate buffer (pH 7.4). Reaction
was initiated by the addition of the NADPH-generating system and was incubated for 2 h at
37°C. The reaction was terminated with two volume of ice-cold dichloromethane. After
centrifugation of the reaction mixture, organic phases from 20 test tubes were combined and
evaporated under nitrogen gas. The chromatographic separation was achieved using a semi-
preparative Gemini C18 column (10 x 250 mm, 10 μm particle size; Phenomenex) with
monitored at A302. The mobile phase was 30% acetonitrile and was delivered at a flow rate of
3.0 ml/min. Retention times for 5'-OH metabolite and OMP were 8.75 and 21.01 min\(^{-1}\), respectively. After collection of the 5'-OH metabolite fraction and drying under nitrogen gas, the dried metabolite was obtained and used for NMR analysis. The 5'-OH OMP was also used as an authentic standard. For typical reaction, about 3.0 mg of the 5'-OH OMP was obtained from the 20 ml reaction.

**LC-MS analysis.** To identify the metabolite of each OMP enantiomer produced by the CYP102A1 mutants, LC-MS analysis was carried out. The oxidation reaction of OMP by CYP102A1 mutants was performed as described above. For the activity assays of human CYP2C19 and CYP3A4, a control experiment of 50 pmol P450, 100 pmol rat NADPH-P450 reductase, and 50 \(\mu\)M L-\(\alpha\)-dilauroyl-sn-glycero-3-phosphocholine was performed in 0.25 ml of 100 mM potassium phosphate buffer (pH 7.4). After extraction and centrifugation, organic phases were evaporated under nitrogen gas. An aliquot (5 \(\mu\)l) 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) including LC-MS solution software. The separation was performed on a Shim-pack VP-ODS column (2.0 × 250 mm; Shimadzu) with a mobile phase of CH\(_3\)CN/H\(_2\)O (70:30, v/v) at a flow rate of 0.10 ml/min. To identify the metabolites, mass spectra were recorded by electrospray ionization in positive mode. The interface and detector voltages were 4.4 and 1.5 kV, respectively. The nebulization gas flow was set at 1.5 l/min. The interface, curve desolvation line, and heat block temperatures were 250°C, 230°C, and 200°C, respectively.

**Identification of OMP metabolites by NMR spectroscopy.** NMR experiments were performed on a Varian VNMRS 600 MHz NMR spectrometer equipped with a carbon-enhanced cryogenic probe (Korea Basic Science Institute, Gwangju, Korea). Acetone-d\(_6\) was used as a solvent, and chemical shifts for the proton NMR spectrum were measured in parts per million (ppm) relative to the TMS. All of the NMR experiments were performed with
standard pulse sequences in VNMRJ (v. 2.3) library and processed with the same software. There have been reports on proton and carbon chemical shifts of racemic omeprazole in acetone-d₆. Spectral assignments of racemic 5′-OH OMP were carried out mainly with one-dimensional proton NMR spectrum of 5′-OH OMP by comparing with reported chemical shift values of racemic omeprazole (Folli et al., 1995; Yang et al., 2003). Redondo et al. (2010) showed that it is possible to differentiate chiral isomers of omeprazole in non-polar NMR solvents by adding (S)-1,10-binaphthyl-2,20-diol as NMR chiral solvating agent.

**Spectral binding titrations.** Spectral binding titrations were used to determine the dissociation constants (Kₛ) for substrates as previously described (Kim et al., 2008). The binding affinities of the ligands to the CYP102A1 enzymes were determined at 23°C by titrating 1.5 μM enzyme with the ligand in a total volume of 1.0 ml of 100 mM potassium phosphate buffer (pH 7.4). The final dimethyl sulfoxide concentration was <2% (v/v). The spectral dissociation constants (Kₛ) were estimated using GraphPad Prism software.

**Results and Discussion**

**Hydroxylation of OMP enantiomers by wild type CYP102A1 and its mutants.** Initially, the catalytic activity of wild type and various CYP102A1 mutants toward S- and R-OMP was investigated at a fixed substrate concentration of 100 μM (Fig. 2). The wild type and mutant enzymes produced only one major metabolite (5′-OH OMP) for both enantiomers. The 5′-OH product formation rates for the mutants for R-OMP were much higher than those for S-OMP (Fig. 2). The turnover numbers from the 26 mutants for 5′-OH OMP formation varied over a wide range. The wild type enzyme as well as mutants #2, #4, #5, #12, #14, and #15 showed lower catalytic activity for both enantiomers (<1.0 min⁻¹). Although 13 mutants showed high catalytic activity (>10 min⁻¹) for R-OMP, only 4 mutants exhibited catalytic activity >10 min⁻¹ for S-OMP. The turnover numbers for mutants #10, #17, and #21 were...
140~220-fold and 90~140-fold higher than those of the wild type enzyme for $S$-OMP (0.16 min$^{-1}$) and $R$-OMP (0.37 min$^{-1}$), respectively. The metabolites were identified by HPLC, LC-MS and NMR. The retention time and fragmentation pattern of the major OMP metabolite from both $S$- and $R$-OMP were matched to the 5'-OH OMP standard (Supplemental Figs. S1 and S2). Mutants #10, #13, #16, #21, and #22 were selected for additional kinetic analysis and binding studies.

When we compared the $S$-OMP 5'-hydroxylation activity with $R$-OMP 5'-hydroxylation activity in the 26 mutants, several statistically significant correlations ($r = 0.97$, $P < 0.0001$) were observed (Supplemental Fig. S3). This result indicates that chirality of the sulfur center is not critical for the hydroxylation reaction at the 5'-C position. Taken together, this suggests that the CYP102A1 mutants can be used to prepare the 5'-OH OMP product regardless of the chirality of the parent substrates.

**Structural characterization of the major metabolite 5'-OH S-OMP generated by CYP102A1.** Mutant #10 was used to prepare the 5'-OH OMP from $S$-OMP for structural analysis by NMR and LC-MS. Racemic 5'-OH OMP (Supplemental Fig. S4a), a metabolite from racemic omeprazole (Supplemental Fig. S4b), was first identified by comparing with reported chemical shift values of racemic omeprazole (Folli et al., 1995; Yang et al., 2003). No structural change in benzoimidazole moiety is evident in $^1$H spectra of the racemic OMP and racemic 5'-OH OMP, while chemical shift changes in the pyridine moiety with conversion of 5'-CH$_3$ ($\delta$ 2.245 ppm) in racemic OMP to 5'-CH$_2$OH ($\delta$ 4.698 ppm) in racemic 5'-OH OMP can be seen in the $^1$H spectrum of racemic 5'-OH OMP (Supplemental Fig. S5). $^1$H chemical shift assignments of racemic 5'-OH OMP are shown (Supplemental Table S2). The position of racemic 5'-hydroxymethyl group was further confirmed with 1-dimensional selective ROE (Rotating Frame NOE) experiments. When already-assigned proton NMR peaks from H-6' ($\delta$ 8.41 ppm) or 4'-OCH$_3$ ($\delta$ 3.81 ppm) were selectively irradiated, proton
NMR peak from 5’-CH₂ (δ 4.70 ppm) appeared (Supplemental Fig. S6). The key ROE results are shown in Supplemental Fig. S4c. Proton NMR spectrum of the metabolite from S-OMP, was identical to that of the metabolite from racemic 5’-OH OMP as we expected (Supplemental Fig. S7).

**Kinetic parameters and total turnover numbers of OMP 5’-hydroxylation by CYP102A1 mutants.** Five mutants were selected to compare the kinetic parameters for the 5’-hydroxylation of S- and R-OMP (Supplemental Table S3 and Supplemental Fig. S8). Although the $k_{cat}$ values of each mutant were similar among the racemate, S-, and R-OMP, the $K_m$ values were in the wide range for all tested mutants. Mutants #10 (R47L/F87V/L188Q mutant) and #22 (F162I/E228K) showed high $k_{cat}$ values (47-122 min⁻¹) for the three OMP substrates. The results indicate that the mutants did not show any preference toward a specific enantiomer. The mutant #10 was used to measure the TTN (mol product/mol catalyst) for the 5’-hydroxylation reaction of S- and R-OMP (Supplemental Fig. S9). The overall values were 950 and 1,200 for 5’-OH product from S- and R-OMP, respectively, when 1 mM of substrate was used.

**Selective conversion of OMP to 5’-OH OMP.** Of the 26 mutants of CYP102A1 that reacted with 1 mM of OMP for 1 h, mutant #10 possessed the most ideal combination of 5’-OH OMP formation activity and regioselectivity. Mutant #10 produced 5’-OH OMP at 93%, 98%, and 88% selectivity with the racemic, S-, and R-OMP, respectively (Fig. 2 and Supplemental Table S4). It also converted up to 97%, 99%, and 100% of the racemic, S-, and R-OMP into 5’-OH OMP, respectively. This result indicates that 5’-OH OMP can be obtained with a high conversion rate and high selectivity from racemic, S-, and R-OMP.

**Binding of OMP to CYP102A1 mutants.** Ferric CYP102A1 exhibits a low-spin state, and the addition of substrate (OMP) produced a conversion rate in the spin-state depending on the different types of CYP102A1 mutant (Butler et al., 2013). Mutants #10, #13, #16, #22,
and #23 (which were the same enzymes used to determine the kinetic parameters) were used for the binding titration. Among the 18 samples tested here for spectral titration, only five samples showed typical conversion (Type I) from the low-spin to high-spin state, with an increase in absorbance at 390 nm and a decrease in absorbance at 420 nm (Supplemental Fig. S10). The binding affinities of mutants #10 and #16 to racemic OMP were determined ($K_s = 109 \pm 5 \mu M$ and $209 \pm 26 \mu M$, respectively). The binding affinities of mutants #10, #13, and #16 to R-OMP were also determined ($K_s = 64 \pm 5 \mu M$, $10 \pm 2 \mu M$, and $48 \pm 2 \mu M$, respectively). Six of the samples did not show apparent conversion. Although seven samples showed a spectral change, the $K_s$ value could be not obtained based on the spectral change.

Binding titration showed that there were no correlations between the spectral change upon substrate binding and catalytic activity. In the cases of mutants showing typical spectral conversion upon OMP binding, the binding affinities of the CYP102A1 mutants tested here were much lower than that of the CYP102A1 F87A/A82F mutant ($K_s = 0.212 \mu M$) (Butler et al., 2013).

Mutants #21 (F162I/K187E) and #22 (F162I/E228K) showed different spectral changes from other mutants that had mutations in the substrate channel and active site. The mutations (F162 and E228) outside of active site and substrate channel appear to have different effects on the conformation of the active site upon binding to the substrates (Supplemental Fig. S11).

It has been reported that 5’-OH OMP was a superior agent for gastric acid hypersecretion disorders with fewer severe adverse effects, reduced potential for drug-drug interactions, and a more predictable dosing regimen compared to OMP (Yelle and Handley, 2001). However, there are no reports to show that 5’-OH OMP is an active metabolite. Although the racemate of 5’-OH OMP could be obtained by chemical synthesis, this synthesis includes 21 steps (Renberg et al., 1989). The individual enantiomers from the
racemate could be separated by additional oxidation and reduction steps (Yelle and Handley, 2001).

It is now possible to study the effects of 5’-OH OMP of each enantiomer on gastric acid hypersecretion disorders in *in vitro*, *in vivo*, and animal model systems because scalable production of the metabolite is possible. Specifically, the effects of the 5’-OH metabolites from racemic, S-, and R-OMP on several diseases caused by gastric acid hypersecretion are of particular interest. The effects of these metabolites on gastric acid hypersecretion disorders may suggest that the metabolites could be used as ‘drug leads’ to avoid interindividual variations in drug metabolizing enzymes and drug-drug interactions. Furthermore, the metabolites can be used as leads and subjected to further structural modifications to obtain improved clinical candidates during the lead optimization phase of the drug discovery process.

In summary, we report that the S- and R-enantiomers of OMP are hydroxylated by a set of 26 CYP102A1 mutants to produce the major metabolite 5’-OH OMP regardless of the chirality of the parent substrates. The 5’-OH product formation rates of the mutants for R-OMP were much higher than those for S-OMP. There were no differences for regioselectivity of the oxidation reaction for both enantiomers. Although the binding of R-OMP to the CYP102A1 active site causes a more obvious change of the heme environment than that of S-OMP, there were no correlations between the spectral change upon substrate binding and catalytic activity. The 5’-OH OMP of racemic, S-, and R-OMP could be obtained with a high conversion rate and high selectivity when the triple R47L/F87V/L188Q mutant was used. These results show that the 5’-OH OMP of each enantiomer could be obtained using a one-step reaction with the CYP102A1 mutants with a high yield.
Authorship Contributions

Participated in research design: Kang, Ahn, and Yun.


Wrote or contributed to the writing of the manuscript: Ryu, K.D. Park, and Yun.
References


Footnotes

S.H.R. and B.Y.P. contributed equally to this work.

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Figure legends

FIG 1. Structures of the omeprazole (OMP) enantiomers and their respective metabolites. R-OMP is primarily metabolized by CYP2C19 for 5’-OH R-OMP. S-OMP is mainly metabolized by CYP3A4 for 5-O-desmethyl OMP and OMP sulfone, which is further hydroxylated by CYP2C19. In this study, we observed that both enantiomers are hydroxylated by CYP102A1 mutants.

FIG 2. Rates of omeprazole (OMP) metabolite production by the 26 CYP102A1 mutants. Assays were performed for 30 min using 100 μM of either S- or R-OMP. The formation rate of the 5’-OH OMP metabolite was determined by HPLC. The values are represented as the mean ± S.E.M. of duplicate measurements.

FIG 3. Conversion of racemic, R-, and S-omeprazole to 5’-hydroxylated metabolites by CYP102A1 mutant #10. Assays were performed for 1 h using 1 mM of each substrate. HPLC chromatograms of racemic (A) S- (C), R- (E) omeprazole and their corresponding 5’-OH metabolites (B, D, and, F, respectively) produced by CYP102A1 mutant #10. The peaks of the substrate and 5’-OH product are indicated. The UV absorbance was monitored at 302 nm.
Figure 3

(a) Racemic omeprazole  
(b) 5′-hydroxylomeprazole  
(c) S-omeprazole  
(d)  
(e) R-omeprazole  
(f)  

$A_{302}$  

$t_R$, min