Generation of Human Chiral Metabolites of Simvastatin and Lovastatin by Bacterial CYP102A1 Mutants

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ABSTRACT:

Recently, the wild-type and mutant forms of cytochrome P450 BM3 (CYP102A1) from Bacillus megaterium were found to oxidize various xenobiotic substrates, including pharmaceuticals, of human P450 enzymes. Simvastatin and lovastatin, which are used to treat hyperlipidemia and hypercholesterolemia, are oxidized by human CYP3A4/5 to produce several metabolites, including 6′-β-hydroxy (OH), 3′-OH, and exomethylene products. In this report, we show that the oxidation of simvastatin and lovastatin was catalyzed by wild-type CYP102A1 and a set of its mutants, which were generated by site-directed and random mutagenesis. One major hydroxylated product (6′-OH) and one minor product (6′-exomethylene), but not other products, were produced by CYP102A1 mutants. Formation of the metabolites was confirmed by high-performance liquid chromatography, liquid chromatography-mass spectrometry, and NMR. Chemical methods to synthesize the metabolites of simvastatin and lovastatin have not been reported. These results demonstrate that CYP102A1 mutants can be used to produce human metabolites, especially chiral metabolites, of simvastatin and lovastatin. Our computational findings suggest that a conformational change in the cavity of the mutant active sites is related to the activity change. The modeling results also suggest that the activity change results from the movement of several specific residues in the active sites of the mutants. Furthermore, our computational findings suggest a correlation between the stabilization of the binding site and the catalytic efficiency of CYP102A1 mutants toward simvastatin and lovastatin.

Introduction

Simvastatin and lovastatin are well known hyperlipidemia and hypercholesterolemia drugs that act as cholesterol-lowering agents (Caron et al., 2007). Simvastatin (marketed under the trade names Zocor, Simlup, Simcard, and Simvacor) is metabolized to at least four primary metabolites, namely 6′-β-OH simvastatin, 6′-exomethylene simvastatin, 6′-hydroxymethyl metabolite, and 3′-OH simvastatin (Fig. 1). Although CYP3A4 is the main enzyme involved in the primary metabolism of simvastatin, CYP2C8 (Tornio et al., 2005), CYP2C9 (Transon et al., 1996), and CYP2D6 (Transon et al., 1996) are also involved in the formation of simvastatin metabolites. The extensive oxidative metabolism of lovastatin (marketed under the trade names Mevacor and Altopen) in the human liver is primarily mediated by CYP3A enzymes, particularly CYP3A4, to generate three known metabolites, 6′-OH lovastatin, 3′-OH lovastatin, and 6′-exomethylene (García et al., 2003; Caron et al., 2007) (Fig. 2).

After oral ingestion, simvastatin and lovastatin, which are inactive lactones, are hydrolyzed to the corresponding β-hydroxyacid form (Vickers et al., 1990a). This molecule is a principal metabolite and an inhibitor of HMG-CoA reductase. This enzyme catalyzes the conver-
sion of HMG-CoA to mevalonate, which is an early and rate-limiting step in the biosynthesis of cholesterol. In addition to the P450-mediated oxidation and β-oxidation processes, glucuronidation constitutes a common metabolic pathway for statins (Prueksaritanont et al., 2002). The metabolites resulting from microsomal oxidation of simvastatin (Vickers et al., 1990b) and lovastatin (Vyas et al., 1990) by P450 enzymes are effective inhibitors of HMG-CoA reductase. Therefore, it has been suggested that the metabolites may contribute to the cholesterol-lowering effect of simvastatin and lovastatin. However, systematic studies of the safety, efficacy, and toxicity of these metabolites have not been performed.

In February 2008, the U.S. Food and Drug Administration (FDA) modified its standards for evaluating drug toxicity, particularly with regard to the toxicity of drug metabolites when it issued the Guidance for Industry: Safety Testing of Drug Metabolites (http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/ucm079266.pdf). According to these guidelines, any human drug metabolites “formed at greater than 10% of parent drug systemic exposure at steady state” should be subject to separate safety testing, which involves the synthesis and administration of these metabolites to test animals (Guengerich, 2009 and references therein). The issue of human metabolites in safety testing

Fig. 1. Structures of simvastatin and its human metabolites. The formation of metabolites is catalyzed by P450 enzymes in the presence of NADPH in the human liver.

Fig. 2. Structures of lovastatin and its human metabolites. The formation of metabolites is catalyzed by P450 enzymes in the presence of NADPH in the human liver.
In 0.25 ml of 100 mM potassium phosphate buffer (pH 7.4) along with a CYP102A1 forms were typically in the range of 1.0 to 2.0 nmol P450/mg yield. The expression levels of wild-type and mutant Escherichia coli work showing their increased catalytic activity toward several human sub-

The heme domain of P450 BM3 (CYP102A1) from Bacillus megaterium, which possesses monoxygenase activity, has strong similarities to eukaryotic members of the CYP4A (fatty acid hydroxylase) family. The P450 BM3 reductase domain, a mammalian-like dithiol reductase, is fused to the C terminus of the P450 domain in a single polypeptide. The fusion of these two enzymatic activities makes soluble CYP102A1 an ideal model for mammalian, particularly human, P450 enzymes. In recent studies, CYP102A1 mutants engineered through rational design or directed evolution were reported to produce human drug metabolites (Kim et al., 2008b, 2009, 2010; Sawayama et al., 2009; Park et al., 2010). In addition, CYP102A1 is a versatile monooxygenase with a demonstrated ability to catalyze a diversity of substrates and an established relevance to biotechnology (Urlacher and Eiben, 2006; Yun et al., 2007; Julsing et al., 2008 and references within).

In this study, we evaluated whether CYP102A1 mutants could be used to produce the human metabolites of simvastatin and lovastatin. To our knowledge, the human metabolites of these statins have not been obtained by chemical synthesis and are not commercially available. Some of the tested mutations enabled the CYP102A1 enzyme to catalyze the oxidation of simvastatin and lovastatin to generate their human metabolites. Furthermore, computational calculations and molecular modeling have been performed to provide further insight into molecular recognition on the basis of free energy decomposition and hydrogen bond analysis.

Materials and Methods

Chemicals. Simvastatin and lovastatin were obtained from Merck (Rahway, NJ). NADPH was purchased from Sigma-Aldrich (St. Louis, MO). Other chemicals were of the highest grade commercially available.

Construction of P450 BM3 Mutants by Site-Directed Mutagenesis. The CYP102A1 mutants used in this study were selected on the basis of earlier work showing their increased catalytic activity toward several human sub-

Expression and Purification of CYP102A1 Mutants. Wild-type and mutant forms of CYP102A1 were expressed in Escherichia coli strain DH5αF−IQ and purified as described previously (Kim et al., 2008b). The CYP102A1 concentrations were determined from CO difference spectra using ε = 91 mM/cm. For all of the wild-type and mutated enzymes, a typical culture yielded 300 to 700 nM P450. The expression levels of wild-type and mutant CYP102A1 forms were typically in the range of 1.0 to 2.0 nmol P450/mg cytotoxic protein.

Oxidation of Simvastatin and Lovastatin. Typical steady-state reactions for the oxidation of simvastatin and lovastatin included 50 pmol of CYP102A1 in 0.25 ml of 100 mM potassium phosphate buffer (pH 7.4) along with a specified amount of substrate. To determine the kinetic parameters of several CYP102A1 mutants, 2 to 200 μM concentrations of statins were used. An aliquot of an NADPH-generating system was used to initiate reactions; final concentrations were 10 mM glucose 6-phosphate, 0.5 mM NADP+, and 1 IU/ml yeast glucose 6-phosphate. A stock solution of statins (20 mM) was prepared in dimethyl sulfoxide and diluted into the enzyme reactions with a final organic solvent concentration of <1% (v/v). For the human CYP3A4 activity assay, a control experiment of 50 pmol of P450, 100 pmol of NADPH-P450 reductase, 100 pmol of cytochrome b5, and 45 μM L-α-dilauroyl-sn-glycero-3-phosphocholine in 0.25 ml of 100 mM potassium phosphate buffer (pH 7.4) was used instead of 50 pmol of CYP102A1.

Reactions were generally incubated for 10 min at 37°C and terminated with a 2-fold excess of ice-cold dichloromethane. After centrifugation of the reaction mixture, the supernatant was carefully removed, and the solvent was evaporated under N2 gas as described previously (Vickers et al., 1990b). The products were analyzed by HPLC using a Gemini C18 column (4.6 × 150 mm, 5 μm; Phenomenex, Torrance, CA) with an acetonitrile-water (70:30, v/v) mobile phase containing 2.5 mM formic acid. Eluates were detected by UV at 238 nm. Authentic metabolites of 6′-β-OH and 6′-exomethylenyl were obtained by HPLC from reaction mixtures of simvastatin and lovastatin with human CYP3A4 as described below.

To determine the total turnover numbers of several P450 enzymes, a 1.0 mM concentration of statin was used. The reaction in the presence of a 500 μM concentration of substrate and 50 pmol of enzyme was initiated by the addition of the NADPH-generating system in 0.25 ml of 100 mM potassium phosphate buffer (pH 7.4) and incubated for 4 h at 37°C. After a 2-h incubation, a 500 μM concentration of each substrate was added to the reaction mixture. The formation rate of statin metabolites was determined by HPLC as described above.

The kinetic parameters (Km and Vmax) were determined using nonlinear regression analysis with GraphPad Prism software (GraphPad Software, Inc., San Diego, CA). The data were fit to the standard Michaelis-Menten equation:

\[ v = \frac{V_{\text{max}}[S]}{K_m + [S]} \]

where v is the velocity of the reaction, S is the substrate concentration, Vmax is the maximum reaction velocity, and Km is the Michaelis constant.

LC-MS Analysis. CYP102A1 mutants and human CYP3A4 were incubated with 100 μM lovastatin and simvastatin at 37°C for 30 min in the presence of an NADPH-generating system. Reactions were terminated by the addition of a 2-fold excess of ice-cold dichloromethane. After centrifugation, the supenatant from each incubated reaction was removed and evaporated to dryness. The reaction residue was reconstituted into 100 μl of mobile phase by vortex mixing and sonication for 20 s. An aliquot (10 μl) of this solution was injected onto the LC column. LC-MS analysis was performed in electrospray ionization (positive) mode on a Shimadzu LCMS-2010 EV system (Shimadzu, Kyoto, Japan) having LC-MS solution software. The separation was performed on a Shim-pack VP-ODS column (2 mm i.d. × 250 mm; Shimadzu) using an acetonitrile-water (70:30, v/v) mobile phase containing 2.5 mM formic acid at a flow rate of 0.16 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 the Metabolites by NMR Spectroscopy. An Agilent model 1100 HPLC system (Agilent Technologies, Santa Clara, CA) was used for the isolation of the 6′-β-OH metabolites of simvastatin and lovastatin in the reaction mixtures. Semi preparative columns were used for the isolation of 6′-β-OH simvastatin (SunFire Prep C18, 5 μm, 10 mm i.d. × 150 mm); Waters, Milford, MA) and 6′-β-OH lovastatin (Pursuit C18, 5 μm, 10 mm i.d. × 250 mm; Varian, Inc., Palo Alto, CA) from the reaction mixtures. Each metabolite mixture, which was approximately 10 mg in CH3OH, was injected onto the semipreparative columns. The 6′-β-OH simvastatin was eluted with a linear gradient (1.5%/min) of 30 to 90% CH3CN after elution with 30% CH3CN for 10 min. The metabolite fractions were collected at 18.2 min. The 6′-β-OH lovastatin was eluted with a series of gradients: H2O-CH3CN (75:25, v/v) for 20 min; 25 to 45% CH3CN for 40 min (0.5%/min); 45 to 90% CH3CN for 40 min (4.5%/min); and 90% CH3CN for 10 min. The metabolite fractions were collected at 63.7 min. The flow rate was 3 ml/min for both columns, and the eluates were monitored at 240 nm.

NMR experiments were performed on a VNMRS 600-MHz NMR spectrometer (Varian) equipped with a carbon-enhanced cryogenic probe. Chloroform-d2 was used as a solvent, and chemical shifts for proton and carbon were measured in parts per million relative to trimethylsilane. All of the one-dimensional and two-dimensional NMR experiments were performed with standard pulse sequences in the VNMRS (version 2.3) library and processed with the same software. Spectral assignments were done with one-dimensional


1H and 13C NMR spectroscopy along with two-dimensional NMR spectroscopic techniques (double quantum correlation spectroscopy, heteronuclear single quantum correlation, and heteronuclear multiple-bond correlation spectroscopy). The stereochemical configurations of the 6/β-OH position of both compounds were determined with 1-dimensional nuclear Overhauser effect spectroscopy.

Effects of 7,8-Benzoflavone on the Oxidation Reactions of Simvastatin and Lovastatin Catalyzed by CYP102A1 Enzymes. Reaction mixtures consisted of 50 pmol of P450, 100 mM potassium phosphate buffer (pH 7.5), an NADPH-generating system, substrate (100 μM of simvastatin or lovastatin), and 200 μM 7,8-benzoflavone (αNF) in 0.25 ml of 100 mM potassium phosphate buffer (pH 7.4). Products were analyzed by HPLC as described above. In the case of the human CYP3A4 activity assay, a control experiment of 50 pmol of P450, 100 pmol of NADPH-P450 reductase, 100 pmol of cytochrome b6, and 45 μM 1,6-dilauroyl-sn-glycero-3-phosphocholine was used instead of 50 pmol of CYP102A1.

Spectral Binding Titrations. Spectral binding titrations were used to determine dissociation constants (Kd) for substrates as described previously (Kim et al., 2008a). Binding affinities of 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). Final CH3CN concentrations were <2% (v/v). Spectral dissociation constants (Kd) were estimated using GraphPad Prism software. Unless the estimated Kd was within 5-fold of the P450 concentration, nonlinear regression analysis was applied using the hyperbolic equation

\[
\frac{B_{\text{max}}[L]}{K + [L]} = A
\]

where A is the absorbance difference extrapolated to infinite ligand concentration, and [L] is the ligand concentration.

Molecular Dynamics and Docking Simulation. Coordinates of the CYP102A1 structure (Protein Data Bank code 2UWH) (Huang et al., 2007) from B. megaterium were taken from the Protein Data Bank. Crystallographic water molecules were removed from all of the structures. The structure 2UWH contains the mutant residue Phe82, which was corrected to Ala82. The rectified CYP102A1 was then mutated to CYP102A1 mutant 16 (R47L/E64G/F81I/E267V) and CYP102A1 mutant 17 (R47L/E64G/F81I/E267V) using MODELLER (Sali and Blundell, 1993). The substrates lovastatin (LOV) and simvastatin (SIM) were prepared for molecular dynamics simulation with CYP102A1 mutants. The substrates were then applied with partial atomic charges derived by fitting ESP obtained by quantum electronic structure calculation. The substrates were geometry-optimized with the 6-31G+(p,d) basis set by the B3LYP method using the Gaussian 03 program (Gaussian Inc., Wallingford, CT). The Antechamber module was used to calculate the atom-centered restrained electrostatic surface potential by fitting ESPs estimated from wave functions (Bayly et al., 1993). Parameters that were missing for the ligands were generated using the general amber force field and the Parmchk module of Antechamber (Wang et al., 2004). Docking was performed using AutoDock (Morris et al., 1998) to determine the correct position for the substrates in place of palmitate. The best position was selected on the basis of the root-mean-square deviation and binding free energy. After applying the force field, all hydrogen atoms were automatically added to all systems. Structures were solvated with a TIP3P water model by creating an isometric water box, in which the distance of the box is 8.5 Å from the periphery of the protein (Jorgensen et al., 1983). Molecular systems were neutralized by the addition of counterions. Systems were then energy-minimized in two steps. In the first step, the CYP102A1 and substrates were kept fixed, and only the water molecules were allowed to move; in the second step, all atoms were allowed to move. For the first step, the energy minimization was performed in 500 and 1500 steps using the steepest descent and conjugate gradient methods, respectively. For the second step, the energy minimizations were performed in 1000 and 1500 steps using the steepest descent and conjugate gradient methods, respectively. Heating was performed with an NVT ensemble for 10 ps; the CYP102A1 mutants complexed with substrates were restrained with a force constant of 10 kcal/mol Å2. Equilibration was performed for 50 ps on an NPT ensemble restraining the CYP102A1 mutants/substrates complex by 1 kcal/mol Å2. Final simulations, i.e., the production phase, were performed for 5 ns on an NPT ensemble at a temperature of 310 K and 1 atm of pressure. Step size was 2 fs for the entire simulation. A Langevin thermostat and barostat was used for temperature and pressure coupling. A SHAKE algorithm was applied to constrain all bonds containing hydrogen atoms (van Gunsteren and Berendsen, 1977). The nonbonded cutoff was kept at 10 Å, and long-range electrostatic interactions were treated by the particle mesh Ewald method with a fast Fourier transform grid having approximately 0.1 nm space (Darden et al., 1993). Trajectory snapshots were taken at each 1 ps, which were then used for analysis. All simulations were performed by the SANDER module of AMBER 10 with amber force field (f03) (Case et al., 2008).

Binding Free Energy Calculation. The molecular mechanics Poisson Boltzmann and surface area (MM/PBSA) methods were used to calculate binding free energies for all of the CYP102A1 mutant complexes, using MM/PBSA implementation in AMBER 10 (Massova and Kollman, 2000). Binding free energy (ΔGbind) is given by the following equation:

\[
\Delta G_{\text{bind}} = \Delta G_{\text{MM}} + \Delta G_{\text{Sol}} + T\Delta S
\]

where ΔGbind is the binding free energy in solution, ΔGMM is the molecular mechanics energy, which is composed of a van der Waals and an electrostatic contribution, and ΔGS is the solvation energy, which consists of electrostatic and nonpolar interactions. Entropy could be calculated by either normal-mode analysis or quasi-harmonic approximation. Estimation of entropy has been shown to be computationally expensive with a low prediction accuracy. In this study, TΔS was not considered for the calculation, as we were mainly interested in protein flexibility and the relative energy contributions of amino acids toward binding. ΔGMM was calculated by the following equation:

\[
\Delta G_{\text{MM}} = \Delta G_{\text{int-elec}} + \Delta G_{\text{int-sasa}}
\]

where ΔGint-elec and ΔGint-sasa are electrostatic and van der Waals interaction energies between substrates and CYP102A1 mutants. These energies were computed with no cutoff. The solvation energy ΔGS was divided into two parts: ΔGsol-calc and ΔGsol-appr. The electrostatic contribution to the solvation free energy ΔGsol-elec was calculated using the generalized Born model (Gohlke and Case, 2004). The hydrophobic contribution to the solvation energy ΔGsol-appr was determined with a function of the solvent accessible surface area by the following equation:

\[
\Delta G_{\text{sol-appr}} = \gamma S_{\text{SASA}} + b, \text{where } \gamma \text{ and } b \text{ are empirical constants.}
\]

The value was set to 0.0072 kcal/mol Å2, whereas b was set to a default value of 0. SASA is the solvent accessible surface area estimated using the linear combination of pairwise overlaps model. Snapshots from 0.1 to 5 ns molecular dynamics (MD) simulation trajectories were taken for the calculation of free energy. For PBSA and generalized Born surface area calculations, dielectric constants for solute and solvent were taken as 1.0 and 80.0, respectively. SASA was calculated using the molsurf program implemented in AMBER 10 by taking the solvent probe as 1.4 Å. Free energy was decomposed using MM/PBSA of AMBER 10 to estimate the contribution of each residue in the binding process.

Results

Oxidation of Simvastatin and Lovastatin by Wild-Type P450 BM3 and Its Mutants. We examined whether CYP102A1 can oxidize simvastatin and lovastatin. First, the ability of wild-type and a set of CYP102A1 mutants to oxidize simvastatin and lovastatin was measured at a fixed substrate concentration (100 μM) (Fig. 3). Whereas simvastatin and lovastatin are known to produce at least four and three metabolites, respectively (Figs. 1 and 2), CYP102A1 mutants produced only two metabolites, one major (6/β-OH statin) metabolite and one minor (6-oxomethylene statin) metabolite. The metabolites were analyzed by HPLC and compared with those of human CYP3A4 (Figs. 4 and 5). Catalytically active mutants of CYP102A1 produced one major product, which had a retention time that exactly matched the 6/β-OH statin and the minor metabolite 6-oxomethylene product. However, other products were not observed under the experimental conditions used here.

The turnover numbers for the oxidation of statins (product formation) by the entire set of 18 mutants varied over a wide range. CYP102A1 wild-type showed very low catalytic activity toward simvastatin (0.0049 min−1 for 6/β-OH simvastatin), and no other metabolites were observed for either substrate. (Fig. 3). Mutant 4 did not show any apparent activity toward either statin (< 0.001 min−1). Mutants 16 and 17 each showed higher activity than that of human
CYP3A4. In the case of mutant 17, the turnover number (10 min⁻¹) was 3.3- and 2040-fold higher than that of human CYP3A4 and wild-type CYP102A1, respectively.

Characterization of the Chiral Metabolites of Simvastatin and Lovastatin. The identities of the major metabolite and substrate were verified by comparing the HPLC results (Figs. 4 and 5), the LC-MS results (Supplemental Figs. S1 and S2), and the absorption spectra (Supplemental Figs. S3 and S4) with those of authentic compounds, which were produced by human CYP3A4. The production of 6′-OH and 6′-exomethylene statins by CYP102A1 mutants was confirmed by LC-MS analysis of the reaction mixture. The retention time and fragmentation pattern of the CYP102A1 metabolites were exactly matched to those of the authentic metabolites, which were produced by human CYP3A4 (Supplemental Figs. S1 and S2).

![HPLC chromatograms of simvastatin metabolites produced by CYP102A1 mutants (13–17) and human CYP3A4. Peaks were identified by comparing the retention times with those of metabolites produced by CYP3A4. The peaks of the substrate and two major products (hydroxylated product and exomethylene product) are indicated. UV absorbance was monitored at 238 nm.](image-url)
Chemical structures of 6′-OH statins were identified by one- and two-dimensional NMR experiments. Spectral assignments were accomplished with two-dimensional $^1$H NMR and $^{13}$C NMR spectroscopy along with two-dimensional NMR spectroscopic techniques. The results are shown in Supplemental Fig. S5 and Supplemental Tables S2 to S5. The stereochemical configurations of the 6′-OH position for both compounds were determined with one-dimensional nuclear Overhauser effect spectroscopy. There have been reports for proton chemical shifts in $^6$H$_1$NMR (Vyas et al., 1990), and, to our knowledge, there have been no publications of C13 chemical shift data for 6′-OH lovastatin and 6′-OH simvastatin. The stereochemical configurations at the 6′-OH position of both compounds were determined with one-dimensional double-pulsed field gradient spin echo nuclear Overhauser effect spectroscopy. When previously assigned proton peaks from H-6′ for 6′-OH lovastatin (Supplemental Figs. S5, E and F). Human CYP3A4, the major enzyme for the hydroxylation reactions of simvastatin and lovastatin in human liver, also shows a preference for the 6′-hydroxylation reaction over the dehydrogenation reaction at the 6′ position (Figs. 4 and 5).

**Kinetic Parameters of Simvastatin and Lovastatin Oxidation by CYP102A1 Mutants.** The two high-activity mutants 16 and 17 were used to measure kinetic parameters for the 6′-hydroxylation of simvastatin and lovastatin (Table 1; Supplemental Fig. S6). Wild-type and some mutant forms of CYP102A1 did not exhibit any or appreciable activities to determine reliable kinetic parameters (Fig. 3). Only mutants 16 and 17 showed significantly elevated $k_{cat}$ values for the 6′-hydroxylation reaction, which yielded the highest $k_{cat}$ values of $>10$ min$^{-1}$ for simvastatin and lovastatin (Fig. 3). Human CYP3A4 exhibited $k_{cat}$ values of 6.6 and 4.2 min$^{-1}$ for 6′-hydroxylation of simvastatin and lovastatin, respectively. The overall range of $K_m$ values for the CYP102A1 mutants was from 37 to 44 μM. Of note, human CYP3A4 displayed a higher $K_m$ value of 130 μM for the 6′-hydroxylation of simvastatin. The catalytic efficiencies ($k_{cat}/K_m$) of 6′-hydroxylation of simvastatin and lovastatin by mutant 17 were

<table>
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<th>P450 Enzyme</th>
<th>Simvastatin</th>
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<tr>
<td></td>
<td>$k_{cat}$</td>
<td>$K_m$</td>
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<tr>
<td>Mutant 16</td>
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<td>37 ± 6</td>
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<tr>
<td>Mutant 17</td>
<td>15 ± 1</td>
<td>42 ± 6</td>
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<tr>
<td>Human CYP3A4</td>
<td>6.6 ± 0.9</td>
<td>130 ± 30</td>
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**TABLE 1**

*Kinetic parameters for the formation of the 6′-OH hydroxylated product by CYP102A1 mutants.*

![HPLC retention time](image)

**FIG. 5.** HPLC chromatograms of lovastatin metabolites produced by CYP102A1 mutants (13–17) and human CYP3A4. Peaks were identified by comparing the retention times with those of substrate and authentic metabolites produced by CYP3A4. The peaks of the substrate and two major products are indicated. UV absorbance was monitored at 238 nm.
Inhibitory Effects of αNF on the CYP102A1-Catalyzed Oxidation of Simvastatin and Lovastatin. Modulation of the catalytic activities of human CYP3A4 by αNF is known (Ueng et al., 1997). In this work, we examined the effect of αNF on the catalytic activities of CYP102A1 mutants that possessed human CYP3A4 activities. αNF inhibited the 6β-hydroxylation of simvastatin and lovastatin in a concentration-dependent manner. When 50 μM αNF was added to incubated reaction mixtures with simvastatin and lovastatin (Supplemental Fig. S7), the 6β-hydroxylation activities of human CYP3A4 for simvastatin and lovastatin were inhibited by 42 and 69%, respectively. The 6β-hydroxylation activities of CYP102A1 mutants 16 and 17 for simvastatin were inhibited by 44 and 77%, respectively. The activities of CYP102A1 mutants 16 and 17 for lovastatin were inhibited by 48 and 76%, respectively, in the presence of αNF. This result shows that αNF can bind to the active site of CYP102A1 mutants to change their catalytic activities.

A triple CYP102A1 mutant 10 (R47L/F87V/L188Q) has been reported to metabolize typical mammalian P450 substrates, such as amodiaquine, dextromethorphan, acetaminophen, testosterone, and 3,4-methylenedioxyxymethamphetamine (van Vught-Lussenburg et al., 2007). Although product formation from these chemicals by CYP102A1 mutant 10 was inhibited from 30 to 60% in the presence of αNF, αNF did not have a significant effect on the metabolism of acetaminophen and 3,4-methylenedioxyxymethamphetamine (van Vught-Lussenburg et al., 2007). This mutant could also oxidize simvastatin and lovastatin to generate 6β-OH and 6′-oxemethyleneproducts at relatively low rates (6β-OH simvastatin, 0.046 ± 0.003 min⁻¹; 6′-oxemethylene simvastatin, 0.00028 ± 0.00068 min⁻¹; 6β-OH lovastatin, 0.22 ± 0.17 min⁻¹; and 6′-oxemethylene lovastatin, 0.057 ± 0.008 min⁻¹) (Fig. 3).

Titration. The addition of simvastatin and lovastatin to a solution with human CYP3A4 produced a typical low- to high-spin conversion (type I difference spectrum) (Supplemental Fig. S8). The binding affinities of human CYP3A4 toward the simvastatin ($K_s = 14 ± 1 \mu M$) and lovastatin ($K_s = 8.8 ± 0.8 \mu M$) substrates were determined from the titration curves. However, in the case of the CYP102A1 mutants, the addition of the substrates tested here did not result in any apparent spectral changes (data not known).

Docking and Molecular Dynamics Simulation Analysis. Binding features observed in the simulation of the substrates docked into the wild-type CYP102A1 (CYP102A1 WT) are schematically represented (Fig. 7, A and B) together with interatomic distances for the 6β-carbon of the substrates and the Fe atom of the heme. Coordination of the 6β-carbon of the substrates to the catalytic iron atom was maintained for the entire simulation time, and none of the oxygen atoms in the substrates formed a stable hydrogen bond to any residues of CYP102A1 WT, suggesting that the substrate oxygen atoms are not the primary determinant; this situation for binding of LOV/SIM into CYP102A1 WT is unlike the case of other CYP102A1 mutant complexes. Because no direct hydrogen bond between CYP102A1 WT and LOV/SIM was retained during the energy minimization stage of the initial complex model, the distance of the 6β-carbon of the substrates moved away from the catalytic iron atom during the equilibrium stage of the MD simulation (8–9 Å). In the subsequent data sampling stage, a direct hydrogen bond was also not observed. Significantly, the movement of the substrates from their original position at the catalytic sites to the channel entrance was observed in the MD simulation; this movement was found to be due to a collision between the fused ring connected to the oxooxane ring of LOV/SIM and flexible or large hydrophobic residues, such as Leu75 and Phe87. The simulations indicated that that the butanonate chain and the oxooxane ring could not fit into the space created by the Thr49, Met185, and Leu437 shift without inducing steric repulsion. Thus, a small increase in the hydroxylation potency for SIM caused by introducing a methyl group on the LOV was only ascribed to the induced fit of SIM. Both simulations yield nearly identical mean values for the distance between the 6β-carbon of the substrates and the Fe iron, 9.07 and 8.62 Å for LOV and SIM (Fig. 7, A and B), respectively, which are comparable to those observed in the experimental results described above.

The replacement of several residues [mutant 16 (R47L/F81I/F87V/E143G/L188Q/E267V) and mutant 17 (R47L/E64G/F81I/F87V/E143G/L188Q/E267V)] that led to quite potent monooxygenase activity induced significant changes in the overall shape of the catalytic sites of CYP102A1, which were determined by the conformations of their side chains (Supplemental Fig. S9). The docking of substrates into CYP102A1 mutants (16 and 17) revealed that coordination of the 6β-carbon of the substrates to the iron atom is possible only when a hydrogen bond is made in the shallow cleft that acts as a catalytic subsite around the heme. The simulations for CYP102A1 WT and its mutant complexes showed a significant difference in the buried surface areas at the interface between the substrates and CYP102A1, suggesting that interaction of the hydrophobic residues in the catalytic subsites with the substrates is very extensive and consequently makes...
an important contribution to the destabilization of substrate binding. We speculate that the mutation of CYP102A1 causes conformational changes in the active site. When coupled with structural information, these conformational changes can be used to interpret the experimental results.

Hydrogen bonds play an essential role in stabilizing the complexes of the CYP102A1 mutants (16 and 17)-LOV/SIM complexes. We examined the geometry and stability of the hydrogen bond network formed by LOV/SIM in the binding pocket of mutants 16/17 on the basis of the trajectories of the MD simulations. Conformational changes in the binding pocket at the active site caused by several mutations affect the LOV and SIM binding (Fig. 7, C–F). Molecular surface visualization showed that there are relatively deep pockets in the CYP102A1 mutants. Interactions of these pockets with the oxooxane moiety of LOV/SIM could enhance their binding ability. The most significant hydrogen bond contacts between the CYP102A1 mutants and LOV/SIM were schematically identified and characterized in terms of distances between heavy atoms and their percentage of occurrence.

The hydroxyl group of the oxooxane ring in LOV complexed with CYP102A1 mutant 16 mainly participates in a hydrogen bond with the Ser72 hydroxyl group (87.3% occupied of the bonds being maintained during the MD simulation) (Fig. 7C). This hydrogen bond possibly plays an important role in enhancing the affinity of the oval-like pocket of CYP102A1 mutant 16. However, the hydroxyl of Ser72 adopted a different conformation in the SIM complex with CYP102A1 mutant 16 and failed to form a hydrogen bond with the hydroxyl group of SIM; it is present in only 17.0% of the computed snapshots (Fig. 7D), indicating that SIM barely interacts with Ser72 in the CYP102A1 mutant 16 active site model. The hydrophobic interaction between the methyl butanoate group and Ile263/Ala264 was shown to contribute to the complex stabilization, whereas the hydrogen bond to Ser72 was frequently observed to be cleaved in the simulation, giving a longer average distance than that for the complex with LOV.

In the model structure of CYP102A1 mutant 17 complex with LOV, Ala330 and Leu437 make a hydrogen bond and an electrostatic interaction through their backbone carbonyl group with a hydroxyl group and carbonyl group located in the oxooxane ring, respectively (95.3% occupied for Leu437 and 91.6% occupied for Ala330). These interactions may stabilize the binding of LOV to CYP102A1 mutant 17. The averaged distances along the 5-ns MD trajectory between the 6′β carbon atom of LOV and the Fe atom of the heme is less than 2.77 Å (Fig. 7E). For the SIM complex with CYP102A1 mutant 17, no interaction exists between the main chain oxygen of the Ala330 backbone and the carbonyl group of the oxooxane ring in SIM (Fig.
7F), but another hydrogen bond forms between the main chain oxygen of the Leu437 backbone and the hydroxyl group of the SIM oxooxane ring (86.0% occupied). This hydrogen bond may also result in the different conformation for bound SIM (Fig. 7F).

The hydrogen bond and the electrostatic interaction in the CYP102A1 mutant 17/LOV make significant contact with the substrate compared with complexes of mutant 16/LOV, mutant 17/SIM, and mutant 16/SIM. According to our newly described binding mode, the carbonyl group of the Ala330 backbone forms a strong electrostatic interaction with the carbonyl group of the oxooxane ring in LOV, whereas a similar interaction does not exist in other complexes. The difference in the hydrogen bonding and electrostatic interaction reveals that LOV binds most strongly with CYP102A1 mutant 17, which explains the significantly higher catalytic activity of the CYP102A1 mutant 17/LOV complex compared with that of other complexes. The large changes at position Leu437 are expected to contribute to substrate binding. These include van der Waals, electrostatic, polar solvation, and nonpolar solvation interaction energies. The difference in the hydrogen bonding and electrostatic interaction between mutants 16 and 17 compared with interaction of LOV with CYP102A1 mutant 17.

Of interest, in CYP102A1 mutant 16 complexed with LOV and SIM, the main chain oxygen atoms of Ala330 and Leu437 lose the hydrogen bond and the electrostatic interaction compared with the CYP102A1 mutant 17 complexes. There is a particularly large difference in orientations of the side chains of Ala330 and Leu437. Rotations occur at the side chains of Ala330/Leu437, which are correlated with changes in the distances of the 6’β-carbon in the substrates from the heme iron. This alteration in the side-chain orientation of the active site residues also implies different interactions between the substrates and the CYP102A1 mutants. Simulations also showed that the butanone moiety did not form any hydrogen bonds to the enzyme. Thus, the oxooxane ring can play an important role in entering the cavity more fully.

**Free Energy Calculation.** To evaluate the different contributions to the binding free energy of LOV and SIM with CYP102A1 WT and the highly active mutants 16 and 17, absolute binding free energies were calculated for four complexes using the MM/PBSA method. The investigation into the forces involved in substrate binding can also be obtained by analyzing the MM/PBSA free energy contributions, which are listed in Table 2 for six complexes. In the present study, we estimated the different components of interaction energy that contribute to substrate binding. These include van der Waals, electrostatic, polar solvation, and nonpolar solvation interaction energies. The entropy was not calculated as we are only interested in the relative binding free energy. The calculated \( \Delta G_{\text{bind}} \) for the mutant 17/LOV complex (\(-19.49 \text{ kcal/mol}\)) was the most negative among the \( \Delta G_{\text{bind}} \) values for all complexes. This result agrees with the previous experimental results, which showed that the energetic analysis performed by MM-PB(generalized Born)SA is applicable to the current case study.

The binding free energy of the mutant complexes 16-LOV, 17-LOV, 16-SIM, and 17-SIM increased by \(-12.75, -16.76, -9.01, \) and \(-10.56 \text{ kcal/mol}\) with respect to the corresponding WT-substrate complex, which indicates that the two mutants 16 and 17 bind more strongly to LOV and SIM than WT does. This result also suggests that mutants 16 and 17 exhibit substrate acceptance for LOV and SIM. The free energy components between the WT complex and each mutant complex were compared to elucidate the mechanism driving substrate acceptance. The differences in the nonpolar solvation energies among the six complexes are relatively small, which indicates that better cavity packing is retained in the mutated complexes. There is a decrease in electrostatic and van der Waals energies in the WT complex of approximately 35 to 70 and 15 to 22 kcal/mol for the LOV and SIM complexes relative to the respective CYP102A1 mutants, which should be essential to the rejection of CYP102A1 WT for LOV and SIM.

Intermolecular van der Waals and electrostatic interactions are both important contributions to substrate binding. Nonpolar solvation terms, which correspond to the burial of solvent accessible surface area upon binding of substrates to the active sites of mutants, contribute slightly favorably. The gas-phase electrostatic values, \( \Delta F_{\text{int,elec}} \), for the four complexes show that electrostatic interactions are in favor of the binding; however, the overall electrostatic interactions energies, \( \Delta G_{\text{elec}} \) (\( \Delta F_{\text{int,elec}} + \Delta G_{\text{elec,water}} \)) are positive and unfavorable for the binding, which is caused by the large desolvation penalty for charged and polar groups that is not sufficiently compensated for upon complex formation. Comparing the van der Waals/nonpolar (\( \Delta F_{\text{int,vdw}} + \Delta G_{\text{elec,water}} \)) contributions with the electrostatic contributions \( \Delta G_{\text{elec}} \), we find that the association between LOV and SIM and mutants 16 and 17 is mainly driven by more favorable van der Waals/nonpolar interactions in the complex than in solution. The electrostatic interactions between mutants 16 and 17 and the butanone chain are strong, but the electrostatic interactions between the solvent (water molecules) and the substrates are much stronger. Thus, when a substrate transfers from the solvent to the binding pocket, the electrostatic contributions for the butanone moiety are unfavorable to substrate binding. The terminal butanyl chain of LOV and SIM is sandwiched between the side chains of Leu75 and Val78 and makes hydrophobic and van der Waals interactions with the side chains of Ala82 and Val87. Overall, this finding suggests that the affinities of the two substrates for the two mutants 16 and 17 are dominated by shape complementarity, but

### Table 2

<table>
<thead>
<tr>
<th></th>
<th>Energy</th>
<th>WT/ Lovastatin</th>
<th>WT/Simvastatin</th>
<th>M16/Lovastatin</th>
<th>M16/Simvastatin</th>
<th>M17/Lovastatin</th>
<th>M17/Simvastatin</th>
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<tbody>
<tr>
<td></td>
<td>kcal/mol</td>
<td></td>
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<tr>
<td>( \Delta F_{\text{int,elec}} )</td>
<td>-6.79 ± 0.65</td>
<td>-7.20 ± 0.28</td>
<td>-38.48 ± 0.26</td>
<td>-80.12 ± 0.57</td>
<td>-48.03 ± 0.28</td>
<td>-73.92 ± 0.43</td>
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<tr>
<td>( \Delta F_{\text{int,vdw}} )</td>
<td>-4.94 ± 0.18</td>
<td>-4.14 ± 0.18</td>
<td>-27.01 ± 0.19</td>
<td>-19.89 ± 0.19</td>
<td>-28.64 ± 0.17</td>
<td>-19.54 ± 0.21</td>
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<tr>
<td>( \Delta F_{\text{elec,water}} )</td>
<td>-0.55 ± 0.17</td>
<td>-0.62 ± 0.04</td>
<td>-3.16 ± 0.02</td>
<td>-4.57 ± 0.01</td>
<td>-4.16 ± 0.09</td>
<td>-3.66 ± 0.02</td>
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<tr>
<td>( \Delta F_{\text{elec,sol}} )</td>
<td>9.55 ± 0.25</td>
<td>9.05 ± 0.19</td>
<td>54.17 ± 0.23</td>
<td>92.65 ± 0.43</td>
<td>60.33 ± 0.22</td>
<td>83.64 ± 0.38</td>
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<tr>
<td>( \Delta F_{\text{elec,elec,water}} )</td>
<td>9.76 ± 0.16</td>
<td>8.42 ± 0.25</td>
<td>55.34 ± 0.24</td>
<td>88.07 ± 0.42</td>
<td>56.18 ± 0.27</td>
<td>79.99 ± 0.31</td>
<td></td>
</tr>
<tr>
<td>( \Delta F_{\text{elec,elec,elec}} )</td>
<td>2.76 ± 0.02</td>
<td>1.84 ± 0.06</td>
<td>15.68 ± 0.21</td>
<td>12.54 ± 0.22</td>
<td>12.31 ± 0.15</td>
<td>9.72 ± 0.27</td>
<td></td>
</tr>
<tr>
<td>( \Delta F_{\text{elec,elec,elec,elec}} )</td>
<td>-2.73 ± 0.24</td>
<td>-2.92 ± 0.04</td>
<td>-15.48 ± 0.24</td>
<td>-11.93 ± 0.24</td>
<td>-19.49 ± 0.18</td>
<td>-13.48 ± 0.23</td>
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M16, mutant 16, R47L/F81D/F87V/E143Q/L188Q/E267V; M17, mutant 17, R47L/E64G/F81D/F87V/E143Q/L188Q/E267V; \( \Delta G_{\text{elec,elec,elec,elec}} \) contribution; \( \Delta F_{\text{elec,elec,elec,elec}} \) contribution.
the total affinity arises from a more complex interplay between all of these energy components. The structural analysis demonstrates that the hydrophobic group in the upper part of the heme takes advantage of the favorable van der Waals and hydrophobic interactions with the binding pocket of CYP102A1 mutants. In the binding pocket, Leu75, Val78, Ala82, Val87, Ile263, and Leu437 residues constitute a relatively large hydrophobic core, which can generate strong van der Waals and hydrophobic interactions with the fused ring of the substrates. The $\Delta E_{\text{int, vdW}}$ value for the complex of mutant 17 with LOV is the highest among the complexes of mutants 16 and 17 with the two substrates because lovastatin fits more snugly within the active site cavity and binds more tightly to mutant 17 than to the other complexes by adding nonpolar packing to the active site to a much greater extent.

**Discussion**

In this work, we have shown that bacterial CYP102A1 mutant enzymes catalyze the same reactions as human CYP3A4 to generate the human metabolites $6\beta$-OH and $6\alpha$-exomethylene from simvastatin and lovastatin. To our knowledge, the production of metabolites of simvastatin and lovastatin by chemical synthesis has never been reported. The $6\beta$-OH products are chiral compounds, which are usually difficult to synthesize by chemical methods. Therefore, an alternative to chemical synthesis of these metabolites is to use CYP102A1 enzymes to generate the metabolites of simvastatin and lovastatin. Of interest, an unknown P450 from Nocardia autotropica has been found to oxidize simvastatin to generate $6\beta$-hydroxymethyl simvastatin (Gbewonyo et al., 1991). Human P450 enzymes are involved in the metabolism of most (>80%) drugs currently available on the market (Guengerich, 2003). We proposed previously that CYP102A1 from B. megaterium could be developed as a biocatalyst with drug oxidation activities comparable to those of human P450 (Yun et al., 2007 and references therein). In recent studies, several lines of evidence supporting our proposal have been reported. CYP102A1 mutants were found to generate human drug metabolites by oxidizing various drugs, including clozapine (Damsten et al., 2008), diclofenac (Damsten et al., 2008), acetaminophen (Damsten et al., 2008), 3,4-methylenedioxyxymethylamphetamine (Sjörenschantz et al., 2008), dextromethorphan (Sjörenschantz et al., 2008), phenacetin (Kim et al., 2010), verapamil (Sawayama et al., 2009), and astemizole (Sawayama et al., 2009). CYP102A1 mutants can also oxidize several human P450 substrates, including 7-ethoxycoumarin (Kim et al., 2008b), coumarin (Park et al., 2010), chlorzoxazone (Park et al., 2010), and resveratrol (Kim et al., 2009); however, the human metabolite piceatannol can be generated from the anticancer agent resveratrol by CYP102A1 (Kim et al., 2009). Several CYP102A1 mutants were generated by directed evolution and rational design and were found to have much higher activity and stability than human P450 enzymes. These mutants could thus be developed as industrial enzymes for cost-effective and scalable production of human drug metabolites. The metabolites could also be developed as lead compounds for new drugs.

Our computational findings suggest that a conformational change in the cavity size of the mutant active sites is related to the activity change. The modeling results further suggest that the changes in activity result from the movement of specific residues in the mutant active sites (Fig. 7; Supplemental Fig. S9). The computational results obtained from highly active mutants with the Fe atom were compared, mutant 17 showed much shorter average distances than those of mutant 16. However, we could not see apparent differences in $K_a$ values between the complexes when experimental error ranges were considered (Table 1).

In summary, this work involved a set of CYP102A1 mutants and statin substrates, which are human P450 substrates, and revealed that bacterial CYP102A1 enzymes catalyze the same reactions as human CYP3A4 to generate the human metabolites $6\beta$-OH and $6\alpha$-exomethylene. The oxidation of simvastatin and lovastatin is catalyzed by wild-type and some mutant forms of CYP102A1. One major hydroxylated product, the $6\beta$-OH product, was produced as a result of a hydroxylation reaction. The other product, the oxymethylene product, was produced by a dehydrogenation reaction. Metabolite formation was confirmed by HPLC and LC-MS by comparing the metabolites with the authentic human metabolites produced by human CYP3A4. Chemical structures of the major $6\beta$-OH products were identified by NMR. Thus, the CYP102A1 mutants efficiently produce metabolites of simvastatin and lovastatin.

**Authorship Contributions**

Participated in research design: K.H. Kim, D. Kim, Jung, Pan, Ahn, and Yun.


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

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