Preparation of Human Metabolites of Propranolol Using Laboratory-Evolved Bacterial Cytochromes P450

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Abstract: Testing the toxicities and biological activities of the human metabolites of drugs is important for development of safe and effective pharmaceuticals. Producing these metabolites using human cytochrome P450s is difficult, however, because the human enzymes are costly, poorly stable, and slow. We have used directed evolution to generate variants of P450 BM3 from *Bacillus megaterium* that function via the ''peroxide shunt'' pathway, using hydrogen peroxide in place of the reductase domain, oxygen and NADPH. Here, we report further evolution of the P450 BM3 heme domain peroxygenase to enhance production of the authentic human metabolites of propranolol by this biocatalytic route. This system offers a versatile, cost-effective, and scaleable route to the synthesis of drug metabolites. © 2005 Wiley Periodicals, Inc.
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INTRODUCTION

The cytochromes P450 (P450s) comprise a ubiquitous superfamily of heme-containing enzymes which perform a variety of oxidative reactions on a wide range of substrates (Lewis, 2001; Sono et al., 1996). In humans, P450s are responsible for the disposition of many xenobiotics, including pharmaceuticals (Guengerich, 2003). During initial clearance, most drugs are metabolized in the liver by P450s (Guengerich, 2001; Lewis, 2004). Some of the metabolites are biologically active themselves, and understanding their effects is crucial in evaluating a drug's efficacy, toxicity, and pharmacokinetics (Johnson et al., 2004). Identification of so-called ''active metabolites,'' as in the case of terfenadine (Markham and Wagstaff, 1998), early on in the drug development process could result in the early identification of safer, more well tolerated therapies. Such studies, however, can require large quantities of the pure metabolites, and these may be difficult to synthesize. An alternative to chemical synthesis is to use P450s to generate the metabolites of drugs or drug candidates. Hepatic microsomes are a source of human P450s, but their limited availability and highly variable expression levels make their use in preparative-scale metabolite synthesis impractical. Some human enzymes can also be obtained by expression in recombinant hosts. As membrane-bound, multi-protein systems, however, they commonly misfold and aggregate or are not expressed in active form. Metabolite preparation has been demonstrated using human P450s expressed in *Escherichia coli* and in insect cells (Parikh et al., 1997; Rushmore et al., 2000; Vail et al., 2005), but these systems are costly and have low productivities due to limited stabilities and slow reaction rates (usually <5 min^{-1} (Guengerich et al., 1996)).

An alternative approach to preparing the human metabolites is to use an engineered bacterial P450 that has the appropriate specificity. Cytochrome P450 BM3, a fatty acid hydroxylase from *Bacillus megaterium*, is expressed at high levels in *E. coli* and exhibits very fast reaction rates (thousands per minute) on favored substrates (Noble et al., 1999). This enzyme has been engineered to accept a variety of new substrates, on which its variants catalyze regio- and enantio-selective oxidations (Graham-Lorence et al., 1997; Kubo et al., 2005; Li et al., 2001b; Meinhold et al., 2005b; Munzer et al., 2005; Peters et al., 2003). In a previous study, we used directed evolution, with iterations of random mutagenesis and high-throughput screening, to generate variants of the P450 BM3 heme domain (BM3-H) which efficiently hydroxylate fatty acids using hydrogen peroxide in place of the reductase domain, oxygen and the costly

NADPH cofactor (P450 ''peroxygenases'') (Cirino and Arnold, 2003). Here, we demonstrate that an engineered P450 BM3 heme domain peroxygenase can be used to produce the authentic human metabolites of propranolol, a multi-function beta-adrenergic blocker used to treat hypertension, arrhythmia, angina, migraine headaches, overactive thyroids, and anxiety (Shand, 1975). Furthermore, this activity can be improved by directed evolution to produce more of the biologically active metabolites.

MATERIALS AND METHODS

All chemicals were purchased from Sigma-Aldrich, Inc. (St. Louis, MO). Solvents were from EM Sciences (Gibbstown, NJ). Enzymes for DNA manipulation were from New England Biolabs (Beverly, MA). DNA purification kits were from Zymo Research (Orange, CA) and Qiagen (Valencia, CA). T4 DNA ligase was from Invitrogen (Carlsbad, CA). Propranolol standards 4′-hydroxypropranolol (4′OHP) and 5′-hydroxypropranolol (5′OHP) were generously provided by Professor Wendel Nelson of the University of Washington, Seattle. (S)-N-desisopropylpropranolol was purchased from ABX Advanced Biochemical Compounds (Radeberg, Germany).

Protein Expression

All P450 BM3 heme domains (BM3-H) were cloned, transformed, and expressed in the catalase-deficient strain of *E. coli SN0037* (Nakagawa et al., 1996) using the isopropyl β-D-thiogalactopyranoside (IPTG)-inducible pCWori vector (Barnes et al., 1991). For protein expression, 50 mL of Terrific Broth (TB) supplemented with 100 µg/mL ampicillin and 25 µg/mL thiamine were inoculated with 500 µL of an overnight culture and incubated for 5 h at 30°C with shaking. P450 expression was induced by adding 0.5 mM IPTG and the heme precursor δ-aminolevulinic acid (δ-ALA) to a final concentration of 1 mM. The cultures were grown for another 18 h and the cells were pelleted and stored at −20°C.

Bioconversion of Propranolol

Cell pellets were resuspended in 100 mM N-[2-hydroxyethyl]piperazine-N′-[3-propanesulfonic acid] (Epps) pH 8.2, sonicated for 3×45 s and centrifuged at $10,000g$ for 30 min. The supernatant was passed through a PD10 size exclusion column and P450 concentration was measured by CO difference spectroscopy (Schenkman and Jansson, 1998).

Bioconversions were done in 400 µL of 100 mM Epps pH 8.2 containing 5 µM P450 and 5 mM propranolol. Reactions were initiated by adding H_2O_2 to a final concentration of 1 mM. An equivalent amount was added every 30 min thereafter. After 180 min at room temperature, the reaction products were analyzed using the 4-aminoantipyrine (4-AAP) assay (Otey and Joern, 2003) and HPLC. Control reactions performed without the addition of enzyme or peroxide produced no detectable products.

HPLC Analysis

Product analysis was similar to that described previously (Upthagrove and Nelson, 2001). Perchloric acid (7%) was added to the reaction mixture at a volume of 10µL/100 µL reaction, followed by addition of 2.5 mg/100 µL ascorbic acid. Samples were vortexed, centrifuged, and 50 µL of the supernatant were analyzed using an Alliance HPLC system (Waters, Milford, MA) equipped with a photodiode array detector. Separation was achieved on a Microsorb-MV phenyl column (250×4.6 mm, particle diameter 5 µm, Varian, Palo Alto, CA). Conditions with solvent A (1% triethylamine (v/v) and 0.8% acetic acid in H_2O) and solvent B (acetonitrile) used to elute propranolol and products were: 0–10 min, A:B 75:25; 10–20 min, linear gradient to A:B 25:75; 20–22 min, A:B 75:25; 22–27 min, linear gradient to A:B 75:25. Metabolites were identified by retention times and absorption spectra and were quantified by comparison to peak areas of known amounts of synthetic standards.

Saturation Mutagenesis

Using BM3-H 9C1 as the parent sequence, a mutant library was generated in which active site residues A74, L75, V78, P81, A82, F87, and T88 were randomly mutated. The primers were designed so that each clone would contain, on average, two amino acid substitutions (Suzuki et al., 1996). The sequence of the forward primer was 5′-CTCGCTTTGA-TAAAAA*CTTAAG*TCAA**RSRSXX**AAATTT**RXW**CGT-GAT**XXXRSW**GGAGACGGGTTG**RSSWSW**AGCT-GGACG-3′, in which R = 85%G, 5% of C, A, and T; S = 85%C, 5% of G, C, and T; W = 85% A, 5% of G, C, and T; X = 85% T, 5% of G, C, and A. The bold letter shows the doped nucleotides, and italic sequence is a *BfrI* restriction site. The reverse primer sequence was 5′-CGTACTAT-GGTTTGCTTTGACGC-3′. Silent mutation A321G was introduced to the parent 9C1 gene to disrupt a *BrfI* restriction site for subsequent cloning purposes. Polymerase chain reaction was performed with *Pfu* Turbo DNA polymerase using these primers and the mutated 9C1 gene (with A321G) as the template. PCR conditions were 94°C (1 min), {94°C (30 s) → 63°C (30 s) → 72°C (2 min)} × 30 cycles, 72°C (10 min). Following agarose gel electrophoresis, the band corresponding to the P450 gene was excised from the gel and purified. The purified DNA fragment was treated with EcoRI and BfrI and ligated into the pCWori plasmid. The ligated DNA was transformed into catalase-deficient *E. coli*, and the resulting clones were screened as described below.

Random Mutagenesis

Error-prone PCR was performed on the BM3-H DE10 gene with the GeneMorph PCR Mutagenesis kit (Stratagene, La Jolla, CA) according to the manufacturers' protocol, with 5–50 ng of target DNA. The reaction mixture was treated with DpnI for template degradation followed by agarose gel purification. The purified DNA fragment was digested with

EcoRI and BamHI, gel purified, and ligated into the pCWori vector. The ligation product was used to transform the catalase-deficient *E. coli* strain, and the transformants were plated on LB agar medium supplemented with 100 µg/mL of ampicillin.

Screening for Increased Activity on Propranolol

Single colonies were picked and inoculated into 500 µL of Luria-Bertani (LB) medium supplemented with 100 µg/mL ampicillin, in 96-well deep-well plates. The plates were incubated overnight at 30°C with shaking and 80% humidity. One hundred and fifty microliters of the pre-cultures were inoculated into 850 µL of TB supplemented with 100 µg/mL ampicillin, 25 µg/mL thiamine, 0.5 mM IPTG, and 1 mM δ-ALA, and grown under the same conditions for another 20 h. The plates were then centrifuged to pellet the cells, supernatants discarded, and frozen overnight at −20°C. Cells were resuspended in 300 µL of 100 mM Epps pH 8.2 containing 0.5 mg/mL lysozyme and 2 U/mL DNaseI. After 60 min at 37°C, the lysates were centrifuged, and the supernatant was used for the activity assay. To 80 µL of the cleared supernatant, 30 µL of Epps pH 8.2 containing propranolol was added followed by 10 µL of 12 mM H_2O_2. Final concentrations in the reaction mixture were 1 mM propranolol, 1% DMSO, 1% acetone, and 1 mM H_2O_2. After 90 min at room temperature, the 4-AAP assay (Otey and Joern, 2003) was used to quantify the products of propranolol bioconversions.

RESULTS

Bioconversion of Propranolol by P450 BM3-H 9C1

In a previous study, we created variants of BM3-H with high peroxygenase activity and thermostability by directed evolution, using multiple rounds of mutagenesis and screening (Cirino and Arnold, 2003). In each round, a mutant library created by error-prone PCR random mutagenesis was screened for H_2O_2-driven hydroxylation of 12-*p*-nitrophenoxycarboxylic acid (12-pNCA) (Schwaneberg et al., 1999). The most active mutants were recombined to obtain the parents for subsequent rounds of evolution. BM3-H variant 21B3 obtained in this way was further evolved during four rounds of error-prone PCR and screening to generate variant 9C1, which has 13 amino acid substitutions relative to the original sequence, wild-type P450 BM3-H with the F87A mutation (BM3-H F87A) (Table I) and catalyzes ~1,000 turnovers on 12-pNCA.

In humans, CYP2D6 and CYP1A2 are the main P450s responsible for the metabolism of propranolol (Masubuchi et al., 1994), forming the three major products shown in Figure 1. The ring-hydroxylated products, 4′OHP and 5′OHP, are of particular importance due to their pharmacological activities (Fitzgerald and O'Donnell, 1971; Oatis et al., 1981). Upon testing different peroxygenase variants of P450 BM3 from our previous study, we found that variant 9C1

Table I. Amino acid substitutions in cytochrome P450 BM3-H variants.

Position (9C1[a])	DE10	D6H10	2C11
K24[b]			R
R47[b,c]			H
A74[c]	V		V
L75[c]		H	
V78[c]		E	
A82[c]	L	P	L
A87[c]	G		G

[a]The 9C1 parent has the following amino acid substitutions compared to wildtype BM3-H: F87A, I58V, H100R, I102T, F107L, A135S, M145A, N239H, S274T, L324I, I366V, K434E, E442K, V446I.
[b]Identified by random mutagenesis.
[c]Targeted active-site residues.

produces a very similar product profile in a bioconversion carried out using *E. coli* cell extracts overexpressing the P450 heme domain. A representative chromatogram showing the products from a propranolol bioconversion by BM3-H 9C1 is shown in Figure 2. In a reaction with 5 µM P450 and 5 mM propranolol, driven by 1 mM H_2O_2 added every 30 min for 180 min, 9C1 converted 18% of the substrate to products. This conversion equates to ~180 turnovers per P450 active site. Under these conditions, the reaction was complete in 180 min, at which point no more product was made, and no P450 remained (as determined by carbon monoxide difference spectroscopy). Control reactions performed without the addition of enzyme or peroxide produced no detectable products.

Directed Evolution to Enhance Activity of BM3-H 9C1 on Propranolol

To test whether the bacterial P450 heme domains can be engineered to increase production of the hydroxylated products 4′OHP and 5′OHP and total activity towards drug compounds such as propranolol, we carried out additional directed evolution, this time with mutations targeted specifically to the active site. Using 9C1 as the parent, a library was created by directing mutations to seven active-site residues (A74, L75, V78, F81, A82, F87, and T88) which are within 5 Å of the substrate, based on the crystal structure of P450 BM3 bound to *N*-palmitoylglycine (Haines et al., 2001). The mutagenic oligonucleotides were doped such that two of the seven residues were mutated in each sequence, on average. This gene library was transformed into catalase-deficient *E. coli* (Nakagawa et al., 1996) to allow screening for activity in the presence of hydrogen peroxide. Propranolol hydroxylation activity was monitored using the 4-AAP assay (Otey and Joern, 2003), which detects the products of aromatic hydroxylation, 4′OHP, 5′OHP, and 1-naphthol. It does not detect the dealkylation product DIP.

Mutants DE10 and D6H10 were selected on the basis of increased formation of detectable products compared to parent 9C1. Sequencing showed that each mutant contained three amino acid substitutions relative to 9C1 (Table I). HPLC analysis of the reaction mixture showed that DE10 and
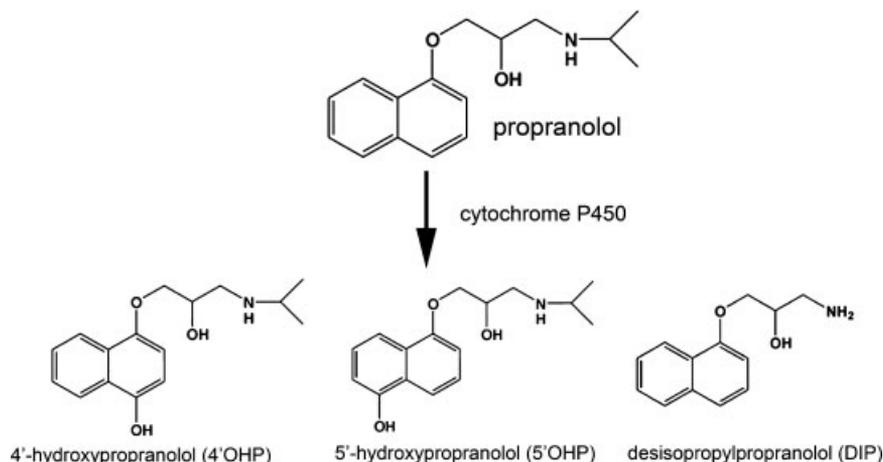
Figure 1. Major metabolites of propranolol formed by human cytochromes P450.

D6H10 convert 13% and 9% of the propranolol, respectively, to products. Although DE10 and D6H10 lead to less conversion than 9C1, they produce more of the metabolites detected by the 4-AAP assay. As shown in Figure 3, DE10 produces twice as much 4′OHP, while D6H10 produces 50% more 5′OHP. These variants make 33% less DIP.

We next generated a second mutant library using error-prone PCR to introduce random mutations throughout the DE10 gene and screened the resulting clones for enhanced product formation. From this library we isolated 2C11. This mutant generates a product profile similar to DE10 but converts more (20%) of the propranolol to the hydroxylated products (Fig. 3). Relative to 9C1, it produces 50% more 5′OHP, 200% more 4′OHP, 100% more 1-naphthol, and 40% of the DIP. The four products 4′OHP, 5′OHP, and 1-naphthol and DIP account for ~75%–80% of the propranolol

converted. Other, minor products were also visible in the HPLC traces, but their identities are unknown. Mutant 2C11 contains an additional two amino acid substitutions compared to DE10 (Table I).

DISCUSSION

We have demonstrated that a bacterial P450 heme domain can be used to prepare the authentic human metabolites of propranolol in a reaction driven by hydrogen peroxide. Furthermore, the activity can be enhanced and product selectivity can be optimized by directed evolution. In the first round of evolution, mutations were targeted to active-site residues believed to contact the substrate during catalysis. In wild-type P450 BM3, phenylalanine 87 sequesters the ω-end of the fatty acid, which is hydroxylated at adjacent positions (Li and Poulos, 1997). Substitution of this residue for alanine increases the activity of wild-type BM3 on polycyclic aromatics (Li et al., 2001a) and affects the regioselectivity
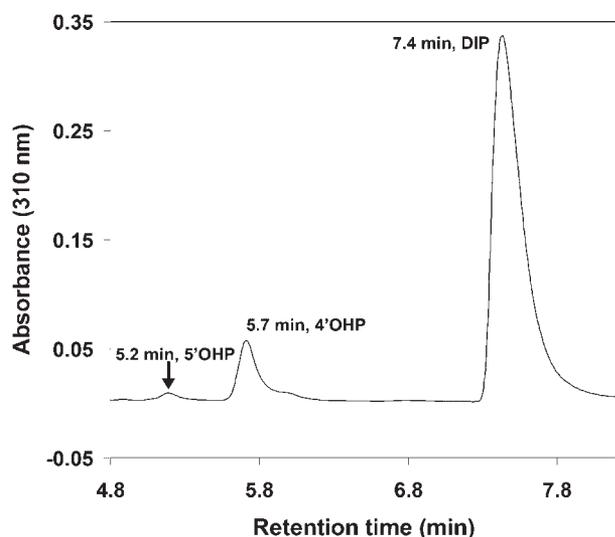

Figure 2. HPLC chromatogram showing the relevant human metabolites of propranolol produced in a bioconversion of (5 mM) propranolol with P450 BM3-H 9C1. Products from left to right are 5′-hydroxypropranolol (5.2 min), 4′-hydroxypropranolol (5.7 min), desisopropylpropranolol (7.4 min). Propranolol (12.9 min) and 1-naphthol (18.5 min) are not shown.
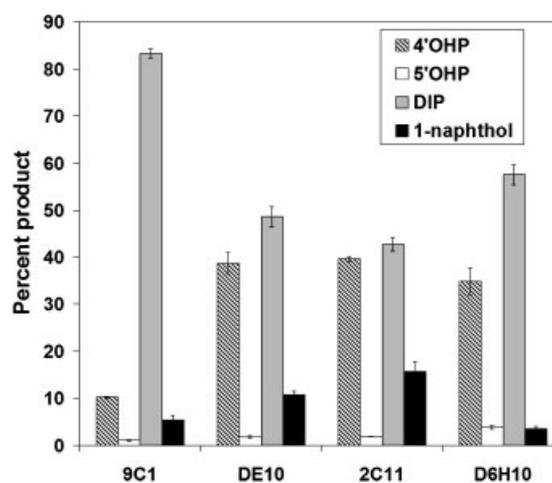

Figure 3. Propranolol metabolites produced by P450 BM3-H 9C1 and variants obtained by further directed evolution. Results are from bioconversions containing 5 μM P450 and 5 mM propranolol, driven by H_2O_2. Error bars represent one standard deviation from three experiments.

of fatty acid hydroxylation (Cirino and Arnold, 2002). It is also known that the residue size at position 87 plays a critical role in H_2O_2 dependent substrate hydroxylation: replacement of phenylalanine 87 with smaller residues such as alanine or glycine increases peroxygenase activity (Cirino and Arnold, 2002; Li et al., 2001c). It is thought that the additional space provided by these mutations allows more water molecules to remain at the active site pocket after substrate binding, promoting H_2O_2 access. We therefore introduced the F87A mutation into the wild-type parent BM3-H during the initial directed evolution efforts to find an efficient peroxygenase. This F87A mutation in the parent 9C1 used for the present study was exchanged for the even smaller, glycine residue after the first round of evolution.

Mutations in the active site have been shown to alter the stereo- and regioselectivity of P450 BM3 (Graham-Lorence et al., 1997; Li et al., 2001b; Peters et al., 2003). Saturation mutagenesis of active-site residues and screening has generated a range of BM3 variants with novel activities, ranging from ethane hydroxylation (Meinhold et al., 2005a) to enantioselective epoxidation of terminal alkenes (Kubo et al., 2005) and enantioselective hydroxylation of protected carboxylic acids (Munzer et al., 2005), demonstrating the ease with which this enzyme's substrate specificity and product selectivity can be modified. The active-site mutations described here produce similar results, altering the regioselectivity of P450 BM3-H in the peroxygenase reaction on propranolol. Activity is shifted away from the dealkylation reaction and towards aromatic hydroxylation (Fig. 3).

The specific effects of each mutation are difficult to rationalize since the substrate likely binds in more than one conformation, as indicated by its multiple products. The mutations in the active site, however, influence the binding geometries available to the substrate and thereby alter the regioselectivity of oxidation. In variants DE10 and 2C11, active-site residues A82 and A74 are both changed to larger hydrophobic amino acids, leucine and valine, respectively, which presumably reduce the volume of the active site. A82L has previously been shown to alter the regioselectivity of substrate hydroxylation (Peters et al., 2003). In contrast, A87G increases the volume of the active site directly above the heme. In 2C11, substitutions R47H and K24R introduced during random mutagenesis increase total activity while retaining a product profile similar to DE10 (Fig. 3). K24R, which is not an active-site residue, is conservative and may be neutral. R47H, in contrast, is located at the mouth of the active site. Mutation of this residue to leucine has been shown to increase activity towards polycyclic aromatic hydrocarbons (Carmichael and Wong, 2001). D6H10 has three mutations which lower total activity relative to parent 9C1 but increase production of 5′OHP. In this variant, the valine at position 78 is mutated to glutamic acid, introducing a charged residue into the hydrophobic active site and likely resulting in weaker binding of the substrate.

In human liver microsomes, ring hydroxylation of propranolol is mediated by CYP2D6, whereas desisopropylation is mediated mainly by CYP1A2 and to a lesser extent by CYP2D6 (Masubuchi et al., 1994). Of these metabolites, the ring-hydroxylated products are of particular importance. 4′OHP has been demonstrated to be equipotent to propranolol as a β-receptor antagonist (Fitzgerald and O'Donnell, 1971) and is believed to contribute to the bioactivity of propranolol in humans (Coltart and Shand, 1970). Other monohydroxylated products have also been shown to produce β-blockade and direct vasodilation in dogs (Oatis et al., 1981). The variants of bacterial P450 BM3-H described here produce all the major products of human propranolol metabolism, but, importantly, the evolved mutants make more of the key ring-hydroxylated products 4′OHP and 5′OHP.

The heme domain of BM3 is expressed at high levels (∼100 mg/L) in a typical shake culture, and this can be further increased in a high density fermentation. Using crude cell lysate containing evolved heme domains capable of 180 turnovers when provided with H_2O_2, one could generate >70 mg of products with the enzyme from 1 L of batch culture. This is similar to the quantities produced in *S. frugiperda* and *E. coli* cells using human P450s (Rushmore et al., 2000; Vail et al., 2005), but the preparation is simpler and far less costly.

An advantage of using the P450 BM3 heme domain, which utilizes H_2O_2 to drive these reactions, is its self-sufficiency. Catalysis does not depend on the addition or regeneration of NADPH, the cofactor required for most P450 reactions. Using the peroxgenase activity significantly decreases the cost and complexity of the preparation of these metabolites. With further optimization of the catalyst and reaction conditions, we anticipate that this system can be used to generate larger quantities of valuable drug metabolites. Additionally, laboratory-evolved P450s may be used for combinatorial biocatalysis of drug scaffolds to further diversify and increase the efficacy of these molecules. Using bacterial P450s to prepare the authentic human metabolites of drugs provides an easily scaleable and improvable system for the biosynthesis of these valuable compounds.

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