Directed Evolution of a Cytochrome P450 Monooxygenase for Alkane Oxidation

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Abstract: Cytochrome P450 monooxygenase BM-3 (EC 1.14.14.1) hydroxylates fatty acids with chain lengths between C_{12} and C_{18}. It is also known to oxidize the corresponding alcohols and amides. However, it is not known to oxidize alkanes. Here we report that P450 BM-3 oxidizes octane, which is four carbons shorter and lacks the carboxylate functionality of the shortest fatty acid P450 BM-3 is known to accept, to 4-octanol, 3-octanol, 2-octanol, 4-octanone, and 3-octanone. The rate is much lower than for oxidation of the preferred fatty acid substrates. In an effort to explore the plasticity and mechanisms of substrate recognition in this powerful biocatalyst, we are using directed evolution – random mutagenesis, recombination, and screening – to improve its activity towards saturated hydrocarbons. A spectrophotometric assay has been validated for high throughput screening, and two generations of laboratory evolution have yielded variants displaying up to five times the specific activity of wild-type P450 BM-3.

Keywords: alkanes; cytochrome P450 BM-3; enzyme catalysis; enzyme engineering; *in vitro* evolution

Introduction

The term paraffin (from Latin *parum affinis* = slight affinity) accurately reflects the nature of these compounds: alkanes are notoriously inert, and activating their C-H bonds presents a difficult chemical obstacle. In fact, one of the great challenges of contemporary catalysis is the controlled oxidation of hydrocarbons.[1] Processes for controlled, stereo- and regioselective oxidation of hydrocarbon feed stocks to more valuable and useful products such as alcohols, ketones, acids, and peroxides would have a major impact on the chemical and pharmaceutical industries.

Enzymes have unique properties that distinguish them from most chemical catalysts. Most impressive is their ability to catalyze specific, and often difficult, chemical reactions in water at room temperature and atmospheric pressure. The cytochrome P450 monooxygenases are a good example. These widely-distributed heme enzymes insert one oxygen atom of O_2 into a diverse range of hydrophobic substrates, often with high regio- and stereoselectivity. The second oxygen atom is reduced to H_2O. The active sites of all cytochrome P450's contain an iron protoporphryin IX with cysteinate as the fifth ligand; the final coordination site is left to bind and activate molecular oxygen.[2] For many of the P450-catalyzed reactions, no chemical catalysts come close in performance.[3] These enzymes, however, are often only poorly active towards non-natural substrates[3] and cannot tolerate normal process conditions, including organic solvents. Simply put, they are a process engineering nightmare.

The cytochrome P450 BM-3 from *Bacillus megaterium* is a soluble, catalytically self-sufficient P450 containing the monooxygenase and reductase domains in a single polypeptide chain.[4] The minimum requirements for activity are substrate, dioxygen, and the cofactor nicotinamide adenine dinucleotide phosphate (NADPH). P450 BM-3 hydroxylates fatty acids of chain length between C_{12} and C_{18} at subterminal positions; the regioselectivity of oxygen insertion depends on the chain length.[5,6] P450 BM-3 reportedly displays no activity towards fatty acids smaller than C_{12}. P450 BM-3 is also known to hydroxylate the corresponding fatty acid amides and alcohols[5] and forms epoxides from unsaturated fatty acids.[7] The enzyme is reported to be inactive towards alkanes and methyl esters lacking the polar functionality of the natural substrates.[5] However, there is some evidence that P450 BM-3 can in fact accept shorter-chain alkanes.[8]

We wish to explore and expand the range of substrates that this enzyme, and this catalytic machinery, can accept, with the long-term goal of creating in-

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dustrially useful oxidation catalysts. In particular, we would like to know whether P450 BM-3 can be converted into an efficient alkane hydroxylase and whether it can be engineered to accept substrates that are shorter than its preferred substrates, the fatty acids. While various alkane hydroxylases are known, for example ω–hydroxylase and methane monooxygenase, none have the practical advantages of P450 BM-3, which is highly expressed in recombinant form in bacteria and contains all its functional domains in a single polypeptide chain. To engineer the enzyme, we are relying on directed evolution[9], in which iterative cycles of random mutagenesis, recombination, and functional screening for improved enzymes accumulate the mutations that confer the desired properties.

Results and Discussion

Activity of P450 BM-3 Towards Octane

In the presence of purified P450 BM-3 and NADPH, octane was consumed within 2 h and gave several products detectable by GC/MS (Figure 1). The major hydroxylated products are 4-octanol, 3-octanol, and 2-octanol. 1-Octanol is not detected under the experimental conditions. The product ratio is approximately 8:9:1; 4-octanol:3-octanol:2-octanol. 4-Octanone and 3-octanone are also present in the product mixture. A possible mechanism for the formation of the ketones is hydroxylation of the alcohol to generate a *gem*-diol which dehydrates to the corresponding ketone.[10] Another possible mechanism is via the pinacol rearrangement.[11] The total yield of products detected is ~50%, with alcohols accounting for ~90% of the product and ketones representing 10%.

We tested whether P450 BM-3 catalyzes the oxidation of 3-octanol to 3-octanone. Upon addition of NADPH to a solution of P450 BM-3 and 3-octanol, the peak at 3.7 min appears. This peak has the same retention time as an authentic sample of 3-octanone. Furthermore, the fragmentation pattern of the peak

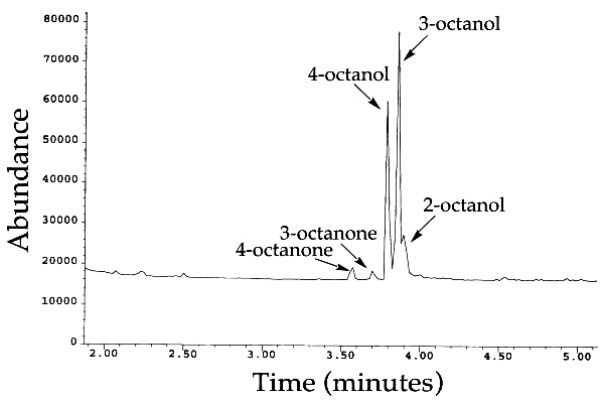

Figure 1. Gas chromatogram of the oxidation products of octane, catalyzed by wild-type P450 BM-3.

matches that of 3-octanone found in the mass spectrum database. Solutions containing only P450 BM-3 and 3-octanol, but no NADPH, do not produce any detectable 3-octanone. Similar results were obtained with 4-octanol as the substrate.

The activity of P450 BM-3 towards octane was measured spectrophotometrically by monitoring the rate of NADPH consumption. For octane, the $k_{cat} = 0.7\ s^{-1}$, $K_m = 2.0 \times 10^{-5}$ M and $k_{cat}/K_m = 3.5 \times 10^4\ M^{-1}\ s^{-1}$. The activity of P450 BM-3 on saturated fatty acids follows the order $C_{15} = C_{16} > C_{14} > C_{17} > C_{13} > C_{18} > C_{12}$.[12] On the C_{16} fatty acid, $k_{cat} = 81\ s^{-1}$ and $K_m = 1.4 \times 10^{-6}$ M ($k_{cat}/K_m = 6.0 \times 10^7\ M^{-1}\ s^{-1}$). Thus on octane the P450 BM-3 enzyme has a k_{cat} 120-fold less and a K_m that is 15 times larger (k_{cat}/K_m ~ 2000 times less) than on its preferred C_{16} fatty acid substrate. With the C_{12} fatty acid, $k_{cat} = 26\ s^{-1}$, $K_m = 136 \times 10^{-6}$ M and $k_{cat}/K_m = 1.9 \times 10^5\ M^{-1}\ s^{-1}$.[12] For comparison, *Pseudomonas oleovorans* is able to oxidize *n*-alkanes using the hydroxylase machinery comprising an integral membrane oxygenase (ω-hydroxylase), a soluble NADH-dependent reductase, and a soluble metalloprotein (rubredoxin) which transfers electrons from the reductase to the hydroxylase.[13] The ω-hydroxylase has been cloned from *P. oleovorans* into *Escherichia coli*, where it has been expressed and purified.[14] The specific activity of ω-hydroxylase for octane (5.2 units/mg hydroxylase [14]) is ~13 times that of P450 BM-3 (0.4 units/mg enzyme). (The specific activity of the complete *P. oleovorans* system, including the rubredoxin and the reductase, is of course less than 5.2 units/mg.)

Hydrogen peroxide production was also monitored in order to determine whether the rate of NADPH oxidation is affected by uncoupling.[12,15] H_2O_2 was not detected under the experimental conditions (data not shown). This is consistent with previous reports that indicate efficient coupling for P450 BM-3 acting on unnatural substrates such as styrene or alkyltrimethylammonium compounds. [12,15]

A tyrosine (Tyr51) at the entrance to the substrate-binding pocket makes a hydrogen bond to the carboxylate group of the substrate in the crystal structure of the enzyme bound with palmitoleic acid.[16] Arg47, also at the entrance to the binding pocket, may form an ionic interaction as well. Non-polar alkane substrates must rely solely on hydrophobic partitioning into the enzyme's extended substrate channel, and poor substrate recognition may contribute to P450 BM-3's sluggish activity on octane.

Directed Evolution of Cytochrome P450 BM-3

To improve the activity of P450 BM-3 towards alkanes by directed evolution requires a rapid, reproducible screen that is sensitive to small changes (< 2-fold) in activity.[9] For this, we prepared an octane analogue,

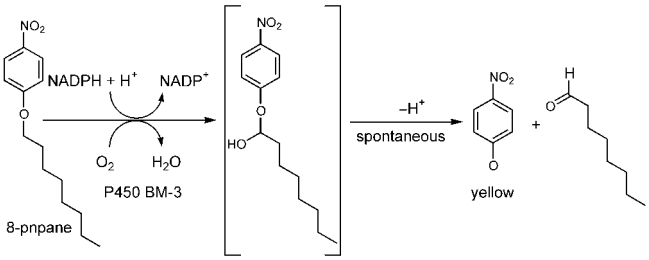

Figure 2. The screening assay for alkane oxidation activity uses the substrate analogue 8-pnpane. Terminal hydroxylation generates the unstable hemiacetal, which decomposes to the aldehyde and *p*-nitrophenolate, which is monitored at 410 nm.

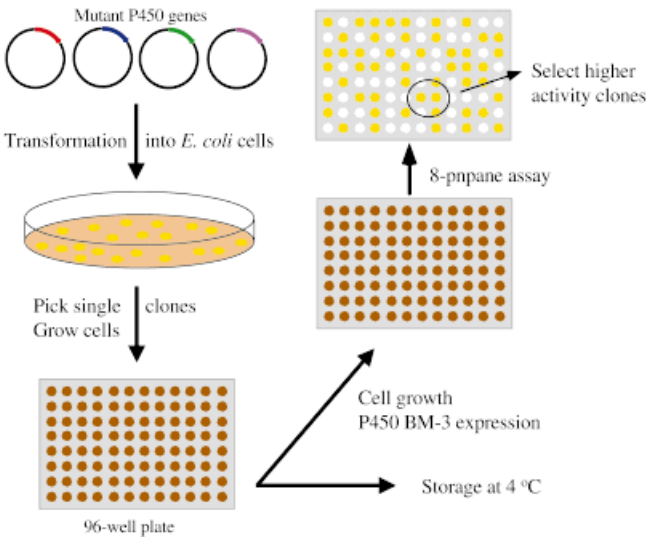

Figure 3. P450 BM-3 screening procedure. Library of P450 BM-3 mutant genes is transformed into *E. coli* and plated on agar, from which single colonies are picked into 96-well plates and grown overnight. From these plates, samples are taken to inoculate fresh 96-well plates, in which the enzymes are expressed and assayed for hydroxylation activity. The plates from the overnight growth are stored at 4 °C and used to isolate active clones identified in the assay.

8-pnpane (Figure 2), that generates a yellow color upon hydroxylation. This "surrogate" substrate with a C_8 backbone and a *p*-nitrophenyl moiety allows us to use a colorimetric assay to screen large numbers of P450 BM-3 mutants for increased hydroxylation activity in microtiter plates.[17] Hydroxylation of 8-pnpane generates an unstable hemiacetal which dissociates to form (yellow) *p*-nitrophenolate and the corresponding aldehyde (Figure 2). The hydroxylation kinetics of hundreds of mutants can then be monitored simultaneously in the wells of a microtiter plate using a plate reader (Figure 3).[17b]

Mutant library construction was limited to the P450 BM-3 heme domain, which contains the substrate binding site and the monooxygenase activity. The libraries were generated by error prone PCR,[18] using $MnCl_2$ concentrations of 0.0, 0.05, and 0.1 mM, which yielded 70, 60, and 50% active transformants, respectively. The library generated with no added $MnCl_2$, corresponding to ~2 base changes per gene (0.15% error rate), [18] was chosen for screening. Two thousand clones were screened and 23 of the most active clones were selected for further analysis. Since the screen is sensitive to total activity, which includes increased expression as well as changes in specific activity, enzyme concentrations were estimated from the CO difference spectra for binding to the reduced P450 BM-3 in order to calculate specific activities.[19] The best variant, VIII 2C9, displayed approximately twice the specific activity of the wild-type enzyme. A second round of error-prone PCR was carried out on this improved gene. Screening 3000 clones produced seven new variants that were 2–3 times more active than VIII 2C9.

The screen we are using is sensitive to hydroxylation at the terminal position of the surrogate *p*-nitrophenoxyoctane substrate. Activity towards octane was then confirmed by measuring the rate of NADPH oxidation, which we assumed to be fully coupled to octane oxidation. By this assay, the best mutants are >5 times as active as wild-type P450 BM-3 on octane. We do not yet know whether the regiospecificity of the reaction is changing during the evolution of the enzyme. This will require further, detailed analysis of the product profiles, which is now in progress.

Our goal is to evolve an enzyme that efficiently oxidizes alkanes. After only two generations, the best P450 BM-3 has close to one-half the specific activity of the alkane hydroxylase from *P. oleovorans*. We believe that we will be able to surpass this activity as well as to target other key properties such as regioselectivity, enantioselectivity, and catalyst stability. The thermostability of P450 BM-3 is quite low, and the enzyme's activity is dramatically decreased in polar organic solvent concentrations greater than 2% (e.g., DMSO, dioxane, or THF, required for substrate solubilization).[20] Our future evolution efforts will target these shortcomings.

Schmid and coworkers recently described the engineering of P450 BM-3 to hydroxylate short-chain (C_8-C_{10}) fatty acids not accepted by the wild-type enzyme.[21] Guided by the X-ray crystal structure, they focused on eight individual amino acids within the binding pocket to perform saturation mutagenesis. This approach successfully discovered mutations that altered the enzyme's substrate specificity. Random mutagenesis, however, can discover other subtle ways to modify activity. For example, sequencing the gene for the P450 BM-3 octane variant VIII 2C9 revealed a mutation that substitutes a glutamine for histidine at amino acid position 236, which is neither in the active site nor in the substrate-binding channel. In fact, the amino acid substitution is found on a helix

situated near the surface of the enzyme, approximately 17 ångstroms from the heme. How this substitution enhances activity towards octane is not clear and would have been impossible to identify using structure-based design methods.

Conclusions

We have shown that P450 BM-3 can oxidize octane, a substrate four carbons shorter than the smallest fatty acid the enzyme is known to accept. The molecular basis for the extremely slow rate of octane hydroxylation is unknown, but we presume that poor substrate recognition is at least partially at fault. Altering the architecture of the enzyme by directed evolution will allow us to observe how activity towards smaller hydrocarbon substrates is acquired; structural and kinetic analysis of the evolved proteins should help elucidate key steps in P450 substrate recognition and catalysis.

We anticipate that recombination of the activating mutations already identified will lead to further functional improvements. The catalytic activity that we can achieve by directed evolution is dictated by the evolutionary search strategy and, ultimately, by what the enzyme and its catalytic machinery are capable of. Engineered P450 BM-3 variants may one day be useful for functionalization of hydrocarbons in chemical synthesis or bioremediation.

Experimental Section

General Methods

All chemical reagents were procured from Aldrich, Sigma, or Fluka. Enyzmes were purchased from New England Biolabs, Stratagene, and Boehringer Mannheim. The ^{1}H NMR spectrum was recorded on a Varian 300 MHz nuclear magnetic resonance spectrometer with a Mercury console. Quantitative Technologies Inc. (Whitehouse, New Jersey) performed elemental analysis.

Expression of P450 BM-3

The P450 BM-3 gene containing a His_6 tag was cloned into expression vector *pCYTEXP1* (pT-USC1BM3)[17] under the control of the strong temperature inducible P_RP_L-promoter, in *E. coli* strain DH5α. For P450 BM-3 expression, a solution containing Terrific Broth (TB) media (500 mL), ampicillin (100 μg/mL), thiamine (5 μg/mL), δ-aminolevulinic acid hydrochloride (0.0125 mg/mL), and 125 μL trace elements [0.5 g $MgCl_2$, 30.0 g $FeCl_2{\cdot}6\,H_2O$, 1.0 g $ZnCl_2{\cdot}4\,H_20$, 0.2 g $CoCl_2{\cdot}6\,H_2O$, 1.0 g $Na_2MoO_4{\cdot}2\,H_2O$, 0.5 g $CaCl_2{\cdot}2\,H_2O$, 1.0 g $CuCl_2$, and 0.2 g H_2BO_3 in 1 L HCl solution (90% v/v distilled water:concentrated HCl)] was inoculated with 0.5 mL of an overnight Luria-Bertani (LB) culture of recombinant *E. coli* DH5α containing pT-USC1BM3. The cells were shaken at 250 rpm at 37 °C. When the $OD_{578} = 1$, the cells were induced by increasing the temperature to 42 °C. After 5 hours, the cells were harvested by centrifugation at 4 °C.

Purification of P450 BM-3

The cell pellet was washed twice with Tris-HCl (15 mL, 0.1 M, pH 8.2), resuspended with Tris-HCl (15 mL, 0.1 M, pH 8.2) and lysed by sonication (3 × 2 minutes; output control = 5, duty cycle 40%; Sonicator, Heat Systems – Ultrasonic, Inc.). The lysate was centrifuged at 23,300 *g* for 1.0 h. The supernatant was further cleared through a 0.45 μM filter. The filtrate was diluted with 15 mL H_2O and purified by published procedures.[22] P450 BM-3 concentrations were measured from the CO-difference spectra as described by Omura and Sato [19] using $\varepsilon = 91\ mM^{-1}\ cm^{-1}$.

Octane Oxidation by P450 BM-3

The oxidation was performed with solutions containing octane in DMSO (1 mM octane; 1% DMSO), P450 BM-3 (2–3 μM), and NADPH (1 – 5 mM) in Tris-HCl (50 mM) containing NaCl (340 mM). A concentrated solution of octane in DMSO was added to the enzyme. The resulting solution was incubated for 30 minutes at room temperature. Octane oxidation was initiated by the addition of NADPH in aqueous solution. After a specific time, the solution was extracted three times with CH_2Cl_2 (333 μL) containing decanol (1 mM) as an internal standard. The organic layer was dried over anhydrous Na_2SO_4 , and the products were analyzed by GC/MS.

The catalytic activity of P450 BM-3 was measured spectrophotometrically by monitoring the rate of NADPH oxidation, as described.[23] The assay solution contained 0.1 nmol P450 BM-3, octane in DMSO, and 0.8 mM NADPH, 50 mM NaCl in 0.1 M Tris-HCl, pH 8.2.

Production of H_2O_2 during the hydroxylation reaction was determined using the iron(III) thiocyanate assay.[15] 1.0×10^{-9} mol P450 BM-3 was incubated with 1.0×10^{-6} mol octane for 5 minutes. The reaction was initiated by addition of 2.5×10^{-7} mol NADPH to the enzyme solution. Every 2 minutes a 0.2-mL aliquot from the reaction was added to 1.0 mL aqueous iron(II) solution [5.0 g $FeSO_4(NH_4)_2{\cdot}6\,H_2O$, 45.0 mL degassed H_2O, 5 mL concentrated H_2SO_4]. Subsequently, 0.4 mL of a 10% aqueous solution of KSCN was added to the solution, and the absorbance was measured at 480 nm.

GC/MS Analysis

The products were identified with GC/MS using a Hewlett Packard 5890 Series II gas chromatograph coupled with a Hewlett Packard 5989A mass spectrometer. The GC was fitted with an HP-1 column (cross-linked methylsilicone gum, 12 m × 0.2 mm × 0.33 μm). The temperature gradient is as follows: 1) 40 to 50 °C at 15 °C/min, 2) 50 to 75 °C at 10 °C/min, 3) 75 to 160 °C at 25 °C/min. Authentic standards were used to identify the retention times of the products. The products were further verified by matching the fragmentation distributions with a database in the software provided by the instrument manufacturer.

Synthesis of *p*-Nitrophenoxyoctane (8-pnpane)

1-Bromooctane (1 g, 5.18 mmol) and 4-nitrophenol, sodium salt (0.92 g, 5.71 mmol) were refluxed in DMSO (30 mL) at 120 °C for 5 hours. The DMSO was distilled off to near dryness. The resulting brown residue was loaded onto a silica column and eluted with 10:1 mixture of petroleum ether and diethyl ether. The yield was 30%. ^{1}H NMR ($CDCl_3$): δ = 8.18 (m, 2H), 6.93 (m, 2H), 4.04 (t, 2H), 1.81 (p, 2H), 1.33 (m, 10H), 0.89 (t, 3H); anal. calcd. for $C_{14}H_{21}O_5N$: C, 66.91; H, 8.42; N, 5.57; found: C, 66.97; H, 8.34; N, 5.52.

Random Library Generation and Screening of P450 BM-3

P450 BM-3 containing a His_6 tag was amplified from pTUSC1BM3 by PCR techniques using a proofreading polymerase Pfu to introduce a *Bam*HI upstream of the start codon and an *Eco*RI site immediately downstream from the stop codon. The two oligonucleotides used were: 5'-cgcggatccatcgatgcttaggaggtcatatgacaattaaagaaatgcctc-3' (*Bam*HI site underlined) and 5'-ccggaattcttaatgatgatgatgatgatgcccagccccacacgtcttttgc-3' (*Eco*RI site underlined). The PCR product was digested with *Bam*HI and *Eco*RI. The P450 BM-3 gene was ligated into expression vector *pCWOri* (+)[24] (pBM3BamSacEco), which is under the control of double P*tac* promoter and contains an ampicillin resistance coding region. A silent mutation was introduced to construct a *Sac*I site 130 bases upstream of the end of the heme domain. The QuikChange (Stratagene) protocol was followed and the primers were: 5'-catacaaactacgagctcgatattaaagaaac-3' (*Sac*I site underlined) and 5'-gtttctttaatatcgagctcgtagtttgtatg-3' (*Sac*I site underlined).

Random mutagenesis: Mutagenic PCR was performed on the heme domain in a 100 μL reaction volume as described[18] with some modifications. The mutated P450 BM-3 fragment was 1291 base pairs. The reaction contained $MgCl_2$ (7 mM), forward and reverse primer (40 pmol each, 5'-acaggatccatcgatgcttaggaggtcatatg-3', and 5'-gtgaaggaataccgccaag-3'), pBM3BamSacEco (10 ng), dNTPs (0.2 mM dGTP, 0.2 mM dATP, 1.0 mM dCTP, 1.0 mM dTTP), and *Taq* polymerase (5 units, Roche), KCl (50 mM), and Tris-HCl (10 mM, pH 8.3, 20 °C). $MnCl_2$ (0.0, 0.05, and 0.1 mM) was added to the PCR mixture to alter the error rate of the polymerase. PCR was performed in a thermocycler (PTC200, MJ Research, Waltham, MA) for 30 cycles (95 °C, 45 s; 50 °C, 30 s; 72 °C, 2 min). The PCR product was restricted with *Bam*HI and *Sac*I and ligated into expression vector *pCWOri* (+). The resulting plasmid was transformed into *E. coli* strain DH5α and the colonies were selected on agar plates containing ampicillin (100 μg/mL).

Screening for hydroxylation activity: A robot (Qpix, Genetix) picked and inoculated colonies into 1-mL deep-well plates containing LB media (400 μL) and ampicillin (100 μg/mL). The plates were incubated at 37 °C, 270 rpm, and 80% relative humidity. After 24 hours, the culture liquid (50 μL) was added to TB (450 μL) containing ampicillin (100 μg/mL), thiamine (5 μg/mL), and trace elements (0.25 μL/mL). After growth at 37 °C for 1 hour, δ-aminolevulinic acid hydrochloride (1 mM) and isopropyl-β-D-thiogalactopyranoside (1 mM) were added. The temperature was shifted to 30 °C and the cultures were grown for 24 hours. The plates were centrifuged and supernatants were discarded. Cell pellets were washed with Tris-HCl (350 μL, pH 8.3) and resuspended in Tris-HCl (350 μL, pH 8.3) containing lysozyme (0.5 mg/mL), deoxyribonuclease I (0.1 μg/mL), and $MgCl_2$ (10 mM). After incubation at 37 °C for 45 minutes, the plates were centrifuged and the lysate (150 μL) was transferred to a 96-well plate. Then 8-pnpane (150 μM) in DMSO (1%) was added to the lysate and incubated at room temperature. After 5 minutes, NADPH (1 mM) was added and the absorbance at 410 nm was measured with a microplate spectrophotometer (SPECTRAmax, Molecular Devices).

CO binding: The most active clones identified from the screen were cultured on a larger scale. Single colonies were cultured at 37 °C overnight in LB (5 mL) containing ampicillin (100 mg/mL). The overnight growth (0.5 mL) was used to inoculate media containing TB (50 mL), ampicillin (100 μg/mL), thiamine (5 μg/mL), and trace elements (0.25 μL/mL). After the OD_{600} reached 0.6 – 1.0, the temperature was decreased to 30 °C, and enzyme expression was induced by adding δ-aminolevulinic acid hydrochloride (1 mM) and isopropyl-β-D-thiogalactopyranoside (1 mM).

After 24 hours, the cells were harvested by centrifugation and the supernatants discarded. The pellets were washed with Tris-HCl (15 mL, pH 8.3). Cells were resuspended in Tris-HCl (5 mL, pH 8.3), sonicated, and centrifuged. The supernatants were further cleared through a 0.45 μM filter. P450 BM-3 concentrations were measured from the CO-difference spectra.

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