Directed Evolution of a Cytochrome P450 Monooxygenase for Alkane Oxidation

Edgardo T. Farinas, Ulrich Schwaneberg, Anton Glieder, Frances H. Arnold

Division of Chemistry and Chemical Engineering 210-41, California Institute of Technology, Pasadena, CA 91125, USA
Fax: (+1) 626–568–8745, e-mail: frances@cheme.caltech.edu

Received March 23, 2001; Accepted May 23, 2001

Abstract: Cytochrome P450 monooxygenase BM-3 (EC 1.14.14.1) hydroxylates fatty acids with chain lengths between C\textsubscript{12} and C\textsubscript{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 5-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 laborotory 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 \textit{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.\textsuperscript{[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 monoxygenases are a good example. These widely-distributed heme enzymes insert one oxygen atom of O\textsubscript{2} into a diverse range of hydrophobic substrates, often with high regio- and stereoselectivity. The second oxygen atom is reduced to H\textsubscript{2}O. The active sites of all cytochrome P450's contain an iron protoporphyrin IX with cysteinate as the fifth ligand; the final coordination site is left to bind and activate molecular oxygen.\textsuperscript{[2]} For many of the P450-catalyzed reactions, no chemical catalysts come close in performance.\textsuperscript{[3]} These enzymes, however, are often only poorly active towards non-natural substrates\textsuperscript{[3]} and cannot tolerate normal process conditions, including organic solvents. Simply put, they are a process engineering nightmare.

The cytochrome P450 BM-3 from \textit{Bacillus megaterium} is a soluble, catalytically self-sufficient P450 containing the monooxygenase and reductase domains in a single polypeptide chain.\textsuperscript{[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\textsubscript{12} and C\textsubscript{18} at subterminal positions; the regioselectivity of oxygen insertion depends on the chain length.\textsuperscript{[5,6]} P450 BM-3 reportedly displays no activity towards fatty acids smaller than C\textsubscript{12}. P450 BM-3 is also known to hydroxylate the corresponding fatty acid amides and alcohols\textsuperscript{[5]} and forms epoxides from unsaturated fatty acids.\textsuperscript{[7]} The enzyme is reported to be inactive towards alkanes and methyl esters lacking the polar functionality of the natural substrates.\textsuperscript{[5]} However, there is some evidence that P450 BM-3 can in fact accept shorter-chain alkanes.\textsuperscript{[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-
directorly 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 \( \omega \)-hydroxylase and methane monoxygenase, 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\(^\text{[9]}\), 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-Octanol and 3-octanol are also present in the product mixture. A possible mechanism for the formation of the ketones is hydroxylization of the alcohol to generate a gem-diol which dehydrates to the corresponding ketone.\(^\text{[10]}\) Another possible mechanism is via the pinacol rearrangement.\(^\text{[14]}\) The total yield of products detected is \( \sim 50\% \), with alcohols accounting for \( \sim 90\% \) of the product and ketones representing \( 10\% \).

We tested whether P450 BM-5 catalyzes the oxidation of 5-octanol to 5-octanone. Upon addition of NADPH to a solution of P450 BM-5 and 3-octanol, the peak at 5.7 min appears. This peak has the same retention time as an authentic sample of 3-octanone. Furthermore, the fragmentation pattern of the peak matches that of 3-octanone found in the mass spectrum database. Solutions containing only P450 BM-3 and 5-octanol, but no NADPH, do not produce any detectable 5-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 \( k_{\text{cat}} = 0.7 \, \text{s}^{-1} \), \( K_m = 2.0 \times 10^{-5} \, \text{M} \) and \( k_{\text{cat}}/K_m = 5.5 \times 10^8 \, \text{M}^{-1} \text{s}^{-1} \). The activity of P450 BM-5 on saturated fatty acids follows the order \( C_{15} > C_{16} > C_{14} > C_{17} > C_{15} > C_{16} > C_{12} \).\(^\text{[12]}\) On the \( C_{16} \) fatty acid, \( k_{\text{cat}} = 81 \, \text{s}^{-1} \) and \( K_m = 1.4 \times 10^{-6} \, \text{M} \) \( (k_{\text{cat}}/K_m = 6.0 \times 10^7 \, \text{M}^{-1} \text{s}^{-1}) \). Thus on octane the P450 BM-5 enzyme has a \( k_{\text{cat}} \) 120-fold less and a \( K_m \) that is 15 times larger \( (k_{\text{cat}}/K_m \sim 2000 \, \text{times less}) \) than on its preferred \( C_{16} \) fatty acid substrate. With the \( C_{12} \) fatty acid, \( k_{\text{cat}} = 26 \, \text{s}^{-1} \), \( K_m = 156 \times 10^{-6} \, \text{M} \) and \( k_{\text{cat}}/K_m = 1.9 \times 10^5 \, \text{M}^{-1} \text{s}^{-1} \).\(^\text{[12]}\) For comparison, *Pseudomonas oleovorans* is able to oxidize \( n \)-alkanes using the hydroxylase machinery comprising an integral membrane oxygenase (\( \omega \)-hydroxylase), a soluble NADH-dependent reductase, and a soluble metalloprotein (rubredoxin) which transfers electrons from the reductase to the hydroxylase.\(^\text{[15]}\) The \( \omega \)-hydroxylase has been cloned from *P. oleovorans* into *Escherichia coli*, where it has been expressed and purified.\(^\text{[14]}\) The specific activity of \( \omega \)-hydroxylase for octane \( (5.2 \, \text{units/mg hydroxylase} \) \( [14] \) is \( \sim 15 \) times that of P450 BM-5 \( (0.4 \, \text{units/mg enzyme}) \). (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.\(^\text{[12,13]}\) \( \text{H}_2\text{O}_2 \) was not detected under the experimental conditions (data not shown). This is consistent with previous reports that indicate efficient coupling for P450 BM-5 acting on unnatural substrates such as styrene or alkyltrimethylammonium compounds.\(^\text{[12,13]}\)

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.\(^\text{[16]}\) 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-5**

To improve the activity of P450 BM-5 towards alkanes by directed evolution requires a rapid, reproducible screen that is sensitive to small changes (< 2-fold) in activity.\(^\text{[9]}\) For this, we prepared an octane analogue,
8-pnpane (Figure 2), that generates a yellow color upon hydroxylation. This “surrogate” substrate with a C8 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 disassociates 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 MnCl₂ 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 MnCl₂, corresponding to ~2 base changes per gene (0.15% error rate),[18] was chosen for screening. Two thousand clones were screened and 25 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 5000 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-nitrophenoxo octane 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 (C₈-C₁₀) 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 ängstroms 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-5 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-5 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. Enzymes were purchased from New England Bioslabs, Stratagene, and Boehringer Mannheim. The 1H NMR spectrum was recorded on a Varian 500 MHz nuclear magnetic resonance spectrometer with a Mercury console. Quantitative Technologies Inc. (Whitehouse, New Jersey) performed elemental analysis.

Expression of P450 BM-5

The P450 BM-5 gene containing a His6 tag was cloned into expression vector pCYTEXP1 (pT-USC1BM5) under the control of the strong temperature inducible PpPp-promoter, in E. coli strain DH5α. For P450 BM-5 expression, a solution containing Terrific Broth (TB) media (500 mL), ampicillin (100 µg/mL), thiamine (5 µg/mL), 5-aminolevulinic acid hydrochloride (0.0125 mg/mL), and 125 µL trace elements [0.5 g MgCl2, 50.0 g FeCl3·6 H2O, 1.0 g ZnCl2·4 H2O, 0.2 g CoCl2·6 H2O, 1.0 g Na2MoO4·2 H2O, 0.5 g CaCl2·2 H2O, 1.0 g CuCl2, and 0.2 g H3BO3] 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-USC1BM5. The cells were shaken at 250 rpm at 57 °C. When the OD578 = 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-5

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 (5 × 2 minutes; output control = 5, duty cycle 40%; Sonicator, Heat Systems – Ultrasonic, Inc.). The lysate was centrifuged at 25,500 g for 1.0 h. The supernatant was further cleared through a 0.45 µm filter. The filtrate was diluted with 15 mL H2O and purified by published procedures.[23] P450 BM-5 concentrations were measured from the CO-difference spectra as described by Omura and Sato[19] using ε = 91 mM⁻¹ cm⁻¹.

Octane Oxidation by P450 BM-5

The oxidation was performed with solutions containing octane in DMSO (1 mM octane; 1% DMSO), P450 BM-5 (2–5 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 50 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 CH2Cl2 (333 µL) containing decanol (1 mM) as an internal standard. The organic layer was dried over anhydrous Na2SO4, and the products were analyzed by GC/MS.

The catalytic activity of P450 BM-5 was measured spectrophotometrically by monitoring the rate of NADPH oxidation, as described.[23] The assay solution contained 0.1 nmol P450 BM-5, octane in DMSO, and 0.8 mM NADPH, 50 mM NaCl in 0.1 M Tris-HCl, pH 8.2.

Production of H2O2 during the hydroxylation reaction was determined using the iron(II) thiocyanate assay.[15] 1.0 × 10⁻⁷ mol P450 BM-5 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-µL aliquot from the reaction was added to 1.0 mL aqueous iron(II) solution [5.0 g FeSO4(NH4)2·6 H2O, 45.0 mL degassed H2O, 5 mL concentrated H2SO4]. 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 5973A mass spectrometer. The GC was fitted with an HP-1 column (cross-linked methylsilicone gum, 12 m × 0.2 mm × 0.35 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-Nitrophenoxoctane (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 50%. 1H NMR (CDCl3): δ = 8.18 (m, 2H), 6.95 (m, 2H), 4.04 (t, 2H), 1.81 (p, 2H), 1.33 (m, 10H), 0.89 (t, 3H); anal. calcd. for C14H21O3N: C, 66.91; H, 8.18 (m, 2H), 6.93 (m, 2H), 4.04 (t, 2H), 1.81 (p, 2H), 1.33 (m, 10H), 0.89 (t, 3H); found: C, 66.97; H, 8.34; N, 5.52.

Random Library Generation and Screening of P450 BM-5

P450 BM-5 containing a His6 tag was amplified from pT-USC1BM3 by PCR techniques using a proofreading polymerase (Pfu) to introduce a BamHI upstream of the start codon and an EcoRI site immediately downstream of the stop codon. The two oligonucleotides used were: 5’-cactgcacgtcttttgc-3’ (BamHI site underlined) and 5’-gtttctttaatatcagctcgatattaaagaaac-3’ (EcoRI underlined). The PCR product was digested with BamHI and EcoRI. The P450 BM-5 gene was ligated into expression vector pCW0r (+) [24] (pBM3BamSacEco), which is under the control of double Plac promoter and contains an ampicillin resistance coding region. A silent mutation was introduced to construct a SacI site 150 bases upstream of the end of the heme domain. The QuikChange (Stratagene) protocol was followed and the primers: 5’-catacataatcagctcgatattaaagaaac-3’ (SacI site underlined) and 5’-gtttctttaatatcactgcacgtcttttgc-3’ (SacI 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 containing a His6 tag was amplified from pT-USC1BM3 by PCR techniques using a proofreading polymerase (Taq) followed and the primer 130 bases upstream of the end of the heme domain. 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%. 1H NMR (CDCl3): δ = 8.18 (m, 2H), 6.95 (m, 2H), 4.04 (t, 2H), 1.81 (p, 2H), 1.33 (m, 10H), 0.89 (t, 3H); anal. calcd. for C14H21O3N: C, 66.97; H, 8.34; N, 5.52.

References