Alkene epoxidation catalyzed by cytochrome P450 BM-3 139-3

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Abstract—We recently reported conversion of cytochrome P450 BM-3, a medium-chain (C_{12}–C_{18}) fatty acid monooxygenase, into a highly efficient alkane hydroxylase by directed evolution [*Nat. Biotechnol.* 2002, *20*, 1135]. P450 BM-3 mutant 139-3 exhibited high activity towards a variety of fatty acid and alkane substrates, including C_3–C_8 alkanes. We report here that mutant 139-3 is also active on benzene, styrene, cyclohexene, 1-hexene, and propylene. Benzene is converted to phenol, while styrene is converted to styrene oxide. Propylene oxidation generates only propylene oxide, but cyclohexene oxidation produces a mixture of cyclohexene oxide (85%) and 2-cyclohexene-1-ol (15%), and 1-hexene is converted to the allylic hydroxylation product, 1-hexene-3-ol. Initial rates of NADPH oxidation for 139-3 in the presence of the substrates greatly (17- to >100-fold) surpass the wild-type in all cases. However, NADPH consumption is only partially coupled to product formation (14–79%). This cytochrome P450 epoxidation catalyst is a suitable starting point for further evolution to improve coupling and activity.
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1. Introduction

Catalyzing a wide range of oxidative reactions under mild conditions in aqueous solutions, cytochrome P450 monooxygenases (P450s) are interesting potential 'green' catalysts.[1] It has been shown that many of their limitations, including poor activities towards non-natural substrates,[2–9] limited stability,[10] inability to withstand organic solvent,[12] and even their cofactor requirements[11,13] can be improved by protein engineering, particularly directed evolution.

This group recently described directed evolution of the highly active, soluble fatty acid hydroxylase P450 BM-3 for selective alkane oxidation.[9] Five generations of random mutagenesis/recombination and screening transformed P450 BM-3 into an efficient alkane hydroxylase, which we named 139-3. The evolved gene contained 13 nucleotide base substitutions in the region coding for the heme domain, resulting in 11 amino acid changes. Mutant 139-3 displayed higher turnover rates than any reported enzyme for the (subterminal) hydroxylation of alkanes with chain lengths between C_3 and C_8 (3600 min^{-1} for hexane). It also exhibited ~2-fold higher initial activity for lauric and

palmitic acid, which are already good substrates for the wild-type. Here we report that the broadly-active 139-3 mutant is also a good epoxidation catalyst. Much more active than the wild-type P450 BM-3, mutant 139-3 is a convenient platform for further directed evolution to optimize epoxidation of specific substrates.
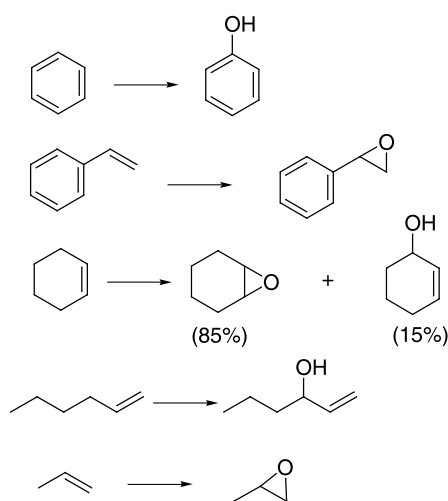
Scheme 1. Substrates oxidized by BM-3 variant 139-3 and the corresponding products. For benzene, styrene, cyclohexene, or 1-hexene oxidations, the reaction mixture contained enzyme, substrate, and methanol in potassium phosphate buffer. For propylene oxidation, the buffer was first saturated with propylene. Reactions were initiated by addition of NADPH, and products were analyzed by GC/MS.

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Table 1. Products, turnover rates, and coupling during oxidation catalyzed by cytochrome P450 BM-3 mutant 139-3

Substrate	Products[a]	Maximum initial rate[b]	Coupling (%)
Benzene	Phenol (100%)[d]	200 ± 64 (0)[e]	14
Styrene	Styrene oxide (100%)	1100 ± 87 (17 ± 15)	35
Cyclohexene	Cyclohexene oxide (85%)	1200 ± 130 (0)	79
	2-Cyclohexene-1-ol (15%)		
1-Hexene	1-Hexene-3-ol (100%)	1300 ± 160 (60 ± 8)	22
Propylene	Propylene oxide (100%)	700 ± 62 (40 ± 31)	nd[c]

[a] The product yields and distributions were determined by gas chromatography/mass spectrometry as described in Section 4.
[b] Maximum initial rates are given in nmoles NADPH consumed/min/nmoles P450.
[c] nd=Not determined.
[d] The value in the parenthesis represents the ratio of product formed/total product.
[e] The value in the parenthesis represents the maximum initial rate for the wild-type.

2. Results and discussion

The activity of BM-3 mutant 139-3 was evaluated against the substrates shown in Scheme 1 and Table 1. Benzene was converted to phenol, presumably via epoxidation of the aromatic ring,[14] with a maximum initial rate of NADPH oxidation of 200 mol/min/mol enzyme. The wild-type enzyme in the presence of benzene shows no NADPH consumption above background (Table 1). For styrene, cyclohexene, 1-hexene, and propylene, the maximum rates of NADPH oxidation by 139-3 also greatly surpassed those for wild-type (between 17- and >100-fold) (Table 1). Styrene and propylene oxidation by 139-3 yielded styrene oxide and propylene oxide, respectively. Cyclohexene oxidation yielded cyclohexene oxide (85%) and 2-cyclohexene-1-ol (15%).

The sole product of 1-hexene oxidation was the allylic hydroxylation product, 1-hexene-3-ol. This was not unexpected, since wild-type P450 BM-3 preferentially oxidizes ω-unsaturated fatty acids at the allylic position, and the corresponding terminal epoxide is not formed.[15] It has been proposed that the structure of the BM-3 substrate binding pocket prevents terminal oxidation.[16] The pocket resembles a long funnel[16–18] at the end of which a small hydrophobic pocket sequesters the substrate terminus, rendering it unavailable for oxidation. The selectivity also reflects the different C–H bond strengths: the ω-2 secondary allylic C–H bond ($\Delta H_{298}^0 \sim 83$ kcal/mol) is weaker than the ω-3 secondary C–H bond ($\Delta H_{298}^0 \sim 98$ kcal/mol).

NADPH oxidation is not necessarily an accurate measure of P450 catalytic activity, because electron equivalents from NADPH can be diverted to produce reduced oxygen species (H_2O or H_2O_2). Using NADPH as a limiting reagent, the 139-3 'uncoupling' reaction was measured for all the substrates (Table 1). In all cases, NADPH oxidation was only partially coupled to substrate oxidation, with efficiencies between 14 and 79%. The amount of H_2O_2 detected using the ABTS/HRP assay was less than 1% in all cases; therefore, it is assumed that H_2O is the uncoupled product. It has been proposed that high substrate mobility in the active site and the presence of excess H_2O near the heme iron cause uncoupling and reflect poor substrate binding.[19]

Substrate binding can be monitored spectroscopically. The substrate-free structure of wild-type P450 BM-3 shows between 17 and 21 water molecules in the substrate access channel.[16] The P450 resting state contains a heme iron as a low-spin six-coordinate ferric species with a dissociable H_2O *trans* to the proximal cysteinate[20] and has a characteristic absorption maximum at 419 nm. The channel dehydrates upon substrate binding, resulting in a high-spin five-coordinate species, and the absorption maximum shifts to 390 nm. In the presence of the alkenes, mutant 139-3 displays only slight spectral shifts, reflecting poor substrate binding or the presence of water in the active site. On the other hand, no detectable spectral shifts are observed when the substrates are added to the wild-type enzyme.

P450s are known to epoxidize a broad range of alkenes and arenes, with rates ranging from 1 min^{-1} for microsomal proteins to 3200 min^{-1} for bacterial enzymes.[1] Using styrene for comparison, P450cam from *Pseudomonas putida* is reported to have an initial rate of NADH consumption of 51 min^{-1}, with only 2% coupling to styrene oxide formation (1.1 min^{-1}).[21] Mutant 139-3, despite its not being 100% coupled, has an initial rate of styrene oxide formation of 385 min^{-1} and is more active than any reported P450 for this reaction.[21,22]

Caldariomyces fumago chloroperoxidase (CPO) performs some P450-like oxygen insertion reactions, including epoxidation.[23] The reported initial rate of CPO-catalyzed styrene epoxidation is 288 min^{-1}.[24] CPO utilizes peroxide in the catalytic cycle and does not require expensive cofactors such as NADPH or additional electron transfer proteins. However, CPO is not functionally expressed in *Escherichia coli*, presumably due to the bacterium's inability to do the processing or post-translational modifications to CPO that occur in the natural, fungal host. Recombinant CPO can be expressed in *C. fumago*, but the fungus does not provide a convenient transformation or expression system for rational protein design or directed evolution experiments.[24–26] In contrast, P450 BM-3 and its mutants, including 139-3, are soluble and easily expressed in *E. coli*. Cirino and Arnold have recently shown that P450 BM-3 heme domain alone can be evolved to use the peroxide shunt pathway, much like CPO.[13] Thus it should be possible to engineer mutants of the P450 heme domain that catalyze epoxidation, using peroxide.

Chiral epoxides are useful synthetic intermediates for the pharmaceutical and chemical industries, and P450s are potential asymmetric epoxidation catalysts.[1] However, slow reaction rates and low expression yields have hampered the synthetic utility of enzymes. For the oxidation of styrene by

P450cam, P450terp from *Pseudomonas* sp., and P450 BM-3, the ratios of *S/R* styrene oxide produced are 12:88, 83:17, and 40:60, respectively.[22] Mutant 139-3, which was evolved to hydroxylate alkanes, shows no enantioselectivity in this reaction (*S/R* styrene oxide=50:50). With a suitable high throughput screen, however, enantioselectivity is also a good target for optimization by directed evolution.[27]

3. Conclusion

The broadly-active laboratory-evolved cytochrome P450 BM-3 mutant 139-3 is far more active than the wild-type enzyme as a catalyst for epoxidation reactions. The activity of wild-type P450 BM-3 on the substrates investigated here is too low to monitor in high throughput and therefore too low for improvement by directed evolution. The evolved enzyme is a suitable parent for further directed evolution to improve rates and coupling efficiency, both of which can be optimized by appropriate changes in the catalyst. It is also possible to modify enantioselectivity. This relatively stable, efficient, and easily-produced enzyme has potential as an epoxidation catalyst.

4. Experimental

All chemicals were purchased from commercial sources and used as received.

4.1. Expression of P450 BM-3 mutant 139-3

The mutant P450 BM-3 gene was cloned behind the double *tac* promoter of the expression vector pCWori.[9,28] For enzyme production, supplemented terrific broth medium[29] (500 ml) was inoculated with 0.5 ml of an overnight culture of *E. coli* DH5α containing the expression plasmid. After shaking for 10 h at 35 °C, δ-aminolevulinic acid hydrochloride (ALA, 0.5 mM) was added, and expression was induced by the addition of isopropyl-β-D-thiogalactoside (IPTG, 1 mM). The cells were cultivated for 30 h at 30 °C. The enzyme was purified following published procedures,[29] and the enzyme concentration was determined from the CO-difference spectra.[30]

4.2. Determination of maximum initial rates of oxidation

The mutant was purified and quantified as described above. The initial rate of oxidation was determined as described.[9] A typical reaction solution contained enzyme (1.0 ml, 1.0 μM) in potassium phosphate buffer (0.1 M, pH 8.0), substrate (2 mM), and methanol (1% v/v). The reaction was initiated by the addition of NADPH (200 μl, 200 μM), and the absorbance was monitored at 340 nm.

4.3. Product characterization

Product distributions and yields were characterized by gas chromatography/mass spectrometry. For benzene, styrene, cyclohexene, or 1-hexene oxidations, the reaction solution contained substrate (2 mM) in methanol (1% v/v) and enzyme (1.0 μM) in potassium phosphate buffer (1.5 ml, 0.1 M, pH 8.0). For propylene oxidation, the buffer was first saturated with propylene. The solution was stirred for 5 min at room temperature, and the reaction was initiated by the addition of NADPH (200 μM). After completion of the reaction, the solution was extracted once with CHCl₃ (300 μl) containing 1-hexanol (0.5 mM) as an internal standard. The organic layer was analyzed by GC/MS using a Hewlett Packard 6890 gas chromatograph coupled to a Hewlett Packard 5973 mass selective detector. The GC was fitted with an Agilent Technologies Innowax column (30 m×0.25 mm with a film thickness of 0.25 μm). The conditions are as follows: (i) 35 °C for 5 min, (ii) 35–200 °C at 20 °C/min, (iii) isothermic at 200 °C for 5 min. Authentic standards were used to identify the retention times and to prepare standard curves. Products were further verified by matching the fragmentation distributions with a database in the software provided with the instrument. Chiral resolution of styrene oxide was determined using a GC fitted with a chiral column (25×0.25 mm Hydrodex β-6 TBDM, Macherey-Nagel), and the conditions are as follows: (i) 100 °C for 10 min, (ii) 100–170 °C at 20 °C/min, (iii) isothermic at 170 °C for 6 min, (iv) 170–100 °C at 20 °C/min, (v) isothermic 100 °C for 1 min.

Percent coupling is determined from the ratio of moles of product formed to the moles of NADPH added to the reaction. NADPH was the limiting reagent, and the substrate concentration was 10× that of NADPH to ensure complete consumption of NADPH. The total product formed was determined by GC/MS, as described above. The concentration of NADPH was determined spectroscopically by measuring the absorbance at 340 nm (ε =6210 M^{-1} cm^{-1}) of the stock solution. The amount of H₂O₂ generated by the reaction was determined using the 2,2′-azino-bis[3-ethyl-benzothiazoline-6-sulfonic acid]/horseradish peroxidase (ABTS/HRP) assay according to published procedures.[31,32]

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