Chapter 2

Engineered Alkane-Hydroxylating Cytochrome P450 BM3 Exhibiting Native-like Catalytic Properties

Material from this chapter appears in: Fasan, R., Chen, M. M., Crook, N. C., and Arnold, F. H. (2007) Engineered alkane-hydroxylating cytochrome P450(BM3) exhibiting native-like catalytic properties, *Angewandte Chemie–International Edition* 46, 8414 – 8418, and is reprinted by permission from Wiley-VCH.

A. Abstract

New functions have been engineered in a variety of pre-existing enzymes using directed evolution, however, examples in which the engineered variants exhibit comparable catalytic properties with the non-natural substrate as the wildtype enzyme with its preferred substrates are rare. Here, we describe the *in vitro* evolution of a proficient P450 propane monooxygenase, $P450_{PMO,}$ starting from a fatty acid hydroxylase CYP102A1 (BM3). Applying only positive selection pressure in combination with a domain engineering mutagenesis strategy, which targeted the heme and reductase domains independently and in combination, re-specialization of BM3 for the non-natural substrate propane was achieved after several rounds of directed evolution. $P450_{PMO}$ supports up to 45,800 propane turnovers with 98.2% coupling of substrate oxidation and cofactor consumption, rivaling those of natural P450s with their preferred substrates. In addition, we were able to demonstrate *in vivo* propane hydroxylation using these BM3 variants in resting *Escherichia coli* cells reaching activities up to 176 U g^{-1} cdw, which surpasses the reported activities of natural alkane hydroxylases acting on their preferred substrates.

B. Introduction

Cytochrome P450 enzymes (P450s) are exceptional oxygenating catalysts (*1 – 2*) with enormous potential in drug discovery, chemical synthesis, bioremediation, and biotechnology (*3 – 4*). Compared to their natural counterparts, however, engineered P450s often exhibit poor catalytic and cofactor coupling efficiencies (*3*). Obtaining native-like catalytic proficiencies is a mandatory first step towards utilizing the power of these versatile oxygenases in chemical synthesis.

Cytochrome P450 BM3 (BM3) isolated from *B. megaterium* catalyzes the subterminal hydroxylation of long-chain (C_{12}–C_{20}) fatty acids (*5*). Its high activity and catalytic self-sufficiency (heme and diflavin reductase domains are fused in a single polypeptide chain) (*4-6*) makes BM3 an excellent platform for biocatalysis. However, despite numerous reports of the heme domain being engineered to accept nonnative substrates, including short-chain fatty acids, aromatic compounds, alkanes, and alkenes (*7 – 15*), reports of preparative-scale applications of BM3 remain scarce (*16 – 19*).

The native BM3 function is finely regulated through conformational rearrangements in the heme and reductase domains and possibly also through hinged domain motions (*5, 20 – 21*). Hydroxylation of fatty acids occurs almost fully coupled to cofactor (NADPH) utilization, 93–96% depending on the substrate (*22 – 23*). In the presence of nonnative substrates or variants containing amino acid substitutions, the mechanisms controlling efficient catalysis in P450s are disrupted (*24 – 25*), leading to the formation of reactive oxygen species and rapid enzyme inactivation (*5*). High coupling efficiencies on substrates whose physicochemical properties are substantially different from the native substrates have not been achieved, and typical coupling

efficiencies range from less than 1% to 40% (*8, 11, 14*). Strategies for addressing this "coupling problem" are needed in order to take engineered P450s to larger-scale applications.

Selective hydroxylation of small alkanes is a long-standing problem, for which no practical catalysts are available (*26 – 28*). In an effort to produce BM3-based biocatalysts for selective hydroxylation of small alkanes, we previously engineered an enzyme variant 35E11, which accepts propane and ethane as substrates (*29*). However, despite greater than 5,000 total turnover supported *in vitro*, the utility of this catalyst remained limited because of its poor *in vivo* performance (see below), which was mostly due to low coupling efficiencies between product formation to cofactor consumption (17.4% for propane and 0.01% for ethane oxidation). The goal of our work was to engineer a BM3 variant with native-like activity and coupling efficiency toward a structurally challenging, non-native substrate, propane, and evaluate the impact of these features on performance in preparative-scale biotransformations.

C. Results and Discussion

C.1. Complete mutagenesis of BM3 through a domain engineering strategy

We used a domain-based protein-engineering strategy, in which the heme, flavin mononucleotide (FMN), and flavin adenine dinucleotide (FAD) domains of variant 35E11 were evolved separately but evaluated in the context of the holoenzymes. As a final step, beneficial mutations identified in each sub-domain were recombined (Figure 2.1). Most previous engineering efforts have focused mutagenesis to the heme (hydoxylase) domain of BM3(*7, 9, 11 – 12, 14*), although mutations in the reductase and linker regions have been shown to also affect catalytic properties, specifically coupling of co-factor consumption with substrate hydroxylation (*29 – 31*). However, no systematic engineering efforts had been undertaken to engineer the complete 1,048 amino acid holoenzyme.

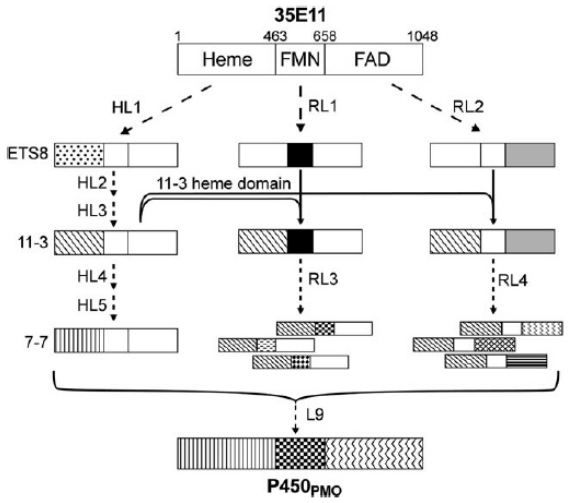

Figure 2.1: Outline of the domain engineering strategy used to improve cytochrome P450 BM3 heme and reductase domain (HL = heme domain library, RL = reductase domain libraries)

Holoenzyme libraries outlined in Figure 2.1 were created using random, saturation, and site-directed mutagenesis and screened for activity on a propane surrogate, dimethyl ether (*14*). Variants with improved dimethyl ether demethylation activity were confirmed by a rescreen,

purified, and characterized for propane hydroxylation activity using sealed head-space vials in the presence of a cofactor regeneration system. As a cumulative measure of both catalytic and coupling efficiency, improvement in total turnover number (TON), i.e., moles of propanol produced per mole of enzyme, was used as the sole selection criterion.

Measurement of the half-denaturation temperature (T_{50}), the temperature at which the enzyme retains 50% of its activity after a 15 minute incubation, of variant 35E11 heme domain demonstrated a considerable reduction in its thermostability as a consequence of the 15 accumulated mutations (T_{50}=43.4 ^{o}C vs. 55.0 ^{o}C for wild-type BM3). We therefore subjected variant 35E11 to an initial thermostabilization step (HL1), in which known stabilizing mutations from a thermostablized BM3 peroxygenase (*32*) were tested singly and in combination in the 35E11 background (see Table 2.1). Variant ETS8 (35E11-L52I-I366V), which showed the best combination of increased stability, ΔT_{50}=+5.1 ^{o}C, with only a small decrease in propane TON, $\Delta TON_{propane}$= -1,250, was selected for further directed evolution.

Table 2.1: Thermostablized variants of 35E11

Variant	Mutations					T_{50}[a] (^{o}C)	ΔT_{50} (^{o}C)	ΔPropane TON[b]
	L52	L234	V340	I366	E442			
35E11	-	-	-	-	-	43.4	n/a	n/a
ETS1	I	-	-	-	-	44.5	1.1	-510
ETS3	-	I	-	-	-	43.2	-0.3	-1,450
ETS4	-	-	M	-	-	46.0	2.6	-1,290
ETS5	-	-	-	V	-	47.1	3.7	-850
ETS6	-	-	-	-	K	45.0	1.6	+170
ETS8	I	-	-	V	-	48.5	5.1	-1,250
ETS9	I	-	-	-	K	46.8	3.4	-2,950
ETS10	-	-	M	-	K	44.2	0.8	-3,180
ETS11	-	-	-	V	K	46.6	3.2	-1750

[a] T_{50} calculated based on a two-state denaturation model using the percentage of 450 nm CO-binding peak of P450 variants remaining after 15-min incubations at varying temperatures

[b] TON determined as nmol product/nmol enzyme. Propane reactions contained 25 – 100 nM protein, potassium phosphate buffer saturated with propane, and an NADPH regeneration system containing 100 μM $NADP^{+}$, 2 U/mL isocitrate dehydrogenase, and 10 mM isocitrate. Errors are at most 10%.

Using ETS8 as parent, heme-domain random mutagenesis libraries were generated by error-prone PCR (HL2). Variant 19A12, ETS8-L188P, was identified from this library with more than a twofold increase in propane TON (Table 2.2). Using 19A12 as the parent, a pool of active-site libraries (HL3) were constructed in which 17 positions along the substrate channel and near the active site (74, 75, 82, 87, 88, 181, 184, 188, 260, 264, 265, 268, 328, 401, 437, and 438) were subjected individually to saturation mutagenesis. From these site-saturation libraries, further improvements in propane-hydroxylating activity were achieved in multiple variants, including 11-3 (19A12-A74S) which supported 13,200 propane TONs. Recombination of the beneficial mutations identified in these active-site variants (HL4, HL5) led to variant 1-3 (19A12-A74S-V184A) and variant 7-7 (19A12-A74E-S82G), supporting 19,200 and 20,500 propane TONs, respectively.

In parallel to the mutagenesis efforts targeting the BM3 heme domain, two libraries were constructed in which random mutations were targeted to the FMN and FAD binding domains of 35E11, RL1 and RL2, respectively. Screening of more than 5,000 members from each library for dimethyl ether demethylation led to the identification of eight beneficial mutations (G443D, V445M, T480M, T515M, P654Q, T664M, D698G, and E1037G). Of these eight mutations, G443D and V445M are actually located in the C-terminus β4 sheet of the heme domain, as RL1 included not only FMN domain but also the last 32 amino acids of the heme domain due to library construction. These eight positions were further optimized through saturation mutagenesis in a holoenzyme construct having the 11-3 heme domain (RL3, RL4). By swapping the heme domains, we aimed to remove mutations whose beneficial effect is solely dependent on the presence of the 35E11 heme domain. With the 11-3 heme domain, improved variants were

found to contain G443A, V445R, P654K, T664G, D698G, and E1037G mutations and supported propane TON between 16,000 and 20,000.

In the final step, a library containing the beneficial reductase domain mutations was fused to the heme domain of variant 7-7 (L9). The most active variant isolated from this library, $P450_{PMO}R2$, supported more than 45,800 turnovers and produced 2- and 1-propanol in a 9:1 ratio. As we expected, the increase in productivity strongly correlated with the increase in coupling efficiency, which in the best variant $P450_{PMO}R2$, 98.2% reaches levels comparable to those measured for wild-type BM3 in the hydroxylation of fatty acids, 88% for myristate, 93% for palmitate, and 95% for laurate (*22 – 23*).

Table 2.2: *In vitro* propane oxidation activities of most representative P450 BM3 variants[a]

Variant	Library	Mutations versus 35E11[b]		Rate[c] (min^{-1})	Coupling[d] (%)	Propane TON
		Heme domain	Reductase domain			
35E11	-	-	-	210	17.4	5,650
19A12	HL2	L52I, L188P, I366V	-	420	44.2	10,550
11-3	HL3	L52I, A74S, L188P, I366V	-	390	55.3	13,200
1-3	HL4	L52I, A74S, V184A, L188P, I366V	-	320	72.1	19,200
7-7	HL5	L52I, A74E, S82G, L188P, I366V	-	150	90.9	20,500
$P450_{PMO}R1$	L9	L52I, A74E, S82G, L188P, I366V, G443A	P654K, E1027G	455	94.4	35,600
$P450_{PMO}R2$	L9	L52I, A74E, S82G, L188P, I366V, G443A	D698G	370	98.2	45,800

[a] Mean values from at least three replicates ± 10 % error
[b] Mutations in 35E11 are R47C, V78F, A82S, K94I, P142S, T175I, A184V, F205C, S226R, H236Q, E252G, R255S, A290V, A328F, E464G, I710T.
[c] Over the first 20 s
[d] Ratio between propanol formation rate and NADPH oxidation rate in propane-saturated buffer

The sequence of mutational events leading to $P450_{PMO}$ generation reveals a continuous rearrangement of substrate channel and active-site residues (Table 2.2). The mutation of L188P

resulted in the single largest increase in propane hydroxylation activity, 2.4-fold relative to its parent, ETS8. Leu 188 is a helix capping residue located at the C-terminus of the F helix, which along with the G-helix forms a lid that undergoes a conformational change during catalysis. In a hinged motion, these two helices move from an "open" state in the absence of a substrates to a "closed" confirmation when substrate is bound (*33*). Accurately assessing the effect of this mutation, which removes the interstrand hydrogen bond provided by the Leu188 amid NH group, is difficult in the absence of an X-ray crystal structure. However, one likely outcome of this mutation could result in an enzyme resting state with the F and B' helix being in closer proximity, mimicking the substrate-bound confirmation. In the subsequent rounds of active site optimization, two different active site configurations, 19A12-A74S-V184A and 19A12-A74E-A82G were found to supported nearly the same number of propane TONs. However, the coupling of substrate oxidation with cofactor consumption differed significantly between these two variants, 72.1% vs. 90.9%. This 18.8% difference in coupling resulted in only a 6.7% difference in propane TON, which suggests that the benefit of higher coupling efficiency for improving enzyme activity diminishes at higher coupling.

Interestingly, the activity-enhancing substitutions in the reductase domain are clustered in the same region in the FAD domain (T664G, D698G, E1037G) and nearby linker to the FMN domain (P654K) (see Figure 2.2). Perturbation of electrostatic charge distribution appears to be a prevailing trend, suggesting a more important role of these forces in BM3 function than previously proposed (*34*). In contrast, no beneficial mutations were identified in FMN domain. This may reflect its higher sensitivity to mutagenesis, as judged by the significantly lower fraction of functional variants in the FMN libraries compared to the FAD libraries. In addition,

chemical and thermal denaturation studies have shown that, among the three cofactors, FMN is the most weakly bound to the enzyme (*35*).

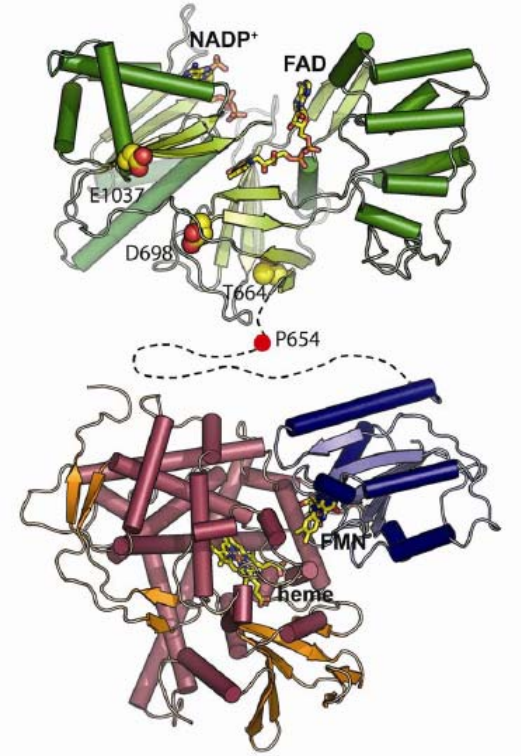

Figure 2.2: Map of the activity-enhancing reductase domain mutations on a homology model of P450BM3 FAD-binding domain prepared on the basis of the rat cytochrome P450 reductase structure (PDB: 1AMO (*36*)). Structural similarity between the two is supported by a preview of the solved but not yet published structure of P450BM3 FAD-binding domain (*6*). Heme domain and FMN domain are represented as in PDB: 1BVY (*37*). A 30 -residue linker connects the C-terminus of the FMN-binding with the N-terminus of the FAD-binding domain (dotted line).

C.2. Whole-cell bioconversion of alkane by BM3 variant with resting cells

A common strategy to reduce the prohibitive costs of NADPH-driven biotransformations is the use of cofactor regeneration systems (*16, 38 – 39*). For bulk chemical transformations such as alkane hydroxylation, these *in vitro* approaches are not economically viable (*40*). The propane-hydroxylating P450 variants were therefore evaluated in whole-cell biotransformations using resting *E. coli* cells. The expression levels of these variants in minimal medium were initially less than 0.5% of total cell mass. After optimization of growth and expression condition,

we were able to achieve expression of soluble P450s at 6–11% of total cell mass. The whole-cell biotransformations were conducted in 100 -mL fermenters using cell suspensions in nitrogen-free M9 minimal medium supplemented with glucose. The cell culture was continuously aerated with a 1:1 propane/air mixture to supply the substrate and oxidant. Under these conditions, cell densities of 0.5 – 0.9 g cdw L^{-1} were used to avoid oxygen-transfer limitations. Activities of 80–120 U g^{-1}cdw (where 1 U = 1 μmol propanol min^{-1}) were measured for $P450_{PMO}$-R1 and-R2 using various *E. coli* strains (Figure 2.3).

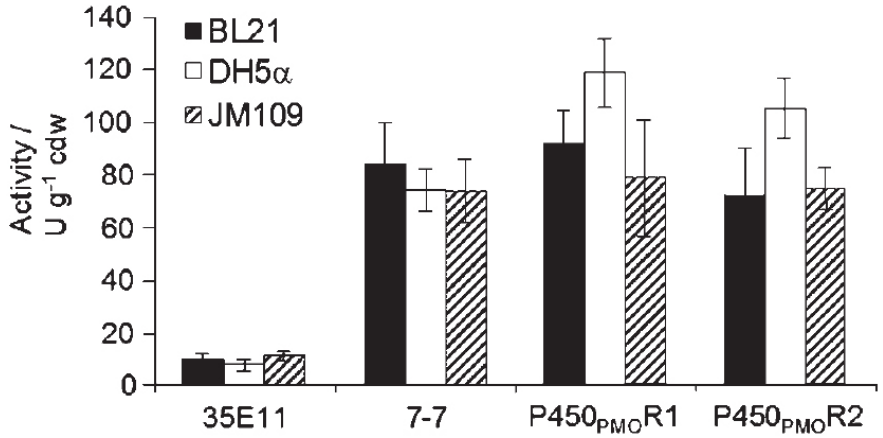

Figure 2.3: Whole-cell biotransformations of propane. Initial activities of selected P450 BM3 variants in different *E. coli* strains using air/propane (1:1) feed (pH 7.2, 25°C) measured after 1 h

The experiment was repeated in a larger fermenter (0.3 L, pH and dissolved oxygen control) with a suspension of P450-expressing DH5α cells, as DH5α was previously shown to be the most productive strain. The cell cultures were fed with a 1:1 mixture of pure oxygen and propane, and propanol formation was monitored for up to 9 h (Figure 2.4(a)). Under these conditions, very high activities (up to 176 U g^{-1} cdw) were obtained (Table 2.3). In comparison, the maximal activities of 30 U g^{-1}cdw on *n*-nonene were reported for the natural AlkB alkane hydroxylase system in both homologous (*P. oleovorans*) and heterologous strains (*E. coli*) (*41*).

Table 2.3: *In vivo* propane oxidation activities of P450BM3 variants[a]

Variant	Oxidant (propane/ oxidant ratio)	Activity [b] (U g^{-1}cdw) 0.5 h	3 h	Productivity [b,c] (mmol propanol g^{-1} P450 h^{-1})
35E11	air (1:1)	9	2	12
19A12	air (1:1)	41	9	44
7-7	air (1:1)	74	n.d.	88
$P450_{PMO}$R1	air (1:1)	118	73	119
$P450_{PMO}$R2	air (1:1)	104	68	106
$P450_{PMO}$R1	O_2 (1:1)	176	63	96
$P450_{PMO}$R2	O_2 (1:1)	119	39	94

[a] Mean values from two biological replicates ±15% error. n.d. = not determined
[b] At 0.5–0.9 g cdw L^{-1} cell density
[c] Calculated from the first hour of biotransformation

At higher cell densities (ca. 4 g cdw L^{-1}), propanol accumulated to a concentration of more than 15 mM over 4 hours (Figure 2.4 (b)). The improved coupling efficiencies resulted in considerably extended periods of whole-cell activity, 6 vs. 0.5 h (Figure 2.4 (a – b)) comparing $P450_{PMO}$ vs. 35E11. To investigate the possible causes for the decrease in productivity over time, we monitored the biocatalyst concentration over the course of the biotransformation (Figure 2.4 (c)). At the end of the experiment, approximately 52% of the initial $P450_{PMO}$R2 was still correctly folded. Control experiments using $P450_{PMO}$R2-expressing cells and propanol concentrations up to 30 mM showed no product inhibition or over-oxidation, suggesting that host related factors, rather than biocatalyst-dependent factors, are limiting. Indeed, 40–60% of the initially measured activity could be restored by resuspending cells from the plateau phase (i.e., after 4–6 h reaction) in fresh medium. In addition, the rate of biocatalyst inactivation could be reduced by varying the relative concentration of oxygen in the gas feed, with more extended whole-cell activity periods obtained at a propane/oxygen ratio of 4:1 compared to 1:1 (Table 2.3). Optimization of this parameter as well as the availability of more robust host strains (*40*) is expected to further enhance the whole-cell productivity of this engineered BM3 variant.

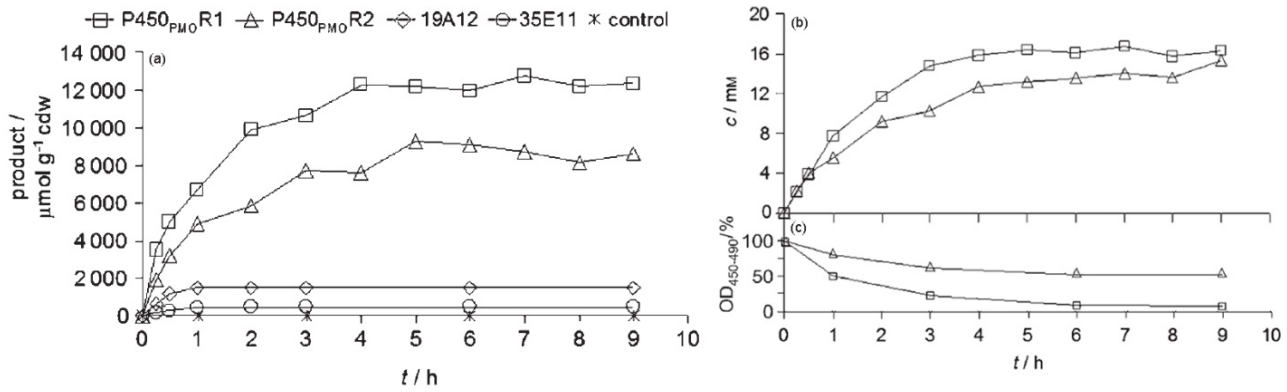

Figure 2.4: (a) Time course of propane biotransformation using recombinant DH5α cells using oxygen/propane (1:1) feed (pH 7.2, 25°C). Product amount is given per gram cell dry weight to facilitate comparison among variants. Control: no propane in the gas feed. (b) Concentration of propanol during biotransformation of propane with DH5α cells expressing P450PMOR1 (Δ) and P450PMOR2 (□) at medium cell density (4g cdw L^{-1}). (b) Relative P450 concentration as determined from CO-binding difference spectra on cell lysate; OD = optical density

D. Conclusion

Overall, a domain-based directed evolution strategy has enabled us to engineer a finely-tuned, multicofactor, multidomain enzyme to exhibit native-like catalytic properties on a substrate significantly different from the native substrate. With this approach, we could use relatively small and targeted libraries to identify beneficial mutations throughout the enzyme, which were recombined to yield the most efficiently engineered P450 reported to date. This strategy should prove useful for engineering other enzymes with multiple, interacting functional domains. With high activity and coupling efficiency for propane oxidation, $P450_{PMO}$s could be used in whole-cell biohydroxylation of propane at room temperature and pressure with air as oxidant. Total activities and product formation rates exceeding those obtained with naturally occurring alkane monooxygenases on their native substrates (*41 – 45*) were achieved in this first report of whole-cell bioconversion of propane to propanol in *E. coli.* These results open the door to considering P450-based oxidations of short-chain alkanes, with promise for green conversion of gaseous hydrocarbons into liquid fuels and chemicals.

E. References

1. Denisov, I. G., Makris, T. M., Sligar, S. G., and Schlichting, I. (2005) Structure and chemistry of cytochrome P450, *Chem. Rev. 105*, 2253-2277.
2. Sono, M., Roach, M. P., Coulter, E. D., and Dawson, J. H. (1996) Heme-containing oxygenases, *Chem. Rev. 96*, 2841-2887.
3. Bernhardt, R. (2006) Cytochromes P450 as versatile biocatalysts, pp 128-145, Elsevier Science Bv.
4. Urlacher, V. B., and Eiben, S. (2006) Cytochrome P450 monooxygenases: perspectives for synthetic application, *Trends Biotechnol. 24*, 324-330.
5. Munro, A. W., Leys, D. G., McLean, K. J., Marshall, K. R., Ost, T. W. B., Daff, S., Miles, C. S., Chapman, S. K., Lysek, D. A., Moser, C. C., Page, C. C., and Dutton, P. L. (2002) P450BM3: the very model of a modern flavocytochrome, *Trends Biochem.Sci. 27*, 250-257.
6. Warman, A. J., Roitel, O., Neeli, R., Girvan, H. M., Seward, H. E., Murray, S. A., McLean, K. J., Joyce, M. G., Toogood, H., Holt, R. A., Leys, D., Scrutton, N. S., and Munro, A. W. (2005) Flavocytochrome P450BM3: an update on structure and mechanism of a biotechnologically important enzyme, *Biochem. Soc. Trans. 33*, 747-753.
7. Appel, D., Lutz-Wahl, S., Fischer, P., Schwaneberg, U., and Schmid, R. D. (2001) A P450BM-3 mutant hydroxylates alkanes, cycloalkanes, arenes and heteroarenes, *Journal of Biotechnology 88*, 167-171.
8. Carmichael, A. B., and Wong, L. L. (2001) Protein engineering of Bacillus megaterium CYP102 - The oxidation of polycyclic aromatic hydrocarbons, *Eur. J. Biochem. 268*, 3117-3125.
9. Glieder, A., Farinas, E. T., and Arnold, F. H. (2002) Laboratory evolution of a soluble, self-sufficient, highly active alkane hydroxylase, *Nat. Biotechnol. 20*, 1135-1139.
10. Kubo, T., Peters, M. W., Meinhold, P., and Arnold, F. H. (2006) Enantioselective epoxidation of terminal alkenes to (R)- and (S)-epoxides by engineered cytochromes P450BM-3, *Chemistry-a European Journal 12*, 1216-1220.
11. Li, Q. S., Ogawa, J., Schmid, R. D., and Shimizu, S. (2001) Engineering cytochrome P450BM-3 for oxidation of polycyclic aromatic hydrocarbons, *Applied and Environmental Microbiology 67*, 5735-5739.
12. Li, Q. S., Ogawa, J., Schmid, R. D., and Shimizu, S. (2001) Residue size at position 87 of cytochrome P450BM-3 determines its stereoselectivity in propylbenzene and 3-chlorostyrene oxidation, *FEBS Lett. 508*, 249-252.
13. Ost, T. W. B., Miles, C. S., Murdoch, J., Cheung, Y. F., Reid, G. A., Chapman, S. K., and Munro, A. W. (2000) Rational re-design of the substrate binding site of flavocytochrome P450BM3, *FEBS Lett. 486*, 173-177.

14. Peters, M. W., Meinhold, P., Glieder, A., and Arnold, F. H. (2003) Regio- and enantioselective alkane hydroxylation with engineered cytochromes P450 BM-3, *J. Am. Chem. Soc. 125*, 13442-13450.

15. Sulistyaningdyah, W. T., Ogawa, J., Li, Q. S., Maeda, C., Yano, Y., Schmid, R. D., and Shimizu, S. (2005) Hydroxylation activity of P450BM-3 mutant F87V towards aromatic compounds and its application to the synthesis of hydroquinone derivatives from phenolic compounds, *Applied Microbiology and Biotechnology 67*, 556-562.

16. Falck, J. R., Reddy, Y. K., Haines, D. C., Reddy, K. M., Krishna, U. M., Graham, S., Murry, B., and Peterson, J. A. (2001) Practical, enantiospecific syntheses of 14,15-EET and leukotoxin B (vernolic acid), *Tetrahedron Lett. 42*, 4131-4133.

17. Maurer, S. C., Kuhnel, K., Kaysser, L. A., Eiben, S., Schmid, R. D., and Urlacher, V. B. (2005) Catalytic hydroxylation in biphasic systems using CYP102A1 mutants, *Advanced Synthesis & Catalysis 347*, 1090-1098.

18. Schneider, S., Wubbolts, M. G., Oesterhelt, G., Sanglard, D., and Witholt, B. (1999) Controlled regioselectivity of fatty acid oxidation by whole cells producing cytochrome P450(BM-3) monooxygenase under varied dissolved oxygen concentrations, *Biotechnology and Bioengineering 64*, 333-341.

19. Sowden, R. J., Yasmin, S., Rees, N. H., Bell, S. G., and Wong, L. L. (2005) Biotransformation of the sesquiterpene (+)-valencene by cytochrome P450(cam) and P450(BM-3), *Org. Biomol. Chem. 3*, 57-64.

20. Haines, D. C., Tomchick, D. R., Machius, M., and Peterson, J. A. (2001) Pivotal role of water in the mechanism of P450BM-3, *Biochemistry 40*, 13456-13465.

21. Murataliev, M. B., and Feyereisen, R. (1996) Functional interactions in cytochrome P450BM3. Fatty acid substrate binding alters electron-transfer properties of the flavoprotein domain, *Biochemistry 35*, 15029-15037.

22. Cryle, M. J., Espinoza, R. D., Smith, S. J., Matovic, N. J., and De Voss, J. J. (2006) Are branched chain fatty acids the natural substrates for P450(BM3)?, *Chemical Communications*, 2353-2355.

23. Noble, M. A., Miles, C. S., Chapman, S. K., Lysek, D. A., Mackay, A. C., Reid, G. A., Hanzlik, R. P., and Munro, A. W. (1999) Roles of key active-site residues in flavocytochrome P450 BM3, *Biochem. J. 339*, 371-379.

24. Kadkhodayan, S., Coulter, E. D., Maryniak, D. M., Bryson, T. A., and Dawson, J. H. (1995) Uncoupling oxygen-transfer and electron-transfer in the oxygenation of camphor analogs by cytochrome P450cam - direct observation of an intermolecular isotope effect for substrate C-H activation, *Journal of Biological Chemistry 270*, 28042-28048.

25. Loida, P. J., and Sligar, S. G. (1993) Molecular recognition in cytochrome-p-450 - mechanism for the control of uncoupling reactions, *Biochemistry 32*, 11530-11538.

26. Labinger, J. A., and Bercaw, J. E. (2002) Understanding and exploiting C-H bond activation, *Nature 417*, 507-514.

27. Shul'pin, G. B., Suss-Fink, G., and Shul'pina, L. S. (2001) Oxidations by the system "hydrogen peroxide-manganese(IV) complex-carboxylic acid" Part 3. Oxygenation of ethane, higher alkanes, alcohols, olefins and sulfides, *J. Mol. Catal. A-Chem. 170*, 17-34.

28. Yamanaka, I., Hasegawa, S., and Otsuka, K. (2002) Partial oxidation of light alkanes by reductive activated oxygen over the (Pd-black + VO(acac)(2)/VGCF) cathode of H-2-O-2 cell system at 298 K, *Appl. Catal. A-Gen. 226*, 305-315.

29. Meinhold, P., Peters, M. W., Chen, M. M. Y., Takahashi, K., and Arnold, F. H. (2005) Direct conversion of ethane to ethanol by engineered cytochrome P450BM3, *Chembiochem 6*, 1765-1768.

30. Govindaraj, S., and Poulos, T. L. (1995) Role of the linker region connecting the reductase and heme domains in cytochrome P450(BM-3), *Biochemistry 34*, 11221-11226.

31. Roitel, O., Scrutton, N. S., and Munro, A. W. (2003) Electron transfer in flavocytochrome P450BM3: Kinetics of flavin reduction and oxidation, the role of cysteine 999, and relationships with mammalian cytochrome P450 reductase, *Biochemistry 42*, 10809-10821.

32. Salazar, O., Cirino, P. C., and Arnold, F. H. (2003) Thermostabilization of a cytochrome P450 peroxygenase, *Chembiochem 4*, 891-893.

33. Arnold, G. E., and Ornstein, R. L. (1997) Molecular dynamics study of time-correlated protein domain motions and molecular flexibility: Cytochrome P450BM-3, *Biophys. J. 73*, 1147-1159.

34. Davydov, D. R., Kariakin, A. A., Petushkova, N. A., and Peterson, J. A. (2000) Association of cytochromes P450 with their reductases: Opposite sign of the electrostatic interactions in P450BM-3 as compared with the microsomal 2B4 system, *Biochemistry 39*, 6489-6497.

35. Munro, A. W., Lindsay, J. G., Coggins, J. R., Kelly, S. M., and Price, N. C. (1996) Analysis of the structural stability of the multidomain enzyme flavocytochrome P-450 BM3, *Biochimica Et Biophysica Acta-Protein Structure and Molecular Enzymology 1296*, 127-137.

36. Wang, M., Roberts, D. L., Paschke, R., Shea, T. M., Masters, B. S. S., and Kim, J. J. P. (1997) Three-dimensional structure of NADPH-cytochrome P450 reductase: Prototype for FMN- and FAD-containing enzymes, *Proceedings of the National Academy of Sciences of the United States of America 94*, 8411-8416.

37. Sevrioukova, I. F., Li, H. Y., Zhang, H., Peterson, J. A., and Poulos, T. L. (1999) Structure of a cytochrome P450-redox partner electron-transfer complex, *Proceedings of the National Academy of Sciences of the United States of America 96*, 1863-1868.

38. Maurer, S. C., Schulze, H., Schmid, R. D., and Urlacher, V. (2003) Immobilisation of P450BM-3 and an NADP(+) cofactor recycling system: Towards a technical application of heme-containing monooxygenases in fine chemical synthesis, *Advanced Synthesis & Catalysis 345*, 802-810.

39. Schwaneberg, U., Otey, C., Cirino, P. C., Farinas, E., and Arnold, F. H. (2001) Cost-effective whole-cell assay for laboratory evolution of hydroxylases in *Escherichia coli*, *J. Biomol. Screen 6*, 111-117.

40. Duetz, W. A., van Beilen, J. B., and Witholt, B. (2001) Using proteins in their natural environment: potential and limitations of microbial whole-cell hydroxylations in applied biocatalysis, *Current Opinion in Biotechnology 12*, 419-425.

41. Staijen, I. E., van Beilen, J. B., and Witholt, B. (2000) Expression, stability and performance of the three-component alkane mono-oxygenase of *Pseudomonas oleovorans* in *Escherichia coli*, *Eur. J. Biochem. 267*, 1957-1965.

42. Fujii, T., Narikawa, T., Takeda, K., and Kato, J. (2004) Biotransformation of various alkanes using the *Escherichia coli* expressing an alkane hydroxylase system from Gordonia sp TF6, *Biosci. Biotechnol. Biochem. 68*, 2171-2177.

43. Furuto, T., Takeguchi, M., and Okura, I. (1999) Semicontinuous methanol biosynthesis by Methylosinus trichosporium OB3b, *J. Mol. Catal. A-Chem. 144*, 257-261.

44. Kubota, M., Nodate, M., Yasumoto-Hirose, M., Uchiyama, T., Kagami, O., Shizuri, Y., and Misawa, N. (2005) Isolation and functional analysis of cytochrome P450 CYP153A genes from various environments, *Biosci. Biotechnol. Biochem. 69*, 2421-2430.

45. Lee, S. G., Goo, J. H., Kim, H. G., Oh, J. I., Kim, Y. M., and Kim, S. W. (2004) Optimization of methanol biosynthesis from methane using *Methylosinus trichosporium* OB3b, *Biotechnol. Lett. 26*, 947-950.