Directed Evolution

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Engineered Alkane-Hydroxylating Cytochrome $P450_{BM3}$ Exhibiting Nativelike Catalytic Properties**

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Cytochrome P450 enzymes (P450s) are exceptional oxygenating catalysts[1] with enormous potential in drug discovery, chemical synthesis, bioremediation, and biotechnology.[2,3] 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 $P450_{BM3}$ (119 kDa, *B. megaterium*) catalyzes the subterminal hydroxylation of long-chain (C_{12}–C_{20}) fatty acids.[4] Its high activity and catalytic self-sufficiency (heme and diflavin reductase domains are fused in a single polypeptide chain)[2,4,5] make $P450_{BM3}$ 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,[6–8] reports of preparative-scale applications of $P450_{BM3}$ remain scarce.[9]

$P450_{BM3}$ function is finely regulated through conformational rearrangements in the heme and reductase domains and possibly also through hinged domain motions.[4,10] Hydroxylation of fatty acids occurs almost fully coupled to cofactor (NADPH) utilization (93–96% depending on the substrate).[11] In the presence of nonnative substrates or when amino acid substitutions are introduced, the mechanisms controlling efficient catalysis in P450s are disrupted,[12] leading to the formation of reactive oxygen species and rapid enzyme inactivation.[4] High coupling efficiencies on substrates whose physicochemical properties are substantially different from the native substrates have not been achieved, and coupling efficiencies ranging from less than 1% to 30–40% are typical.[7,8] Strategies for addressing this "coupling problem" are needed in order to take engineered P450s to larger-scale applications.

Selective hydroxylation of short alkanes is a long-standing problem, for which no practical catalysts are available.[13] In an effort to produce $P450_{BM3}$-based biocatalysts for selective hydroxylation of small alkanes, we previously engineered this enzyme to accept propane and ethane (35E11 variant).[14] Despite greater than 5000 total turnover (TTN) supported in vitro, the utility of this catalyst remained limited because of its poor in vivo performance (see below), which was mostly due to the low efficiencies for coupling the product formation to cofactor consumption (17.4% for propane and 0.01% for ethane oxidation).

Our goal was to engineer a $P450_{BM3}$ variant with native-like activity and coupling efficiency towards a structurally challenging, nonnative substrate (propane) and evaluate the impact of these features on performance in preparative-scale biotransformations. To this end, we used a domain-based protein-engineering strategy, in which the heme, flavin mononucleotide (FMN), and flavin adenine dinucleotide (FAD) domains of the 35E11 variant were evolved separately in the context of the holoenzyme, and beneficial mutations were recombined in a final step (Figure 1). Previous work suggested that mutations in the reductase and linker regions can affect catalytic properties.[14,15] However, no systematic engineering efforts had been undertaken to engineer the complete 1048 amino acid holoenzyme.

Holoenzyme libraries outlined in Figure 1 were created using random, saturation, and site-directed mutagenesis and

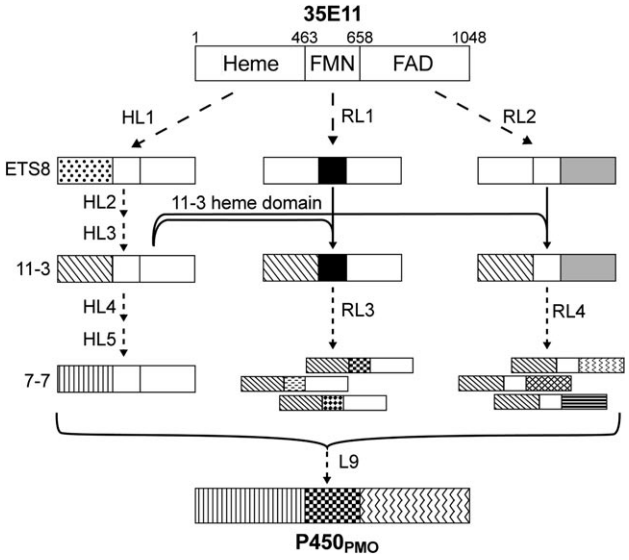

Figure 1. Outline of the domain engineering strategy used to improve cytochrome $P450_{BM3}$ heme and reductase domains. HL = heme domain libraries, RL = reductase domain libraries.

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Supporting information for this article is available on the WWW under http://www.angewandte.org or from the author.

screened for activity on a propane surrogate, dimethyl ether.[8] Positives were confirmed in a re-screen, purified, and challenged with propane in sealed vials in the presence of a cofactor regeneration system. As a cumulative measure of both catalytic and coupling efficiency, improvement in total turnover (moles of propanol produced per mole of enzyme) was used as the sole selection criterion.

Measurement of the half-denaturation temperature of 35E11 heme domain demonstrated a considerable reduction in its stability as a consequence of the 15 accumulated mutations ($T_{50} = 43.4\,^{\circ}C$ vs. $55.0\,^{\circ}C$ for wild type). We therefore subjected 35E11 to an initial thermostabilization step (HL1), in which stabilizing mutations from a thermostable $P450_{BM3}$ peroxygenase[16] were tested singly and in combination in the 35E11 background (see the Supporting Information). Variant ETS8 ($\Delta T_{50} = +5.1\,^{\circ}C$, $\Delta TTN_{propane} = -1250$) showed the best combination of increased stability with little decrease in TTN and was selected for further directed evolution. Using ETS8 as parent, heme-domain random mutagenesis libraries were generated by error-prone PCR (HL2). Variant 19A12, with about twofold increase in TTN (Table 1), was then used to create a pool of active-site libraries (HL3) in which 17 positions along the substrate channel (Figure 2a) and near the active site (Figure 2b) were subjected individually to saturation mutagenesis. Further improvements in propane-hydroxylating activity were achieved in multiple variants, including 11-3. Recombination of the beneficial mutations from the active-site libraries (HL4) led to variant 1-3. Further fine-tuning of the active site was pursued with a series of recombination/site-saturation libraries (HL5, see Supporting Information). From these libraries, 7-7 emerged as the most active variant, supporting 20500 turnovers with propane.

Meanwhile, 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 5000 members from each library led to the identification of eight beneficial mutations (G443D, V445M, T480M, T515M, P654Q, T664M, D698G, and E1037G).[17] These positions were further optimized by saturation mutagenesis in a holoenzyme construct having the 11-3 heme domain (RL3, RL4). Swapping the heme domains this way serves to remove mutations whose beneficial effect is solely dependent on the presence of the 35E11 heme domain. Improved 11-3-derived variants were found to contain G443A, V445R, P654K, T664G, D698G, and E1037G mutations and showed TTN between 16000 and 20000. 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 45000 turnovers and produced 2- and 1-propanol in a 9:1 ratio. As we expected, the increase in productivity strongly correlates 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 in the hydroxylation of myristate (88%), palmitate (93%), or laurate (96%).[11]

The sequence of mutational events leading to $P450_{PMO}$ generation reveals a continuous rearrangement of substrate-channel and active-site residues (Table 1), presumably in search of an optimal configuration for accommodating propane. Additional beneficial mutations in the hydroxylase domain include L188P and G443A. Leucine 188 is located along helix F, which together with helix G forms a lid covering

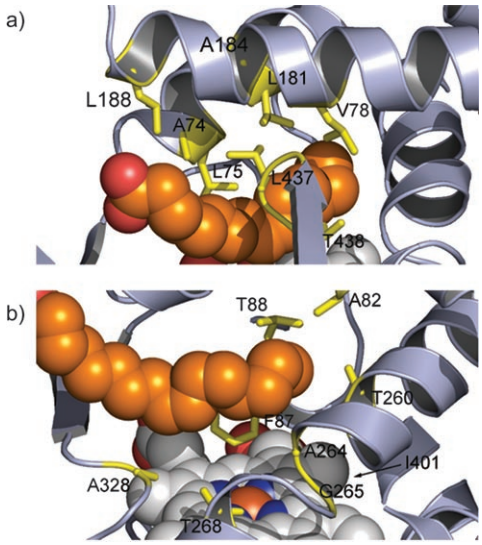

Figure 2. a) Substrate channel and b) active-site residues targeted for saturation mutagenesis mapped on the palmitate-bound structure of $P450_{BM3}$ heme domain (PDB 1FAG[26]). Heme (white) and fatty acid (orange) are shown in space-filling mode.

Table 1: In vitro propane oxidation activities of most representative $P450_{BM3}$ variants.[a]

Variant	Library	Mutations versus 35E11[b]		Rate[c] [equiv min^{-1}]	Coupling[d] [%]	Total turnovers
		heme domain	reductase domain			
35E11	–	–	–	210	17.4	5650
19A12	HL2	L52I, L188P, I366V	–	420	44.2	10550
11-3	HL3	L52I, A74S, L188P, I366V	–	390	55.3	13200
698E5	RL3	L52I, A74S, L188P, I366V	D698G	295	65.3	17300
1-3	HL4	L52I, A74S, V184A, L188P, I366V	–	320	72.1	19200
7-7	HL5	L52I, A74E, S82G, A184V, L188P, I366V	–	150	90.9	20500
$P450_{PMO}R1$	L9	L52I, A74E, S82G, A184V, L188P, I366V, G443A	P654K, E1037G	455	94.4	35600
$P450_{PMO}R2$	L9	L52I, A74E, S82G, A184V, L188P, I366V, G443A	D698G	370	98.2	45800

[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, L353V, E464G, I710T. [c] Over the first 20 s. [d] Ratio between propanol formation rate and NADPH oxidation rate in propane-saturated buffer.

the active site.[18] Glycine 443 lies on a loop at the C-terminal end of hairpin β4, which inserts into the active site.[18]

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 map in the Supporting Information). Perturbation of electrostatic charge distribution appears to be a prevailing trend, suggesting a more important role of these forces in $P450_{BM3}$ function than previously proposed.[19] A smaller contribution was obtained by mutating the FMN domain. This effect 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 (data not shown). Chemical and thermal denaturation studies have shown that, among the three cofactors, FMN is most weakly bound to the enzyme.[20]

A common strategy to reduce the prohibitive costs of NADPH-driven biotransformations is the use of cofactor regeneration systems.[9c,21] For bulk chemical transformations such as alkane hydroxylation, these in vitro approaches are not viable.[22] The propane-hydroxylating P450 variants were therefore evaluated in whole-cell biotransformations using resting *E. coli* cells (Figure 3).

The expression levels of these variants in minimal medium (initially less than 0.5% of total cell mass) were first optimized to achieve 6–11% of total cell mass as soluble P450 enzyme. Experiments were carried out in a 100-mL fermenter using cell suspensions in nitrogen-free minimal medium (supplemented with glucose) and a propane/air mixture as substrate and oxidant feed. Under these conditions, cell densities less than 1 g cdw L^{-1} (typically 0.5–0.9 g cdw L^{-1}; cdw = cell dry weight) 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 $P450_{PMO}R2$ in various *E. coli* strains (Figure 3a, Table 2). The experiment was repeated in a larger fermenter (0.3 L, pH and dissolved oxygen control). A suspension of P450-expressing DH5α cells was fed with a 1:1 mixture of pure oxygen and propane, and propanol formation was monitored for up to 9 h (Figure 3b). Under these conditions, very high activities (up to 180 U g^{-1} cdw) were obtained. In comparison, 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*).[23a]

Table 2: In vivo propane oxidation activities of $P450_{BM3}$ 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 cell density = 0.5–0.9 g cdw L^{-1}. [c] Calculated from the first hour of biotransformation.

At moderately higher cell densities (ca. 4 g cdw L^{-1}), propanol accumulated to a concentration of more than 15 mM over 4 h (Figure 4, upper panel). The improved coupling efficiencies result in considerably extended periods of whole-cell activity (6 vs. 0.5 h, Figures 3b and 4). To investigate the possible causes of the decrease in productivity over time, we monitored the biocatalyst concentration over the course of the biotransformation (Figure 4, lower panel). At the end of the experiment, approximately 52% of the

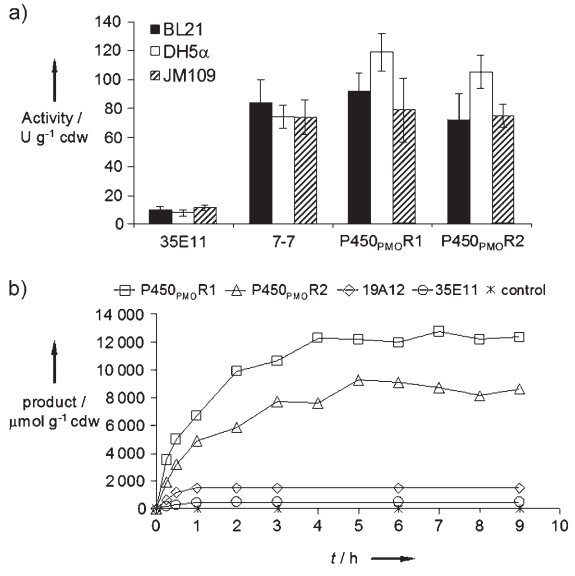

Figure 3. Whole-cell biotransformation of propane. a) Initial activities of selected $P450_{BM3}$ variants in different *E. coli* strains using air/propane (1:1) feed (pH 7.2, 25 °C). b) Time course of propane biotransformation using recombinant DH5α cells using oxygen/propane (1:1) feed (pH 7.2, 25 °C). Product amount is given per gram cell dry weight to facilitate comparison among variants. Control: no propane in the gas feed.

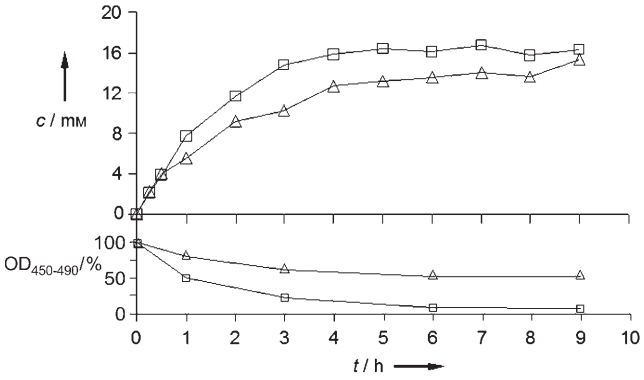

Figure 4. Concentration of propanol during biotransformation of propane with DH5α cells expressing $P450_{PMO}R1$ (□) and $P450_{PMO}R2$ (△) at medium cell density (4 g cdw L^{-1}). In the lower panel, relative P450 concentration as determined from CO-binding difference spectra on cell lysate; OD = optical density.

initial $P450_{PMO}R2$ was still correctly folded in the cells. Control experiments using $P450_{PMO}R2$-expressing cells and propanol concentrations up to 30 mM showed no product inhibition nor overoxidation to acetone, suggesting that host-related 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). Optimization of this parameter as well as the availability of more robust host strains[22] is expected to further enhance the whole-cell productivity of this engineered $P450_{BM3}$.

Overall, a domain-based directed evolution strategy has enabled us to engineer a finely-tuned, multicofactor, multidomain enzyme to exhibit nativelike 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 efficient 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[23,24] were achieved in this first report of whole-cell bioconversion of propane to propanol in *E. coli*.[25] 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.

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[1] a) M. Sono, M. P. Roach, E. D. Coulter, J. H. Dawson, *Chem. Rev.* **1996**, *96*, 2841–2888; b) I. G. Denisov, T. M. Makris, S. G. Sligar, I. Schlichting, *Chem. Rev.* **2005**, *105*, 2253–2277.

[2] V. B. Urlacher, S. Eiben, *Trends Biotechnol.* **2006**, *24*, 324–330.

[3] R. Bernhardt, *J. Biotechnol.* **2006**, *124*, 128–145.

[4] A. W. Munro, D. G. Leys, K. J. McLean, K. R. Marshall, T. W. Ost, S. Daff, C. S. Miles, S. K. Chapman, D. A. Lysek, C. C. Moser, C. C. Page, P. L. Dutton, *Trends Biochem. Sci.* **2002**, *27*, 250–257.

[5] A. J. Warman, O. Roitel, R. Neeli, H. M. Girvan, H. E. Seward, S. A. Murray, K. J. McLean, M. G. Joyce, H. Toogood, R. A. Holt, D. Leys, N. S. Scrutton, A. W. Munro, *Biochem. Soc. Trans.* **2005**, *33*, 747–753.

[6] a) T. W. Ost, C. S. Miles, J. Murdoch, Y. Cheung, G. A. Reid, S. K. Chapman, A. W. Munro, *FEBS Lett.* **2000**, *486*, 173–177; b) W. T. Sulistyaningdyah, J. Ogawa, Q. S. Li, C. Maeda, Y. Yano, R. D. Schmid, S. Shimizu, *Appl. Microbiol. Biotechnol.* **2005**, *67*, 556–562; c) D. Appel, S. Lutz-Wahl, P. Fischer, U. Schwaneberg, R. D. Schmid, *J. Biotechnol.* **2001**, *88*, 167–171; d) A. Glieder, E. T. Farinas, F. H. Arnold, *Nat. Biotechnol.* **2002**, *20*, 1135–1139; e) Q. S. Li, J. Ogawa, R. D. Schmid, S. Shimizu, *FEBS Lett.* **2001**, *508*, 249–252; f) T. Kubo, M. W. Peters, P. Meinhold, F. H. Arnold, *Chem. Eur. J.* **2006**, *12*, 1216–1220.

[7] a) Q. Li, J. Ogawa, R. D. Schmid, S. Shimizu, *Appl. Environ. Microbiol.* **2001**, *67*, 5735–5739; b) A. B. Carmichael, L. L. Wong, *Eur. J. Biochem.* **2001**, *268*, 3117–3125.

[8] M. W. Peters, P. Meinhold, A. Glieder, F. H. Arnold, *J. Am. Chem. Soc.* **2003**, *125*, 13442–13450.

[9] a) S. Schneider, M. G. Wubbolts, G. Oesterhelt, D. Sanglard, B. Witholt, *Biotechnol. Bioeng.* **1999**, *64*, 333–341; b) R. J. Sowden, S. Yasmin, N. H. Rees, S. G. Bell, L. L. Wong, *Org. Biomol. Chem.* **2005**, *3*, 57–64; c) J. R. Falck, K. Y. Reddy, D. C. Haines, K. M. Reddy, U. M. Krishna, S. Graham, B. Murry, J. A. Peterson, *Tetrahedron Lett.* **2001**, *42*, 4131–4133; d) S. C. Maurer, K. Kuhnel, L. A. Kaysser, S. Eiben, R. D. Schmid, V. B. Urlacher, *Adv. Synth. Catal.* **2005**, *347*, 1090–1098. Among these, only two examples involve a nonnative substrate, with the highest TTN (12850) achieved by Maurer et al. on cyclohexane over a 100-h period using an isolated $P450_{BM3}$ variant, a cofactor regeneration system, and a cyclohexane/buffer biphasic system.

[10] a) D. C. Haines, D. R. Tomchick, M. Machius, J. A. Peterson, *Biochemistry* **2001**, *40*, 13456–13465; b) M. B. Murataliev, R. Feyereisen, *Biochemistry* **1996**, *35*, 15029–15037.

[11] a) M. A. Noble, C. S. Miles, S. K. Chapman, D. A. Lysek, A. C. Mackay, G. A. Reid, R. P. Hanzlik, A. W. Munro, *Biochem. J.* **1999**, *339*, 371–379; b) M. J. Cryle, R. D. Espinoza, S. J. Smith, N. J. Matovic, J. J. De Voss, *Chem. Commun.* **2006**, 2353–2355.

[12] a) P. J. Loida, S. G. Sligar, *Biochemistry* **1993**, *32*, 11530–11538; b) S. Kadkhodayan, E. D. Coulter, D. M. Maryniak, T. A. Bryson, J. H. Dawson, *J. Biol. Chem.* **1995**, *270*, 28042–28048.

[13] a) J. A. Labinger, J. E. Bercaw, *Nature* **2002**, *417*, 507–514; b) G. B. Shul'pin, G. Suss-Fink, L. S. Shul'pina, *J. Mol. Catal. A* **2001**, *170*, 17–34; c) I. Yamanaka, S. Hasegawa, K. Otsuka, *Appl. Catal. A* **2002**, *226*, 305–315.

[14] P. Meinhold, M. W. Peters, M. M. Chen, K. Takahashi, F. H. Arnold, *ChemBiochem* **2005**, *6*, 1765–1768; Wong and coworkers have recently reported an engineered P450cam for in vitro hydroxylation of propane and ethane (F. Xu, S. G. Bell, J. Lednik, A. Insley, Z. Rao, L.-L. Wong , *Angew. Chem.* **2005**, *117*, 4097–4100; *Angew. Chem. Int. Ed.* **2005**, *44*, 4029–4032) using a reconstituted redox system containing $P450_{cam}$/putidaredoxin/putidaredoxin reductase in a 1:16:1 ratio.

[15] a) S. Govindaraj, T. L. Poulos, *Biochemistry* **1995**, *34*, 11221–11226; b) O. Roitel, N. S. Scrutton, A. W. Munro, *Biochemistry* **2003**, *42*, 10809–10821.

[16] O. Salazar, P. C. Cirino, F. H. Arnold, *ChemBiochem* **2003**, *4*, 891–893.

[17] Residues G443 and V445 are in the heme domain. The region targeted for construction of FMN libraries comprises 32 residues located at the C-terminus of $P450_{BM3}$ heme domain, which are not included in the heme domain libraries (see the Supporting Information).

[18] O. Pylypenko, I. Schlichting, *Annu. Rev. Biochem.* **2004**, *73*, 991–1018.

[19] D. R. Davydov, A. A. Kariakin, N. A. Petushkova, J. A. Peterson, *Biochemistry* **2000**, *39*, 6489–6497.

[20] A. W. Munro, J. G. Lindsay, J. R. Coggins, S. M. Kelly, N. C. Price, *Biochim. Biophys. Acta Protein Struct. Mol. Enzymol.* **1996**, *1296*, 127–137.

[21] a) U. Schwaneberg, C. Otey, P. C. Cirino, E. Farinas, F. H. Arnold, *J. Biomol. Screening* **2001**, *6*, 111–117; b) S. C. Maurer, H. Schulze, R. D. Schmid, V. B. Urlacher, *Adv. Synth. Catal.* **2003**, *345*, 802–810.

[22] W. A. Duetz, J. B. van Beilen, B. Witholt, *Curr. Opin. Biotechnol.* **2001**, *12*, 419–425.

[23] a) I. E. Staijen, J. B. Van Beilen, B. Witholt, *Eur. J. Biochem.* **2000**, *267*, 1957 – 1965; b) T. Fujii, T. Narikawa, K. Takeda, J. Kato, *Biosci. Biotechnol. Biochem.* **2004**, *68*, 2171 – 2177; c) M. Kubota, M. Nodate, M. Yasumoto-Hirose, T. Uchiyama, O. Kagami, Y. Shizuri, N. Misawa, *Biosci. Biotechnol. Biochem.* **2005**, *69*, 2421 – 2430.

[24] a) T. Furuto, M. Takeguchi, I. Okura, *J. Mol. Catal. A* **1999**, *144*, 257 – 261; b) S. G. Lee, J. H. Goo, H. G. Kim, J.-I. Oh, Y. M. Kim, S. W. Kim, *Biotechnol. Lett.* **2004**, *26*, 947 – 950.

[25] Previous whole-cell bioconversions of gaseous alkanes have relied on partial inhibition (i.e. blockage of methanol dehydrogenase activity) of methanotrophic bacteria carrying multicomponent nonheme methane monooxygenases.[24] Accumulations of 5–8 mM methanol over periods of 40 h were reported.

[26] H. Li, T. L. Poulos, *Nat. Struct. Biol.* **1997**, *4*, 140 – 146.

