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# Engineering Cytochrome P450 BM3 for Terminal Alkane Hydroxylation

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**Abstract:** Enzymes that catalyze the terminal hydroxylation of alkanes could be used to produce more valuable chemicals from hydrocarbons. Cytochrome P450 BM3 from *Bacillus megaterium* hydroxylates medium-chain fatty acids at subterminal positions at high rates. To engineer BM3 for terminal alkane hydroxylation, we performed saturation mutagenesis at selected active-site residues of a BM3 variant that hydroxylates alkanes. Recombination of beneficial mutations generated a library of BM3 mutants that hydroxylate linear alkanes with a wide range of regioselectivities. Mutant 77-9H exhibits 52% selectivity for the terminal position of octane. This regioselectivity is octane-specific and does not transfer to other substrates, including shorter and longer hydrocarbons or fatty acids. These results show that BM3 can be readily molded for regioselective oxidation.

**Keywords:** alkanes; biocatalysis; cytochrome P450 BM3; directed evolution; high throughput screening; terminal hydroxylation

## Introduction

Efficient routes for regio- and stereoselective hydroxylation of non-activated carbon atoms are sought after for production of higher value chemicals from alkanes.[1] Synthetic catalysts are plagued by low selectivity leading to overoxidation[2] and often require harsh reaction conditions. Enzymes, in contrast, can catalyze the selective oxidation of alkanes to alcohols at room temperature and pressure, using dioxygen or peroxide as the oxidant and producing no side products other than water. The first step of alkane degradation by alkane-assimilating microorganisms involves selective hydroxylation of the alkane at the terminal carbon. Biochemical and genetic studies have focused on a limited number of enzymes that catalyze this reaction,[3] the best known of which is the *Pseudomonas putida* GPo1 alkane hydroxylase.[4] Enabling growth on $C_6$ to $C_{12}$ *n*-alkanes, this enzyme system consists of an alkane hydroxylase (AlkB), rubredoxin (AlkG), and rubredoxin reductase (AlkT). AlkG and AlkT transfer electrons to AlkB, a non-heme diiron integral membrane protein that carries out the hydroxylation reaction[5–7] at the terminal alkane carbon exclusively, at rates of ~200 $min^{-1}$.[8]

Cytochromes P450 catalyze the insertion of oxygen into a wide variety of substrates, including *n*-alkanes, *via* a reactive iron-oxo species chelated by a porphyrin. Some members of the CYP4[9,10] CYP52,[11–14] CYP86,[15] and CYP153 families[16] hydroxylate terminal methyl groups of fatty acids or *n*-alkanes. Like AlkB, these enzymes are membrane-bound and contain multiple protein components, which complicates their structural and kinetic characterization and makes them difficult to use in engineered pathways. (Only recently has a soluble, terminal alkane hydroxylating cytochrome P450, classified as CYP153A6, been reported.[17]) The reported rates on alkanes are relatively low. Alkane oxidation by CYP52A3 from alkane-assimilating *Candida maltosa*, for example, proceeds at a rate of 27 $min^{-1}$ for hexadecane.[14] Similar rates have been reported for mammalian P450s such as CYP4B1 (33 $min^{-1}$, 26 $min^{-1}$, 31 $min^{-1}$, 11 $min^{-1}$, 67 $min^{-1}$ for heptane, octane, nonane, decane and 4-methylheptane, respectively).[10] No crystal structures are available for any of these P450s.

   

Cytochrome P450 BM3 (CYP102A1) from *Bacillus megaterium* hydroxylates medium-chain ($C_{12}$ to $C_{20}$) fatty acids at extraordinarily high rates of up to 17,100 $min^{-1}$.[18] Oxygen is inserted at subterminal (ω-1, ω-2, and ω-3) positions in approximately equal amounts (the exact regioselectivity varies with the chain length of the substrate).[19] BM3 is a soluble enzyme whose hydroxylase and reductase domains are contained in a single polypeptide. Expressed in functional form at high levels in *Escherichia coli*, this enzyme can be incorporated into engineered pathways for whole cell biotransformations[20] or can be used *in vitro* for selective oxidations.[21,22] Attracted by the possibility of transferring the high rate of fatty acid hydroxylation to other substrates, various groups have engineered BM3 substrate specificity[23–28] as well as regio- and enantioselectivity.[21,29–31] In previous work, our group converted BM3 into a fast and productive alkane hydroxylase with specificity for different subterminal carbons.[21,32,33] To extend the spectrum of BM3-catalyzed reactions we wanted to engineer BM3 for terminal alkane hydroxylation.

Protein engineering to obtain high selectivity for terminal hydroxylation is made challenging by the fact that BM3 undergoes large conformational changes during catalysis[34] that are not fully captured by studying the crystal structures of the heme domain in its substrate-bound and substrate-free forms.[35–39] Directed evolution involving sequential rounds of mutagenesis and high throughput screening has therefore been our method of choice. Laboratory-evolved BM3 alkane hydroxylase mutant 9-10A[21] contains 14 mutations in the heme domain, only one of which, V78A, is located in the active site. We recently described two other active-site mutations, A82L and A328V, that shifted the regioselectivity of alkane hydroxylation predominantly to a single, subterminal (ω-1) position.[21] Prompted by these results, we decided to introduce further mutations in the active site in order to achieve the desired selectivity for terminal hydroxylation. Starting with BM3 mutant 9-10A, we performed saturation mutagenesis on eleven residues that should be in close proximity to the substrate. We assayed these mutants using a screen that is sensitive to the regioselectivity of hydroxylation. Beneficial mutations were recombined to generate a library of ~9,000 functional BM3 mutants, from which mutants exhibiting a range of regioselectivities for alkane hydroxylation have been isolated.

## Results and Discussion

### Screening for Regioselective Alkane Hydroxylation

Screening enzyme libraries for alkane hydroxylation is challenging because the desired (alcohol) products are colorless and are formed at concentrations too low to detect by their *in situ* chemical conversion to dye-sensitive compounds such as aldehydes and ketones. Spectroscopic monitoring of NADPH consumption rates in the presence of the alkane is not useful either, because it often selects for mutants with NADPH oxidation rates that are uncoupled to product formation. We circumvented these issues in previous work by using surrogate substrates that mimic desired substrates but yield products that are more easily detected. For example, we used dimethyl ether (DME) to screen for mutants more active on propane.[21] Here we chose hexyl methyl ether (HME) as a surrogate for octane. HME shares most of the physical properties of octane, including solubility in buffer, size, shape, and C–H bond strength. This screen is also sensitive to terminal hydroxylation. As shown in Figure 1, hydroxylation at the HME methoxy carbon produces an equivalent of formaldehyde, which is detected at concentrations as low as 10 μM with the dye Purpald.[40] Hydroxylation at the α-carbon, on the other hand, produces hexanal, which is practically unreactive towards Purpald at the concentrations encountered in the screens (formaldehyde is approximately 42 times more sensitive to Purpald than is hexanal). Because using a surrogate screen always runs the risk of identifying mutants that are more active on the surrogate but not the desired substrate, it is important to verify that activity on the surrogate (HME) correlates with activity on the desired substrate (octane) (see below).

### Saturation Mutagenesis of Cytochrome P450 BM3 9-10A and Recombination of Beneficial Mutations

Changing substrate specificity or regioselectivity often requires multiple mutations within the active site, some of which may be coupled. Such mutations are not likely to be found by random mutagenesis of the full protein.[41,42] We therefore used the BM3 heme domain crystal structures with the bound substrates palmitoleic acid[36] and *N*-palmitoylglycine[38] to identify 11 residues with side chains that are within 5 Å of the terminal eight carbons of the alkyl chains of the bound substrates: A74, L75, V78, F81, A82, F87, T88, T260, I263, A264, and A328. Mutations at six of these positions (74, 78, 82, 87, 264, and 328) were previously known to influence the activity of BM3. For example, changes to F87 in wild-type BM3 affect the distributions of hydroxylated fatty acid products[30,43–45] and activity towards non-natural substrates.[29] Residues at positions 74, 87, and 264 have been altered in combination with other mutations to increase the activity of BM3 towards a variety of substrates, including octane and polyaromatic compounds.[23,25,26,46,47] Mutation of A328 to valine in the wild-type enzyme has been reported to affect substrate binding and activity on fatty acids.[48] We generated 11 saturation mutagenesis libraries in which all twenty amino acids were introduced at each of the 11 identi-

  

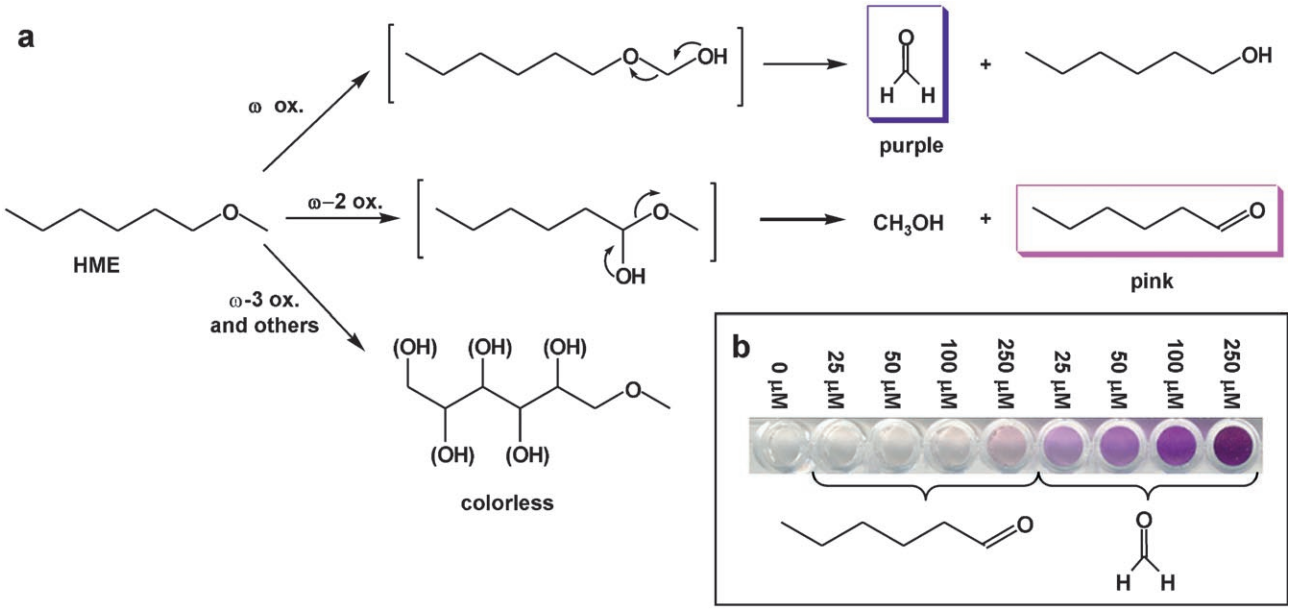


**Figure 1. a)** Screening for terminal alkane hydroxylation using hexyl methyl ether (HME). Terminal (ω) oxidation of HME results in the formation of formaldehyde, which turns dark purple in the presence of Purpald. Hydroxylation of the ω-2 carbon forms hexanal, which turns pink upon reaction with Purpald. Other hydroxylation products do not react with Purpald. **b)** The Purpald assay is much more sensitive to formaldehyde than to hexanal.

fied positions in 9-10A. From each library, 174 clones were screened for activity towards DME and HME.

Mutations that improved activity towards HME or DME were found at seven positions: L75 (W), A78 (T, F), A82 (T, S, F, I, C, G), F87 (I, V, L), T88 (C), T260 (N, L) and A328 (L, F, M) (see Table S1 in Supporting Information). The improved enzymes exhibited different levels of activity and regio- and enantioselectivity for hydroxylation of octane (Table 1). The primary products are 2-, 3-, and 4-octanol, but limited amounts of 1-octanol are also produced (typically $<10\%$ of the total products). Only mutations at position 328 lead to fractions of 1-octanol higher than 10%: A328L and A328M produce 13 and 24% 1-octanol, respectively.

The beneficial mutations of Table 1 and the wild-type residues at these positions were recombined by overlap extension PCR with degenerate primers or a mixture of primers to generate a library containing all possible combinations of these mutations. This relatively involved strategy was favored over other DNA recombination techniques such as DNA shuffling,[49] because it does not bias recombination events towards those separated by longer stretches of DNA. Mutation L75I, which affects regioselectivity but not activity, was also included in the recombination, as were previously known beneficial mutations A82L and A328V.[21] Mutation A82V was included as well, due to constraints in the design of the degenerate primer used to construct the library. The resulting recombination library contains approximately 9,000 different mutants of P450 BM3, each of which contains a characteristic active site. More than 70% of these were active towards DME or HME (data not shown). A total of 16 mutants with improved activity on DME or HME compared to the best BM3 alkane hydroxylase to date, mutant 1-12G,[21] were selected for purification and further characterization. The active-site amino acid substitutions and activities on DME and HME for four of these mutants are presented in Table 2.

## Correlation between Activity on HME and Terminal Hydroxylation of Octane

Rates of product formation, total turnover numbers (ttn), and regioselectivities for hydroxylation of octane were determined for the mutants in Table 2. The total turnover number describes the number of turnovers the enzyme catalyzes before it is inactivated. Rates and mechanism of inactivation were not further examined for this report.

Mutant 53-5H, which shows increased activity on DME but not on HME (Table 2), exhibits the least terminal hydroxylation (3% of the product is 1-octanol) but produces 2-octanol with high selectivity (87%), similar to 1-12G. The mutants that show increased HME activity exhibit a range of terminal hydroxylation capabilities, with 1-octanol accounting for as much as 52% of the product for 77-9H. In general, mutants exhibiting high total turnover on HME in the screen also produced more 1-octanol. The total amount of 1-octanol formed (% 1-octanol $\times$ ttn) correlates with the amount of HME converted for the different BM3 mutants (Fig-

  

**Table 1.** Regioselectivities, rates of product formation and total turnover numbers of octane hydroxylation reactions catalyzed by variants of cytochrome P450 BM-3 9-10A containing single active-site mutations.[a]

| Mutation[b] | 1-ol [%] | 2-ol [%] | 3-ol [%] | 4-ol [%] | Others[c] | Rate[d] | ttn[e] |
|---|---|---|---|---|---|---|---|
| **(9-10A)** | **1** | **51** | **21** | **26** | **1** | **540** | **3000** |
| L75I | 3 | 42 | 18 | 17 | 20 | 210 | 4500 |
| L75W | 3 | 6 | 18 | 24 | 9 | 200 | 4000 |
| A78T | 4 | 22 | 27 | 45 | 2 | 500 | 4000 |
| A78F | 4 | 37 | 17 | 31 | 11 | 380 | 3000 |
| A78S | 5 | 48 | 18 | 20 | 9 | 260 | 2500 |
| A82T | 5 | 23 | 25 | 44 | 3 | 340 | 2700 |
| A82S | 6 | 40 | 19 | 26 | 9 | 300 | 2600 |
| A82F | 4 | 26 | 20 | 48 | 2 | 140 | 3000 |
| A82I | 4 | 11 | 21 | 59 | 5 | 600 | 3500 |
| A82C | 5 | 26 | 26 | 42 | 1 | 200 | 3000 |
| A82G | 7 | 52 | 17 | 23 | 1 | 360 | 2000 |
| F87I | 8 | 70 | 6 | 3 | 13 | 60 | 2600 |
| F87V | 5 | 52 | 13 | 4 | 26 | 240 | 3600 |
| F87L | 7 | 55 | 22 | 12 | 4 | 60 | 2300 |
| T88C | 4 | 51 | 21 | 22 | 2 | 460 | 4000 |
| T260L | 7 | 67 | 11 | 10 | 5 | 260 | 2500 |
| T260S | 6 | 39 | 26 | 29 | 0 | 160 | 3000 |
| T260N | 6 | 29 | 23 | 42 | 0 | 300 | 2500 |
| A328F | 6 | 88 | 4 | 0 | 2 | 40 | 2800 |
| A328M | 24 | 71 | 0 | 0 | 5 | 10 | 700 |
| A328L | 13 | 87 | 0 | 0 | 0 | 20 | 1000 |

[a] Transformations were carried out using cell lysates. Refer to Experimental section for reaction conditions.
[b] Mutations are in variant 9-10A (with amino acid mutations R47C, V78A, K94I, P142S, T175I, A184V, F205C, S226R, H236Q, E252G, R255S, A290V, L353V).
[c] Overoxidation products (ketones and aldehydes) with a similar product distribution as the alcohols.
[d] Initial rates of product formation were measured by GC over 60 s and reported as nmol total products/min/nmol protein. The reactions contained P450 (100 nM), NADPH (500 μM), and octane (4 mM) in ethanol (1%) and potassium phosphate buffer (0.1 M, pH 8.0). Errors are at most 10%.
[e] Total turnover numbers are reported as nmol product/nmol protein. The reactions contained P450 (25 nM), NADPH (500 μM), and octane (4 mM) in ethanol (1%) and potassium phosphate buffer (0.1 M, pH 8.0). The reactions were allowed to proceed for 4 hours, well past the lifetime of the enzymes under these conditions. Errors are at most 10%.

**Table 2.** Mutations[a] in selected cytochrome P450 BM3 mutants made by recombining active-site mutations and relative activities towards dimethyl ether (DME) and hexyl methyl ether (HME). Product distributions,[b] rates and total turnover numbers (ttn) of octane hydroxylation are reported.

| Mutant | A78 | A82 | F87 | A328 | DME[c] | HME[c] | 1-ol [%] | 2-ol [%] | 3-ol [%] | 4-ol [%] | ttn[d] | Rate[e] [$min^{-1}$] |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 9-10A | | | | | 0.6 | 0.5 | 0 | 51 | 21 | 27 | 3000 | 540 |
| 1-12G | | L | | V | 1.0 | 1.0 | 3 | 79 | 11 | 4 | 7500 | 150 |
| 77-9H | T | G | | L | 0.9 | 1.5 | 52 | 45 | 3 | 0 | 1300 | 160 |
| 68-8F | F | G | | L | 1.9 | 1.2 | 31 | 58 | 8 | 2 | 1400 | 180 |
| 13-7C | T | | | L | 1.4 | 1.1 | 17 | 74 | 8 | 1 | 2600 | 310 |
| 53-5H | F | S | | F | 1.4 | 0.7 | 3 | 87 | 5 | 1 | 7400 | 660 |

[a] Mutations were made in parent 9-10A which contains amino acid substitutions R47C, V78A K94I, P142S, T175I, A184V, F205C, S226R, H236Q, E252G, R255S, A290V, and L353V.
[b] Product distribution determined as ratio of a specific alcohol product to the total amount of alcohol products (given in %). Errors are at most 3%. The formation of ketones was also observed but is less than 10% of the total amount of product.
[c] Activities for terminal hydroxylation of dimethyl ether and hexyl methyl ether are reported relative to variant 1-12G.
[d] Measured after 4 hours and reported as nmole octanols/nmole P450 produced (as described in Table 1).
[e] Initial rates, in nmole octanol/nmole P450/min as described in Table 1.

ure 2), showing that activity on HME is a good surrogate for terminal hydroxylation of octane. Mutant 77-9H, for example, although not improved relative to 1-12G in terms of overall rate or ttn on octane, is highly active towards HME in the screen due to its propensity for terminal hydroxlylation.

  

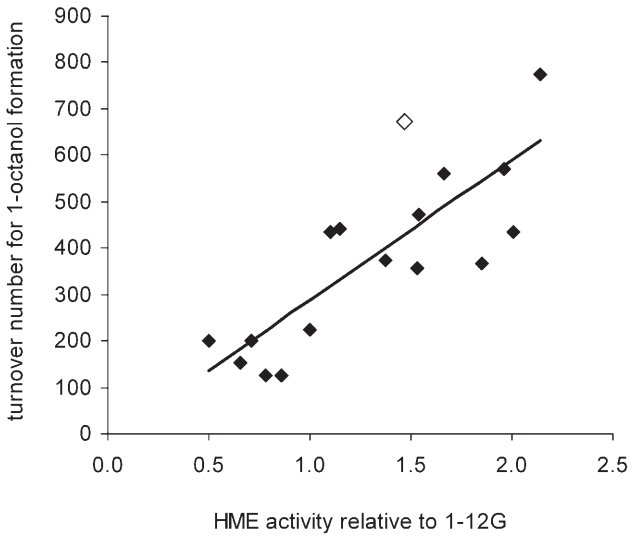


**Figure 2.** The amount of 1-octanol formed during an octane hydroxylation reaction correlates with activity on HME measured during screening ($R^2 = 0.64$). Mutant 77-9H is depicted with ◇.

**Table 3.** Product distributions[a] for alkane hydroxylation catalyzed by cytochrome P450 BM3 77–9H.

| Substrate | 1-ol [%] | 2-ol [%] | 3-ol [%] | 4-ol [%] | 5-ol [%] |
|---|---|---|---|---|---|
| hexane | 11.4 | 83.8 | 4.9 | | |
| heptane | 21.3 | 74.3 | 4.2 | 0.2 | |
| octane | 51.8 | 45.1 | 2.8 | 0.3 | |
| nonane | 9.7 | 88.1 | 1.5 | 0.7 | 0.0 |
| decane | 5.5 | 91.8 | 2.0 | 0.7 | 0.0 |

[a] Product distribution determined as ratio of a specific alcohol product to the total amount of alcohol products (given in %). Errors are at most 3%. The formation of ketones was also observed but is less than 10% of the total amount of product.

Variants other than 77-9H also exhibited increased activity on HME (Table 2). None has the selectivity of 77-9H for terminal hydroxylation and instead favors conversion of octane into 2-octanol. Because the total amount of 1-octanol formed correlates with the signal intensity in the HME screen, an enzyme that catalyzes 10× more total turnovers than 77-9H but produces only one-tenth the fraction of 1-octanol would yield approximately the same signal in the screen. In fact, the enzymes with the highest regioselectivities for 2-octanol were also the most active in terms of ttn (data not shown).

## Regioselectivity of Mutant 77-9H on n-Alkanes

Mutant 77-9H with the highest selectivity for terminal hydroxylation was characterized in reactions with alkanes of chain length $C_6$ to $C_{10}$. Product distributions summarized in Table 3 show that mutant 77-9H hydroxylates only octane primarily at the terminal position. Alkanes shorter or longer than octane are hydroxylated mainly at the 2-position.

Wild-type BM3 hydroxylates lauric and palmitic acid at subterminal positions. Lauric acid is hydroxylated mainly (48%) at the ω-1 position, while palmitic acid is hydroxylated at the ω-2 position (48%).[19] On fatty acids, mutant 77-9H shows a similar trend, with selectivity for specific subterminal positions (data not shown). We altered residues that contact the terminal eight carbons of the substrate based on the crystal structure with bound palmitoylglycine.[38] Amino acid residues outside this region have not been modified, particularly those interacting with the carboxyl moiety, and we might expect changes in regioselectivity towards fatty acids to be less dramatic than with alkane substrates.

## Total Turnover Numbers, Rates and Coupling Efficiency

Total turnover numbers and rates of product formation were measured for hydroxylation of the different alkanes by 77-9H (Table 4). Rates of product formation range from 112 $min^{-1}$ (on heptane) to 742 $min^{-1}$ (on nonane), similar to that reported for AlkB on octane (200 $min^{-1}$).[8] The rates of cytochromes P450 that carry out terminal hydroxylation reactions are an order of magnitude lower than those of 77-9H.[10,14] Wild-type BM3 also hydroxylates alkanes at similarly low rates (e.g., 30 $min^{-1}$ for octane[28]).

To determine ttn, the enzyme was diluted to a concentration at which the P450 is neither oxygen-limited nor inactivated (at low enzyme concentrations, a decrease in activity is observed which is likely due to dialysis of the flavin cofactors, a possible inactivation mechanism at low P450 concentrations[50]). The ttns and rates of hydroxylation were found to be substrate-dependent and generally increase with the alkane's chain length. The observed decrease in ttn and rate for decane hydroxylation might be an artefact of the limited solubility of decane in the reaction buffer (~0.4 mM in water with 2% ethanol).[51]

What limits the ttn of 77-9H is currently not understood. It is well known that unproductive dissociation of reduced oxygen species can lead to the formation of superoxide or peroxide, which inactivate the enzyme. In fact, the enzyme exhibits the most turnovers on nonane, the substrate for which the coupling efficiency is the highest (66%, Table 4).

Wild-type P450 BM3 tightly regulates electron transfer from the cofactor (NADPH) to the heme. In the absence of substrate, a weakly-bound water molecule acts as the sixth, axial ligand of the heme iron. Substrate replaces this water molecule, perturbing the spin-state

  

**Table 4.** Total turnovers, product formation rates, NADPH oxidation rates, and coupling efficiencies for alkane hydroxylation catalyzed by cytochrome P450 BM3 mutant 77-9H.

| Substrate | ttn[a] | Rate of product formation [$min^{-1}$][b] | Rate of NADPH oxidation [$min^{-1}$][c] | Coupling efficiency [%][d] |
|---|---|---|---|---|
| hexane | 1800 ± 130 | 251 ± 29 | 1560 ± 60 | 16.1 ± 2.0 |
| heptane | 1730 ± 110 | 112 ± 9 | 1630 ± 20 | 6.9 ± 0.6 |
| octane | 3040 ± 150 | 236 ± 24 | 1630 ± 30 | 14.5 ± 1.7 |
| nonane | 4240 ± 680 | 742 ± 30 | 1150 ± 100 | 65.6 ± 9.3 |
| decane | 2831 ± 225 | 318 ± 47 | 940 ± 50 | 33.4 ± 7.2 |

[a] Total turnover numbers determined as nmol product/nmol protein. The reactions contained P450 (25 nM), an NADPH regeneration system (166 μM $NADP^+$, 6.6 U/mL isocitrate dehydrogenase, 41.6 mM isocitrate) and octane (4 mM) in ethanol (2%) and potassium phosphate buffer (0.1 M, pH 8.0).

[b] Rates of product formation were measured by GC over 20 s as nmol total products/min/nmol protein. The reactions contained P450 (200 nM), NADPH (500 μM) and octane (2 mM) in ethanol (2%) and potassium phosphate buffer (0.1 M, pH 8.0).

[c] Rates of NADPH oxidation were measured over 20 s at 340 nm as nmol NADPH/min/nmol protein. The reactions contained P450 (200 nM), NADPH (160 μM), and octane (2 mM) in ethanol (2%) and potassium phosphate buffer (0.1 M, pH 8.0). The background NADPH oxidation rate (without substrate) rate was 220/min.

[d] Coupling efficiency is the ratio of product formation rate to NADPH oxidation rate.

equilibrium of the heme iron in favor of the high-spin form and also increases the heme iron reduction potential by approximately 130 mV.[52,53] These events trigger electron transfer to the substrate-bound heme and start the catalytic cycle. However, with mutant BM3 enzymes and non-natural substrates, NADPH consumption is not necessarily coupled to the formation of product. Instead, electrons from NADPH reduce heme-bound dioxygen to water or to peroxide and superoxide.[50] To determine the coupling efficiency of mutant 77-9H, rates of NAPDH oxidation upon addition of the alkane substrates were measured and compared to product formation rates (Table 4). The rate of product formation is correlated to coupling efficiency, i.e., the more the reaction is coupled to NADPH oxidation, the higher is the rate of product formation.

## Engineering the Active Site of P450 BM3 for Selective Substrate Oxidation

Linear alkanes are challenging to hydroxylate selectively because they contain no functional groups that help fix their orientation with respect to the reactive heme iron-oxo group. Model studies with metalloporphyrins show that the regioselectivity of *n*-alkane hydroxylation is determined by the relative C–H bond dissociation energies if access to the reactive oxygen species is not restricted.[54,55] Engineering a P450 for terminal hydroxylase activity must therefore override the inherent specificity of the catalytic species for the methylene groups so that only the terminal methyl carbon is activated.

Wild-type cytochrome P450 BM3 hydroxylates its preferred substrates, medium-chain fatty acids, at subterminal positions. The terminal carbon is not hydroxylated.[56] P450 BM3 mutant F87A was reported to support ω-hydroxylation of lauric acid,[43] although more recent work showed that F87A broadens regioselectivity and shifts hydroxylation away from the terminal position.[45,57] The F87A substitution in 9-10A does not lead to terminal hydroxylation activity on alkanes (data not shown).

By directing mutations to 11 residues in the active site, we generated a set of P450 BM3 mutants that exhibit a range of regioselectivities for alkane hydroxylation. Single mutations within the active site mainly result in relatively small changes in the distribution of the alcohol products (Table 1). When these mutations are combined, however, more dramatic changes are observed (Table 2). Mutant 77-9H, whose activity on HME is even higher than that of 1-12G, has three mutations in the active site, A78T, A82G, and A328L. All three mutations individually increase the fraction of terminal hydroxylation (Table 1). Mutation A328L exerts the most dramatic effect in 9-10A, increasing the terminally hydroxylated product from 1% to 13%. In contrast, A78T produces predominantly 4-octanol (with 4% 1-octanol), while A82G produces 7% 1-octanol. In combination, mutations A78T and A328L (mutant 13-7C in Table 2) increase terminal hydroxylation of octane to 17%. Addition of A82G further increases the selectivity for terminal hydroxylation, to 52% in 77-9H.

Mutant 77-9H exhibits terminal hydroxylation activity only for octane. Other alkane substrates, in comparison, are hydroxylated primarily at the subterminal position. For nonane and decane, the 2-alcohols account for ~90% of the products. The active site of 77-9H is therefore not restricted near the activated oxygen to prevent subterminal hydroxylation in general. The specificity for the terminal methyl group of octane probably reflects specific interactions between active site residues and octane methylene groups and/or the methyl group at the

  

unreacted end. The hexyl methyl ether substrate used for screening terminal hydroxylation activity has the same chain length as octane. The mutations that enable terminal hydroxylation of HME likely act in a similar fashion to promote terminal hydroxylation of octane. This result is fully consistent with the first law of directed evolution: "you get what you screen for."[58]

## Conclusion

The BM3 active-site mutants described here also selectively oxidize substrates other than alkanes and fatty acids. We recently reported enantioselective terminal alkene epoxidation[31] as well as regio- and enantioselective hydroxylation of 2-cyclopentylbenzoxazole,[22] yielding potentially valuable intermediates for chemical synthesis. Mutant 53-5H, which exhibits the highest regioselectivity for octane hydroxylation (Table 2), selectively oxidizes ethane to ethanol, an activity unknown in naturally-occurring P450s.[28] These findings demonstrate that the BM3 active site can be efficiently molded for enantio- and regioselective oxidation of a wide range of substrates. The effects of mutations, however, are hard to predict. Because the BM3 active site is highly amenable to mutation, focusing mutagenesis to the active site and identifying improved mutants using high throughput screening or selection systems will continue to be an effective strategy for engineering selective BM3 catalysts.

## Addendum

While this paper was under review, Urlacher and collaborators reported an engineered homologue of P450 BM3 (CYP102A3 from *Bacillus subtilis*) with terminal octane hydroxylation activity.[59] None of the mutations in the CYP102A3 variant are equivalent to those of 77-9H.

## Experimental Section

### General Remarks

All liquid alkanes and product standards and solvents, as well as isocitrate and isocitrate dehydrogenase were purchased from Sigma-Aldrich, Inc. (St. Louis, MO). Propane and dimethyl ether were purchased from Special Gas Services (Warren, NJ). Hexyl methyl ether was purchased from TCI America (Portland, OR). NADPH and $NADP^+$ were obtained from Biocatalytics, Inc. (Pasadena, CA). Enzymes for molecular biology experiments were purchased from Invitrogen (T4 DNA ligase), Roche (BamHI, SacI) and Stratagene (Pfu turbo DNA polymerase)

### Expression and Purification of P450 BM3

The genes encoding P450 BM3 mutants described in this report were expressed and purified as described previously.[21] Enzyme concentrations were determined in triplicate from the CO-difference spectra.[60]

### Construction of Saturation Mutagenesis Libraries

Mutations were introduced into cytochrome P450 BM3 mutant 9-10A[21] by PCR overlap extension mutagenesis using Pfu turbo DNA polymerase.[61] The following positions were chosen for saturation mutagenesis: A74, L75, A78, F81, A82, F87, T88, T260, L263, I264 and A328. The primers for each saturation library contained all possible combinations of bases, NNN (N = A, T, G, or C), at the codon for a particular residue. The primers in the forward direction for each library were: 74NNNfor (5′-GTCAANNNCTTAAATTTGCACG-3′); 75NNNfor (5′-GTCAAGCGNNNAAATTTGCACG-3′); 78NNNfor (5′-GCTTAAATTTNNNCGTGATTTTGCAGG-3′); 81NNNfor (5′-CGTGATNNNGCAGGAGAC-3′); 82NNNfor (5′-CGTGATTTTNNNGGAGAC-3′); 87NNNfor (5′-GAGACGGGTTANNNACAAGCTGGAC-3′); 88NNNfor (5′-GGAGACGGGTTATTTNNNAGCTGGACG-3′); 260NNNfor (5′-CAAATTATTNNNTTCTTAATTGCGGGAC-3′); 263NNNfor (5′-ACATTCTTANNNGCGGGACACGAAAC-3′); 264NNNfor (5′-ACATTCTTAATTNNNGGACACGAAAC-3′); and 328NNNfor (5′-CCAACTNNNCCTGCGTTTTCC-3′).

The reverse primers for each of these libraries complement their corresponding forward primers. For each mutation, two separate PCRs were performed, each using a perfectly complementary primer, BamHI-forw (5′-ggaaacaggatccatcgatgc-3′) and SacI-rev (5′-gtgaaggaataccgccaagc-3′), at the end of the sequence and a mutagenic primer. The resulting two overlapping fragments that contain the mutations were then annealed in a second PCR to amplify the complete mutated gene. The full gene was cut with BamHI and SacI restriction enzymes and ligated with T4 DNA ligase into pBM3_WT18-6, previously cut with BamHI and SacI to remove the wild-type BM3 gene. The ligation mixtures were transformed into E. coli DH5α electrocompetent cells and plated onto Luria-Bertani (LB) agar plates to form single colonies for picking into 96-well plates. A library of 174 mutants in two 96-well plates (eight wells containing parent 9-10A, one well not inoculated) was prepared. Assuming perfect primers, the library size is large enough to cover all amino acids encoded by at least two codons with a probability of 99.5%. Only for methionine is the probability lower (93.5%).

### Construction of Recombination Libraries

Beneficial mutations found at seven positions (L75W, A78T/F, A82T/S/F/I/C/G, F87I/V/L, T88C, T260N/L and A328L/F/M) and the wild-type amino acids at these positions were recombined sequentially by overlap extension PCR with degenerate primers (see above) or a mixture of primers (mutation sites in the primers are in bold letters; the reverse primers for each library complement corresponding forward primers). The mutation L75I which was not found to increase activity was selected for its change in regioselectivity and added to the library to in-

  

crease its diversity. The mutations A82L and A328V were added to the library because of their previously described beneficial effects.[21] Mutation A82V was added due to constraints in designing the degenerate primer used to construct the library.

First, mutations at positions 78 and 82 were introduced into 9–10A using primers 78A/T/S 82A/T/S/F/I/C/G/L/V for* (5′-GCTTAAATTC**RCG**CGTGATTTT**DBC**GGAGACG), and 78F 82A/T/S/F/I/C/G/L/V for (5′-GCTTAAATTC**TTT**CGTGATTTT**DBC**GGAGACG).

Next, mutations at position 260 were introduced using primers 260Tfor* (5′-CAAATTATT**ACA**TTCTTAATTGCGGGAC), 260Nfor (5′-CAAATTATT**AAC**TTCTTAATTGCGGGAC), and 260Lfor (5′-CAAATTATT**CTT**TTCTTAATTGCGGGAC).

Next, mutations at position 87 and 88 were introduced using primers 87F/I/V/L 88Tfor* (5′-GAGACGGGTTA**NTYACA**AGCTGGAC), and 87F/I/V/L 88Cfor (5′-GAGACGGGTTA**NTYTGT**AGCTGGAC).

Next, mutations at position 328 were introduced using primers 328Afor* (5′-CCAACT**GCT**CCTGCGTTTTCC), 328L/F/Vfor (5′-CCAACT**BTT**CCTGCGTTTTCC), and 328Mfor (5′-CCAACT**ATG**CCTGCGTTTTCC).

Finally, mutations at position 75 were introduced using primers 75L/Ifor* (5′-CTTAAGTCAAGCG**MTT**AAATTC), and 75Wfor (5′-CTTAAGTCAAGCG**TGG**AAATTC).

While building the library, we screened and sequenced ten improved members of a partial library containing mutations at positions 78, 82 and 260 to verify that it had been constructed properly. Mutations were identified at all of these sites. We found all possible amino acids at positions 78 and 260, and four out of nine amino acids at position 82.

## High Throughput Screening with Alkyl Methyl Ethers

Cell lysates for high throughput screening were prepared as described.[21] Screening for activity on dimethyl ether was performed as described.[21] For screening on hexyl methyl ether (HME), phosphate buffer (120 μL, 0.1 M, pH 8.0) and then 2 μL of 400 mM HME in ethanol was added to 30 μL of *E. coli* supernatant. After 1 min of incubation at room temperature, NADPH (50 μL, 0.8 mM) was added to the diluted lysate and substrate. Purpald (168 mM in 2 M NaOH) was added 15 min after initiating the reaction to form a purple product with the formaldehyde that was generated upon demethylation of the substrate. The purple color was quantified approximately 15 min later at 550 nm using a Spectramax Plus microtiter plate reader (Molecular Devices, Sunnyvale, CA). Improved mutants were selected based on an increased signal compared to 9-10A (for the saturation mutagenesis libraries) and 1-12G (for the recombination library).

## Alkane Hydroxylation Reactions

For determination of total turnover numbers and regioselectivities, reactions with liquid alkanes (hexane, heptane, octane, nonane and decane) were performed for 12 hours at 25 °C in 20 mL headspace vials, stirred at low speed using magnetic stirring bars. The reactions were carried out in potassium phosphate buffer (0.1 M, pH 8.0, 3 mL) containing purified protein (25 nM). Substrate was added to this solution as 60 μL of 200 mM ethanol solutions to give 4 mM total substrate and 2% ethanol. After 1 min, an NADPH regenerating system (300 μL, 1.66 mM $NADP^+$, 66 U/mL isocitrate dehydrogenase, 416 mM isocitrate) was added to the reaction before the vial was capped and pressurized with oxygen (1.4 bar). At the end of the reaction, an aliquot (1400 μL) was removed from the vial.

To determine product formation rates, the reactions (5 mL) were performed similarly, except more protein (200 nM) was used, and NADPH was added (500 μM) instead of a regeneration system. Additionally, the vials were not pressurized with oxygen. Aliquots of the reaction (1400 μL) were removed at 20, 40 and 60 s.

In all cases, the aliquots were quenched with $CHCl_3$ (300 μL) and HCl (6 M, 100 μL) in a 2 mL microcentrifuge tube. An internal standard containing 1-hexanol (14 μL, 10 mM) for octane, nonane and decane reactions or 1-octanol (14 μL, 10 mM) for hexane and heptane reactions was added to the tube. The tube was vortexed and then centrifuged at 10,000 g for 2 minutes in a microcentrifuge. The chloroform layer was removed with a pipette and analyzed by gas chromatography to determine product concentrations. Control reactions performed by repeating these steps without the addition of substrate revealed no detectable background levels of these specific products.

## Gas Chromatography

Identification of analytes was performed using purchased standards. Quantification was based on 5-point calibration curves using internal standards. All samples were injected at a volume of 1.0 μL and analyses were performed at least in triplicate. Analyses of alkane hydroxylation products were performed on a Hewlett-Packard 5890 Series II Plus gas chromatograph with a flame ionization detector (FID) and fitted with an HP-7673 autosampler system. Direct analysis of hexane, heptane, octane, nonane and decane hydroxylation products was performed on an HP-5 capillary column (cross-linked 5% phenylmethylsiloxane, 30 m length, 0.32 mm ID, 0.25 μm film thickness) connected to the FID detector. A typical temperature program for separating the alcohol products is 250 °C injector, 300 °C detector, 50 °C oven for 3 minutes, then 10 °C/minute gradient to 200 °C, 25 °C/minute gradient to 250 °C, then 250 °C for 3 minutes.

## NADPH Oxidation Rates

Rates of NADPH oxidation were measured at 25 °C using a BioSpec-1601 UV-Vis spectrophotometer (Shimadzu, Columbia, MD) and 1 cm pathlength cuvettes. A typical reaction solution contained enzyme (800 μL, 200 nM) in potassium phosphate buffer (0.1 M, pH 8.0) and 2 mM or 4 mM substrate in ethanol (2%, vol/vol). The reaction was initiated by the addition of NADPH (200 μL, 800 μM), and the decrease in absorption at 340 nm was monitored. Rates were calculated based on the extinction coefficient of NADPH ($6.22\ mM^{-1}\ cm^{-1}$). Background NADPH consumption rates were measured without substrate.

  

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