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Regio- and Enantioselective Alkane Hydroxylation with Engineered Cytochromes P450 BM-3

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Abstract: Cytochrome P450 BM-3 from *Bacillus megaterium* was engineered using a combination of directed evolution and site-directed mutagenesis to hydroxylate linear alkanes regio- and enantioselectively using atmospheric dioxygen as an oxidant. BM-3 variant 9-10A-A328V hydroxylates octane at the 2-position to form *S*-2-octanol (40% ee). Another variant, 1-12G, also hydroxylates alkanes larger than hexane primarily at the 2-position but forms *R*-2-alcohols (40−55% ee). These biocatalysts are highly active (rates up to 400 min^{-1}) and support thousands of product turnovers. The regio- and enantioselectivities are retained in whole-cell biotransformations with *Escherichia coli*, where the engineered P450s can be expressed at high levels and the cofactor is supplied endogenously.

Introduction

Cytochromes P450 comprise a superfamily of enzymes with well over a thousand members that are, as a whole, capable of oxidizing an immense variety of organic molecules in vivo using atmospheric dioxygen as an oxidant.[1,2] Conversion of even a small fraction of these P450 systems into useful synthetic catalysts, however, is limited by several factors, including the multicomponent nature of most of these enzymes, the fact that most are membrane-bound, their limited stabilities, and their generally slow rates. The few bacterial cytochrome P450s that have been characterized are in general faster, soluble (i.e., not membrane-bound), more stable, expressible in *Escherichia coli*, and structurally characterized.[3] In addition to possessing these useful properties, the bacterial cytochrome P450 BM-3 from *Bacillus megaterium*, in contrast to almost all other characterized P450 systems, is comprised of a single polypeptide chain.[4] Inspired by the diversity of activities supported by the P450 scaffold in nature, our laboratory has focused on engineering BM-3 to generate practical P450-based oxidation catalysts. In particular, we are interested in creating useful biocatalysts for the controlled oxidation of alkanes.

Linear alkanes are difficult to hydroxylate: the alkane C−H bond is notoriously inert because of its high (∼97 kcal/mol) bond strength, making alkanes ideal solvents for use with very reactive oxidation catalysts.[5] Additionally, the activation energies for subsequent oxidations of an alcohol are similar to the energy required for the initial hydroxylation of the starting alkane, resulting in mixtures of alcohol, ketone/aldehyde, and carboxylic acid products in most alkane oxidation reactions. The similarity of methylene C−H bond strengths in a linear alkane and the lack of functional groups that can serve to direct catalysis make selective hydroxylation of these compounds especially challenging. Limited selective alkane hydroxylation has been reported for bulky cycloalkanes and aryl alkanes using biomimetic transition-metal complexes as catalysts and peroxides as oxidants, but these complexes do not produce hydroxylated products in useful amounts (the catalysts support very few (<20) total turnovers).[6−9]

Several biological systems are capable of dioxygen-supported alkane hydroxylation and, in some cases, even regioselective alkane hydroxylation. For example, the particulate methane monooxygenase from *Methylococcus capsulatus* (Bath) can hydroxylate butane and pentane to yield primarily *R*-2-alcohols.[10] This enzyme does not accept larger alkanes. Soluble methane monooxygenase from the same organism hydroxylates several alkanes with little or no selectivity.[11] Neither enzyme is likely to serve as a practical biocatalyst because of their inability to be overexpressed in a suitable host organism and their rather low intrinsic activities. In contrast, alkane hydroxylase B (AlkB) from *Pseudomonas oleovorans* hydroxylates octane selectively at the terminal (or ω) position to make

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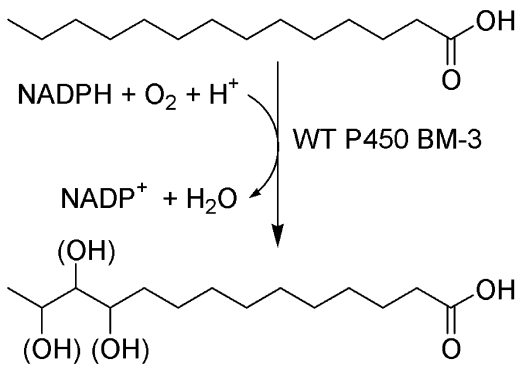

Figure 1. General hydroxylation reaction catalyzed by cytochrome P450s. Hydroxylation of myristic acid by wild-type cytochrome P450 BM-3 results in 54% ω-1, 25% ω-2, and 20% ω-3 hydroxylation product.[24]

1-octanol, and is used in engineered whole cells to generate this product on an industrial scale.[12] Terminal alkane hydroxylation was also reported in some mammalian liver P450s, including rabbit liver P450 CYP4B1.[13] Nonselective cytochrome P450 alkane hydroxylation systems include the site-directed mutants of $P450_{cam}$ of Wong and co-workers[14−16] and an engineered P450 BM-3 reported by our group.[17,18] Enantioselective (but not regioselective) hydroxylation of alkanes has been reported with whole-cell systems of soil bacteria genera such as *Bacillus*,[19] *Pseudomonas*,[20,21] and *Rhodococcus*,[22,23] but the selectivity observed in these systems is most likely due to metabolism of specific isomers rather than the action of selective oxygenases.

The natural substrates of cytochrome P450 BM-3 are medium-chain (C12 to C18) fatty acids, which are hydroxylated at their ω-1, ω-2, and ω-3 positions using atmospheric dioxygen and nicotinamide adenine dinucleotide phosphate (NADPH) (Figure 1).[24] Substrate is bound and hydroxylated in a hydrophobic pocket positioned directly above the heme cofactor in the P450 heme, or hydroxylase, domain. A peptide linker connects the P450 BM-3 heme domain to the reductase domain, where NADPH is reduced and flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD) cofactors are used to transfer electrons to the heme active site for catalysis. In previous work, we used directed evolution to convert wild-type BM-3 into a fast, but nonselective, alkane hydroxylase called 139-3.[17,18] A large increase in activity toward alkanes as small as propane was observed after five rounds of directed evolution experiments, which accumulated 11 amino acid substitutions in the heme domain.

We have begun to explore the further evolution of P450 BM-3 to hydroxylate very small alkanes such as ethane and methane, activities never reported in a cytochrome P450, by applying a combination of directed evolution and site-directed mutagenesis. The mutants obtained from these experiments hydroxylate propane at faster rates and reach higher total turnover numbers than 139-3. Of particular interest, we found that two of these mutants are highly regioselective and enantioselective toward longer alkanes. One mutant forms *R*-2-alcohols from alkanes bigger than heptane, and the other forms *S*-2-octanol from octane. The engineered P450 BM-3 enzymes described here are, to our knowledge, the only systems capable of direct, regio-, and enantioselective hydroxylation of linear alkanes expressed in hosts suitable for large-scale biocatalytic processes.

Results and Discussion

Directed Evolution. In our previous work with this enzyme, five rounds of directed evolution starting with wild-type cytochrome P450 BM-3 yielded the alkane hydroxylase 139-3.[18] In each round, a library of randomly mutated BM-3 enzymes was screened for octane hydroxylation activity on the "surrogate" substrate *p*-nitrophenyl octyl ether. Hydroxylation of this substrate at the carbon atom containing the *p*-nitrophenoxy moiety results in the formation of *p*-nitrophenolate, which was used for colorimetric identification of active mutants. Active mutants were then tested for octane hydroxylation activity, and the most active ones were used as parents for subsequent rounds of evolution. In some cases, several active mutants were isolated from a single round of screening and recombined using DNA shuffling to obtain the parent for the next round. In these and the evolution experiments described in this work, random mutagenesis and recombination were applied only to the heme domain (residues 1−429, with 429 chosen for the convenience of introducing a *Sac*I restriction site into the heme domain gene); the reductase domain was left untouched. The complete BM-3 enzyme (modified heme domain attached to the unmodified reductase domain) was used in all the experiments described here.

Of the 11 amino acid substitutions in the alkane hydroxylase 139-3 heme domain, only one (V78A) occurs at a residue that contacts the fatty acid substrate in the (wild-type) enzyme.[18] The mechanisms by which the mutations contribute to the new activity are difficult to rationalize. The rather broad distribution of 139-3's hydroxylated alkane products, however, suggests that its active site is rather large and that its alkane substrates are "loosely" bound. This is not surprising since the surrogate substrate used to identify these mutants is quite large relative to octane, the intended substrate. The one active-site substitution, in fact, replaces valine with a smaller, alanine side chain.

P450 BM-3 mutant 139-3 shows significant activity on propane, even though small alkane substrates were not used to screen the mutant libraries in the directed evolution experiments. To improve this activity further, and perhaps even achieve activity toward ethane, we continued to apply iterative rounds of mutagenesis and screening, using propane. The BM-3-catalyzed hydroxylation reaction results in the oxidation of 1

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Table 1. Amino Acid Substitutions in P450 BM-3 Mutants

position (WT)	DNA mutation	139-3 aa	J aa	9-10A aa	9-10A-A82L aa	9-10A-A328V aa	1-12G aa
R47	C142T			C	C	C	C
V78	T236C	A	A	A	A	A	A
A82	247−249[a]				L		L
K94	A284T			I	I	I	I
F107	C324T	F	F	F	F	F	F
H138		Y					
P142	C427T			S	S	S	S
T175	C527T	I	I	I	I	I	I
V178		I					
A184	C554T	V	V	V	V	V	V
F205	T617G		C	C	C	C	C
S226	C681G		R	R	R	R	R
H236	T711G	Q	Q	Q	Q	Q	Q
E252	A758G	G	G	G	G	G	G
R255	C766A	S	S	S	S	S	S
A290	C872T		V	V	V	V	V
A295		T					
A328	C986T					V	V
L353	C1060G	V	V	V	V	V	V
E372	A1119G		E	E	E	E	E

[a] A82L DNA mutation was GCA to CTT.

equiv of NADPH for each equivalent of hydroxylated substrate. Using a 96-well plate reader, we can monitor spectrophotometrically at 340 nm the rate of NADPH oxidation in the presence of BM-3-containing cell lysate and substrate to quickly identify mutants with high activity toward any given substrate.[25] It is possible that the reducing equivalents of NADPH become uncoupled to substrate oxidation, leading to false positives in a mutant library, but gas chromatographic analysis of reaction mixtures can confirm when an increased NADPH oxidation rate is accompanied by more or faster product formation.

For the first round of directed evolution, a library of P450 BM-3 variants was generated by recombining mutant 139-3 with 15 other mutants from the same generation that also exhibited increased activity toward *p*-nitrophenyl octyl ether and octane. The shuffled gene library was transformed into *E. coli* (DH5α) competent cells, where the enzymes were overexpressed. Aliquots of the cell-free extracts from individual clones were transferred to 96-well plates, where NADPH consumption was monitored in the presence of propane. Positive clones were grown up and their enzymes purified for comparative analysis using gas chromatography. Mutant J was selected on the basis of its increased rate of NADPH consumption in the presence of propane. This increased rate corresponded to an increase (relative to 139-3) in total production of hydroxylated products with the substrates propane (800 turnovers) and octane (3000 turnovers).

Error-prone PCR of the gene for P450 BM-3 mutant J, performed under conditions designed to yield 1−2 mutations on average per gene, generated the library for the second round of directed evolution. Mutant 9-10A was selected from this library for its increased NADPH consumption rate in the presence of propane; it was subsequently shown to support more turnovers of propane (1100). The sequences and properties of these mutants are detailed in Tables 1−3 and Figure 2. Neither J nor 9-10A acquired active-site mutations, nor did they show major changes in regioselectivity toward longer alkanes, compared to 139-3. Although these mutants supported more turnovers, their coupling efficiencies (see below) were not improved.

Random mutagenesis of 9-10A generated the library for a third round of evolution. This time, a second screen was applied

Table 2. Product Distributions (% total alcohols[a]) and % ee of Selected Products

mutant	product	propane	hexane	% ee[c]	heptane	% ee[c]	octane	% ee[c]	nonane	% ee[c]	decane	% ee[c]
	1-alcohol	3	0		0		1		0		0	
	2-alcohol	97	14	14(*S*)	30	15(*S*)	61	58(*S*)	30	83(*S*)	15	
139−3	3-alcohol		86	39(*S*)	42	15(*S*)	20		50		37	
	4-alcohol				29		17		21		49	
	ketones[b]		<1		3		5		5		7	
	1-alcohol	4	0		1		1		0		2	
	2-alcohol	96	23	20(*S*)	29	12(*S*)	52	57(*S*)	29	67(*S*)	16	
J	3-alcohol		77	46(*S*)	42	11(*S*)	25		48		35	
	4-alcohol				28		22		23		48	
	ketones[b]		<1		2		5		5		5	
	1-alcohol	8	0		1		1		0		1	
	2-alcohol	92	6	14(*S*)	26	7(*S*)	53	50(*S*)	39	60(*S*)	16	
9-10A	3-alcohol		95	41(*S*)	41	8(*S*)	20		59		32	
	4-alcohol				33		26		3		51	
	ketones[b]		<1		3		5		5		6	
	1-alcohol	5	0		0		0		1		0	
	2-alcohol	95	35	39(*S*)	27	4(*S*)	22	10(*S*)	16	7(*S*)	21	
9-10A-A82L	3-alcohol		65	42(*S*)	46	30(*S*)	25	17(*R*)	16		19	
	4-alcohol				29		53		67		60	
	ketones[b]		<1		2		5		5		5	
	1-alcohol	10	6		14		10		3		1	
	2-alcohol	90	64	21(*R*)	62	15(*R*)	80	40(*S*)	76	0	79	5(*S*)
9-10A-A328V	3-alcohol		30		17		8		19		17	
	4-alcohol				6		2		3		2	
	ketones[b]		<1		<1		<1		<1		<1	
	1-alcohol	11	9		5		5		3		1	
	2-alcohol	89	77	4(*R*)	76	40(*R*)	82	39(*R*)	86	52(*R*)	86	55(*R*)
1-12G	3-alcohol		14		15		11		7		9	
	4-alcohol				3		3		5		4	
	ketones[b]		<1		<1		<1		<1		<1	

[a] Product distribution determined as ratio of a specific alcohol product to the total amount of all alcohol products (given in %). Errors are at most ±1%. [b] Product distribution for ketones was similar to alcohol product distribution. The numbers reported here are the (%) total of all ketones relative to total products (alcohols + ketones). [c] Favored enantiomer is listed in parentheses. Errors are at most ±5%.

Table 3. Catalytic Properties of Mutants of P450 BM-3

mutant	substrate	rate of NADPH consumption (min^{-1})[a]	rate of product formation (min^{-1})[c]	coupling to NADPH (%)[d]	total turnovers[e]	fold improvement[g]
139-3	octane	2200[b]	480	22	1000	1
	propane	140[b]	12	9	500	1
J	octane	2900	660	23	3000	3
	propane	610	30	5	800	1.6
9-10A	octane	2600	540	21	3000	3
	propane	420	23	5	1100	2.2
9-10A-A82L	octane	1400	530	38	6000	6
	propane	190	54	28	2400	4.8
9-10A-A328V	octane	1200	50	4	2000	2
	propane	320	35	11	100	0.2
1-12G	octane	410	150	37	7500	7.5
	propane[f]	20	160	100[f]	6000	12

[a] Initial NADPH consumption rates were measured over 15 s at 340 nm as nmol NADPH/min/nmol protein. Octane reactions contained 100 nM P450, 166 μM NADPH, and 4 mM octane in 1% ethanol and potassium phosphate buffer. Propane reactions contained 200 nM P450, 166 μM NADPH, and propane-saturated potassium phosphate buffer. Errors are at most 10%. [b] The difference between these numbers and ones we reported previously are due to the longer time period used to measure rates in this work (15 s compared to 2 s). [c] Initial rates were measured by GC over 1 min as nmol total products/min/nmol protein. Octane reactions contained 100 nM P450, 500 μM NADPH, and 4 mM octane in 1% ethanol and potassium phosphate buffer. Propane reactions contained 1 μM P450, 500 μM NADPH, and propane-saturated potassium phosphate buffer. Errors are at most 10%. [d] Coupling determined by ratio of product formation rate to NADPH consumption rate. [e] Total turnover numbers determined as nmol product/nmol enzyme. Octane reactions contained 10−25 nM P450, 500 μM NADPH, and 4 mM octane in 1% ethanol and potassium phosphate buffer. Propane reactions contained 10−25 nM protein, potassium phosphate buffer saturated with propane, and an NADPH regeneration system containing 100 μM $NADP^+$, 2 units/mL of isocitrate dehydrogenase, and 10 mM isocitrate. Errors are at most 10%. [f] The NADPH consumption rate with propane was only 20 min^{-1} faster than the background rate without substrate. The higher propanol formation rate suggests that propane hydroxylation with this mutant outcompetes the background NADPH consumption process. [g] Fold improvement is based on total turnovers of hydroxylated products compared to 139-3.

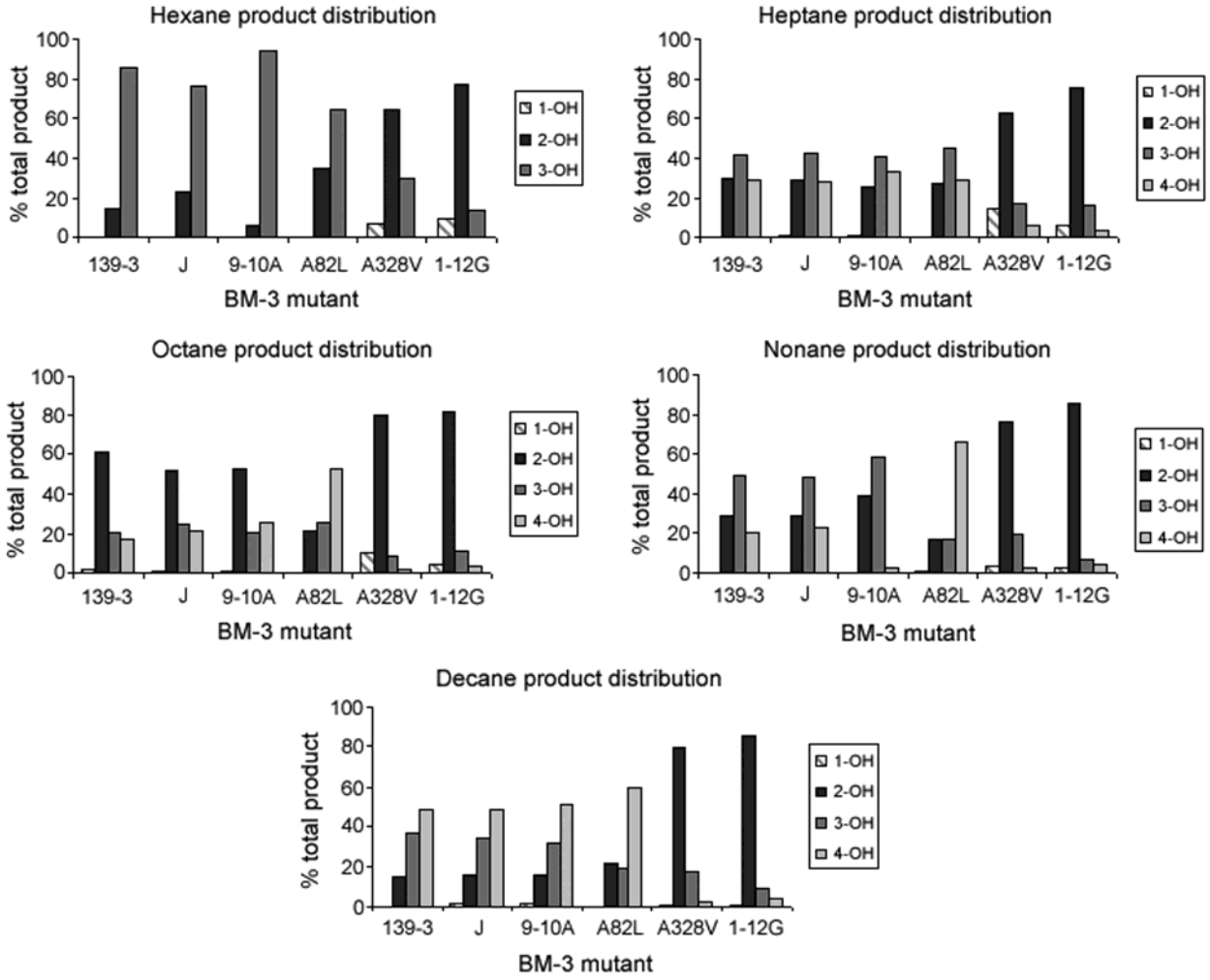

Figure 2. Hydroxylation product distribution of BM-3 mutants. Mutants 9-10A-A82L and 9-10A-A328V are abbreviated A82L and A328V, respectively.

to assess the amount of hydroxylation products generated by each mutant. This screen uses the surrogate substrate dimethyl ether, which is similar to propane in size and C−H bond strength. Upon hydroxylation, dimethyl ether forms formaldehyde, which can be detected with Purpald dye[26] (Figure 3). This third round failed to produce a mutant with either increased

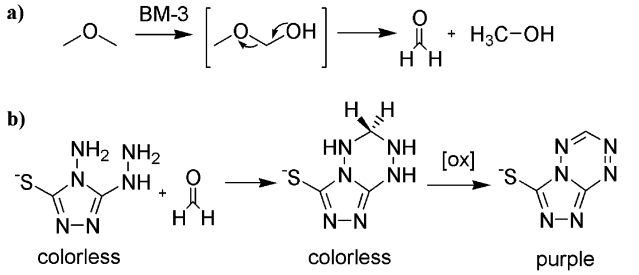

Figure 3. (a) Hydroxylation of surrogate substrate dimethyl ether produces formaldehyde. (b) Purpald reacts with formaldehyde to form a purple adduct upon air oxidation.

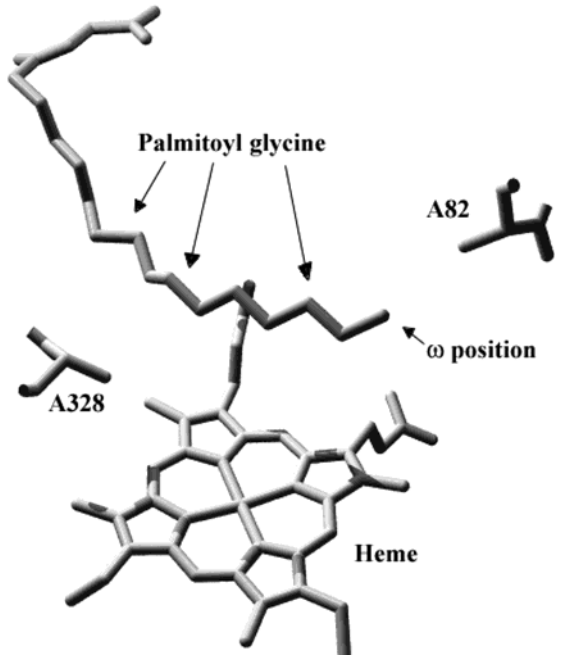

Figure 4. Positions of A328 and A82 in the active site of wild-type cytochrome P450 BM-3. The picture was made from the coordinates of the crystal structure 1JPZ in the Protein Database. The substrate is palmitoyl glycine.

propane hydroxylation activity or more propane hydroxylation products. A possible explanation is that further increases in activity require two or more simultaneous, or coupled, genetic mutations. Such events occur with very low probability and will not be found by screening a few thousand clones.

Site-Directed Mutagenesis. Wong et al. achieved hydroxylation activity toward butane and propane in the camphor hydroxylase cytochrome $P450_{cam}$ by reducing the size of the enzyme's active site using site-directed mutagenesis.[15] We reasoned that decreasing the volume of the active site of 139-3 might serve to enhance its activity toward smaller substrates. Tightening up the active site might also confer regioselectivity toward longer alkanes; if the substrate is bound more tightly, fewer hydroxylation products should be possible. We therefore turned to site-directed mutagenesis of residues in the active site for further improvements.

Using a crystal structure of the heme domain of wild-type BM-3 containing a bound substrate,[27] we identified two residues that should influence substrate binding (Figure 4). Alanine 328 sits directly above the heme cofactor and is the residue closest to the proximal side of the heme iron. Mutation of this residue to valine in the wild-type enzyme had been reported to affect substrate binding and turnover rates on fatty acids.[28] We substituted alanine 328 in 9-10A with the larger, hydrophobicvaline and determined the activity of this mutant (termed 9-10A-A328V) toward several alkanes (Tables 2 and 3; Figure 2). Neither the propane hydroxylation activity nor the total propane turnovers improved relative to its parent. However, we observed a dramatic shift in the regioselectivity of hydroxylation on longer alkanes (Figure 2). Wild type and all mutants of BM-3 generated in this laboratory by directed evolution hydroxylate longer alkanes such as heptane, octane, and nonane with roughly equivalent distributions of the 2-, 3-, and 4-alcohols. Mutant 9-10A-A328V, on the other hand, forms primarily (>80%) 2-alcohols from these substrates. With octane, the resulting 2-alcohol is ~70% *S*-2-octanol (40% ee) (Tables 2 and 3; Figure 2). Other alkanes are not hydroxylated enantioselectively. We rationalize that these changes in regioselectivity and enantioselectivity result from a reduction in possible binding geometries for these substrates in the active site.

The alkane hydroxylation product distributions for 9-10A-A328V clearly show its selectivity for the alkane 2-position. The fact that only 2-octanol is formed enantioselectively, but not 2-heptanol or 2-nonanol, suggests that a substrate−protein contact specific to the methyl group of octane furthest from the heme induces enantioselectivity toward this lone substrate. The other nonregioselective mutants, 139-3, J, and 9-10A, also exhibit similar enantioselectivity toward octane; this contact must therefore function independently of residue 328. It may be possible to engineer a mutant that is enantioselective for the *S*-2-position of other alkanes by changing other residues in the binding pocket (or outside) to mimic this contact.

The second side chain we chose to alter is located near the active site that forms after the conformational change associated with substrate binding occurs. In the crystal structure of BM-3 with bound palmitoyl glycine, alanine 82 is located within 3.5 Å of the substrate ω-terminus.[27] We reasoned that changing this residue to a larger, hydrophobic amino acid would decrease active-site volume upon substrate binding. Lacking information to choose an appropriate replacement, we prepared a small library containing the four large, hydrophobic amino acids leucine, isoleucine, valine, and phenylalanine at position 82 and screened the library using dimethyl ether. Mutant 9-10A-A82L was identified as more active, and subsequent gas chromatographic analysis of the reaction mixtures revealed that this mutant supports more turnovers with propane (~2400) than the 9-10A parent. The rate of propane hydroxylation and its coupling efficiency with this mutant were also improved. Hydroxylation of longer alkanes using this mutant occurred with a small shift in regioselectivity, in this case favoring the formation of primarily 3- and 4-alcohols (Figure 2).

Recombination. Next, we shuffled the (heme domain) genes of J, 9-10A, 9-10A-A328V, and 9-10A-A82L and screened that library for activity on propane and dimethyl ether. Mutant 1-12G was selected from this library. As with mutant 9-10A-A328V, 1-12G hydroxylates alkanes at the 2-position (>80%). However,

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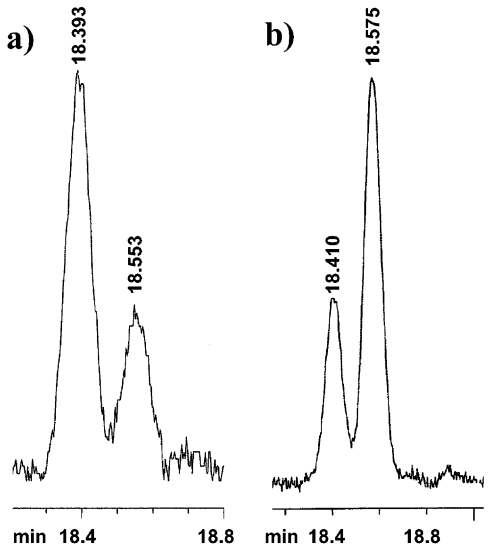

Figure 5. GC/FID analysis of the (−)-menthyl carbonate diastereomers of the 2-octanol produced by mutant BM-3-catalyzed alkane oxidation. The *S*-2-octanol derivative elutes at 18.4 min, the *R*-2-octanol derivative elutes at 18.6 min. (a) Reaction catalyzed by 9-10A-A328V (40% ee). (b) Reaction catalyzed by 1-12G (40% ee).

chiral GC analysis of the products revealed that 1-12G is as enantioselective as 9-10A-A328V, generating the *R*-2-alcohols (40−55% ee) of heptane, octane, nonane, and decane (Tables 2 and 3; Figure 2). Mutant 1-12G also supports thousands of turnovers on the substrates propane (6000) and octane (7500), resulting in approximately 1 order of magnitude improvement compared to 139-3. Sequencing revealed that this mutant contains all of the mutations introduced into the recombination library: it is the double mutant A328V + A82L of 9-10A. Addition of the A82L mutation to 9-10A-A328V has shifted the octane substrate in the active site such that the opposite enantiomer of its 2-alcohol is now favored (Figure 5). Residue 82 was chosen for its proximity to the substrate ω-terminus, but it is not clear whether the larger leucine side chain "pushes" the substrate further up the active site or blocks the channel such that the substrate is flipped relative to its position in the A328V mutant.

In addition to its effect on selectivity, the A82L substitution, both with and without the A328V mutation, at least doubles the stability of the catalyst, as determined by total turnover number and increases its coupling to NADPH oxidation (Table 3). It is possible that this mutation protects the heme cofactor from degradation by reduced oxygen species such as peroxide and superoxide (see below), but further analysis beyond the scope of this report will be required to determine the cause.

Coupling of NADPH-Reducing Equivalents to Product Formation. Under oxygen-limiting conditions, i.e., substrate and NADPH concentrations in large excess of the 225−250 μM dioxygen present in oxygen-saturated buffer, hydroxylation reactions catalyzed by the mutants in this work form 225−250 μM of products. This upper limit to product formation suggests, as we have reported in our previous work, that substrate oxidation is tightly coupled to the consumption of dioxygen in these mutants. In general with P450 systems, NADPH oxidation not coupled to the formation of product is assumed to be wasted on reducing heme-bound dioxygen to active oxygen species such as peroxide and superoxide (which may subsequently inactivate the enzyme) or to water. If oxygen reduction is the only mechanism for NADPH uncoupling, then our mutants should be fully coupled. When we compare the NADPH consumption rates of these mutants (in the presence of substrate) with their product formation rates, however, we discover that a large fraction of the NADPH oxidized is not used for product formation or uncoupled oxygen reduction (Table 3). We are currently investigating the fate of these lost electrons, but have noticed that when we screen for product formation using the dimethyl ether/purpald screen, the resulting mutants, 1-12G and 9-10A-A82L, are more coupled than mutants obtained by screening for NADPH consumption only and also support more total turnovers.

Whole-Cell Biotransformations. Two major practical obstacles to implementing a biocatalytic process are the need for large amounts of purified protein and the expensive cofactors. Lysates of *E. coli* (DH5α) containing overexpressed cytochrome P450 BM-3 mutants exhibit the same activity as purified protein (data not shown) but still require the addition of NADPH. We have used an isocitrate dehydrogenase-based NADPH-regenerating system[29] to perform reactions using both cell lysate and purified protein, with results indistinguishable from using NADPH alone (consistent with the fact that these reactions are generally oxygen-limited; data not shown). We were curious to see if we could use our *E. coli* system as a whole-cell catalyst, since the alkane substrates and alcohol products should be permeable to the cell membrane. To test this, we prepared cultures of DH5α cells that overexpress the 9-10A-A328V and the 1-12G mutants. Without optimizing the system, we prepared the cells and supplied them octane according to a procedure published by Witholt's group that had used *E. coli* K27 expressing wild-type BM-3 and myristic acid as a substrate.[30,31] We used isopropyl-β-D-thiogalactopyranoside (IPTG) to induce expression. Extraction of these whole-cell reaction mixtures with methylene chloride and analysis of the products revealed that both the regio- and enantioselectivity of the alkane hydroxylation products are preserved in the whole-cell system (Figure 6). Optimization of these reaction conditions, perhaps using a system analogous to the two-phase system described by Witholt and co-workers for AlkB octane hydroxylation,[12] could lead to cost-effective use of our *E. coli* cells for alkane hydroxylation.

Conclusions

Cytochrome P450 BM-3 is a highly "evolvable" catalyst: protein engineering by directed evolution and site-directed mutagenesis has generated variants with wide-ranging properties, including the ability to utilize different oxidants such as hydrogen peroxide for fatty acid hydroxlation,[32,33] activity on non-natural substrates[34−37] and in organic solvents,[38] and greater

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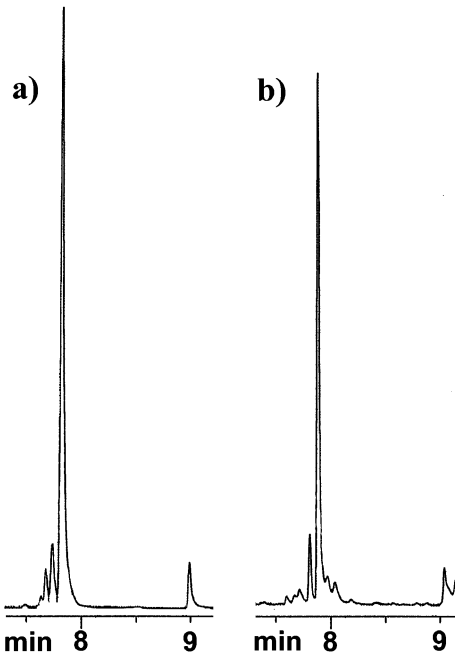

Figure 6. GC/FID analysis of the octane hydroxylation product using 9-10A-A328V as (a) purified protein and (b) a whole-cell catalyst. 3-Octanol elutes at ~7.7 min, 2-octanol elutes at ~7.8 min, and 1-octanol elutes at ~9.0 min.

thermostability.[39] The regio- and enantioselective alkane hydroxylation activities reported here further demonstrate the functional flexibility of the BM-3 scaffold. We might (perhaps optimistically) regard BM-3 as a "one-enzyme-fits-all" oxidation catalyst.

Regioselective alkane hydroxylation requires a catalyst that can discriminate between very similar methylene carbons. In addition, there must be a mechanism to remove the hydroxylated substrate to prevent overoxidation. With the modifications presented in this work, the hydrophobic channel that serves as the active site of BM-3 provides the perfect environment for binding and oxidizing alkanes. It should be possible to make further improvements in the enantioselectivity of the 9-10A-A328V and 1-12G biocatalysts, using directed evolution, as has been done for several other enzyme systems.[40]

Experimental Procedures

Materials. All liquid alkanes, product standards, and solvents were purchased from Sigma-Aldrich, Inc. (St. Louis, MO). Propane and dimethyl ether were purchased from Advanced Gas Technologies (Palm, PA). Isocitrate dehydrogenase and $NADP^+$ were purchased from Sigma-Aldrich, Inc. (St. Louis, MO). NADPH was obtained from BioCatalytics, Inc. (Pasadena, CA).

Expression and Purification of P450 BM-3. P450 BM-3 was expressed and purified as described.[18] The P450 BM-3 gene or mutants thereof, which include a silent mutation to introduce a *Sac*I site 130 bp upstream of the end of the heme domain, was cloned behind the double *tac* promoter of the expression vector pCWori[17] (pBM3_WT18-6). *E. coli* DH5α, transformed with these plasmids, was used for expression of P450 BM-3 on a 500-mL scale as well as for expression in 96-well plates.

For protein production, supplemented terrific broth (TB) medium (500 mL, 100 μg/mL of ampicillin, 50 μg/mL of thiamine) was inoculated with an overnight culture (1 mL) and incubated at 30 °C and 250 rpm shaking. After 12 h of incubation, the rotation speed was lowered to 200 rpm, δ-aminolevulinic acid hydrochloride (ALA; 0.5 mM) was added, and expression was induced by addition of isopropyl-β-D-thiogalactoside (IPTG; 1 mM). Cells were harvested by centrifugation 20−24 h after induction. The enzymes were purified following published procedures.[17] Enzyme concentration was measured in triplicate from the CO-difference spectra.[41]

Recombination of P450 BM-3 Variants. The first generation of mutants was created by StEP recombination of mutant 139-3 [18] with 15 other mutants from the same generation, as described.[42] Mutant J was isolated from a library of ~350 mutants on the basis of its increased NADPH depletion rate using propane as a substrate.

Random Mutagenesis of P450 BM-3. Libraries for the second (~1000 mutants) and third (~1500 mutants) generations were created by error-prone PCR using the GeneMorph Kit (Stratagene, La Jolla, CA) according to the manufacturer's protocol, using approximately 50 ng of plasmid DNA as template and primers *Bam*HI-forw (5′-ggaaacaggatccatcgatgc-3′) and *Sac*I-rev (5′-gtgaaggaataccgccaagc-3′).

Site-Directed/Saturation Mutagenesis. Base mutations were introduced into mutant 9-10A by PCR overlap extension mutagenesis.[43] Position A82 was mutated to L, I, V, and F using mutagenic primers A82-forw (5′-ggagacgggttatttacaagc-3′) and A82-rev (5′-gcttgtaaataaccgctctccaanaaaatcacg-3′). Position A328 was mutated to V using mutagenic primers A328V-forw (5′-gcttatggccaactgttcctgc-3′) and A328V-rev (5′-gcaggaacagttggccataagc-3′). For each mutation, two separate PCRs were performed, each using a perfectly complementary primer (*Bam*HI-forw and *Sac*I-rev) at the end of the sequence and a mutagenic primer. The resulting two overlapping fragments that contained the base substitution were then annealed in a second PCR to amplify the complete mutated gene.

Recombination of P450 BM-3 Variants. The fifth and last generation of mutants was created by recombination of mutants 139-3, J, 9-10A, 9-10A-A82L, and 9-10A-A328V using a modification of Stemmer's DNA shuffling protocol.[44]

Preparation of Cell Lysates for High-Throughput Screening. Single colonies were picked and inoculated by a Qpix robot (Genetix, Beaverton, OR) into 1-mL, deep-well plates containing Luria−Bertani (LB) medium (350 μL, supplemented with 100 mg/mL of ampicillin). The plates were incubated at 30 °C, 250 rpm, and 80% relative humidity. After 24 h, clones from this preculture were inoculated using a 96-pin replicator into 2-mL, deep-well plates containing TB medium (400 μL, supplemented with 100 mg/mL of ampicillin, 10 μM IPTG and 0.5 mM ALA). The cultures were grown at 30° C, 250 rpm, and 80% relative humidity for another 24 h. Cells were then pelleted and stored frozen at −20 °C until they were resuspended in 500 μL 0.1 M phosphate buffer (0.1 M, pH = 8, 500 μL, containing 0.5 mg/mL of lysozyme, 2 units/mL of DNaseI, and 10 mM $MgCl_2$). After 60 min at 37 °C, the lysates were centrifuged, and the supernatant was diluted for activity measurements in 96-well microtiter plates.

High-Throughput NADPH Consumption Assay. The recombination library (resulting in mutant J) and first mutant library (resulting in mutant 9-10A) were screened in 96-well plates for NADPH depletion using propane as substrate. To 30 μL of *E. coli* supernatant, 170 μL of phosphate buffer (0.1 M, pH 8.0) saturated with propane was added. The reaction was initiated by addition of 50 μL of NADPH (0.8 mM), and NAPDH oxidation was monitored at 340 nm for 5 min using a Spectramax Plus microtiter plate reader (Molecular Devices, Sunnyvale, CA). The second library (no improved mutants found) was also screened

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for $NADP^+$ formation using propane as substrate, as described.[25,45] In brief, residual NADPH was destroyed with acid after an appropriate amount of time, followed by conversion of $NADP^+$ to a highly fluorescent alkali product at high pH, which was then measured fluorometrically. Mutants were selected from these libraries based upon increased NADPH consumption.

High-Throughput Product-Based Screen. For direct measurement of product formation, a screen based on the demethylation of dimethyl ether was used in the later generations. To 30 μL of *E. coli* supernatant, 120 μL of phosphate buffer (0.1 M, pH = 8) saturated with dimethyl ether was added. After 2 min of incubation at room temperature, NADPH (50 μL, 1.0 mM) was added, and NADPH depletion was monitored as described above. 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 read approximately 15 min later at 550 nm using a Spectramax Plus microtiter plate reader. Mutants were selected from these libraries based upon their absorbance at 550 nM.

Alkane Hydroxylation Reactions. To measure alkane hydroxylation activities, ethanol solutions of liquid alkanes (hexane to decane) were added to buffer solutions containing the enzymes such that the total ethanol in the reaction mixture was 1%. Several solvents, including ethanol, methanol, acetone, and dimethyl sulfoxide, were tested, and ethanol was shown to support the most product turnovers. Reactions with liquid alkanes that contained no cosolvent produced no detectable products. In the absence of substrate, NADPH has been reported to inactivate BM-3 by over-reducing the flavin cofactors in its reductase domain.[46] To avoid this problem, substrate was added to the enzyme first and incubated for a few seconds before NADPH was added. Dioxygen was not added to the reactions, limiting the amount of possible product formed to the 225−250 μM of oxygen present in air-saturated buffer. The addition of excess dioxygen to the reactions by direct bubbling or rapid stirring did not increase and often decreased the total product turnover, possibly by denaturing the protein. Reaction mixtures for all BM-3 mutants containing 0.5−1.0 μM enzyme produced 225−250 μM products. We also noticed that total turnover numbers measured in systems containing less than ~50 nM protein were less than expected, i.e., 10 nM protein in a reaction gave much less than half the total turnovers of a 20 nM reaction. At very low concentrations of BM-3, the FMN cofactor has been reported to diffuse out of the protein and inactivate it.[47] Given this fact and the dilution effects on total turnover that we witnessed, mutant 1-12G might be capable of even more turnovers than the ~7500 reported.

Reactions with the liquid alkanes, hexane, heptane, octane, nonane, and decane were performed in closed 20-mL scintillation vials and stirred at low speed using magnetic stirring bars. In a typical reaction, purified protein (or cell lysate) was added to 4.45 mL of 0.1 M potassium phosphate buffer (pH = 8.0) such that the total protein concentration equaled 50 nM. The substrates were added to this solution as 50 μL of 400 mM ethanol solutions to give 4 mM total substrate and 1% ethanol. After a few seconds, 500 μL of 5 mM NADPH in 0.1 M potassium phosphate buffer (pH = 8.0) was added to the reaction, and the vial was capped. After 1−2 h of stirring at room temperature, a 1.5-mL aliquot of the reaction was removed from the vial and quenched with 300 μL of chloroform in a 2-mL microcentrifuge tube. An internal standard containing 15 μL of 10 mM 1-pentanol or 3-octanol was added to the tube. The tube was vortexed and then centrifuged at 10000g for 2 min in a microcentrifuge. The chloroform layer was removed with a pipet and analyzed by gas chromatography to determine total turnover numbers and product distributions. Control reactions performed by repeating these steps without the addition of substrate revealed no detectable background levels of these specific products.

Chiral Analysis of Alkane Reactions. Chiral analysis of liquid alkane hydroxylation products was performed with a slight modification of an existing method,[48] starting with extracting 9-mL alkane reactions (using the reaction conditions above) with 2 mL of CH_2Cl_2 in a 15-mL centrifuge tube. After centrifugation at 2800g for 15 min, the organic layer was removed with a pipet and dried over a small amount of anhydrous $MgSO_4$. The $MgSO_4$ was removed by filtration, and 1 μL of pyridine and 2.5 μL (−)-menthyl chloroformate were added. After an hour, 1 mL of deionized water was added to the reaction. After vortexing and letting the layers separate, the organic phase was removed with a pipet and dried with anhydrous $MgSO_4$. The drying agent was again removed with a pipet filter and the remaining solution analyzed by gas chromatography. Control reactions were performed by repeating these steps without the addition of substrate and revealed no background levels of these specific products.

Propane Hydroxylation Reactions. Reactions using propane did not contain cosolvent because of potential competition between the solvent and the small substrate. These reactions were performed in propane-saturated buffer under an atmosphere of propane and dioxygen (provided by a balloon filled with the two gases). The addition of this atmosphere ensured that both gaseous substrates were saturated in the reaction solution, since a balloon of just propane or oxygen would dilute the concentration of the other gas. Total turnovers determined with this system were not dependent on oxygen concentration in the balloon (data not shown), illustrating that only the original ~250 μM of dioxygen in the buffer was available to the reaction. Additionally, we discovered that NADPH could neither be purchased nor easily purified in a form that contained less than 2−3% ethanol, which interfered with analysis of the reaction products. For this reason, an NADPH-regeneration system was used for propane reactions.[29]

Propane hydroxylation reactions were performed in 25-mL Schlenk flasks. In a typical reaction, enzyme (either purified or in cell lysate) was added to 4.5 mL of propane-saturated 0.1 M potassium phosphate buffer (pH = 8.0) to a final concentration of 50−100 nM. To this mixture, 500 μL of NADPH-regeneration system containing 1 mM $NADP^+$, 100 mM sodium isocitrate, and 20 units/mL of isocitrate dehydrogenase was quickly added. The flask was topped with a balloon filled with equal amounts of propane and dioxygen. After stirring for 2 h at room temperature, the propane hydroxylation products were derivatized to alkyl nitrites using a published method.[49] To the reaction mixture, 0.3 g of $NaNO_2$ and 2 mL of 10 μM chloroform in hexane was added, and the mixture cooled on ice. While on ice, 0.2 mL of concentrated H_2SO_4 was added. The flask was stoppered with a rubber stopper and stirred on ice for 15 min. The reaction was rinsed into a separatory funnel with 20 mL of deionized water. The organic phase was washed twice with 20 mL of water and analyzed by gas chromatography. Control reactions were performed by repeating these steps without the substrate to correct for background levels of propanol.

Whole-Cell Reactions. The procedure for whole-cell reactions of *E. coli* (DH5α) overexpressing mutants 9-10A-A328V and 1-12G was only slightly modified from Witholt's published method.[30] An overnight culture of cells (in 3 mL of LB with 100 μg/mL of ampicillin) was used to inoculate 75 mL of M9-minimal medium containing 0.5% w/v glucose, 0.2 mM $CaCl_2$ 5 mM $MgSO_4$, and 100 μg/mL of ampicillin. The culture was then shaken for 24 h at 37 °C and 250 rpm. The cells were collected by centrifugation at 2200g for 10 min and resuspended in 20 mL of 0.2 M potassium phosphate buffer (pH = 7.4) containing 0.5% glucose, 100 μg/mL of ampicillin, 1 mM IPTG, and 0.5 mM ALA, 5 mM alkane (from a 500 mM stock of alkane in dimethyl sulfoxide). This mixture was shaken for 8 h at 37 °C and 250 rpm. Product distributions were measured by gas chromatography after

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extracting this culture with 1 mL of chloroform. Chiral analysis of the reaction products was performed after extracting the culture with 2 mL of CH_2Cl_2 and derivatizing the organic layer with (−)-menthyl chloroformate.

Gas Chromatography. Identification and quantification of analytes were performed using purchased standards and 5-point calibration curves with internal standards. All samples were injected at a volume of 1.0 μL, and analyses were performed at least in triplicate. Analyses of hydroxylation products were performed on a Hewlett-Packard 5890 Series II Plus gas chromatograph with both a flame-ionization (FID) and electron-capture detector (ECD) and fitted with a 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% phenyl methyl siloxane, 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 inlet, 300 °C detector, 50 °C oven for 3 min, then 10 °C/min gradient to 200 °C, 25 °C/min gradient to 250 °C, and then 250 °C for 3 min. The (−)-menthyl chloroformate-derivatized chiral products were separated as diastereomers on a CycloSil-B chiral capillary column (Agilent Technologies, 30-m length, 0.32-mm ID, 0.25-μm film thickness) connected to the FID detector. Each pair of diastereomers required a different temperature program for full resolution, but a typical program is as follows: (chiral heptanol analysis) 250 °C inlet, 300 °C detector, 100 °C oven for 1 min, then 10 °C/min gradient to 180 °C, hold at 180 °C for 10 min, 10 °C/min gradient to 250 °C, and then 250 °C for 3 min. The propyl nitrite products were analyzed with an HP-1 capillary column (cross-linked 1% phenyl methyl siloxane, 30-m length, 0.32-mm ID, 0.25-μm film thickness) connected to an ECD detector. The temperature program for separating 1- and 2-propyl nitrites was: 250 °C inlet, 300 °C detector, 30 °C oven for 3 min, 20 °C/min gradient to 200 °C, and then 200 °C for 5 min.

Determination of NADPH Consumption Rates. The enzymes were purified and quantified as described above. Initial rates of NADPH consumption were measured at 25 °C in a BioSpec-1601 UV/vis spectrophotometer (Shimadzu, Columbia, MD). For the liquid alkanes, substrate stock solutions in ethanol (10 μL) were added to the protein solution (100 nM, final concentration) in 0.1 M potassium phosphate buffer (pH = 8) and incubated for 2 min before initiating the reaction by addition of 200 μL of NADPH (0.8 mM), and the absorption at 340 nm was monitored. Rates were corrected for background NADPH consumption measured without substrate. Rates using propane were measured using 200 nM protein, propane-saturated 0.1 M potassium phosphate buffer (pH = 8), and the same amount of NADPH. Rates were determined at least in triplicate.

Product Formation Rates: Oxidation of Octane. To determine octanol (and octanone) formation rates (measured at 21°C), 5-mL reactions containing 100 nM P450 and 4 mM octane in 1% ethanol in 0.1 M potassium phosphate buffer, pH = 8, were initiated by the addition of 500 μM NADPH. Aliquots (1 mL) were removed from the reaction at 15-s intervals and quenched in 2-mL microcentrifuge tubes containing 300 μL of chloroform, 100 μL of 6 N HCl, and 10 μL of 10 mM 1-pentanol in ethanol. The tubes were vortexed and centrifuged at ∼10000g for 2 min to separate the layers. The chloroform layer was removed and analyzed for total hydroxylated products by gas chromatography. Rates were determined in triplicate.

Product Formation Rates: Oxidation of Propane. Propanol formation rates were measured in 4-mL reactions containing 1 μM P450 and propane-saturated 0.1 M potassium phosphate buffer, (pH = 8) which were inititated with 500 μM NADPH. Aliquots of this mixture (933 μL) were removed at 20-s intervals and quenched with 67 μL of 6 N HCl containing 3 mM 1-pentanol internal standard (200 μM final concentration). The samples were analyzed by GC-FID fitted with an HP FFAP (cross-linked FFAP, 30-m length, 0.25-mm ID, 0.25-μm film thickness) using the temperature program: 250 °C inlet, 300 °C detector, 50 °C oven for 3 min, then 10 °C/min gradient to 200 °C, 25 °C/min gradient to 220 °C, then 220 °C for 3 min. Rates were determined in triplicate.

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