Laboratory evolution of a soluble, self-sufficient, highly active alkane hydroxylase

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We have converted cytochrome P450 BM-3 from *Bacillus megaterium* (P450 BM-3), a medium-chain (C12–C18) fatty acid monooxygenase, into a highly efficient catalyst for the conversion of alkanes to alcohols. The evolved P450 BM-3 exhibits higher turnover rates than any reported biocatalyst for the selective oxidation of hydrocarbons of small to medium chain length (C3–C8). Unlike naturally occurring alkane hydroxylases, the best known of which are the large complexes of methane monooxygenase (MMO) and membrane-associated non-heme iron alkane monooxygenase (AlkB), the evolved enzyme is monomeric, soluble, and requires no additional proteins for catalysis. The evolved alkane hydroxylase was found to be even more active on fatty acids than wild-type BM-3, which was already one of the most efficient fatty acid monooxgenases known. A broad range of substrates including the gaseous alkane propane induces the low to high spin shift that activates the enzyme. This catalyst for alkane hydroxylation at room temperature opens new opportunities for clean, selective hydrocarbon activation for chemical synthesis and bioremediation.

Selective oxyfunctionalization of hydrocarbons remains one of the great challenges for contemporary chemistry. Despite decades of effort, including recent advances[1–3], chemical catalysts for alkane functionalization are characterized by low yields, poor selectivity, and harsh conditions. Biocatalysts that oxidize alkanes allow organisms to use hydrocarbons as a source of energy and cellular building blocks[4,5]. Forty years of screening alkane-assimilating organisms[6] have identified a variety of multisubunit, membrane-associated enzyme complexes, which have inspired curiosity and mimicry for their ability to catalyze selective oxidations at room temperature and ambient pressure[7–11]. However, low catalyst turnover rates and limited stability make applications of biocatalytic C-H bond activation feasible only in a very few industrial processes that produce high-value compounds[12].

P450 BM-3 consists of a hydroxylase domain (64 kDa) and a reductase domain (54 kDa) in a single polypeptide chain[13–15]. This heme enzyme inserts oxygen into subterminal C-H bonds of fatty acids of chain length C12–C18 in the presence of dioxygen and NADPH[13]. We have used laboratory evolution methods consisting of sequential rounds of random mutagenesis, *in vitro* recombination, and high-throughput screening to convert this highly efficient fatty acid monooxygenase into one that can hydroxylate hexane and other alkanes similarly well. In a preliminary study[16], we verified that P450 BM-3 shows very low activity toward octane[17]. A colorimetric screen using *p*-nitrophenoxy octane (8-pnpane) as an alkane substrate analog identified more active clones. Unfortunately, the enzyme's activity on alkanes reached a plateau after a few rounds of evolution, and further screening yielded no improvements. Here we describe evolution of the enzyme to achieve the highest activity yet reported for alkane hydroxylation.

Results and discussion

To identify mutants of the wild-type cytochrome P450 BM-3 active on alkanes, we required an assay sensitive enough to observe improvements in activities that were still very low. The colorimetric assay on the surrogate substrate 8-pnpane nicely fulfilled this role[16,18,19]. The risk in using a surrogate substrate such as 8-pnpane, however, is that the enzyme may become "addicted" to that particular substrate. Activity toward the desired substrate may not be increasing in the same proportion (or not at all). By the third generation of mutagenesis and screening, the most active BM-3 variant acquired sufficient activity to allow us to screen mutant libraries for activity directly on an alkane (octane) using a high-throughput NADPH consumption assay. NADPH oxidation, however, may also not provide an accurate measure of hydroxylase activity because reducing equivalents from NADPH can be diverted into forming reduced oxygen intermediates (H_2O or H_2O_2). Therefore, we screened all subsequent generations using a combination of the 8-pnpane assay, sensitive to product formation, and the NADPH consumption assay in the presence of octane.

By monitoring cell growth under conditions in which the P450 enzyme was expressed, we also determined that the enzyme had become toxic to the *Escherichia coli* cells during the process of acquiring higher activity toward alkanes. This increased toxicity placed an artificial barrier on how active the enzyme could become and still appear as a positive during high-throughput screening for alkane hydroxylase activity. By altering the growth and expression conditions to limit enzyme production during growth, we were able to identify P450 BM-3 mutants with very high alkane hydroxylation activities.

A total of five generations of mutagenesis and screening yielded P450 BM-3 mutant 139-3. As shown in Figure 1, the enzyme evolved

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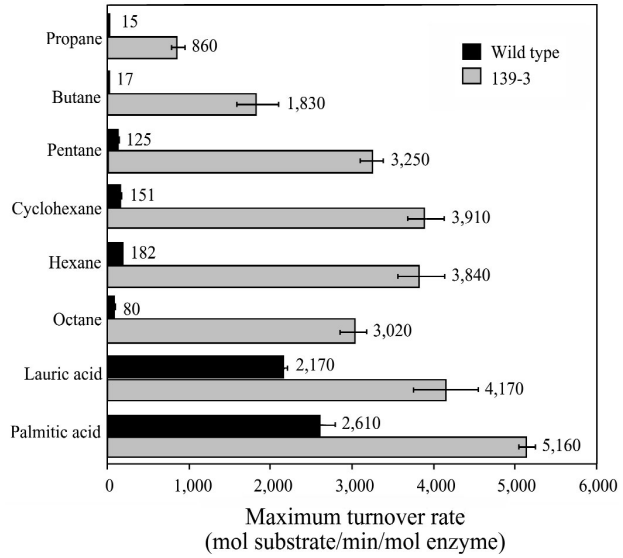

Figure 1. Maximum turnover rates (mol substrate/min/mol enzyme) for P450 BM-3 wild type and 139-3. Substrate concentrations for maximum initial rates of 139-3 (shaded bars) and wild type (black bars) are as follows: propane (saturated solution), butane (saturated solution), pentane (2.5 and 2.5 mM, respectively), hexane (2.5 and 2.5 mM), cyclohexane (2.5 and 2.5 mM), octane (2.5 and 5.0 mM), lauric acid (0.5 and 1.0 mM), palmitic acid (0.5 and 0.5 mM).

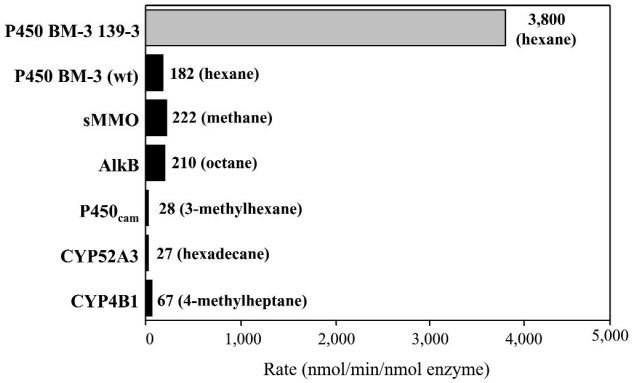

Figure 2. Maximum rates reported for alkane hydroxylation by alkane monooxygenases. Rates for CYP4B1 (ref. 10), CYP52A3 (ref. 7), $P450_{cam}$ (ref. 8), AlkB (ref. 20), and sMMO (ref. 9) were obtained from the literature. Rates for P450 BM-3 wild type and mutant 139-3 were determined in this work.

using 8-pnpane and octane to screen for more active clones was highly active on octane, hexane, cyclohexane, and pentane. The rates for hydroxylation of all the liquid alkanes exceed those of wild-type P450 BM-3 on its fatty acid substrates (Fig. 1). Surprisingly, the evolved enzyme is also more active (approximately twofold) on palmitic acid, which is already a very good substrate for P450 BM-3.

Analysis of the products of reaction with *n*-alkanes showed hydroxylation at subterminal positions (Table 1), similar to the wild-type enzyme's regioselectivity on fatty acids. No further oxidation to diols or ketones was observed. Cyclohexanol was the sole product of hydroxylation of cyclohexane. We found that the ratio of total product, as determined by gas chromatography–mass spectrometry (GC/MS), to the initial amount of dioxygen present in the reaction solution, as measured with a Clark electrode, was 1:1 for octane and hexane and 0.9:1 for cyclohexane in these oxygen-limited reactions. H_2O_2 was not detected with any of the substrates tested. This demonstrates that the BM-3 catalysis remains tightly coupled: reducing equivalents from NADPH result primarily in substrate hydroxylation, and the enzyme does not waste electrons to produce reduced oxygen intermediates. Coupling is slightly reduced during the hydroxylation of cyclohexane, when a small amount of water may be formed.

We also measured the enzyme's activity toward its oxidation products, hexanol and cyclohexanol, to assess its propensity to catalyze multiple oxidations on the same substrate when oxygen is not limiting. For 2- and 3-hexanol, the rates were only 5% and 3%, respectively, of that on hexane; on cyclohexanol, it was 7% relative to cyclohexane. At low conversions, oxidation of the alcohol product does not contribute to the rates reported for alkane oxidation, and the reaction selectivity should remain high at high conversions.

P450 BM-3 139-3 is a better catalyst than known, naturally occurring alkane monooxygenases acting on their most preferred substrates (Fig. 2). For example, the preferred substrate for the AlkB from *Pseudomonas oleovorans* is octane; its reported maximal turnover rate is 210 min^{-1} (ref. 20). In contrast to P450 BM-3, AlkB is membrane-bound and requires two additional proteins for catalytic activity: NADPH-rubredoxin reductase and rubredoxin. Soluble (sMMO) and particulate methane monooxygenases (pMMO) are large (~300 kDa), multimeric iron (and in the case of pMMO, iron–copper) enzymes with turnover rates of 200–250 min^{-1} for gaseous alkanes (methane, 222 min^{-1})[9,21,22]. Rates on alkanes larger than C4 are much lower[23]. Furthermore, AlkB and MMO have not been produced with high activity in heterologous hosts suitable for protein engineering[23–25]. Among the best known of the cytochrome P450 alkane hydroxylases is P450 Cm1 (CYP52A3), which is involved in alkane metabolization in *Candida maltosa*[26]. This enzyme is also membrane-bound, requires a separate reductase, and has turnover rates lower than AlkB (27 min^{-1} for the purified protein and 40 min^{-1} for microsomal preparations)[7]. A number of other P450s also show low levels of activity for alkanes[8,10,17].

To investigate the possibility that P450 BM-3 could be engineered to accept the small, gaseous hydrocarbon substrates preferred by MMO, we determined the ability of the 139-3 mutant to hydroxylate butane and propane. Because the screen identified

Table 1. Product distribution for alkane oxidation by wild-type P450 BM-3 and its 139-3 variant

Substrate	Product	Product distribution for 139-3 (%)	Product distribution for wild type (%)
Octane	2-Octanol	66	17
	3-Octanol	32	40
	4-Octanol	2	43
Hexane	2-Hexanol	19	20
	3-Hexanol	81	80
Cyclohexane	Cyclohexanol	100	100
Butane	2-Butanol	100	ND
Propane	2-Propanol	100	ND

ND, not determined.

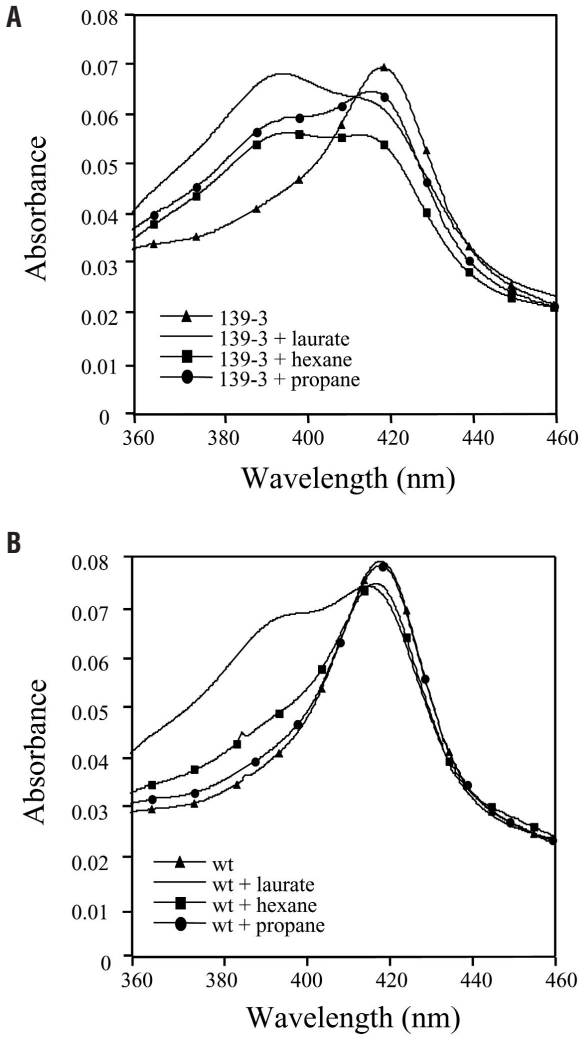

Figure 3. Optical spectra for 139-3 and wild-type P450 BM-3. (A) Mutant 139-3 (0.5 μM) in potassium phosphate buffer (0.1 M, pH 8; ▲); with laurate (0.5 mM, solid line) in 1% methanol; hexane (1.0 mM, ■) in 1% methanol; propane (saturated solution, ●). (B) Wild-type P450 BM-3 in potassium phosphate buffer (0.1 M, pH 8; ▲); with laurate (0.5 mM, solid line) in 1% methanol; hexane (1.0 Mm; ■) in 1% methanol; propane (saturated solution, ●).

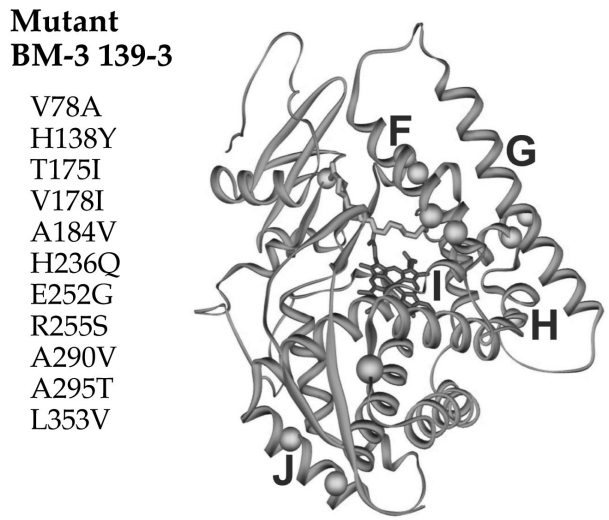

Figure 4. Positions of the amino acid substitutions in P450 BM-3 mutant 139-3. The variant contains 11 amino acid substitutions, represented by spheres on the crystal structure of the substrate-bound enzyme (Protein Data Base accession no. 1FAG). Five are clustered on the highly flexible F–G helix–loop–helix structure and the I helix along which it slides during substrate binding and release.

mutants more active on 8-pnpane and longer-chain alkanes, high activities on propane and butane were not necessarily expected. Initial rates of NADPH consumption with butane and propane are 1,800 and 860 min^{-1}, respectively (Fig. 1), results that compare favorably to those of the much larger MMO. Oxidation of propane and butane by P450 BM-3 139-3 leads to single products, 2-propanol and 2-butanol (Table 1), with no terminal hydroxylation.

The P450 resting state contains an iron protoporphyin IX as a low-spin six-coordinate ferric species with a dissociable water molecule ligated *trans* to the proximal cysteinate[27]. Substrate binding displaces water and generates a high-spin five-coordinate species. The spin-state shift causes the redox potential of the heme to increase, activating the protein for hydroxylation. The heme's absorption maximum at 419 nm corresponds to the low-spin species; a shift to 390 nm is characteristic of the high-spin form. This spectral shift is induced in mutant 139-3 by all the substrates (Fig. 3A), allowing us to estimate K_d values for octane, hexane, and laurate of 10 μM, 27 μM, and 19 μM, respectively. Only the fatty acids produce a spin shift in the wild-type enzyme (laurate: K_d = 260 μM).

Crystal structures of wild-type P450 BM-3 with and without substrate reveal large conformational changes upon substrate binding at the active site[28–31]. The substrate-free structure displays an open access channel with 17–21 ordered water molecules. Substrate recognition serves as a conformational trigger to close the channel, which dehydrates the active site, increases the redox potential, and allows dioxygen to bind to the heme. Five of the 11 amino acid substitutions in mutant 139-3 occur in the region that undergoes the largest conformational change, the F and G helices and the loop connecting them, as well as the I helix across which the F and G helices must slide (Fig. 4). The F and G helices serve as a lid, which closes over the substrate access channel upon substrate binding.

Attempts to engineer catalyst specificity are often limited to altering amino acids directly involved in substrate recognition and binding. Only one residue that is in direct contact with substrate in the wild-type enzyme has been mutated in 139-3 (V78A). Amino acids Arg47, Tyr51, Phe42, and Phe87 have been proposed to be essential for fatty acid substrate recognition[32]. Arg47, Tyr51, and Phe42 are located at the mouth of the substrate-binding pocket. Arg47 and Tyr51 bind the substrate carboxylate moiety through electrostatic and hydrogen-bonding interactions, while Phe42 serves as a hatch covering the binding pocket. All of these are retained in mutant 139-3, suggesting that their interactions are not specific to fatty acids. A salt bridge between Arg255 and Asp217 in the substrate-free structure (T. Poulos, personal communication) is disrupted by the R255S mutation in 139-3. This mutation may facilitate conformational changes that permit alkanes to bind more favorably. Rational engineering of the substrate-binding pocket of P450 BM-3 produced a triple mutant (F87V, L188Q, A74G) that shows improved activity on octane as well as aromatic substrates[33]. The evolutionary process that produced mutant 139-3 did not find beneficial mutations at these sites and instead solved the problem in an entirely different way.

The finding that a small number of amino acid substitutions can produce a broadly specific and highly active hydroxylase indicates that there is no selective advantage to maximizing this enzyme's

activity. It is possible that such a broadly active enzyme is toxic to the host organism, as it is to *E. coli*. Natural selection clearly has not maximized the activities of many cytochrome P450s, particularly the mammalian enzymes, which are one to two orders of magnitude less active than P450 BM-3. Even the BM-3 enzyme appears not to have explored its full catalytic potential. Laboratory evolution of the enzyme uncoupled from its biological context can access functions and traits that are either not biologically relevant or possibly harmful. These traits can be useful in biotechnological contexts.

P450 BM-3 mutant 139-3 is the fastest alkane hydroxylase known. Highly active, easily overexpressed in *E. coli,* soluble, and requiring no additional electron transfer proteins for catalysis, this P450 exhibits many of the properties of a good industrial biocatalyst. Additional features such as high stability, ability to function in organic solvents, and even the ability to function without the expensive cofactor are all being addressed in several laboratories with considerable success by parallel directed evolution[34,35] and reaction engineering[36,37] efforts. We predict that enzyme-based hydrocarbon oxidations will one day provide ecologically and economically attractive alternatives to the current energy- and resource-intensive processes that are the backbone of the chemicals industry.

Catalyst engineering has allowed us to direct the cytochrome P450s powerful oxidative chemistry to include hydrocarbon substrates as small as propane. Related small hydrocarbons such as propylene are also likely to be good substrates. An obvious question is whether an evolved P450 enzyme could convert ethane to ethanol, or methane to methanol. Such transformations could create new opportunities for exploiting one of the world's largest and most underutilized natural resources, methane. Variant 139-3 provides an evolutionary platform for exploring whether a cytochrome P450 can function as a methane monooxygenase.

Experimental protocol

All chemical reagents of highest purity were procured from Aldrich (Milwaukee, WI), Sigma (St. Louis, MO), or Fluka (Ronkonkoma, NY). Enzymes were purchased from New England Biolabs (Beverly, MA), Stratagene (La Jolla, CA), and Boehringer Mannheim (Mannheim, Germany).

Expression and purification of P450 BM-3 variants. The P450 BM-3 gene, which includes 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[16] (pBM3_WT18-6). This plasmid was used for production of wild-type protein and as a starting clone for directed evolution. For protein production, supplemented terrific broth (TB) medium[34] (500 ml) was inoculated with 500 µl of an overnight culture of *E. coli* BL21 containing the expression plasmid. After shaking for 10 h at 35°C and 250 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 after a total cultivation time of 30 h. The enzymes were purified following published procedures[16]. Enzyme concentration was measured from the CO-difference spectra[38].

Random mutagenesis of P450 BM-3. For the first two generations, mutagenic PCR of the heme domain was carried out as described[16], using primers *Bam*for (5′-acaggatccatcgatgcttaggaggtcatatg-3′) and *Sac*rev (5′-gtgaaggaataccgccaag-3′) and *Taq* polymerase (Roche, Indianapolis, IN). The PCR product was cloned by replacing the *Bam*HI-*Sac*I fragment of pBM3_WT18-6. Nine mutants from generation 2 showing at least twofold improved activity on 8-pnpane were recombined by staggered extension process (StEP)[39] using the same primers and 10 s extension time. A variant with three mutations (V78A, H236Q, E252G) with at least twofold improvement in activity relative to the parents was isolated. The fourth and fifth generations were generated by error-prone PCR using the Genemorph kit (Stratagene) according to the manufacturer's protocol, using ~1–10 ng of template DNA. The most active mutant, 139-3, was isolated from the fifth generation. Sequencing of the gene revealed 13 point mutations, 11 of which lead to amino acid substitutions (V78A, H138Y, T175I, V178I, A184V, H236Q, E252G, R255S, A290V, A295T, L353V) and 2 of which are synonymous (F107F, Q397Q).

Preparation of cell lysates for high-throughput screening. For high-throughput screening, clones from the first three generations were cultivated as described[16]. For subsequent generations, colonies were picked and inoculated by a Qpix (Genetix, Beaverton, OR) robot into Luria–Bertani (LB) medium (350 µl, 100 mg/L ampicillin) into 1 ml deep-well plates. 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 replicator pin into 2 ml deep-well plates containing TB medium (400 µl), ampicillin (100 mg/L), IPTG (10 µM), and ALA (0.5 mM). The clones were cultivated at 30°C for 24–30 h. Cell pellets were frozen at –20°C and resuspended in phosphate buffer (1 ml, 0.1 M, pH 8.0) containing lysozyme (0.5 mg/ml), DNase I (0.1 µg/ml), and $MgCl_2$ (10 mM). 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 determination of enzymatic activity. Mutant libraries were screened on 8-pnpane as described[16]. A cofactor (NADPH) depletion assay was also used to determine relative activities. *E. coli* lysates of the mutants were diluted into 96-well microtiter plates containing phosphate buffer (200 µl, 0.1 M, pH 8.0), alkane substrate (0.5–1 mM), and dimethyl sulfoxide (DMSO; 1%, vol/vol). The liquid alkanes were added to the buffer using alkane stock solutions in DMSO, whereas the gaseous alkanes were bubbled into buffer for ~45 min to obtain saturated solutions. The reaction was initiated by addition of NADPH (200 µM), and the oxidation of NADPH was monitored at 340 nm. A total of ~10,000 colonies were screened over five generations.

Determination of maximum initial rates of hydroxylation. The enzymes were purified and quantified as described above. The substrate concentration corresponding to the maximum turnover rate was determined by monitoring NADPH consumption with a plate reader in the presence of enzyme in phosphate buffer (0.1 M, pH 8.0) and varying amounts of substrate in methanol (1%, vol/vol). Rates were measured using an UV-Vis spectrophotometer and 1 cm pathlength quartz cuvettes. A typical reaction solution contained enzyme (700 µl, 0.35–3.5 µM) in potassium phosphate buffer (0.1 M, pH 8.0) and substrate in methanol (1%, vol/vol). The reaction was initiated by the addition of NADPH (300 µl, 200 µM), and the absorption at 340 nm was monitored. The reaction solutions for determining the rate of NADPH consumption by the mutant for alcohols (2-hexanol, 3-hexanol, and cyclohexanol) are identical to the above conditions, except a substrate concentration of 200 µM was used. H_2O_2 was determined using the 2,2'-azino-di-[3-ethylbenzothiazidine]-6-sulfonic acid–horseradish peroxidase assay according to published procedures[40].

GC/MS analysis. Biocatalytic oxidations were carried out under oxygen-limited conditions in sealed vials with no headspace. For octane, hexane, or cyclohexane conversions, the solution containing alkane (1 mM) in DMSO (DMSO, 1%, vol/vol) and enzyme (1 µM) in potassium phosphate buffer (100 mM, pH 8.0) was stirred at room temperature for 5 min, and the reaction was initiated by addition of NADPH (1 mM). Reactions with gaseous alkanes were carried out in a sealed 20 ml vial containing enzyme (10 µM) in potassium phosphate buffer (5 ml, 100 mM, pH 8.0). The headspace was filled with either propane or butane. The reaction was initiated by addition of NADPH (1 mM).

The reaction mixture was transferred to a screw-top vial with a septum using a gas-tight syringe. Before addition of the reaction solution, the vials were purged with nitrogen. The reaction mixture was analyzed directly by GC/MS using a Hewlett Packard 5890 Series II gas chromatograph coupled with an Hewlett Packard 5972 Series Mass Selective Detector. The GC was fitted with HP FFAP column (crosslinked FFAP, 30 m × 0.25 mm × 0.25 µm film thickness). The conditions for the various alkanes are as follows: for octane, isothermic at 120°C for 6 min; for hexane and cyclohexane (i) 100°C for 5 min, (ii) 100°C to 200°C at 25°C/min, (iii) isothermic at 200°C for 2 min; for propane and butane (i) 30°C for 3 min, (ii) 30°C to 150°C at 20°C/min. Authentic standards were used to identify the products by retention time. Products were further verified by matching the fragmentation distributions with a database in the software provided with the instrument.

To quantify the product yields for hydroxylation of octane, hexane, and cyclohexane, we generated calibration curves for the corresponding alcohols using the peak areas from the GC/MS chromatogram. Each conversion was carried out in duplicate, and each reaction sample was injected

into the GC/MS three times. The initial amount of oxygen in the reaction mixture was measured using a Clark electrode (Hansatech Instruments Ltd., Haverhill, MA).

Substrate binding. Dissociation constants for octane, hexane, and lauric acid were determined at 25ºC as described[41] from the change in absorption at 418 nm upon substrate binding. For the alkanes, an enzyme solution (3–5 μM) in buffer (0.1 M potassium phosphate, pH 8.0) was titrated with a stock solution of alkane (octane, 2 mM in methanol; hexane, 10 mM in methanol). Methanol (1%, vol/vol) does not induce a spin-state shift. For laurate, the reaction contained enzyme (3–5 μM) and laurate (1 mM) in buffer (20 mM MOPS, 100 mM KCl, pH 7.4). Aliquots of the enzyme–substrate solution were removed and replaced with an equal volume of an enzyme solution.

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Competing interests statement

The authors declare that they have no competing financial interests.

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