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Direct Conversion of Ethane to Ethanol by Engineered Cytochrome P450 BM3

Peter Meinhold,[a] Matthew W. Peters,[a] Michael M. Y. Chen,[a] Katsuyuki Takahashi,[a, b] and Frances H. Arnold*[a]

Most natural gas sources remain untapped as energy and chemical feedstocks. A catalyst for the selective oxidation of gaseous alkanes into more easily transported alcohols could allow these reserves to be exploited. Although catalytic alkane hydroxylation has been reported by many groups,[1] selective conversion of ethane and methane mainly to their corresponding alcohols has yet to be demonstrated.[2] For example, limited (i.e. $<$ 500 total turnovers) catalytic ethane oxidation is supported by a variety of transition metal catalysts, but these systems produce mixtures containing significant amounts of acetaldehyde and acetic acid in addition to ethanol.[3,4] Harsh oxidants such as hydrogen peroxide or sulfuric acid are usually required, although catalytic systems that make use of dioxygen have also been reported.[4] The ideal catalyst should convert ethane to ethanol with high selectivity and productivity, be easily prepared from relatively common, nontoxic materials, function at low temperature and pressure, use dioxygen from the air as the oxidant, and produce little or no hazardous waste.

Biological systems have evolved metalloenzymes that convert alkanes into alcohols with many of the features of the ideal catalyst. The well-studied methane monooxygenase (MMO), for example, catalyzes the conversion of methane to methanol and has long been a source of inspiration for catalyst designers.[5] Unfortunately, these structurally complex enzymes have never been functionally expressed in a heterologous organism suitable for bioconversion and process optimization, and therefore have proven to be of little practical use for producing alcohols.

We chose cytochrome P450 BM-3 as our target for generating an enzyme that can convert ethane and methane to alcohols because it possesses properties that make it both an ideal catalyst and straightforward to engineer: BM-3 is highly soluble, exhibits high catalytic rates on preferred substrates (thou-

[a] P. Meinhold, M. W. Peters, M. M. Y. Chen, K. Takahashi, Prof. F. H. Arnold
California Institute of Technology
Division of Chemistry and Chemical Engineering
M/C 210-41, Pasadena, CA 91125 (USA)
Fax: (+1) 626-568-8743
E-mail: frances@cheme.caltech.edu

[b] K. Takahashi
Current address:
Biocatalysis & Bioprocess Group, Catalysis Science Laboratory
Mitsui Chemicals, Inc. 1144 Togo, Mobara, Chiba 297-0017 (Japan)

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sands of turnovers per minute),[6] and is readily expressed in convenient heterologous hosts such as *Escherichia coli*. Additionally, all of the machinery required for catalysis—hydroxylase and reductase domains—is included on a single polypeptide chain,[7] unlike MMOs and most other P450 enzymes. Neither BM-3 nor any other of the thousand-plus naturally occurring members of the P450 enzyme superfamily, however, is known to oxidize ethane or methane. We and others have previously engineered cytochromes P450 that hydroxylate alkanes as small as propane.[8–10]

Communication between the heme and reductase domains of BM-3 is substrate-dependent and fine-tuned to transfer a pair of electrons from an NADPH cofactor through two reductase-bound flavins and then shuttle the electrons, one at a time, into the hydroxylase domain during catalysis.[11] Engineering the protein for activity on a new substrate such as ethane must conserve this regulated communication, and poses significant technical challenges. The engineered enzyme must bind ethane, a substrate considerably smaller than a fatty acid, directly above the heme in the active site of the hydroxylase domain. Substrate binding must initiate electron transfer from the reductase domain to the heme domain during catalysis. Once electron transfer occurs, the active iron–oxo species must be capable of breaking the high-energy C–H bond in ethane (101.1 kcal mol^{-1} vs. 95–99 kcal mol^{-1} for the secondary C–H bonds of the fatty acid substrates[12] of wild-type BM-3). Finally, the singly hydroxylated product must be released from the active site before further oxidation can occur.

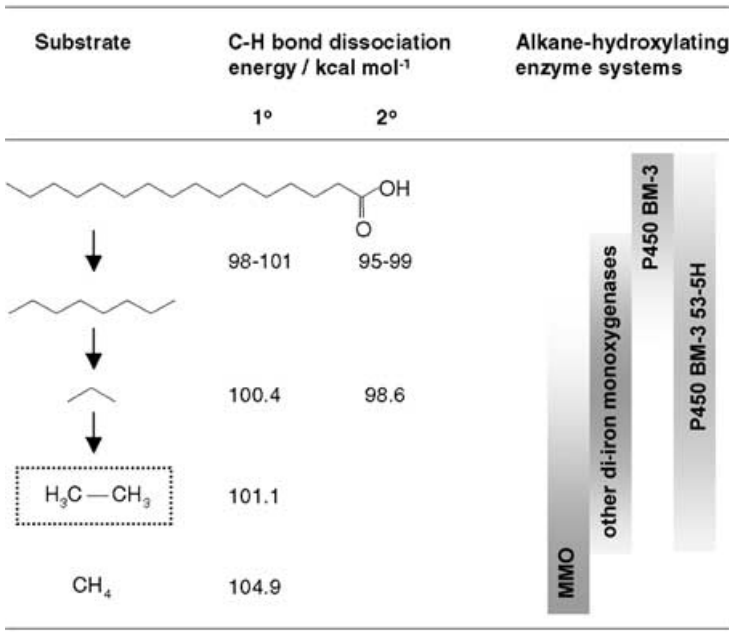

Figure 1. Variants of P450 BM-3 exhibit activities towards smaller alkane substrates, which are characterized by higher C–H bond dissociation energies. Directed evolution was used to convert wild-type P450 BM-3 stepwise from a fatty acid hydroxylase into an enzyme capable of activating the C–H bond of ethane. The figure shows the substrate range of wild-type P450 BM-3, variant 53-5H, and, for comparison, the range of substrates of other naturally occurring alkane monooxygenases.

Although crystal structures of the BM-3 heme domain with and without substrate are available,[13,14] a structure of the reductase domain is not. Furthermore, large conformational changes in both domains during catalysis[15] make it difficult to identify amino acid substitutions that can address all these issues. To engineer an ethane hydroxylase, we therefore utilized an "evolutionary" strategy in which mutations are accumulated over multiple generations of random mutagenesis and screening.[16] Our approach has been to adapt the enzyme to exhibit higher turnover on smaller and smaller alkanes (Figure 1), since efficient product formation is indicative of good solutions to the multiple engineering problems described above. In the first generations, we increased the activity of BM-3 towards octane and also acquired a small but appreciable activity on propane, producing propan-2-ol and a very small amount (less than 3%) of propan-1-ol.[9] The propane-hydroxylating activity was further increased by randomly mutating the heme domain and screening the resulting libraries for activity towards the (propane surrogate) substrate dimethyl ether. Mutant 9-10A obtained in this way was much more active towards propane than 139-3 and produced significantly more propan-1-ol (8%).[10] The increase in propan-1-ol was particularly interesting because the propane terminal C–H bond energy (100.4 kcal mol^{-1}) is similar to the C–H bond energy of ethane (101.1 kcal mol^{-1}).[12]

To obtain an ethane-hydroxylating P450, we continued directed evolution of 9-10A, this time targeting mutations to the active site rather than the entire heme domain. Guided by the high-resolution crystal structure of P450 BM-3 with bound palmitoylglycine substrate,[14] we chose for mutagenesis eleven amino acid residues in this channel that lie within 5 Å of the terminal eight carbon atoms of the bound substrate, positing that these residues are likely to contact small alkanes in the active site during catalysis. Saturation mutagenesis libraries constructed for each of these residues were screened for mutants showing improved activity towards dimethyl ether. Single mutants selected from these libraries were then recombined to form a large library containing all possible combinations of the beneficial active-site mutations, and this library was screened for activity towards dimethyl ether. The mutant with the highest activity on propane, 53-5H, was isolated and purified, and shown to catalyze 5000 turnovers of propane to propanol at a rate of 370 min^{-1} (Table 1). This enzyme also catalyzes at least 8000 turnovers of octane hydroxylation.[17]

Notably, P450 BM-3 mutant 53-5H also hydroxylates ethane to generate ethanol as the sole product. 53-5H consistently produces at least 50 equivalents of ethanol at a rate of 0.4 min^{-1}, independent of the starting concentration of enzyme (see Supporting Information). We tested for overoxidation by supplying ^{13}C-labeled ethanol as a substrate and monitoring ethanol depletion by gas chromatography and the production of acetaldehyde and acetic acid by ^{13}C NMR. No (i.e.

Table 1. Alkane hydroxylation activities of wild-type and mutant cytochromes P450 BM-3.

Enzyme	Number of amino acid substitutions	Active site amino acid substitutions	Ethane		Propane		Octane[a]			
			Rate[b]	TTN[c]	Rate[d]	TTN[c]	Rate[d]	TTN[c]	octan-2-ol [%]	*ee* [%][e]
Wild-type BM-3	–	–	–	–	–	–	30	150	17	n.d.[f]
9-10A	13[g]	V78A	–	–	23	1100	540	3000	53	50 (*S*)
53-5H	15	V78F, A82S, A328F	0.4	50	370	5000	660	8000	89	65 (*S*)
35-E11	17	V78F, A82S, A328F	0.4	250	210	6000	420	8000	89	65 (*S*)

[a] Octane reactions were performed in the presence of 1% ethanol. [b] Rates of ethanol formation were measured over 30 min by using GC coupled to an electron-capture detector and are reported as nmol ethanol per min per nmol of enzyme. Errors are at most 10%. [c] Total turnover number (TTN) was measured by using GC after completion of the reaction and is reported as nmol product per nmol protein. Errors are at most 10%. [d] Initial rates of propanol and octanol formation were measured over 1 min by GC and are reported as nmol product per min per nmol protein. Errors are at most 15%. [e] *ee* of octan-2-ol (main product) was measured by GC; the favored enantiomer is listed in parenthesis. [f] Wild-type P450 BM-3 primarily produces octan-3- and -4-ol. The yields were not sufficient for the determination of *ee* for wt BM-3. [g] Mutant 9-10A contains amino acid substitutions R47C, V78A, K94I, P142S, T175I, A184V, F205C, S226R, H236Q, E252G, R255S, A290V, and L353V.

less than 10%, the detection limit of these experiments) overoxidation products were detected in these experiments (see Supporting Information). In general, the hydrophobic active sites in P450s assist in the release of singly hydroxylated products, and we have witnessed very little (less than 5%) overoxidation of longer and more hydrophobic alcohols in any BM-3 mutants. In addition, we have observed that alkane-hydroxylating mutants generally overoxidize longer-chain alkanes such as decane to a higher extent (~7%) than shorter ones such as hexane (~1%).[10] 53-5H also catalyzes the rapid (660 min^{-1}) and efficient (80% coupled, see Supporting Information) conversion of octane to (primarily) octan-2-ol in the presence of 1% ethanol; this demonstrates that ethanol does not inhibit the catalyst.

53-5H contains three active-site mutations, A78F, A82S, and A328F, all of which replace alanine with a larger side chain and presumably reduce the volume of the active site and position small alkanes above the heme during catalysis. In addition to its activity towards ethane, 53-5H exhibits the highest regioselectivity (89% octan-2-ol) and enantioselectivity (65% *S*-octan-2-ol) towards octane that we have encountered in any BM-3 variant (Table 1), this provides further evidence of tighter substrate binding in the engineered active site.

In the presence of ethane, 53-5H oxidizes NADPH at a rate of 660 min^{-1} (see Supporting Information); this indicates inefficient utilization of the electrons transferred to the heme during catalysis. NADPH and enzymatic systems for its regeneration are expensive. In vitro, uncoupled oxidative biotransformations would not be economical; in vivo, the enzyme would deplete the cell of its energy currency. We therefore performed a further round of directed evolution to increase the ethane activity of 53-5H; this time, we targeted mutations to the entire reductase domain, including the polypeptide linker that connects it to the heme domain. In previous work (unpublished), we had identified that amino acid substitution E464G in the linker region increased total turnover in selected BM-3 mutants, presumably by facilitating communication between the highly mutated hydroxylase domain and the as yet unmodified reductase domain. Alone, this mutation does not enhance the production of ethanol by 53-5H, but further improvement was found upon screening a 53-5H library containing E464G and random mutations in the reductase domain for high activity towards dimethyl ether accompanied by reduced NADPH consumption rates in the absence of substrate. The resulting mutant 35-E11 contains all 15 of the hydroxylase-domain substitutions found in 53-5H, the linker mutation E464G, and a new mutation I710T located in the reductase domain. Compared to 53-5H, mutant 35-E11 exhibits a fivefold increase in total turnover of ethane to ethanol (total turnover number (TTN) = 250). In a typical reaction with ethane, 200 nM of mutant 35-E11 produces over 50 μM of ethanol (see Supporting Information). The rate of product formation by 35-E11 equals that of 53-5H, while the NADPH consumption rate in the presence of ethane has decreased to ~520 min^{-1} (see Supporting Information). The increased productivity probably reflects a prolonged catalyst lifetime, achieved by reducing unproductive cofactor oxidation, which inactivates the protein by forming various reactive species. Amino acid residue I710, by comparison to the crystal structure of the homologous rat P450 reductase,[18] is located near the FAD cofactor.

These results demonstrate that the 101.1 kcal mol^{-1} C–H bond dissociation energy of ethane does not pose a fundamental barrier to a cytochrome P450 and that a P450-based biocatalyst can cleanly convert ethane into ethanol without measurable side reactions. Although the rate, total turnover number, and coupling efficiency of ethane hydroxylation are too low for practical purposes, we have shown that continual improvements can be achieved, and we anticipate that further directed evolution will generate a biocatalyst with similar productivity to what we have obtained with propane (6000 turnovers).

While this paper was under review, Wong and co-workers also reported that a cytochrome P450 is capable of ethane hydroxylation. This activity was achieved by engineering the active site of P450cam.[19]

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