C–H Activation

DOI: 10.1002/anie.201006587

Tuning a P450 Enzyme for Methane Oxidation**

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Cytochrome P450 (CYP) enzymes are heme-dependent monooxygenases that catalyze the oxidation of C–H bonds of endogenous and exogenous organic compounds with formation of the respective alcohols.[1] The mechanism involves the intermediacy of a high-spin oxyferryl porphyrin radical cation which abstracts a hydrogen atom from the substrate, and the short-lived alkyl radical then undergoes C–O bond formation. The binding pockets of CYPs are relatively large, therefore small compounds do not have a statistically high enough probability of being properly oriented near the oxyferryl moiety for rapid oxidation to occur; additionally there are other effects that slow down or prevent catalysis. A notorious challenge is the oxidation of methane to methanol by chemical catalysis[2] or using enzymes of the type methane monooxygenases (MMOs).[3] It is not only the smallest alkane, but also has the strongest C–H bond (104 kcal mol^{-1}). Although CYPs represent a superfamily of monooxygenases, none have been shown to accept methane, whereas MMOs are complex enzymes (many membrane bound) that have not been expressed in heterologous hosts in any significant quantities, among other problems.[3] Herein we show that chemical tuning of a CYP, which is based on guest/host activation using perfluoro carboxylic acids as chemically inert guests, activates the enzyme for oxidation of not only medium-sized alkanes such as *n*-hexane, but also of small gaseous molecules such as propane and even methane as the ultimate challenge.

In the present study we chose, for practical reasons, the enzyme P450 BM3 (CYP102A1) from *Bacillus megaterium*, which is a self-sufficient fusion protein composed of a P450 monooxygenase and an NADPH diflavin reductase.[4] Several crystal structures of this CYP harboring a fatty acid or fatty acid derived inhibitors, as well in the absence of such compounds have been published.[5] To engineer mutants of P450 BM3 and of other CYPs for enhanced activity and selectivity toward a variety of different compounds, including such difficult substrates as small alkanes, rational design as well as directed evolution have proven to be successful to some extent.[1c,6] For example, P450 BM3 variants characterized by numerous point mutations were obtained in extensive laboratory evolution, and showed for the first time notable activity toward propane by formation of the respective alcohols (2-propanol/1-propanol = 9:1);[6a] however, the ethane to ethanol conversion remains problematic[6b] and methane oxidation has not been achieved to date. Higher activity in ethane oxidation was accomplished using mutants of P450cam,[6c] but here again methane oxidation was not reported. Our chemical approach involves a chemically inert compound that serves as a guest in the binding pocket of P450 BM3, thereby filling the space and reducing the translational freedom of small alkanes or of any other substrate. On the basis of previous reports involving CYPs harboring various substrates,[1] such guest/host interactions can be expected to induce other modes of activation effects as well, specifically water displacement at the Fe/heme site accompanied by a change in the electronic state from the inactive low-spin state to the catalytically active high-spin states.[1,5d] Moreover, many studies have shown that P450 enzymes and mutants thereof can harbor two different substrates simultaneously, thus leading to cooperative effects;[1,7–9] one example is lauric acid and palmitic acid in which cooperativity has been demonstrated by isotope labeling experiments.[8] In yet another study regarding the metabolism of bilirubin, the addition of lauric acid or the perfluorinated analogue was reported to facilitate NADPH oxidation and substrate degradation, a finding that has implications for the treatment of jaundice, uroporphyria, and possibly cancer.[9] It has also been shown for the case of a distantly related H_2O_2-dependent P450 enzyme that its peroxidase activity can be influenced by the addition of fatty acids, wherein increased or decreased activity is observed depending upon their chain length.[10]

In our endeavor we were guided by the binding mode of the natural substrates, fatty acids, of P450 BM3. The binding includes H-bonds originating from their carboxy function and residues Arg 47 and Tyr 51, as well as hydrophobic interactions.[5] The use of perfluoro carboxylic acids such as **1a–h** as chemically inert, yet activating guests was therefore envisioned, because perfluoro alkyl groups are known to be resistant to oxidation while having a hydrophobic character.[11] Moreover, it is known that a CF_3 residue is sterically comparable to a $CH(CH_3)_2$ group,[11a] which means that a perfluoro fatty acid fills much more space in a P450 binding pocket than a traditional fatty acid, and can additionally induce the crucial low-spin to high-spin conversion of Fe/heme.

$CF_3(CF_2)_nCO_2H$

1a: $n = 0$
1b: $n = 5$
1c: $n = 7$
1d: $n = 8$
1e: $n = 9$
1f: $n = 10$
1g: $n = 11$
1h: $n = 12$

In exploratory studies, the oxidation of *n*-octane and *n*-hexane as well as isomers thereof was studied using P450

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[**] We thank Heike Hinrichs, Frank Kohler, and Sylvia Ruthe for help with the chromatographic measurements.

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/anie.201006587.

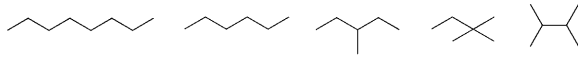
BM3 as the catalyst in both the absence and presence perfluoro carboxylic acid **1** ($n \geq 7$) under defined reaction conditions. A small amount of dimethylformamide (DMF; 1% v/v) served as the solubilizing cosolvent as part of the standard reaction conditions (Table 1). The results reveal

Table 1: Oxidation of alkanes catalyzed by P450 BM3 in the presence and absence of a perfluoro carboxylic acid **1**.[a]

Alkane	Additive	Total product formed [mM]	TON	Regioselectivity
2	none	0.15	150	2-/3-/4-octanol = 12:44:44
2	**1c**	1.16	1184	2-/3-/4-octanol = 10:42:48
3	none	0.43	149	2-/3-hexanol = 83:17
3	**1e**	1.50	525	2-/3-hexanol = 77:23
4	none	0.36	126	2-hydroxy-3-methyl-/3-hydroxy-3-methylpentane = 89:11
4	**1c**	1.36	476	2-hydroxy-3-methyl-/3-hydroxy-3-methylpentane = 88:12
5	none	0.12	111	only 2-hydroxy-3,3-dimethylbutane
5	**1c**	0.96	891	only 2-hydroxy-3,3-dimethylbutane
6	none	0.02	20	only 2-hydroxy-2,3-dimethylbutane
6	**1e**	0.19	3241	only 2-hydroxy-2,3-dimethylbutane

[a] Reaction conditions: 3.2 mM alkane, 1% v/v DMF, 2% v/v ethanol, 100 mM glucose, 1 mM $NADP^+$, 1 U mL^{-1} GDH, 1 mM additive, 100 mM KPi, pH 8.0, total volume 20 mL, 1 h, 20°C.

some remarkable effects. In all cases the presence of a perfluoro carboxylic acid leads to an increase in the total amount of alcohol product formed, and the turn over number (TON) increases by factors ranging between 4 and 12. In contrast, regioselectivity increases by the presence of an inert guest only to a small extent. Apparently, the presence of perfluoro carboxylic acids in the binding pocket does not lead to a single well-defined orientation of such alkanes, which are relatively small and lack functional groups for additional anchoring. In one case we also focused on stereoselectivity by measuring the enantiomeric excess (% *ee*) of 2-hexanol formed in the oxidation of *n*-hexane (**3**). In the absence of



the activator, the *ee* value was found to be 35% in favor of the *R* alcohol, whereas when using **1e** as an activator the enantioselectivity increased to 44% *ee* (*R*). The difference is small, but measurable, indicating that stereoselectivity can in fact be influenced even when reacting such small nonfunctionalized compounds. When subjecting functionalized compounds to P450-BM3-catalyzed hydroxylation in the presence of perfluoro carboxylic acids, notable effects on regio- and stereoselectivity are more likely. When testing iso-hexanes, products arising from the oxidation of methyl groups could not be detected.

We then turned to the smaller gaseous substrates *n*-butane and propane, and this time tested a wider range of perfluoro carboxylic acids **1**. As before, significant increases in product formation and in TON were observed (Table 2), and they depend to some extent on the nature of the perfluoro carboxylic acid. For example, in the case of *n*-butane, the efficiency is highest when using perfluoro carboxylic acid **1b**

Table 2: Oxidation of *n*-butane and propane catalyzed by P450 BM3 in the presence and absence of an additive **1** under standard reaction conditions.[a]

Alkane	Additive	Total product formed [mM]	TON	*ee* [%]
n-butane	none	1.2	527	24
n-butane	**1a**	1.1	469	32
n-butane	**1b**	8.4	3632	22
n-butane	**1c**	2.0	879	23
n-butane	**1d**	3.9	1699	20
n-butane	**1e**	5.8	2519	19
propane	**1e**	3.0	1021	–
propane	**1f**	0.67	227	-
propane	**1h**	0.50	170	–

[a] Reaction conditions: 2.5 mL reaction volume, 5 mL gas volume, 100 mM KPi, pH 8.0, 10 bar pressure (10% butane, 8% O_2, 82% N_2; 7% propane, 8% O_2, 85% N_2), 25°C.

(TON = 3632). Notably, trifluoroacetic acid (**1a**) exerts no activating effect, and the turnover is approximately the same (TON = 469) as in that for the reaction lacking this additive (TON = 527). Products resulting from the oxidation of methyl groups in *n*-butane or propane were not formed (< 1%). In the case of propane, which is essentially not accepted by P450 BM3 in the absence of an activating additive, the most striking effect was observed when using **1e**, which resulted in highest activity (TON = 1021). This is still lower than the reported activity of a P450 BM3 mutant obtained by directed evolution,[6a] but our approach is simple to perform and does not require protein engineering. Moreover, unlike the results of the protein engineering study,[6a] we observe complete regioselectivity in favor of 2-propanol.

In ultimate experiments, we considered the oxidation of methane, which is also not accepted by P450 BM3. To obtain reliable data regarding small amounts of methanol formation in an aqueous medium, the sensitive LaCourse method of pulsed amperometric detection of aliphatic alcohols was used[12] and coupled with GC/MS measurements (see the Supporting Information). Gratifyingly, high turnover numbers were observed as a consequence of adding the appropriate perfluoro carboxylic acids (Table 3). As before, the activity depends upon the chain length of the perfluoro fatty acid and achieves a maximum of TON = 2472 in the case of **1d**. Presently it is difficult to interpret the observed "trend", inter alia because perfluoro fatty acids are sterically quite different from their nonfluorinated counterparts.

In an effort to unveil the origin of enhanced activity induced by the appropriate perfluoro carboxylic acids **1**, biophysical and computational investigations were carried out. It was particularly important to obtain evidence showing that the perfluoro carboxylic acids do in fact exert their activating effects as chemically inert guests in the binding

Table 3: Oxidation of methane into methanol catalyzed by P450 BM3 in the presence of an additive **1** under standard reaction conditions.[a]

Additive	Total product formed [mM]	TON
1c	2.75	2053
1d	3.31	2472
1e	1.81	1353
1f	1.25	933
1g	0.75	560
1h	0.28	210

[a] Reaction conditions: 2.5 mL reaction volume, 5 mL gas volume, 100 mM KPi, pH 8.0, 10 bar pressure (7% methane, 8% O_2, 85% N_2), 25 °C.

pocket of P450 BM3. It is known that long-chain substrates, once in the binding pocket of P450 BM3 (and of other CYPs), lead to the displacement of water at the Fe/heme which causes a shift from the low-spin resting state to the catalytically active high-spin state of the metalloenzyme, as indicated by UV/Vis and UV/Vis difference spectra.[1,5d,7] In contrast, most inhibitors either stabilize the water or directly ligate to the Fe/heme, thus favoring the inactive low-spin state. We therefore applied this technique to P450 BM3 in the absence and presence of perfluoroundecanoic acid (**1e**), and for comparison we included the analogous C_{11} fatty acid (undecanoic acid). The anticipated effect was indeed observed, with undecanoic acid and the perfluoro analogue **1e** leading to similar UV/Vis difference spectra (see the Supporting Information). In both cases the spectra show an absorption decrease at 420 nm (low-spin complex) and concomitant increase at 390 nm (high-spin species). This behavior is typical of activated, substrate-bound P450 BM3. In additional experiments, the titration curves resulting from using **1e** (0.1 mM to 16 mM) and P450 BM3, and competition experiments involving *n*-octylamine as an inhibitor (which is known to exert its effect by entering the binding pocket of CYPs[13]) support this conclusion (see the Supporting Information).

We surmise at this stage that two phenomena are likely to be responsible for the guest/host activating effect of perfluoro carboxylic acids. The occurrence of such compounds in the binding pocket not only reduces its effective volume, but it also leads to partial conversion of the low-spin species into a catalytically active high-spin Fe species, probably induced by the displacement of water at the Fe/heme.[1,5d,7] In line with this proposition is the observation that trifluoroacetic acid (**1a**) fails to show any activating effect.

To gain additional insight, we turned to molecular dynamics (MD) simulations.[14] Following MD calculations of P450 BM3 in the absence of any ligand, the Gromos algorithm was applied,[15] leading to the visualization of an ensemble of different conformers. Subsequently, perfluorodecanoic acid (**1d**), which caused the highest activity in methane oxidation (Table 3), was successfully docked in the average minimized structure of the most populated cluster of P450 BM3 conformers. A second MD calculation was then performed of the enzyme harboring the activator **1d** and simulating the experimental conditions of methane concentration and pressure (see the Supporting Information). It was found that **1d** adopts two different binding modes of essentially equal energy. In binding mode I the carboxy function interacts with Tyr51 and Ser72 through hydrogen bonds that allow the end of the perfluoro chain to lie very deeply in the binding pocket near the Fe/heme, thereby distinctly reducing space. Binding mode II is characterized by hydrogen bonds between the carboxy function and Arg47, which in this case prevents such deep penetration of the perfluoro chain. Space around and near the active Fe site is therefore considerably larger. The simulations do not provide accurate information as to whether the water at Fe/heme is displaced by activator **1d** as actually indicated by the UV/Vis difference spectra.

In the final step of the computational analysis, docking experiments using methane in the presence and absence of activator **1d** were performed (see the Supporting Information), and they led to some remarkable results. In the case of **1d** undergoing binding mode I with a reduced space around the active site, a cluster of methane molecules lies directly above Fe/heme, perfectly juxtaposed for rapid oxidation (Figure 1). In the case of binding mode II, methane distributes throughout the distinctly larger cavity, presumably leading to lower or no activity. In neither of the cases above nor in the absence of an activating guest was any reorientation of the phenyl group of Phe87 with concomitant shielding of the oxyferryl porphyrin radical cation observed; this shielding would slow down catalysis as reported for certain other substrates.[1,4–7]

In the absence of the activator **1d**, the binding pocket is dramatically larger, and the Glide algorithm[15] does not predict any specific positioning of methane clusters (see the

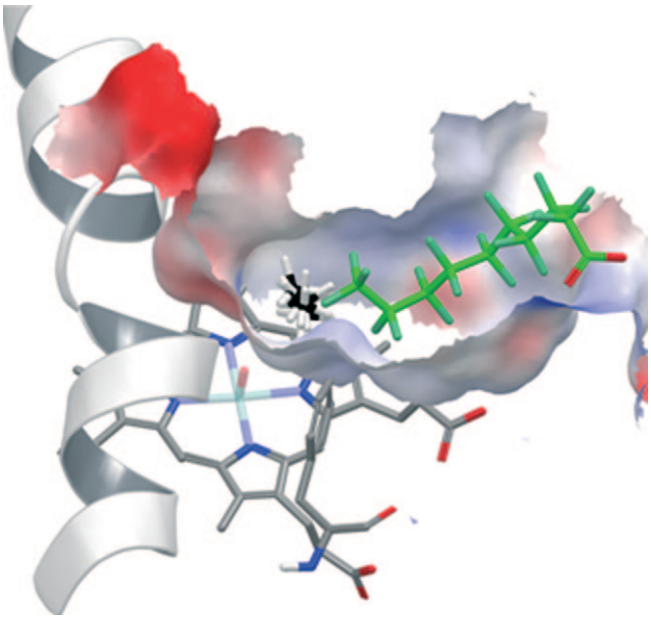


Figure 1. Binding pocket representation of P450 BM3 harboring activator **1d** (perfluoro chain in green) and docking poses of a methane cluster (carbon atoms in black and hydrogen atoms in white) hovering above Fe=O (red) in the porphyrin ring. Binding pocket surface is colored according to its electrostatic potential (red: positive, blue: negative, white: neutral). Helix I is shown as a point of reference.

Supporting Information). It remains to be seen whether such extensive computational analyses of the other perfluoro carboxylic acids used in the present study will reflect the observed dependency on chain length (Table 3).

In conclusion, we have implemented a chemical strategy for tuning the catalytic profile of the monooxygenase P450 BM3 so that the enzyme accepts such notoriously difficult substrates as propane and methane and results in the formation of the respective alcohols. We expect ethane[6b,c] to behave similarly. In contrast to time-consuming protein engineering,[6] the present approach simply requires the addition of an appropriate chemically inert perfluoro fatty acid to the enzyme, thereby triggering a catalytically activating effect which originates from specific guest/host interactions in the binding pocket. A shift from an inactive low-spin state to a catalytically active high-spin state and a decrease in the effective volume of the binding pocket appear to be the crucial factors as shown by UV/Vis difference spectra as well as a theoretical analysis based on MD simulations and docking experiments. The present approach not only allows methane to be oxidized with notable enzyme activity, but also opens the door for using perfluoro carboxylic acids, which can be expected to bind to most CPYs, to influence the catalytic profile of monooxygenases as catalysts in the functionalization of more complex organic compounds, including the control of regio- and stereoselectivity.[16] The size of the perfluoro carboxylic acid can then be matched to the specific requirements, which depend upon the steric and electronic nature of the to-be-oxidized substrate, with MD simulations serving as a guide. The use of chiral perfluoro carboxylic acids or of other types of chemically inert perfluoro compounds as additives for influencing stereoselective oxidative hydroxylation is yet another perspective. Finally, we anticipate that the combination of the present tuning method with protein engineering techniques[6] constitutes a powerful tool for controlling regio- and stereoselective P450-catalyzed C–H activation.

Received: October 20, 2010
Revised: January 1, 2011
Published online: February 17, 2011

Keywords: alkanes · C–H activation · host–guest systems · methane oxidation · P450 enzymes

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