Alkane Activation

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Use of Perfluorocarboxylic Acids To Trick Cytochrome P450BM3 into Initiating the Hydroxylation of Gaseous Alkanes**

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The catalytic hydroxylation of inert C–H bonds under mild conditions is a major challenge in synthetic chemistry.[1] Despite important advances in this area,[2–4] the direct activation of alkanes is still a key research topic. Such transformations are of great value to the chemical industry. In particular, the hydroxylation of gaseous alkanes has become an increasingly important approach for producing liquid fuel or chemical precursors from natural gas.[5] Biocatalysts provide an alternative to conventional chemical processes for alkane hydroxylation.[6] Iron-containing monooxygenases, such as soluble methane monooxygenase (sMMO), alkane ω-hydroxylase (AlkB), and cytochrome P450s (P450s), show remarkable catalytic activity towards the oxidation of a variety of alkanes with dioxygen to give alcohols.[6,7] P450BM3 (CYP102A1) isolated from *Bacillus megaterium* is a promising enzyme, as it has the highest catalytic rate reported so far for a P450 ($>15000\ min^{-1}$ with arachidonic acid). The high catalytic ability of P450BM3 is attributed to the location of its heme and reductase domains on a single polypeptide chain.[8] The hydroxylation rate is even faster than those of sMMO ($222\ min^{-1}$ with methane) and AlkB ($210\ min^{-1}$ with octane).[9,10] P450BM3 exclusively catalyzes hydroxylation at the terminus of alkyl chains (ω-1, ω-2, and ω-3; see Figure 1) of long-alkyl-chain fatty acids, such as myristic acid and palmitic acid, as well as some unsaturated fatty acids.[11,12]

The crystal structure of the bound form of P450BM3 with palmitoleic acid shows that palmitoleic acid is fixed by two major interactions: 1) the ionic interaction of the substrate carboxylate group with Arg47 and Tyr51, and 2) the hydrophobic interaction of the alkyl chain with amino acids at the substrate-binding site. The catalytic cycle of P450BM3 is initiated by fatty-acid binding to the heme cavity of P450BM3. This binding induces a structural change in P450BM3 and the removal of a water molecule coordinated to the heme iron atom (Fe^{3+}). It results in a positive shift in the reduction potential of the heme iron atom,[8,13,14] followed by electron transfer from NADPH (the reduced form of nicotinamide adenine dinucleotide phosphate) to reduce Fe^{3+} to Fe^{2+}. After the reductive activation of molecular oxygen to generate a highly active oxidant species, the oxoferryl(IV) porphyrin π cation radical (so-called compound I),[15] the bound substrate is oxidized by compound I (Figure 1a). According to this reaction mechanism of P450BM3, substrate binding is crucial for initiation of the catalytic cycle. Thus, substrates whose structures are different from that of the fatty acid cannot enable the formation of compound I from P450BM3. Therefore, P450BM3 does not catalyze the hydroxylation of gaseous alkanes, such as methane, ethane, or propane, because gaseous alkanes cannot bind to the heme active site of P450BM3. Since the substrate specificity in an enzymatic reaction is governed by the local chemical environment of the enzyme active site, mutants of P450BM3 have been prepared as biocatalysts for the hydroxylation of gaseous alkanes.[16,17]

Although the mutagenesis of P450BM3 to construct a binding pocket suitable for gaseous-alkane hydroxylation is regarded as a promising method, we propose a simple and unique strategy for the hydroxylation of gaseous alkanes with wild-type P450BM3 without the replacement of any amino acid residues. We assumed that compound I could be formed from P450BM3 by the addition of a "dummy substrate" with a chemical structure similar to that of a fatty acid. Studies on the binding of its inhibitors have shown that P450BM3 has a large heme cavity that can accommodate two different molecules at the same time[18,19] and thus indicate that the simultaneous binding of a gaseous alkane and a dummy substrate would be possible. If the dummy substrate had stable chemical bonds, such as C–F bonds, that were not oxidizable,[20] and if gaseous alkane molecules could penetrate into the active site of P450BM3 in the presence of such a dummy substrate, then gaseous alkane molecules could be hydroxylated. Herein, we report that the addition of perfluorocarboxylic acids (PFs) as dummy substrates to wild-type P450BM3 results in the hydroxylation of gaseous alkanes to the corresponding alcohols.

Our strategy for the hydroxylation of gaseous alkanes and a plausible active-site structure of P450BM3 containing both perfluorodecanoic acid and propane are shown in Figure 1c. We selected a series of PFs with alkyl chains containing 8–14 carbon atoms (PFC8–PFC14) as dummy substrates. We expected that PFs with shorter alkyl chains than those of

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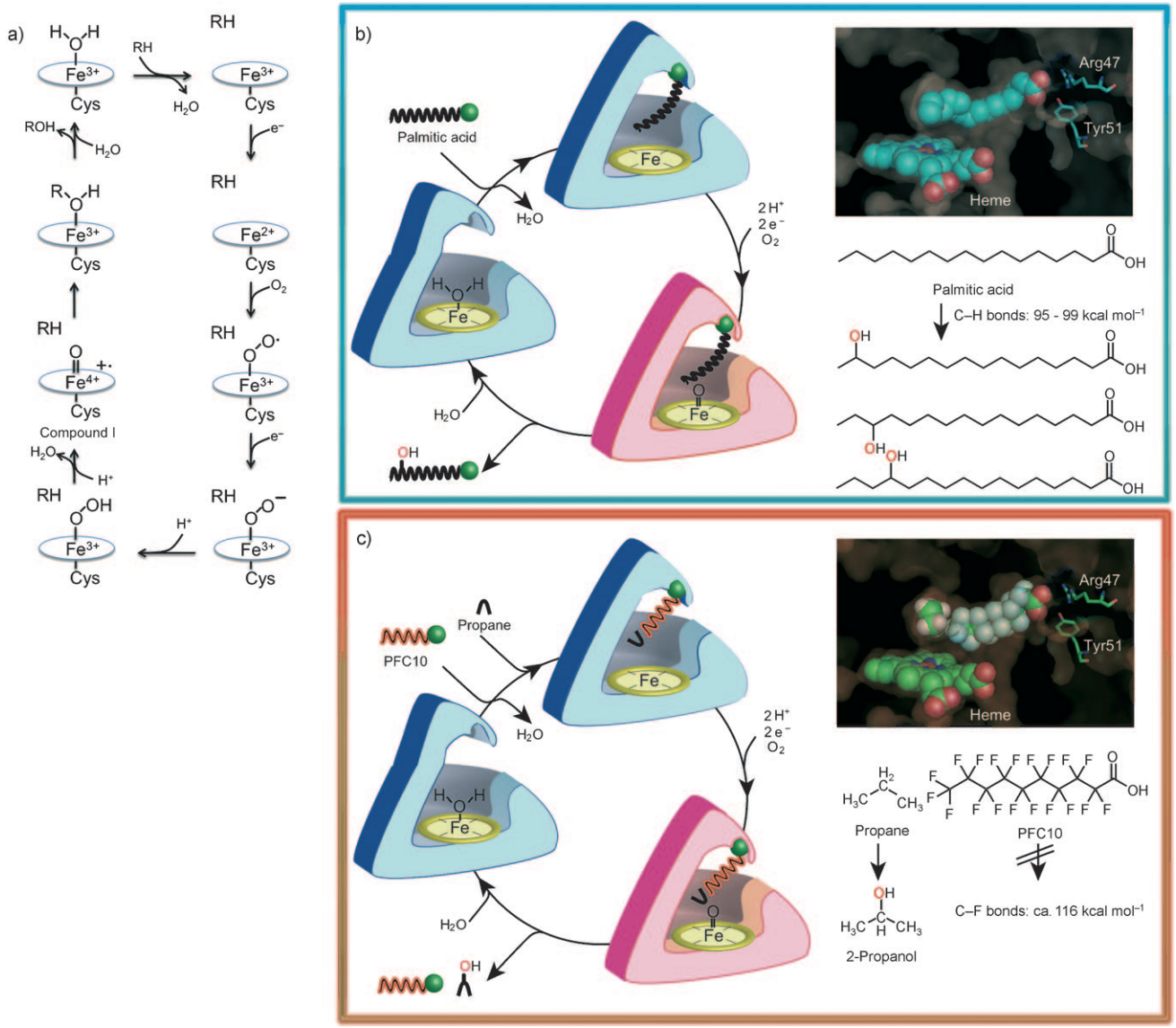

Figure 1. a) General catalytic reaction mechanism of P450s. b) The natural and c) our proposed reaction of P450BM3. In (b) and (c), the respective reaction model is shown on the left, and the image at the top right shows the active site of P450BM3 containing palmitoleic acid (PDB accession No. 1FAG) and a plausible active-site structure containing manually docked propane and PFC10, respectively (O red, N dark blue, Fe brown; C pale blue in (b); H white, C green, F aqua blue in (c)). The atoms of the heme group, substrates, and PFC10 are shown as spheres. Hydrogen atoms are omitted for clarity, except those on the propane molecule. The respective substrate and hydroxylated product are shown at the bottom right of (b) and (c); the oxygen atom inserted into the substrate is shown in red.

natural substrates would provide space for gaseous alkanes because of the incomplete occupation of the heme cavity; the substrate-binding site of P450BM3 naturally accommodates a fatty acid containing 16 carbon atoms.

Initially, we investigated whether a series of PFs would serve as dummy substrates to remove the water molecule coordinated to the heme iron atom of P450BM3 as the first step of the catalytic cycle (Figure 1a) by examining the change in the UV/Vis spectrum of P450BM3 on the addition of PFs with alkyl chains containing 8–14 carbon atoms. A spectral change would be indicative of binding to the dummy substrate, and the dissociation constant of the PFs was estimated to be in the range $K_d = 1.8$–$1900\ \mu M$ (Table 1; see also Figure S1 in the Supporting Information).

Table 1: Dissociation constants of the PFs and NADPH-consumption rate in the presence of the PFs.

Dummy substrate	K_d [μM]	NADPH consumption [min^{-1}][a]
PFC8	1900	72 ± 19
PFC9	980	190 ± 6
PFC10	290	310 ± 8
PFC11	91	330 ± 20
PFC12	30	430 ± 20
PFC13	1.8	440 ± 20
PFC14	13	390 ± 4

[a] The unit for NADPH consumption is (nmol NADPH consumed) min^{-1} (nmol P450)$^{-1}$. Values are averages ± standard deviation calculated from three different experiments.

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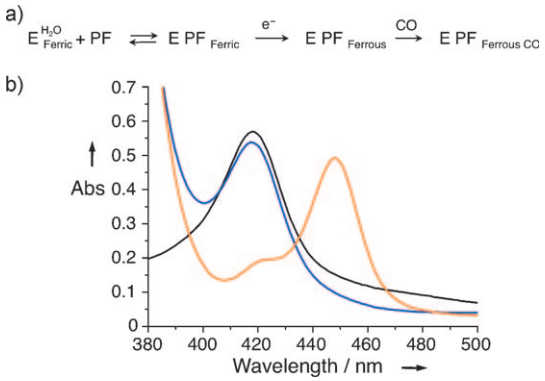

Figure 2. a) Reaction mechanism for the formation of a ferrous CO complex. The terms E and E PF denote the P450BM3 and P450BM3/PF complexes, respectively. b) UV/Vis spectra of P450BM3 in a buffer saturated with CO gas before the addition of NADPH (black) and after the addition of NADPH in the absence (blue) and presence (orange) of PFC10.

Although the UV/Vis spectral changes induced by the PFs were small, the elimination of the ligated water molecule initiated the consumption of NADPH (Table 1). This NADPH oxidation, coupled with the production of H_2O through the four-electron reduction of the iron-atom-bound oxygen molecule only in the presence of the PFs,[21] indicated that the binding of the PFs induces the formation of compound I, followed by its reduction by NADPH in the absence of a gaseous substrate. The reduction of the ferric heme of P450BM3 with PFs ($E\,PF_{ferric}$) to ferrous heme ($E\,PF_{ferrous}$) by NADPH provided further confirmation of the formation of a ferrous CO complex ($E\,PF_{ferrous\,CO}$; Figure 2a).

The formation of a ferrous CO complex of P450BM3 ($E\,PF_{ferrous\,CO}$) with a UV/Vis absorption maximum at 448 nm was observed when NADPH was added to a CO-saturated reaction mixture containing PFC9–PFC14, whereas no ferrous CO complex was observed in the absence of PFs (Figure 2b). In contrast to PFC9–PFC14, PFC8 did not induce the formation of the ferrous CO complex, although a small amount of NADPH was consumed, which implies that PFC8 is not effective for the elimination of the iron-bound H_2O because of its short alkyl chain. These results showed that the removal of iron-bound H_2O and the subsequent electron transfer from NADPH to the heme iron atom was initiated by PFC9–PFC14.

We next investigated the hydroxylation of gaseous alkane molecules (methane, ethane, propane, and butane) and cyclohexane with P450BM3 in the presence of a series of PFs (Figure 3; see also Table S1). All gaseous alkanes were supplied as a gas-saturated buffer maintained at a pressure of 5–8 kPa; the mixed gas contained the gaseous alkane and oxygen in an 8:2 ratio during the reaction. We examined the rate of product formation and NADPH oxidation, as well as the coupling efficiency (i.e., the ratio of product formation to NADPH consumption), for a reaction time of 10 min (Figure 3). Propane, butane, and cyclohexane were hydroxylated with P450BM3 (500 nM) in the presence of a PF (100 μM) to yield 2-propanol, 2-butanol, and cyclohexanol, respectively. The reactions with methane and ethane did not lead to any detectable amount of the corresponding alco-

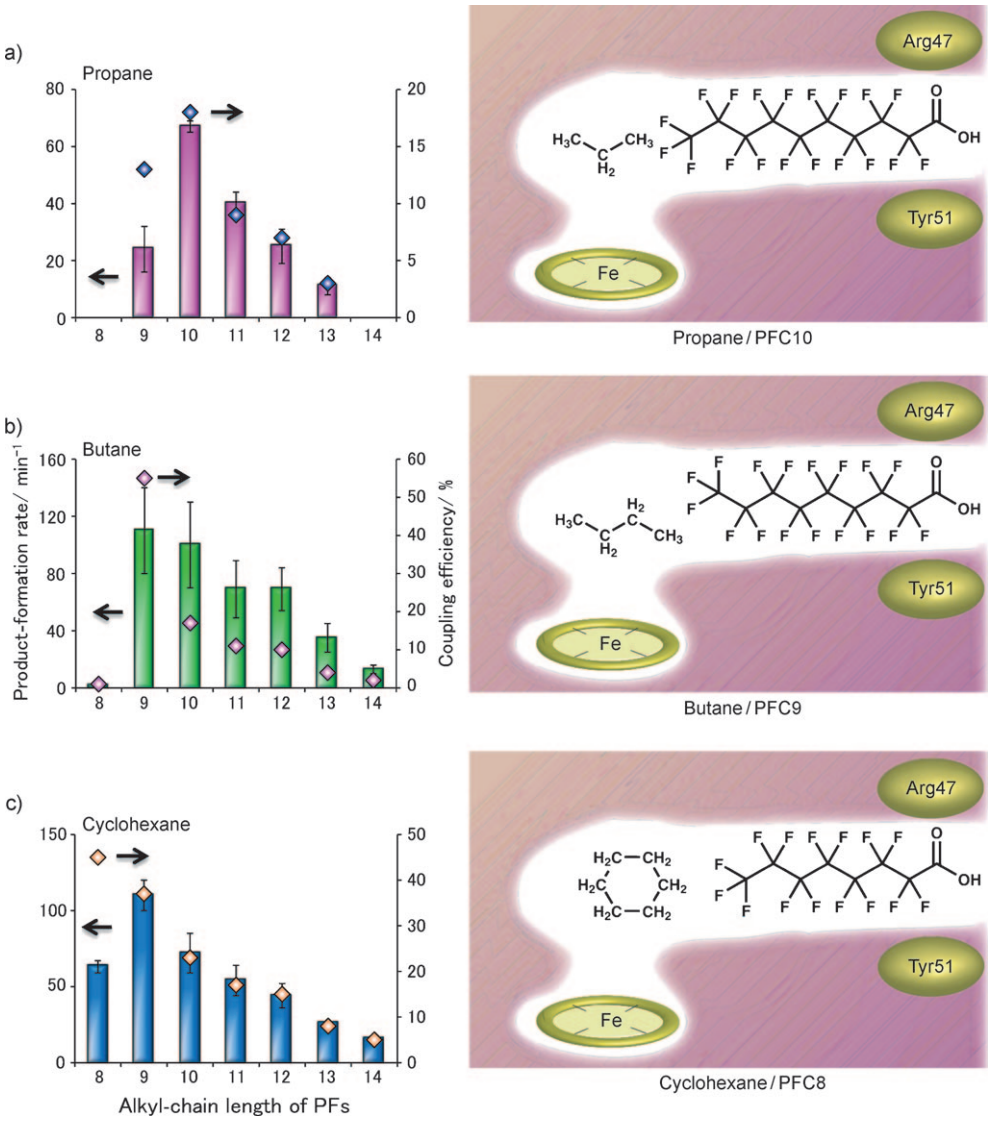

Figure 3. Histograms showing the formation rate of a) 2-propanol, b) 2-butanol, and c) cyclohexanol produced by P450BM3 with various PFs. The square symbols denote the coupling efficiency. The error bars denote the standard deviation calculated from three different experiments. The model on the right of each graph shows the occupation of the heme cavity by a combination of the preferred PF with the corresponding alkane.

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hols.[22] In the absence of PFs, no product was detected for any of these alkanes.

These results clearly show that the addition of the dummy substrate to wild-type P450BM3 conferred the ability to catalyze the hydroxylation of gaseous alkanes. Interestingly, the rates of product formation and NADPH oxidation were significantly influenced by the alkyl-chain length of the PFs. PFC10 showed the highest rate of product formation ($67\ min^{-1}$) and the highest coupling efficiency (18%) of the PFs examined for propane hydroxylation.[23] The fact that decanoic acid did not induce the formation of 2-propanol clearly indicates that the substitution of hydrogen with fluorine in the carboxylic acid is crucial for hydroxylation. A decrease in the formation of 2-propanol was observed when the length of the PF was changed. This result indicates that the size of the heme cavity of P450BM3 depends on the alkyl-chain length of the bound PF, and that the cavity size provided by PFC10 is the most suitable for fixing propane molecules. In accord with our hypothesis, the larger alkanes tended to prefer the PFs with shorter alkyl chains for an efficient reaction (Figure 3, right). PFC9 and PFC10 were almost equally effective for butane hydroxylation and showed the maximum rate of formation of 2-butanol (100 and $113\ min^{-1}$), whereas PFC9 showed the highest coupling efficiency of 57%. In the hydroxylation of cyclohexane, PFC9 showed the highest rate of cyclohexanol formation ($110\ min^{-1}$) with a coupling efficiency of 35%.[24] Although PFC8 did not induce the formation of the ferrous CO complex, PFC8 was effective for the hydroxylation of cyclohexane with a coupling efficiency of 45%. This result suggests that a cooperative effect operates. In fact, the ferrous CO complex was formed in the presence of both PFC8 and cyclohexane (see Figure S2). These findings show that a combination of the alkyl-chain length of the PFs and the size of the alkane governs the efficiency of the hydroxylation reaction catalyzed by P450BM3 with a dummy substrate.

Although we have focused herein on a series of perfluorinated fatty acids with a linear alkyl chain (PFC8–PFC14) as the dummy substrate, PFs with a branched alkyl chain and/or partially fluorinated carboxylic acids may also be potent dummy substrates. Furthermore, our system is compatible with reported protein-engineering techniques, such as mutagenesis and chemical modification.

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[22] The solubilities of methane, ethane, propane, and butane in water at 293 K and 0.1 MPa are 1.4, 1.8, 1.6, and 1.2 mM, respectively, which suggests that the solubility of a gaseous alkane is not a major factor in determining whether it can be hydroxylated.[25]
[23] P450BM3 with PFC10 catalyzed the hydroxylation of propane for a period of more than 1 h, and the reaction stopped after all of the added NADPH had been consumed (see the Supporting Information).
[24] We examined the origin of the oxygen atom in cyclohexanol in ^{18}O labeling experiments and confirmed that, in most cases, the oxygen atom in cyclohexanol was derived from molecular oxygen (see the Supporting Information).
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