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How Do Perfluorinated Alkanoic Acids Elicit Cytochrome P450 to Catalyze Methane Hydroxylation? An MD and QM/MM Study

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Abstract

Recent experimental studies show that usage of perfluoro decanoic acid (PFDA), as a dummy substrate, can elicit P450_{BM3} to perform hydroxylation of small alkanes, such as methane (ref. 17) and propane (ref. 17 and ref. 18). To comprehend the mechanism whereby PFDA operates to potentiate P450_{BM3} to catalyze the hydroxylation of small alkanes, we used molecular dynamics (MD) and hybrid quantum mechanical / molecular mechanical (QM/MM) calculations. The MD results show that without the PFDA, methane escapes the active site, while the presence of PFDA can potentially induce a productive Cpd I-Methane juxtaposition for rapid oxidation. Nevertheless, when only a single methane molecule is present near the PFDA, it still escapes the pocket within less than a nanosecond. However, when three methane molecules are present in the pocket, they alternate quasi-periodically such that at all times (within 10 ns), a molecule of methane is always present in the proximity of Cpd I in a reactive conformation. Our results further demonstrate that the PFDA does not exert any electrostatic catalysis, whether the PFDA is in the protonated or deprotonated forms. Taken together, we conclude that methane hydroxylation requires, in addition to PFDA, a high partial pressure of methane that will cause a high methane concentration in the active site. Further study of ethane and propane hydroxylations demonstrates that higher alkane concentration is helpful for all the three small alkanes. Thus for the smallest alkane, methane, at least three molecules are necessary whereas for the larger ethane, two molecules are needed to force one ethane to be closer to Cpd I. Finally, for propane a second molecule is helpful but not absolutely necessary; for this molecule the PFDA may well be sufficient to keep propane close to Cpd I for efficient oxidation. We therefore propose that high alkane pressure should assist small alkane hydroxylation by P450 in a manner inversely proportional to the size of the alkanes.

Introduction

Cytochrome P450s are versatile heme-containing monooxygenases that use O₂, and two proton and electron equivalents, to generate the active species, iron(IV)-oxo porphyrin π cation radical species, so-called Compound I (Cpd I), which functions as a catalyst in detoxification of xenobiotics and biosynthesis of endogenous compounds.¹ One of the most important reactions catalyzed by P450s is alkane hydroxylation.^{2, 3} The consensus mechanism of this reaction,⁴ shown in Scheme 1, involves initial hydrogen abstraction from the alkane by Cpd I, followed by radical rebound to generate the ferric-alcohol complex.

Although theory^{5,6} and experiment¹ have shown that P450s are capable of activating C–H bonds of organic compounds very efficiently, the oxidation of small-sized gaseous alkanes such as methane, ethane and propane, has still eluded P450s and has posed a challenge of both intellectual and practical dimensions. Three types of difficulties always seemed to be in

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the way of achieving this goal. First, P450s have generally a relatively larger binding pocket compared with the size of small alkanes, and this may prevent the requisite juxtaposition between the alkane and Cpd I, and lead to sluggish activation.^{5, 6} Second, being gaseous molecules, small alkanes may not be present in the active site in sufficient concentration to afford much oxidative products. Third, smaller-sized alkanes have high C–H bond dissociation energies (BDEs),⁷ and hence their oxidation is presumably more difficult. As such, being the smallest alkane and having the highest BDE, methane poses the greatest difficulties. Indeed, theoretical calculations have predicted a relatively high activation barrier in the H-abstraction step of methane hydroxylation by P450.^{8,9} For all these reasons, and for the practical importance of methane-to-methanol conversion,¹⁰ the search for methods that enable P450 to perform methane hydroxylation has become the Golden Fleece of the field.

Mutagenesis and chemical modifications of P450s by replacing selected amino acid residues in active site have been shown to enhance the activity and selectivity toward various small and medium-sized alkanes, but still none of these mutated P450s has been shown to accept methane.^{11–16} The breakthrough has been made recently by Reetz¹⁷ and Watanabe,¹⁸ who have successively reported a unique approach whereby the wild-type P450_{BM3}, a fatty acid monooxygenase from *Bacillus megaterium*,^{19, 20} was elicited to oxidize the intractable small alkanes including methane.¹⁷ In their implemented strategy, perfluoro carboxylic acids (PFCAs) were inserted into the binding pocket of P450_{BM3} to serve as nonactivated dummy substrates, and thereby exerting two major effects: The dummy substrates initiated the catalytic cycle of the enzyme, and at the same time they consumed space and restricted the active site.^{17, 18} The so diminished space of the active site, admitted in turn alkanes that underwent hydroxylation to the alcohol products.

Additionally, the PFCAs induced catalytic activity, which depended on the tuning effect between the size of alkanes and the length of perfluoro alkyl-chain of PFCA vis-à-vis the size of heme cavity of P450_{BM3}. Generally, the smaller were the alkanes and the longer the chain of PFCA was, the more efficient was the alkane hydroxylation reaction. Although the oxidation activity was still lower than that obtained by the protein-engineering techniques, the PFCAs-embedding approach is appealingly simple and not time-consuming. Even more important is the fact that the most challenging small alkanes, like propane,^{17,18} and methane,¹⁷ were admitted and activated by P450_{BM3} without the need to modify amino acid residues of the enzyme. Curiously though, while Watanabe et al,¹⁸ could not observe the oxidation of methane and ethane, Reetz and coworkers,¹⁷ reported the oxidation of a variety of alkanes, including methane and propane.

This breakthrough achievement is the focus of the present study. While we cannot resolve the disparity of the results of the two groups,^{17,18} theory can still investigate the requisites under which P450_{BM3} is made capable of hydroxylating methane and propane. Thus, we seek to elucidate the manner whereby the inserted PFCA induces the desired activity and to explore the mechanism of the small alkane oxidation by P450_{BM3} in such a system. Does the oxidation occur simply because of the packing of the small alkane by the PFCA into the active pocket of the enzyme? Is there an electrostatic catalysis by the negatively charged fluorines of the PFCA, as found recently the epoxidation with H₂O₂ in the presence of perfluorinated alcohols?²¹ Is the PFCA sufficient to induce hydroxylation? These are the main questions that are explored in this paper using MD and QM/MM calculations of methane hydroxylation vis-à-vis ethane and propane by P450_{BM3} in the presence of perfluoro decanoic acid (PFDA) that afforded the highest activity.¹⁷

Methods

The QM/MM study was carried out in the usual software combination^{22–24} used in the group, and followed well established protocols,^{6,25} employed in previous studies.^{26–28} Details are given in the Electronic Supporting Information (ESI) document, and the ones more specific to the paper are given here briefly. Closely related QM/MM approaches have been employed successfully by other research groups.^{29, 30}

Preparation of the System

The initial coordinates were taken from the X-ray crystal structure (PDB Code: 1JPZ, resolution 1.65 Å) wherein the heme domain of P450_{BM3} is bound to N-Palmitoylglycine.³¹ We used only chain B from the crystallographic dimer, and the unresolved residues in this chain were omitted in our calculations. The associated crystal water molecules were included in the model. N-Palmitoylglycine was removed and replaced by an oxygen atom at a distance of 1.65 Å above the iron and keeping an O-Fe-S angle to 170.1 degrees to generate Cpd I. We then added missing hydrogen atoms, took care of the protonation states of acidic and basic residues, using the established protocols based on the pK_a values given by the empirical PROPKA³² procedure and verified through visual inspection. To build a neutral system with zero net charge and avoid thereby artificial electrostatic effects some titrable residues on the surface of the protein were also selected to be protonated (see the detailed protonation scheme in the ESI).

MD Equilibration and Production

Figure 1a shows the active site of P450_{BM3}. Thus, based on Reetz et al,¹⁷ PFDA was selected as the dummy substrate and docked into the P450_{BM3} binding pocket, using Autodock 4.2,³³ in the manner described in previous work.³⁴ This was followed by molecular dynamics (MD) runs using the highest populated docking mode wherein the carboxylic group of PFDA interacts directly with the side chain of Ser72 but indirectly with Arg47 and Tyr51. Methane was added manually into the resulting binding space close to the ferryl-oxo group with a C---O distance of 3.0 Å.

A partial solvation scheme was used to solvate a region of 35 Å around the ferryl-oxo oxygen atom center by overlaying a water ball on the enzyme. A potential was imposed on the water sphere to prevent the free water molecules from escaping into the vacuum. The solvation process was repeated several times until no more than 50 water molecules will be added in the next step.

After solvation, we performed a preliminary MD run to test whether methane could be retained in the pocket. However, *after a very short time, methane escaped the pocket to a position too far from the oxo ligand* to have any reactivity (see later). Inspection showed still significant vacant space around the active site in the vicinity of oxo, methane and PFDA. Therefore, we manually added additional water into these spaces (see Figure 2a), to fill this gap, and obtained a system having two additional solvent waters around the active site. However, these additional solvent waters could not keep methane in the active site for a long time (see later). Thus, we decided to replace these two water molecules by two methanes, since obviously the pocket is still large enough to accommodate more than one molecule. Finally we got a system consisting of 19755 atoms including 10851 atoms in the solvent and three methane molecules in the active site (see Figure 2b).

The solvated system was then relaxed by performing classical energy minimization and MD simulations, i.e. 100 ps equilibration and 10 ns production, at the MM level using the CHARMM22 force field³⁵ as implemented in the CHARMM program.²³ During the energy minimizations and MD simulations, an active region was defined to include the residues

within 25 Å, and the water molecules within 35 Å around the ferryl-oxo oxygen atom. The residues and water outside the active region as well as the Cpd I unit including coordinating Cys400 were kept fixed. The MD equilibration and production procedures were repeated with the P450_{BM3}-methane system in the absence of PFDA for testing whether methane is retained in the pocket without PFDA (see later). After the MD procedure, on the system containing the enzyme, PFDA and the methane molecules, we selected two different snapshots for subsequent QM/MM calculations. As the snapshots gave similar results, we discuss here only the snapshot after 1823 ps of production; the other one studied can be found in the ESI.

In order to explore the reactivity of ethane and propane, we manually replaced one of the methane molecules, which is closest to oxo, by ethane and propane respectively, and removed the other two methane molecules. The so generated systems were subject to 2 ns MD production. As will be shown below, to maintain one of the molecules close to Cpd I, ethane needs two molecules in the active site. However, while a second molecule is also helpful to propane, it is not necessary since propane seems sufficiently large to pack in the proximity of Cpd I. The snapshots after 500 ps of these two MD simulations were taken randomly for the subsequent QM/MM calculations.

QM Regions

Three QM regions were employed in our QM/MM calculations. The smallest one contains the iron-oxo-porphine, the sulfur of Cys400, the capping hydrogen atoms and substrate (see R1 for methane system in Figure 1b). While the larger QM region (R2 in Figure 1b), included also the PFDA. The largest QM region was extended to include side chain of Arg47 which was modeled as methylguanidinium cation (see R3 in Figure 1b). The QM/MM optimization included R1/R2/R3 and the MM region with all residues and water molecules within 4 Å around the active site (about 930 atoms in total, see ESI). The model of the deprotonated PFDA in MM or QM region was investigated by QM/MM calculation as well, using the R1 or R3 QM regions.

QM/MM Methodologies

As done before,^{26,27} the QM subsystem was computed with the unrestricted B3LYP^{36,37} functional using two basis sets; for geometry optimization we used a split valence basis set (B1) consisting of LACVP³⁸ for iron and 6-31G³⁹ for the rest of the atoms, while the energy was corrected by use of a triple- ζ valence polarized basis set, LACV3P++*(Fe)/6-311++G** (rest) basis set, (B2).⁴⁰ The extended all-electron basis set, def2-TZVP⁴¹ (B3), was used as well to test the convergence of B3LYP. The QM calculations were carried out using the TURBOMOLE program,²⁴ and the MM part was described by CHARMM22 force field by using DL_POLY program.⁴² The charge shift model^{43, 44} was employed to treat the QM/MM boundary and the HDLC optimizer⁴⁵ was taken for geometry optimization. The transition states (TS) were located by initial potential energy surface (PES) scan followed by full TS optimizations using P-RFO optimizer implemented in HDLC code.⁴⁵ All TSs were characterized as having one imaginary vibrational corresponding for the expected mode along the corresponding reaction coordinates, using numerical frequency calculations on the QM region. All calculations were performed with the ChemShell package²² that integrates the TURBOMOLE and DL_POLY programs.

Gas Phase Calculations

To assess the effect of the protein and the PFDA, we carried out also gas phase calculations of alkane hydroxylation using GAUSSIAN 09⁴⁶ with the B2 basis set, and Turbomole with B3 basis set. For QM-only calculations we also tested LACV3P+*(Fe)/6-311+G*(rest), henceforth B4. The results for B1, B3 and B4, and other basis sets are collected in the ESI.

Results and Discussion

A. What is Necessary to Cause P450_{BM3} to Retain Methane?

As shown above in Figure 1a, in the highest populated docking mode, the carboxylic group of PFDA interacts with side chain of Ser72 by direct hydrogen bond. Another two neighboring residues with polar side chain, i.e. Arg47 and Tyr51, interact indirectly with PFDA by the linking water molecules between them. This binding mode is quite similar, but not exactly identical, to the binding mode obtained by Reetz *et al*¹⁷ in which the ligand's carboxy group interacts with Ser72 and Tyr51 directly. The difference may be caused by different docking softwares and algorithms employed in the two studies. Careful visual inspection of our obtained binding mode, shows that the perfluorinated tail of PFDA had penetrated into the reactive pocket above the heme with the distances between the fluorine atoms of the CF₃ terminal group and the oxo atom of Cpd I varying from 5.86 to 7.13 Å. This leaves sufficient space to accommodate at least another small substrate in the active site. Based on this binding mode, we tentatively inserted one methane molecule into the remaining cavity and ran the subsequent MD simulation. In parallel we ran the MD simulations in the absence of PFDA.

Figure 3 summarizes the results of the MD simulations in the absence and presence of PFDA in the active site of P450_{BM3}. It is seen that in the absence of PFDA (Figure 3a), methane is roaming throughout the reactive cavity of P450_{BM3} and is eventually outside of the cavity after about 700 ps. In some snapshots, for example near 500 ps, methane returns to the vicinity of Cpd I, but it does not stay around Cpd I for sufficiently long time and leaves the active site quickly. This is no doubt because the empty cavity in P450_{BM3} without PFDA is considerably larger than the size of methane, such that the substrate is zooming around the free space without finding any bound mode near Cpd I. We also ran production MD by packing the empty space, in the absence of PDFA, by water molecules. This however did not change the situation for methane, which kept freely fluctuating about the space without finding a reactive pose for further oxidation.

Is One Molecule of Methane Sufficient?—In the presence of PFDA, we explored three different situations described in Figures 3b–3d. Firstly, we just manually put one methane molecule into the vacant space close to Cpd I, after letting PFDA occupy the large channel of the long-chain natural substrate of P450_{BM3}. Figure 3b shows the mobility of the added methane in this case. Although the fluctuation is smaller than that in Figure 3a, the methane still could not be retained in the pocket. Just after about 100 ps in equilibration, it flies to the position which is too far from oxo to have reactivity. Obviously more packing is needed to keep methane in the active site. Thus, in addition to the methane, we added two water molecules into the vacancy around the active cite by careful inspection of the space. In this situation, shown in Figure 3c, the residence time of methane in the reactive site was extended. However, still the fluctuation of the methane mobility is as large as in Figure 3b. Thus, these two solvent molecules could not keep methane in the pocket for a long time. After about 1 ns equilibration, methane flies out again and loses its bound mode to Cpd I.

Finally, we replaced the two solvent molecules by methane molecules and the 10 ns MD of the three-methane loaded P450_{BM3} is shown in Figure 3d. We can see that in the presence of PFDA, the methane molecules are kept in the active site in a rolling style during the 10 ns simulation. For example, at the beginning of the equilibration, one of the methanes (METH1, the black line in Figure 3d, see Figure 2 for the definition) stays in a position near Cpd I, while the other two (METH2/METH3, red/blue lines in Figure 3d) are located relatively far apart. After a short MD running-time, METH1 flies away but is replaced by METH3 that approaches Cpd I. In the subsequent MD simulation time, METH3 leaves the reaction center, while METH2 enters instead. This quasi-periodic residence, even if not in

an exact same order, is kept throughout the whole 10 ns MD simulation. As such, *there is always at least one methane staying in the vicinity of Cpd I and maintaining a reactive mode in the simulation time*, as may be confirmed by the average distance between the carbon atom of methane and the oxygen atom of Cpd I around 4 Å. The fluctuation of the reactive methane is not large and is within the margin of MD deviation. Moreover, as shown in Figure 3d, in many snapshots, a methane molecule approaches Cpd I to within 3 Å or even closer than that.

As can be seen later, in the juxtaposed snapshots, the methane molecule is being held through its positively charged hydrogen atoms by two of the negatively charged fluorine substituents of the PFDA. Thus the PFDA induces perfect juxtaposition of Cpd I and methane for rapid oxidation. However, the additional methane molecules are essential for keeping the system in this mode. This basically means that methane hydroxylation requires in addition to PFDA also a high concentration of methane molecules, which in our study is mimicked by the three methane molecules. Thus, while we cannot resolve the differences between the two experimental results,^{17,18} nevertheless our MD study shows that *a single methane molecule will not remain in the active site, and three are necessary to keep methane in the active site*. As such, methane can be hydroxylated only under sufficient pressure that forces a few molecules into the active site.

B. More than One Alkane Molecule is Helpful also for Ethane and Propane

Figure 4 collects the 2ns MD results of ethane and propane in the reactive site of P450_{BM3} with PFDA. As shown in Figure 4a, when there is only one ethane molecule in the pocket, the fluctuation of the C(ethane)-O(Cpd I) distance is large, i.e., 3 to 8.5 Å. However, the ethane molecule does not fly out of the active site within the 2ns MD. In the single-propane case (Figure 4b), the molecule keeps almost a fixed distance from oxo moiety of Cpd I with two methyl carbon atoms approaching oxo in turn and the secondary carbon atom staying constantly about 5 Å from Cpd I.

When we admit another ethane molecule into the pocket, the ethanes alternately approach Cpd I, keeping at least one methyl carbon atom within 3.5 Å from the oxo moiety of Cpd I during the 2n MD (see Figure 4c). The fluctuations of these two ethanes are sufficiently small and there are many snapshots in which an ethane molecule approaches Cpd I to within 3 Å or even closer than that. This means that the double-ethane system is helpful for maintaining reactive juxtaposed poses for ethane oxidation.

In the double-propane system (Figure 4d), one of the propanes keeps quite close to Cpd I. The other propane keeps staying 7 Å from Cpd I. Thus our MD results show that the higher alkane concentration is helpful for all the three small alkanes. However, for methane it is absolutely necessary to have at least three molecules. For the larger ethane, one needs two molecules to force one of them closer to Cpd I. But for propane a second molecule is helpful but not absolutely necessary. Apparently, usage of high pressure should benefit the small alkanes hydroxylation in a manner that depends on the size of the alkanes; the smaller the alkane the higher the requisite pressure.

C. The Hydroxylation Mechanism Elucidated by QM/MM Calculations

Figure 5 shows the calculated QM/MM energy profile for methane hydroxylation by Cpd I of P450_{BM3} (for more detailed energy profiles and other snapshots, see Table S2 in the ESI). The energy data out of parentheses are obtained by single-point calculations at the B3LYP/B2//B1 level, while the data within parentheses involve zero-point energy (ZPE) correction.

Much like all previous studies of C–H hydroxylation of methane by P450 Cpd I,^{8,9} here too, the reaction exhibits a typical multi-state reactivity (MSR) mechanism^{5,6,47,48} and a two-

phasic process, first hydrogen abstraction and then radical rebound. The mechanism starts with a pair of degenerated quartet and doublet reactant clusters, $^{2,4}\text{RC}$, which are nascent from the high spin (HS) and low spin (LS) state of Cpd I, and it passes through several closely lying hydrogen abstraction transition states (TS_H), which differ in their spin states (doublet and quartet) and their electromeric heme characters (PorFe^IV and $\text{Por}^{\bullet+}\text{Fe}^\text{III}$). The initial PES scan from $^{2,4}\text{RC}$ (see Figure S2 in the ESI) leads to two virtually degenerated $\text{Por}^{\bullet+}\text{Fe}^\text{III}$ -type TSs, labeled as $^{2,4}\text{TS}_\text{H}(\text{III})$, which subsequently lead to the corresponding radical intermediates, $^{2,4}\text{IC}(\text{III})$. By swapping electrons in the $\text{TS}_\text{H}(\text{III})$ and $\text{IC}(\text{III})$, we got PorFe^IV -type $^2\text{TS}_\text{H}(\text{IV})$ and $^{2,4}\text{IC}(\text{IV})$ with slightly lower energy. All attempts to optimize $^4\text{TS}_\text{H}(\text{IV})$ converged back to $^4\text{TS}_\text{H}(\text{III})$ automatically. The order of these electronic states is confirmed by even larger all electron basis set B3 (see Table S2 for B3 data in ESI). Thus, our QM/MM results show that the barrier and the reaction energy of the hydrogen abstraction step in methane hydroxylation, are 18.2 and 13.0 kcal mol $^{-1}$ respectively at B3LYP/B2//B1 level with ZPE correction. And the entire energy spread of at the $\text{TS}_\text{H}/\text{IC}$ states is 1.8/2.4 kcal mol $^{-1}$. The TS_H and IC species were identified as before using their spin density and orbital occupancies. Thus, as seen from Figure 6, $^2\text{TS}_\text{H}(\text{IV})$ is typified by spin density of 1.99 on Fe, and a small spin density of the porphyrin, while $^{2,4}\text{TS}_\text{H}(\text{III})$ possess spin density of ~ 1 on Fe and on the porphyrin.

Interestingly, unlike P450_{cam} where the lowest transition states were generally $\text{TS}_\text{H}(\text{III})$ types,⁶ here in P450_{BM3} the lowest species is $^2\text{TS}_\text{H}(\text{IV})$, resembling more the gas phase situation. As further seen from Figure 6, $^2\text{TS}_\text{H}(\text{IV})$ has shorter C–H distance and longer H–O bond compared with the other two TSs. With these features $^2\text{TS}_\text{H}(\text{IV})$ has an earlier geometry compared with other TS_H types, which is also in line with the finding that $^2\text{IC}(\text{IV})$ is the most stable of all the intermediate states. Thus, the LS Fe^IV route has the earliest TS and the lowest barrier in according with the Hammond postulated⁴⁹ and the Bell-Evans-Polanyi principle.^{50, 51}

Past the H-abstraction step, we can see in Figure 5 that the three lowest-energy intermediate radical complexes undergo barrier free rebound, while high spin Fe^III -type radical intermediate, $^4\text{IC}(\text{III})$, encounters a sizeable barrier, ca. 4.3 kcal mol $^{-1}$. The extended all-electron basis set, B3, gives the same energy trends (see ESI). These features are similar to what one generally finds in alkane hydroxylation by P450 .^{5, 6} The PFDA do not alter the normally found rebound scenario.

In a nutshell, based on the QM/MM energy profile shown in Figure 5, methane oxidation by Cpd I of P450_{BM3} in the presence of PFDA is a feasible effectively concerted process with ultra-short life radical intermediates.

Up to now, the PFDA seems to act as a packing molecule that fixes the methane in a configuration that enables oxidation. This effect is similar to *the cooperative binding* of two substrates, where one substrate is far and serves as packing material to juxtapose the second one that has to undergo oxidation, as observed in the human $\text{P450}_{3\text{A4}}$ (CYP3A4) enzyme,^{52, 53} as well as in P450_{BM3} .¹⁷

D. Does PFDA Exert any Electronic Effect on Methane Hydroxylation?

Since it has been demonstrated that the fluorinated species can promote reactions by its electrostatic catalytic effect,^{21, 54} and since even a water molecule was shown to affect the barrier by electrostatic stabilization of the TS,⁵⁵ we thought to explore a potential catalytic effect by the PFDA on methane hydroxylation, using the R1, R2 and R3 models (see Figure 1b above). The deprotonated PFDA molecule was also investigated in the R1 and R3 models where the negative charge in deprotonated PFDA is screened by a salt bridge with Arg47.

Consider first Figure 7, which displays the charge distribution in the ^2RC and $^2\text{TS}_\text{H}(\text{IV})$ species in the R1/R2 model. Inspecting the ^2RC in R2 model, it is seen that two of the negatively charged fluorine atoms of PFDA claw the methane molecule via its positively charged H atoms, by $\text{F}^{\delta-} \cdots \text{H}^{\delta+}$ bonds, and hence fix the methane near the ferryl-oxo unit. Compared with ^2RC , the $^2\text{TS}_\text{H}(\text{IV})$ species in the model R2 possesses longer distance between methane and PFDA. Additionally, the partial positive charges on the H's of methane and the negative charges on the F's of PFDA remain unchanged. The same charge distribution exists in R1 model as well. Thus, there is no charge transfer between PFDA and methane in $^2\text{TS}_\text{H}(\text{IV})$.

Second, as can be seen from Table 1 where the methane hydroxylation barriers are collected, including protonated or deprotonated PFDA with and without Arg47 into QM region the barrier will be changed by only 0.1~0.8 kcal mol⁻¹ in QM/MM and QM calculations (see R1_A, R2_A, R3 and R1_E, R2_B in Table 1).

In additional tests, we performed the QM/MM calculation with deletion of all the point charges of the PFDA or removing PFDA out of MM region. The results produced a change of ~0.6 kcal mol⁻¹ compared with the PFDA with all its point charges intact (see R1_A, R1_B and R1_C in Table 1). However, replacing PFDA by water molecules raises the reaction barrier by 1.3 kcal mol⁻¹ (see R1_A and R1_D in Table 1). This may mean P450_{BM3} protein favors PFDA over water when the large empty binding pock needs to be shrunk.

The other QM calculations, using PFDA and its charges, do not show much effect on the barrier (R1_E–R1_G in Table 1). As such the PFDA does not exert much, if any, electrostatic catalysis^{21, 54, 55} on methane hydroxylation. Most likely, the stabilizing interactions of the negatively charged fluorines with the positively charged hydrogen atoms of the methane moiety are counterbalanced by the repulsion with the negatively charged oxo group in Cpd I, which acquires more negative charge in the TS, thus leading to negligible effect on the barrier.

E. Does PFDA Exert any Electronic Effects on Ethane and Propane Hydroxylations?

To further test the PFDA effect we studied the H-abstraction of ethane and *i*-propane using QM/MM and QM calculations. The QM/MM calculated energy profiles are shown in Figure 8 and the barriers of hydrogen abstraction in various situations are collected in Table 1. Other computational data can be found in the ESI.

Much like the data for methane in Figure 5, the QM/MM energy profiles calculated for hydroxylations of ethane and *i*-propane in Figure 8 exhibit MSR and two-phasic mechanisms. Here too, the LS Fe^{IV}-type $^2\text{TS}_\text{H}(\text{IV})$ and $^2\text{IC}(\text{IV})$ are the lowest transition state and intermediate, respectively, in the several closely-lying spin- and electromeric-states in the hydrogen abstraction step. The hydrogen abstraction barriers of the three alkanes are close to the gas phase results^{5, 9, 10} and follow the order, $\Delta E^\ddagger(\text{CH}_4) > \Delta E^\ddagger(\text{C}_2\text{H}_6) > \Delta E^\ddagger(i\text{-C}_3\text{H}_8)$. Thus, the barrier of *i*-propane is only 10.5 kcal mol⁻¹ which is lower than that of ethane, 13.9 kcal mol⁻¹, and of methane, 18.2 kcal mol⁻¹. Subsequently, only the rebound on the HS Fe^{III} manifold encounters the transition state, $^4\text{TS}_\text{reb}(\text{III})$, with a significant barrier, while from all the other HS/LS manifolds the barrier is negligible. As such, the QM/MM energy profiles show that for all the three small alkanes the hydrogen abstraction is the rate-determine step during the hydroxylation process by Cpd I.

Inspection of Table 1 shows that PFDA does not affect the barrier for hydrogen abstraction from ethane. However in the case of *i*-propane, the barrier is lowered by 1.2 kcal mol⁻¹ in the presence of PFDA. Although this effect is not large and may be within the DFT error margin, the 1.2 kcal mol⁻¹ difference could in principle reflect that PFDA is more helpful to

the hydroxylation of *i*-propane than that of methane or ethane. Be this as it may, it is clear that the major role of PFDA is as a packing molecule that consumes space.

F. Relative Reactivity of the Alkanes

Inspection of the barriers in Table 1 shows that the relative reactivity of the alkanes is still largely determined by the relative BDEs of the activated C-H bonds. As shown by Senn et al,⁵⁶ free energy calculations of the hydrogen abstraction step during camphor hydroxylation by P450_{cam} yield a result that is very close to the QM/MM calculated barrier with ZPE correction, and for good reasons.⁶ Therefore, the barrier ordering determined in the present study is not likely to change by free energy calculations. Thus, if one were to consider only the relative barriers of hydrogen abstraction for the three alkanes, one might have concluded that the hydroxylation of *i*-propane should have been the most efficient while that of methane the least. We must recall however, that generally the rate-determining step in the catalytic cycle P450 is the second electron transfer from the reductase and prior to the formation of Cpd I,⁵⁷ and therefore the experimental turnover numbers (TONs) may not reflect the barriers to hydroxylation.

Conclusion

In summary, our MD and QM/MM study supports the observed experimental results that small alkanes are accepted and oxidized by P450_{BM3} in the presence of dummy substrate PFDA.^{17,18} PFDA plays a role in juxtaposing the small alkanes vis-à-vis Cpd I of P450_{BM3}. This may explain why the PFDA could by itself elicit the hydroxylation of *i*-propane by P450_{BM3}.^{17,18} PFDA does not exert any electrostatic catalysis, and it plays here a similar role to the cooperative binding effect on hydroxylation in P450_{3A4}.^{52, 53}

Different from *i*-propane, in the case of methane, at least two additional methane molecules are required to keep at least one of them near Cpd I. A single molecule will not remain in the active site to be hydroxylated even in the presence of PFDA. The larger ethane also needs one more molecule to force one of them closer to Cpd I, while for the largest propane a second molecule is helpful but not absolutely necessary. Thus, the most significant finding in our study is that using the PFDA strategy, methane can be hydroxylated only under sufficient pressure that forces a few molecules into the active site. Ethane may need high pressure to facilitate oxidation as well. The higher alkane concentration is helpful for all the three small alkanes in an inverse manner to their size. This conclusion can stir interest in the use of pressure and packing molecules like PFDA for performing catalytic conversions of small molecules by P450s.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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(a)

(b)

Figure 1.

(a) The binding mode from docking results by Autodock 4.2, in which the carboxylic group of PFDA directly interacts with Ser72, and indirectly interacting with Tyr51 and Arg47. (b) The QM models (R1, R2 and R3) employed in QM/MM calculation.

Figure 2.
The active site of P450_{BM3} in the presence of PFDA and methane (a) with two additional water molecules, (b) with two additional methanes.

Figure 3.
The mobility of methane in P450_{BM3} binding pocket in the presence and absence of PFDA: (a) one methane without PFDA; (b) one methane with PFDA; (c) one methane and two additional water molecules with PFDA; (d) three methane molecules with PFDA; the molecules are colored differently to emphasize that one of them is close to Cpd I throughout the simulation.

Figure 4.
The mobility of ethane and propane in P450_{BM3} binding pocket in the presence of PFDA:
(a) one ethane; (b) one propane; (c) two ethanes; (d) two propanes.

Figure 5.

Energy profiles of methane hydroxylation by Cpd I. The relative energies are given as B3LYP/B2//B1(B3LYP/B2//B1+ZPE) in kcal mol⁻¹. The oxidation state of iron is given as Roman numerals in parentheses.

Figure 6.

Key geometric features (geom), group spin densities (ρ), NBO charges (Q) of the hydrogen abstraction transition states in the C-H hydroxylation of methane by P450_{BM3} Cpd I. The data are given in the order of ²TS_H(III)(²TS_H(IV))[⁴TS_H(III)]. Bond lengths are in angstroms and dihedral angles (D) are in degrees.

Figure 7. NBO charges of ^2RC and $^2\text{TS}_\text{H}(\text{IV})$ in R1 and R2 models (in black). The distances between hydrogen atoms of methane and fluorine atoms of PFDA are shown as well (in red).

Figure 8.

Energy profiles for ethane (a) and *i*-propane (b) hydroxylation by Cpd I. The relative energies are given as B3LYP/B2//B1(B3LYP/B2//B1+ZPE) in kcal mol⁻¹. The oxidation state of iron is given as Roman numerals in parentheses.

Scheme 1.
Schematic representation of the rebound mechanism.

Table 1

B3LYP/B2//B1 calculated hydrogen abstraction barriers (kcal mol⁻¹) of small alkanes by Cpd I.

	QM/MM calculations					QM calculations (gas phase)				
	R1 ^a	R1 ^a _B	R1 ^a _C	R1 ^a _D	R2 ^a _A	R3 ^a	R1 ^{b,c} _E	R1 ^{b,c} _F	R2 ^{b,c} _B	R1 ^{b,d} _G
methane	21.7(22.0)	21.1	22.1	23.0	21.6	21.5(21.2)	19.8	19.8	19.2	19.1
ethane	17.9	17.7	18.0	18.9	17.2		18.1	18.1	17.7	15.6
<i>n</i> -propane	14.5	14.1	15.1	17.3	13.9		13.6	13.7	12.4	13.8

^aR1A: R1 QM/MM optimization keeping PFDA in MM part; R1B: R1 QM/MM single-point removing PFDA's point charges; R1C: R1 QM/MM single-point removing PFDA totally; R1D: R1 QM/MM single-point replacing PFDA by water molecules; R2A: QM/MM single-point including PFDA into QM region; R3: QM/MM single-point including PFDA and Arg47 in QM region. All data are from protonated PFDA model except that the two values in parentheses are from deprotonated PFDA model. No zero point energy correction is included in these single point energy calculations.

^bR1E: R1 QM single-point using QM/MM optimized geometry; R1F: R1 QM single-point using QM/MM optimized geometry and adding PFDA's point charges; R2B: R2 QM single-point using QM/MM optimized geometry; R1G: R1 full QM optimization. All are relative energies from ²TSH(IV) vs ²RC.

^cBy Turbomole 6.3.1.

^dBy Gaussian 09.