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Utilizing Terminal Oxidants to Achieve P450-Catalyzed Oxidation of Methane

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Abstract: Terminal oxidant-supported P450 reactions alleviate the need for substrate binding to initiate catalysis by chemically generating "compound I." This allows investigation of the innate substrate range of the enzyme active site. Using iodosylbenzene as the oxidant, CYP153A6, a medium-chain terminal alkane hydroxylase, exhibits methanol formation in the presence of methane demonstrating that P450-mediated methane hydroxylation is possible.

Keywords: alkanes; C–H activation; compound I; enzyme catalysis; metalloenzymes; methane oxidation

The selective conversion of methane, the smallest and most inert alkane, to a liquid fuel such as methanol remains one of the great challenges in hydrocarbon chemistry.[1] The only known biological solution for catalytic methane oxygenation is the carboxylate-bridged di-iron methane monooxygenases (MMOs), which convert methane to methanol using dioxygen at ambient conditions with rates up to 220 min^{-1}.[2] However, despite decades of research, these complex multi-component enzymes have yet to be functionally expressed in heterologous hosts, which together with other drawbacks have limited their use as practical biocatalysts.[3] Cytochrome P450s (P450s) are heme-thiolate enzymes also capable of hydroxylating unactivated C–H bonds.[4] The more than 11,500 known P450s (data source http://drnelson.utmem.edu/CytochromeP450.html) include many soluble, monomeric, bacterial P450s that have been shown to be amenable to protein engineering and useful in industrial applications.[5] Utilizing a high-valent ferryl-porphyrin cation radical species (compound I, **CMP I**) as the active oxidant, members of the P450 superfamily have been demonstrated to oxidize a variety of organic substrates, including liquid and gaseous alkanes, although none has been shown to accept methane as a preferred substrate.[6] Under natural turnover conditions, **CMP I** is generated through a series of electron transfer and protonation events initiated and modulated by substrate binding within the enzyme active site.[7] However, **CMP I** synthesis can be simplified by reacting the resting state enzyme with terminal oxidants, such as hypervalent iodine reagents and peroxides. Herein, we utilized this approach to survey five P450s for their ability to hydroxylate hydrocarbons as small as methane. We report that reaction of iodosylbenzene with CYP153A6 in the presence of methane leads to small amounts of methanol formation, demonstrating that P450-mediated methane hydroxylation is possible.

Our group and others have been engineering P450s for activity on small alkanes.[8] Using both directed evolution and rational design, propane and ethane hydroxylation activities have been successfully achieved with two bacterial P450s, CYP102A1 (BM3) and CYP101 ($P450_{cam}$).[8d,e] However, activity for methane remained elusive for the laboratory-engineered catalysts. Methane presents two major challenges as a P450 substrate: (i) its small molecular size and apolar nature limit its ability to initiate the P450 catalytic cycle, which normally occurs upon the displacement of a heme-coordinated water ligand by substrate binding, and (ii) the strength of its C–H bond (104.9 kcal mol^{-1}) exceeds those of known P450 substrates. In this study, we dissected the problem posed by the small size of methane from the challenge of the higher activation barrier presented by the methane C–H bond by assaying the reactivity of the P450 **CMP I** directly through terminal oxidant-supported P450 reactions (see Figure 1). In PhIO-supported reactions, **CMP I** is formed directly, while a ferric hydroperoxo complex (**CMP 0**) is formed in reactions with peroxides.[9] The generation of **CMP I** from

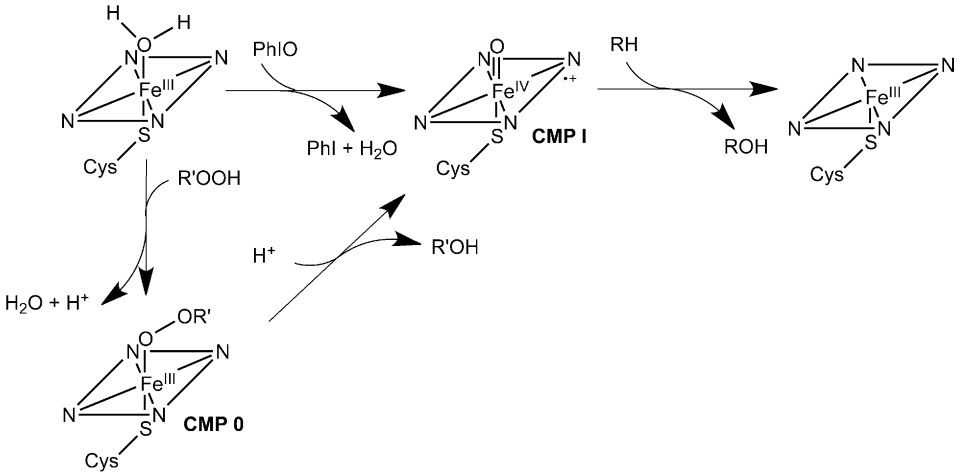

Figure 1. CMP I is formed directly in PhIO-supported reactions, whereas a ferric hydroperoxo complex (**CMP 0**) is formed in reactions with peroxides. The generation of **CMP I** from this complex requires protonation followed by heterolytic O–O bond cleavage.

CMP 0 requires protonation at the distal oxygen followed by heterolytic O–O bond cleavage. The protonation of **CMP 0** can also occur at the proximal oxygen resulting in unproductive release of peroxide. This latter process is one of several uncoupling mechanisms in the P450 catalytic cycle that are especially prevalent for substrates which do not expel the water from the active site upon binding.

Iodosylbenzene (PhIO), 3-chloroperoxybenzoic acid (*m*-CPBA), and hydrogen peroxide (H_2O_2) were used to determine the ability of the **CMP I** of BM3, $P450_{PMO}$ (PMO),[8b] $P450_{cam}$, CYP153A6 (A6),[10] and CYP153A6 BMO-1(A6 BMO-1)[11] to hydroxylate alkanes ranging from methane to octane (Table 1). Wild-type BM3 and $P450_{cam}$ were found to hydroxylate alkanes as small as propane using all three oxidants. Only sub-terminal alcohols were produced. This indicates that the native active sites of these two enzymes are capable of accommodating alkanes as short as propane in a productive orientation. In the case of BM3, alkane hydroxylation has been observed for hexane but nothing smaller under turnover conditions utilizing NADPH/O_2.[8c] The lack of BM3 activity for smaller alkanes under turnover conditions is solely due to poor substrate binding, which results in insufficient activation of the catalytic cycle and uncoupling at **CMP 0**. PMO, a laboratory-evolved BM3 variant exhibiting wild-type like coupling and catalytic efficiency for propane as its preferred substrate,[8b] displays the same substrate range as BM3 with similar propane turnover numbers (TONs), in terminal oxidants-supported reactions, 0.8 compared to 1.0 for BM3. This implies that the laboratory evolution from BM3 to PMO enabled propane binding to activate

Table 1. Alkane hydroxylation by P450s utilizing terminal oxidants.

		Product formed[a], μmol (μmol P450)$^{-1}$			
P450	Oxidant	Methane	Ethane	Propane	Octane
$P450_{cam}$	PhIO	–[b]	–	0.83	0.38
	MCPBA	–	–	0.78	0.22
	H_2O_2	–	–	0.96	0.07
BM3	PhIO	–	–	1.0	1.4
	MCPBA	–	–	0.35	0.12
	H_2O_2	–	–	0.30	0.20
PMO	PhIO	–	–	0.77	0.29
A6	PhIO	0.05 (0.02)	2.5	3.9	0.51
	MCPBA	–	0.34	1.6	0.15
	H_2O_2	–	0.23	0.41	0.30
A6 BMO-1	PhIO	0.02 (0.01)	2.1	3.0	n.d.[c]

[a] Alkanes (2.5 mM, or saturated at 20 psi) were incubated with P450 (100 μM) and terminal oxidant (5 mM) at 25 °C for 10 min. The data represent the averages of at least two experiments and do not correct for P450 inactivation. Standard errors are within 20% of the reported average with exceptions given in parentheses.

[b] A dash (–) indicates a lack of detectable amounts of product.

[c] n.d. = not determined.

the catalytic cycle and generate **CMP I** efficiently but did not alter the H-atom abstraction reaction, i.e., similar yields are obtained from reactions between the **CMP I** of BM3 and PMO with propane.

A6, a natural terminal alkane hydroxylase that prefers medium-chain-length alkanes (C_6–C_8),[10] hydroxylates all alkane substrates, even methane, with PhIO as the oxidant. This demonstrates that direct methane-to-methanol conversion by a cytochrome P450 catalyst at ambient conditions is possible. The low TONs of the PhIO-mediated A6 oxidation of methane, 0.05 TON, show that although methane can be oxidized by A6, it is a poor substrate with minimal reactivity even in the presence of a pre-generated **CMP I**. As a comparison, the A6 methane TON is 50-fold lower than the A6 ethane TON, which may reflect both a lower binding affinity for methane, since the generation of **CMP I** is substrate-independent, and a higher reaction barrier due to the difference in C–H bond strength. A6 methane reactions supported by *m*-CPBA and H_2O_2 did not yield detectable methanol product, as well as control experiments using Hemin as the catalyst. Considering the low yield of the methane reaction with PhIO and the general trend of peroxide reactions being less efficient, presumably due to uncoupling at compound 0, the absence of methanol product in these reactions could simply reflect the limited ability to detect the product (2.0 μM, Figure S1, Supporting Information).

Surprisingly, reactions with the preferred substrate of A6, octane, yielded far less product as compared to reactions with ethane and propane. This may be the result of competition between substrate and PhIO for access to the active site. Finally, A6 BMO-1, a laboratory-evolved A6 variant with improved butane hydroxylation activity obtained through selection for growth on butane,[11] also exhibited methane oxidation with PhIO, but with only 0.02 TON. Its propane TON also decreased compared to wild-type A6, from 3.9 to 3.0, which reflects a similar diminished activity as observed previously under turnover conditions.[11]

Isotope labeling experiments using $^{13}CH_4$ and $^{18}OH_2$ were conducted to verify the sources of carbon and oxygen in the methanol product generated in PhIO reactions with A6. Reactions with ^{13}C-methane produced $m/z = 33$ ions unique to ^{13}C-methanol, which corresponds to a $+1$ m/z shift of the ^{12}C-methanol ion of $m/z = 32$ (Figure S2, Supporting Information). Quantification against authentic ^{13}C-methanol standards showed a yield of $0.035 + 0.009$ TON in A6 PhIO-mediated reactions with ^{13}C-methane. Reactions in the presence of 50% $^{18}OH_2$ also produced $m/z = 33$ ions corresponding to a $+2$ m/z shift of the $m/z = 31$ ion of ^{12}C-methanol, confirming solvent oxygen incorporation which is a hallmark of PhIO-mediated reactions.[12] Quantification for ^{18}O incorporation was not possible due to the low yield. These results confirm that the methanol product is generated through a PhIO-mediated P450 reaction with methane.

Convinced that the **CMP I** of A6 can hydroxylate methane, we investigated A6 for oxidation of methane and other alkanes under turnover conditions with reconstituted A6 reductase proteins utilizing NADH/O_2. *In vitro* A6 hydroxylation of octane proceeded with a rate of octanol formation of 75 min^{-1}, which is slightly higher than the reported activity determined in *P. putida* GPo12 cell extracts.[10] Reactions with ethane at 20 psi headspace pressure resulted in ethanol formation rates of 32 min^{-1}, but reactions with both ^{12}C- and ^{13}C-methane did not produce detectable amounts of methanol, even at methane headspace pressures as high as 880 psi, corresponding to *ca.* 90 mM dissolved methane. The absence of A6 methane hydroxylation activity under turnover conditions appears to be analogous to the absence of BM3 propane hydroxylation activity which can be rationalized by a lack of **CMP I** formation. Methane is unable to trigger a type I UV-Vis binding shift for A6 (Figure S3, Supporting Information) just as wild-type BM3 exhibits no appreciable type I binding for propane.[13] This lack of spin-shift indicates that the binding of these substrates is unable to displace the resting state water ligand, which is necessary to initiate catalysis.[14]

To gain more insight into how A6-catalyzed oxidation of a preferred substrate differs from a smaller,

Table 2. A6 alkane hydroxylation under turnover conditions.[a]

	k_{cat} (min^{-1})	K_M (mM)	k_{cat}/K_M ($M^{-1}s^{-1}$)	KIE (k_H/k_D)
Methane	0	n.d.[b]	n.d.	n.d.
Iodomethane	58 (5.1)	17.7 (1.4)	55	5.8
Ethane	61 (8.3)[c]	n.d.	n.d.	n.d.
Hexane	98 (7.0)	0.78 (0.04)	2,100	1.0
Octane	75 (7.2)	0.32 (0.02)	3,900	1.0

[a] Reaction conditions: 0.5 μM A6, 0.5 μM FdrA6, 5 μM FdxA6,1 mM NADH, 0.1 M KPi, pH 8.0, various substrate concentrations. The data represent the averages of three replicates; values in parentheses are the standard errors.

[b] n.d. = not determined.

[c] Ethane k_{cat} was determined at 40 psi head-space pressure, corresponding to the maximum rate of ethanol formation observed.

non-natural substrate, the kinetic parameters (K_M and k_{cat}) and the intermolecular kinetic isotope effects (KIE) were determined for the oxidation of hexane, octane, and oxidative dehalogenation of iodomethane (Table 2). Attempts to characterize ethane hydroxylation kinetics were unsuccessful because saturating kinetics were not observed over the investigated pressure range (data not shown). Therefore, iodomethane was chosen as a surrogate for the small gaseous alkanes, since it possesses both a molecular size and a C–H bond strength (102.9 kcal mol^{-1}) intermediate to those of methane (104.9 kcal mol^{-1}) and ethane (101.0 kcal mol^{-1}). That iodomethane is a liquid offers the additional benefit that saturating kinetics can be observed.

Comparing the reaction kinetics for octane hydroxylation with iodomethane dehalogenation of A6, a 50-fold increase in K_M from 0.32 mM to 17.7 mM was observed. Surprisingly, despite a *ca.* 3 kcal mol^{-1} difference in C–H bond strength between these substrates, only a small difference in the k_{cat} values was observed, 75 min^{-1} for octane *vs.* 58 min^{-1} for iodomethane. The overall 70-fold decrease in catalytic efficiency from 3,900 M^{-1} s^{-1} for octane hydroxylation to 55 M^{-1} s^{-1} for iodomethane dehalogenation is thus largely due to the higher K_M. Intermolecular KIEs of near unity were observed for the hydroxylation of the preferred A6 substrates of hexane and octane, which indicates that the H-atom abstraction step is not rate-limiting for these substrates. This is expected as the second electron transfer step is generally rate-limiting for P450s acting on their preferred substrates.[15] In contrast, a KIE of 5.8 was observed for iodomethane dehalogenation, which demonstrates that the H-atom abstraction reaction has become rate-limiting. A KIE of 5.8 falls within the classical limit and indicates an absence of hydrogen atom tunneling, which has been suggested to occur during sMMO oxidation of methane.[16] As a comparison, a similar KIE of 6.4 was observed for the hydroxylation of (1*R*)-5,5-difluorocamphor by $P450_{cam}$,[17] where blocking of the preferred C-5-exo site forces hydroxylation to occur at the C-9 position in a non-optimized geometry, making C–H activation rate-limiting. The higher mobility of iodomethane within the A6 active site may have a similar effect such that the substrate C–H bond is not properly oriented near **CMP I** for reaction.

Recently, methane hydroxylation has been reported with wild-type BM3, with the use of perfluorocarboxylic acid additives.[18] This "chemical tuning" approach is thought to generate a catalytically active enzyme complex with reduced active site volume using an inert molecule as an external trigger to initiate catalysis. Accordingly, we investigated the ability of perfluoroalkanes (C_8F_{18}, C_7F_{16}) to trigger methane hydroxylation for A6 under turnover conditions. However, we were unable to detect any activity towards methane. This could be due to the lower solubility of perfluoroalkanes relative to perfluoro acids, as no type I spin-shift of A6 in the presence these effectors was observed. Furthermore, it is noteworthy that none of the five P450s used in this study displayed evidence of binding to iodobenzene, the by-product of iodosylbenzene oxidations, which implies that the activities described in Table 1 are not the result of modulation of active site geometry but rather of a direct survey of the **CMP I** active site. Importantly, both our terminal oxidant approach with A6 and the "chemical tuning" approach with BM3[18] suggest that the barrier for P450 methane oxidation is poor activation of the P450 catalytic cycle due to low methane binding affinity rather than the strength of the methane C–H bond.

In conclusion, we have used terminal oxidants to evaluate the innate substrate specificity of P450s, independent of the requirement for substrate binding to initiate catalysis. Using this assay, we were able to show that the **CMP I** of A6 can support methane oxidation, just as the **CMP I** of wild-type BM3 is poised for propane oxidation, despite the fact that neither activity is observed under turnover conditions. This result confirms that the methane C–H bond can be oxidized by a P450 and suggests that A6 could be a good starting point for the engineering of a P450 methane monooxygenase.

Experimental Section

Protein Expression and Purification

E. coli Dh5α cells were transformed with the plasmids containing P450 BM3 and PMO, and *E. coli* BL21(DE3) cells were transformed with plasmids containing P450cam, CYP153A6, CYP153A6 BMO-1, CYP153A6 ferredoxin, and CYP153A6 ferredoxin reductase. An aliquot of an overnight culture in LB medium, supplemented with ampicillin (100 μg mL^{-1}) was used to inoculate TB medium to an initial optical density (OD_{600}) of 0.5. After 3.5 h of incubation at 37 °C and 250 rpm shaking, the cultures were cooled to 25 °C, and protein expression was induced with the addition of IPTG (1 mM). The cells were harvested by centrifugation at 4 °C, 5,000 rpm for 10 min, 20 to 24 h after induction and stored at −20 °C. A three-step purification was performed following a published protocol.[19] P450 concentrations were measured from the CO-difference spectra as described by Omura and Sato[20] using $\varepsilon = 91$ mM^{-1} cm^{-1}.

Terminal Oxidant-Supported Reactions

In oxidation reactions of liquid alkanes, the substrates were added from an ethanol stock solution to a 0.27 mL reaction mixture containing 50–250 μM P450, in 0.1 M phosphate buffer, pH 8.0, to yield a final solution containing 2.5 mM alkane, 2% ethanol. In oxidation reactions of gaseous alkanes, lyophilized protein was added to a 10 mL crimp-top headspace vial. The vial was sealed and flushed with the

substrate for 2 min before the addition of 0.27 mL of pre-saturated 0.1 M phosphate buffer, pH 8.0. The head-space was then pressurized to 20 psi with the gaseous alkane. All reactions were initated by the addition of the oxidant and allowed to proceed for 10 min at 25 °C. Reactions with liquid alkanes were extracted with 150 μL of chloroform and analyzed by GC-FID with the addition of 2-nonanol as an internal standard. Reactions with gaseous alkanes were quenched with 20 μL of 3.0 M HCl and neutralized with 75 μL of 1.0 M phosphate buffer, pH 8.0 to precipitate the enzymes in solution. After centrifugation at 14,000 *g* for 2 min, the resulting solution was analyzed by GC-MS.

A6 *in vitro* Hydroxylation Reactions

For the *in vitro* A6 alkane hydroxylation reactions, purified reductase components, ferredoxin reductase (fdrA6) and ferredoxin (fdxA6) were quantified using known extinction coefficients for their FAD and [Fe_2-S_2] cofactors.[21] A ratio of reductase components of 1:1:10 for A6:fdrA6:fdxA6 was used for all reactions. The 0.3 mL reaction mixtures contained 0.5–2.0 μM A6 and 0.5–2.0 μM fdrA6 and 5.0–20.0 μM fdxA6 at a 1:1:10 ratio in 0.1 M phosphate buffer, pH 8.0. For liquid substrates, additions from stock solutions in ethanol were used to reach final solutions with 25 μM to 10 mM substrate and 2% ethanol. For gaseous alkanes, the reaction was carried out in crimp-top head-space vials pressurized to 20–60 psi with the alkane. The reactions were initiated with the addition of 1–2 mM NADH. Reaction work-up and analysis were performed as described above for the terminal oxidant reactions.

For more details see the Supporting Information.

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