

Directed Evolution of Cytochrome P450 for
Small Alkane Hydroxylation

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ABSTRACT

Methane is an ideal alternative to petroleum refining as a chemical feedstock source since it is highly abundant and inexpensive. However, the lack of selective methane oxidation catalysts has limited such utilization. Starting from cytochrome P450 CYP102A1 (BM3) from *Bacillus megaterium*, which prefers C₁₂-C₂₀ fatty acids as its substrates, I investigated several protein engineering approaches to shift the enzyme's substrate specificity toward small gaseous alkanes, with the ultimate goal of methane. By continuing previous directed evolution efforts in our group, a variant with wild-type-like affinity and catalytic efficiency for propane, P450_{PMO}, was isolated. To alleviate the loss of protein thermostability (~ 10 °C) as a result of this approach, mutations were targeted to the BM3 active site with site saturation mutagenesis, targeted mutagenesis with a reduced set of amino acids, and computationally guided library designs. From these enzyme libraries, variants were identified that replicated much of the P450_{PMO} activities with a minimal number of mutations while maintaining wild-type thermostability.

Continuing the protein engineering with a high throughput ethane hydroxylation screen, variants with improved *in vitro* ethane hydroxylation activity were obtained. However, in whole-cell ethane bioconversions, BM3-derived variants could not match the activity of a natural P450 alkane hydroxylase, CYP153A6. To investigate the oxidation capability of the P450 oxo-ferryl porphyrin radical intermediate directly, I employed a variety of terminal oxidants to support P450 alkane hydroxylation reactions abridging the P450 catalytic cycle. In this study, the CYP153A6 oxo-ferryl intermediate was able to oxidize methane in reactions using iodosylbenzene, which demonstrated that direct methane-to-methanol conversion by a P450 heme porphyrin catalyst at ambient conditions is possible and does not necessarily require the use of additional effectors to alter the active site geometry.

THESIS SUMMARY

Selective hydroxylation of small alkanes is a long-standing problem for which few practical catalysts are available. The lack of catalysts that can efficiently convert gaseous alkanes into transportable liquid commodities has been a barrier to broader utilization of these resources. In particular, methane, the principal component of natural gas, is an ideal alternative to petroleum as a chemical feedstock source since it is highly abundant and inexpensive. Currently, methane is converted to methanol via an energy intensive, endothermic, and costly process that first converts methane into synthesis gas, followed by methanol synthesis from this intermediate. This process is economically feasible only on a large scale, which in combination with general transportation limitations of a gas commodity prevent methane recovery from many sources.

The selective oxidation of small alkanes is difficult because of the inertness of the alkane C-H bond, which requires highly reactive radical or ionic species to cleave. However, as the desired partial oxidation products, alcohol and aldehyde, have weaker C-H bonds compared to the alkane, they are susceptible to further oxidation to CO₂. While this transformation has been achieved only by a limited set of transition-metal-based catalyst systems, a variety of alkane hydroxylases found in alkanotrophic microorganisms support selective alkane oxidation at ambient conditions using oxygen as the oxidant. Chapter 1 of this thesis provides an introduction to enzymatic alkane oxidation by monooxygenases, highlighting the structure and mechanisms of three major enzyme classes, methane monooxygenases (MMOs), non-heme (di-iron) monooxygenases, and cytochrome P450s (P450s).

MMOs enable methanotrophic bacteria to use methane as their sole carbon source, while non-heme (di-iron) monooxygenases and P450s enable microorganisms to grow on medium- and long-chain alkanes. These monooxygenases selectively oxidize alkanes into alcohols as the first

step in hydrocarbon metabolism. Unfortunately, since the majority of these hydroxylases function as a part of a larger enzyme complex and are membrane associated, their potential for industrial applications is limited. For these reasons, we have been engineering well-expressed, soluble, bacterial P450s, in particular CYP102A1 (BM 3) isolated from *Bacillus megaterium*, for small alkane hydroxylation.

In addition to being well-expressed and soluble, BM 3 was chosen as the starting point for this protein engineering effort because it is a rare self-sufficient P450 with its heme (hydroxylase) and reductase domains fused on a single polypeptide. This unique domain architecture has been credited for BM3's high catalytic rate acting on its preferred C₁₂ to C₂₀ fatty acids substrates. Previous work in our laboratory (by Ulrich Schwaneberg, Edgardo T. Farinas, Anton Glieder, Matthew Peters, and Peter Meinhold) aimed at converting BM3 into a methane monooxygenase applied directed evolution –iterations of mutagenesis, recombination, and screening, to generate BM3 variants with improved alkane hydroxylation activity. Their overall strategy for shifting BM3's substrate specificity was to enhance the promiscuous alkane hydroxylation activity of BM3 and subsequent variants starting with octane as the target substrate. Using a colorimetric screen with *p*-nitrophenoxy octane as a surrogate substrate in combination with monitoring cofactor consumption in the presence of octane, variants with improved activity for octane hydroxylation were identified. Limited activity toward propane, a substrate not hydroxylated by wild-type BM3 was also observed in later generation variants. At this point, selection pressure was shifted toward propane hydroxylation by screening for dimethyl ether demethylation in high-throughput as the surrogate activity. Using this screen and a combination of mutagenesis techniques, ethane hydroxylation activity was obtained with variant 35E11. At this point, I took over the P450 alkane hydroxylase project.

Chapter 2 describes the continuation of laboratory evolution efforts aimed at converting BM3 into a small alkane hydroxylase, starting from variant 35E11. As a result of the previous ten rounds of mutagenesis and screening, variant 35E11 displayed a significantly lower thermostability ($\Delta T_{50} = -11.6$ °C) compared to the wild-type enzyme. To reverse this loss in thermostability, which is known to reduce the ability of a protein to acquire beneficial mutations that are destabilizing, known stabilizing mutations from a P450 peroxygenase were grafted onto variant 35E11 singly and in combination. The resulting thermostablized variant was subjected to a domain-based protein-engineering strategy (developed by Rudi Fasan), in which the three domains of BM3 were mutated individually using both random and site-saturation mutagenesis. Beneficial mutations identified through high-throughput screening for dimethyl ether demethylation were verified to improve propane and ethane hydroxylation in the context of the holoenzymes. Using this strategy, re-specialization of BM3 for propane hydroxylation was achieved with variant P450_{PMO}, a proficient P450 propane monooxygenase. This variant displays substrate affinity and coupling of cofactor consumption rivaling those of the natural P450s with their preferred substrates. In addition, we were able to demonstrate *in vivo* propane hydroxylation using these BM3 variants in resting *E. coli* cells reaching activities surpassing those reported for natural alkane hydroxylases acting on their preferred substrates.

In Chapter 3, we explored alternative mutagenesis approaches to engineer BM3 for small alkane hydroxylation. Instead of gradually shifting the BM3's substrate specificity by enhancing its promiscuous alkane hydroxylation activity as done previously, we applied several semi-rational library design approaches to mutate the BM3 active site in an attempt to acquire activity for small alkane hydroxylation directly from the wild-type enzyme. From screening of mutagenesis libraries created by combinatorial active site saturation with a reduced set of amino

acids and two structure-based computational library design approaches, we identified variants supporting both propane and ethane hydroxylation. Although, none of the obtained variants reached the level of specialization that was previously obtained with P450_{PMO}, the range of obtained propane TON and coupling of cofactor consumption corresponds to those values of generalist intermediates of P450_{PMO} lineage obtained after 10 – 12 rounds of mutagenesis and screening. These results suggest semi-rational library design can be an effective strategy to move away from a specialist enzyme toward generalist enzymes, but functional specialization still requires optimization through several rounds of random mutagenesis and screening.

The BM3 variants we obtained with high activity on small alkanes hydroxylate propane and longer chain alkanes predominantly at the more energetically favorable subterminal position. In contrast, sMMO and other alkane hydroxylases utilized by microorganisms for alkane metabolism selectively oxidize at the terminal carbon to produce 1-alcohols. Since selective terminal hydroxylation has been difficult to achieve by engineering BM3, a sub-terminal hydroxylase, we investigated in Chapter 4 whether a small-alkane terminal hydroxylase could be obtained by directed evolution of a longer-chain alkane hydroxylase that exhibits this desirable regioselectivity. For this study, we engineered two alkane hydroxylases that prefer medium-chain-length alkanes (C₆ – C₁₀), AlkB from *P. putida* GPo1 and CYP153A6 from *Mycobacterium* sp. HXN-1500, for enhanced butane hydroxylation activity using an *in vivo* growth-based selection system (developed by Daniel Koch). This system enabled selection for terminal alkane hydroxylase activity based on enhanced growth complementation of an adapted strain of *P. putida*. The resulting enzymes, AlkB-BMO1, -BMO2, and CYP153A6-BMO1 conferred improved growth on butane as the sole carbon source and exhibited higher rates of 1-butanol production in whole-cell butane bioconversions while maintaining their preference for

terminal hydroxylation. These results demonstrated the usefulness of this *in vivo* selection system, which could be generally applied to directed evolution of enzymes for small alkane hydroxylation.

To apply selection pressure for the main goal of this research, selective hydroxylation of ethane and methane, we developed a high-throughput screen to directly assay for P450 alkane hydroxylation, described in Chapter 5. With the use of a pressurizable 96-well reactor, the P450 alkane hydroxylation reaction was conducted in high throughput and the alcohol product was quantified spectroscopically by a coupled enzyme assay. Applying this screen to BM3 variants generated in our laboratory, we identified variant E31 as the best candidate for further engineering, since it displayed both the highest activity in the screen and wild-type-like thermostability. Subsequent rounds of site-saturation and random mutagenesis resulted in improved variants demonstrating the efficacy of the screen. However, none of the identified BM3 variants were able to produce ethanol or methanol in whole-cell alkane bioconversions using growth-arrested *E. coli* cells. In contrast, CYP153A6, a natural terminal alkane hydroxylase, was able to produce ethanol in whole-cell alkane bioconversions. The inability of BM3 variants to produce ethanol *in vivo* reflects their poor affinity for ethane and indicates they still lag behind a natural P450 alkane hydroxylase in terminal hydroxylation of small alkanes.

The complete absence of methane oxidation activity in numerous BM3 variants evolved for propane and ethane hydroxylation activity led us to question if the P450 oxo-ferryl porphyrin radical intermediate, compound I, can oxidize the 105 kcal/mol methane C-H bond. In Chapter 6, we separated the substrate binding problem presented by the small size of methane from the challenge of the higher activation barrier of the reaction presented by the methane C-H bond by assaying the reactivity of compound I directly through terminal oxidant-supported P450

reactions. Using iodosylbenzene, 3-chloroperoxybenzoic acid, and hydrogen peroxide as oxidants, we investigated the ability of the compound I of five P450s (BM3, P450_{PMO}, P450_{cam}, CYP153A6, and CYP153A6-BMO1) to hydroxylate alkanes ranging from methane to octane. From these terminal oxidant-supported P450 reactions, we found the compound I of CYP153A6, and CYP153A6 BMO-1 to be able to break the methane C-H bond using PhIO as the oxidant. This demonstrates both the feasibility of P450 methane oxidation and the use of terminal oxidant-supported P450 reactions as an assay to investigate the compatibility of P450 active sites for small alkane oxidation. By chemically generating the active radical, we eliminated the requirement for substrate binding to initiate P450 catalysis, which enabled us to determine the innate substrate range of each P450 active site.

Although the BM3 variants we generated could not hydroxylate methane, we have found other applications for which they excel, such as regioselective hydroxylation of non-activated carbon centers. The involvement of human P450s in the degradation of most drug compounds made us wonder if BM3 variants can be used to replicate or predict the metabolism patterns of human P450s and produce these metabolites on a preparative scale. In Chapter 7, we demonstrated that a small panel of BM3 variants covers the breadth of reactivity of human P450s by producing 12 of 13 mammalian metabolites for two marketed drugs, verapamil and astemizole, and one research compound. The most active enzymes could support preparation of individual metabolites for preclinical bioactivity and toxicology evaluations. Underscoring their potential utility in drug lead diversification, engineered BM3 variants also produce novel metabolites by catalyzing reactions at carbon centers beyond those targeted by animal and human P450s. Finally, we enhanced the production of a specific metabolite by directed evolution of the enzyme catalyst.

Chapter 8 details experimental procedures and materials used throughout the studies described in this thesis.

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ABBREVIATIONS

MMO	Methane monooxygenase
<i>M. c.</i> Bath	<i>Methyloccus capsulatus</i> Bath
<i>M. t.</i> OB3b	<i>Methylosinus trichorium</i> OB3b
<i>E. coli</i>	<i>Escherichia coli</i>
BM3	Cytochrome P450 BM3 (CYP102A1)
A6	CYP153A6
CAM	CYP101
PMO	P450 _{PMO}
ET	Electron transfer
PCET	Proton coupled electron transfer
KIE	Kinetic isotope effect
SRS	Substrate recognition site
TON	Turnover number
EPPCR	Error-prone polymerase chain reaction
SOEPCR	Splicing by overlap extension polymerase chain reaction
SSM	Site-saturation mutagenesis
CAST(ing)	Combinatorial active site saturation test
NADH	Nicotinamide adenine dinucleotide, reduced form
NAD ⁺	Nicotinamide adenine dinucleotide, oxidized form
NADPH	Nicotinamide adenine dinucleotide phosphate, reduced form
NADP ⁺	Nicotinamide adenine dinucleotide phosphate, oxidized form
FAD	Flavin adenine dinucleotide
FMN	Flavin mononucleotide
DME	Dimethyl ether
BDE	Bond dissociation energy
DFT	Density functional theory

Chapter 1

Introduction: Enzymatic Alkane Oxidation by Monooxygenases

A. Introduction

Petroleum and natural gas are the primary energy resources currently utilized to meet the world's energy needs (1). In addition to its use as a fuel source, the conversion of crude oil to olefins and aromatics through refining has also allowed petroleum to act as a major feedstock for the chemical industry. This ability to generate chemical precursors—through processes such as cracking, dehydrogenation, and reforming—differentiates petroleum from natural gas, which has been limited to usage as a fuel. However, as the world's known reserves of crude oil are shrinking (2), the need to find alternative sources for chemical feedstocks, such as natural gas, is becoming more pressing. This search for alternative feedstocks is also motivated by the environmental impact of petroleum refining. As the reactions to produce olefins and aromatics from petroleum are endothermic, CO₂ is released during both the generation of these chemical precursors and in the subsequent partial oxidation steps to produce the desired oxygenated compounds (e.g., aldehydes, alcohols, carboxylic acids).

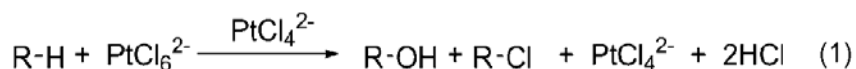
Methane, the principal component of natural gas, is an ideal alternative to petroleum refining, since it fulfills all the requirements for a chemical feedstock, including high abundance, low cost, and lower carbon footprint (CO₂ emission) compared to petroleum refining. In addition to the methane available in known natural gas and coal sources, it can also be produced via biogas (3), by fermentation of organic matter (3), and vast quantities are stored as methane hydrates at the ocean floor (4). There are also economic incentives to convert methane into oxygenated products, as it is less expensive than petroleum-generated olefins and aromatics. Finally, the methane oxidation reaction is exothermic. Therefore replacing the highly endothermic petroleum refining processes with methane oxidation would also result in concurrent energy production with the chemical products instead of energy consumption. Despite

all these favorable factors, methane is still underutilized as a feedstock owing to a lack of economical and sustainable strategies for its selective oxidation (5).

The selective oxidation of methane to oxygenated products represents a significant challenge, as the methane C-H bond is extremely inert (105 kcal/mol) (6). Therefore, highly reactive radical or ionic species are required to cleave the methane C-H bond. However, as the desired partial oxidation products, methanol and formaldehyde, have weaker C-H bonds compared to methane, they are susceptible to further oxidation to CO₂. To overcome these challenges, research toward partial methane oxidation and improved methane utilization has taken several different approaches: (1) the one-step oxidation of methane to methanol or formaldehyde, (2) oxidative and non-oxidative coupling of methane, (3) Fischer-Tropsch synthesis of hydrocarbons from synthesis gas (syngas), generated from steam reformation of methane. Currently, industrial conversion of methane to methanol falls into the latter category, utilizing an energy intensive, endothermic, and costly process to first convert methane into syngas, followed by methanol synthesis from this intermediate (1, 7). While there is a variety of mixed metal-oxide heterogeneous catalysts capable of the desired methane partial oxidation (8) and coupling reactions (9 – 10), these catalysts currently lack the reactivity and selectivity necessary for commercialization (5).

The most hopeful strategy for selective methane oxidation is through electrophilic activation by late transition metal ions, such as Pt(II) (11), Pd (II) (12), Rh (13), and Hg(II) (14). These systems are derived from the landmark study by Shilov demonstrating the production of alcohol and alkyl-chloride using Pt(II) salts in aqueous solution (11) (see equation (1)). These systems have been shown to be capable of both stoichiometric and catalytic oxidation of methane. Their most attractive feature is a high selectivity for the partially oxidized product: i.e.,

the reactivity for the methane C-H bond is substantially greater than that of a product C-H bond, such as H-CH₂OH or H-CH₂SO₄H (15). The mechanism of the Shilov systems occurs in three steps: (1) electrophilic activation of the R-H bond by Pt(II) to form a Pt(II)-alkyl intermediate, (2) oxidation of the Pt(II)-alkyl complex by [PtCl₆]²⁻ to give a Pt(IV)-alkyl species, (3) nucleophilic S_N2 attack of water at Pt-C bond results in the formation of the alcohol product and regenerates the Pt(II) catalyst.



Advancement of the original system has been made by Periana et al., which has replaced the oxidant [PtCl₆]²⁻ with sulfuric acid (15). Using an Hg²⁺ complex in sulfuric acid, a one-pass yield of 40% conversion of methane to methyl hydrogensulfate was obtained at > 90% selectivity (14). An improved system utilizing Pt(II) chelated by 2,2'-bipyrimidine, which is more thermodynamically robust, resulted in a one-pass yield of greater than 73% (15). While these yields are the highest reported for direct partial oxidation of methane, several key disadvantages have prevented commercialization: low turnover frequency (16), costly methanol recovery from concentrated sulfuric acid, and catalyst poisoning by water and oxidation products (5).

In contrast to the difficulties for transition metal catalysts to selectively oxidize methane, metalloenzymes, specifically methane monooxygenases (MMOs) with metal centers composed of abundantly available metals (iron and copper) are able to convert methane to methanol at room temperature, atmospheric pressure, in water, and using O₂ as the oxidant (17). Alkane hydroxylases, including MMOs, are discussed in detail in the next section. As the structures of these metalloenzymes have become available, they have inspired chemists to make “biomimetic” catalysts (18) in attempts to capture the metal-centers in a functional form using a variety of

scaffolds. The synthesis and characterization of multiple di-iron $\text{Fe}^{\text{IV}}=\text{O}$ complexes modeled after the Q intermediate of MMOs have been reported (19 – 22). To date, these complexes have been shown to activate C-H bonds as strong as 100 kcal/mol, however, the obtained reaction rates were much lower than those observed with metalloenzymes (20, 22).

B. Alkane Oxidizing Enzymes

B.1. Methane monooxygenases (MMOs)

While a catalyst that supports efficient conversion of methane to methanol has so far eluded transition metal chemistry, Nature found a solution to utilize methane as an energy source long ago. Methanotrophic bacteria found in a variety of environments including methane vents in the deep sea, gastrointestinal tracts of cows, and landfills are unique in their ability to utilize methane as their sole carbon and energy source (23). Methanotrophs, comprising 13 different genera within the α and γ protobacteria (24), are defined by their expression of a methane monooxygenase (MMO) that directly converts methane to methanol. The methanol product is further oxidized to formaldehyde by a methanol dehydrogenase and is used both for biomass synthesis (23) and as a source of ATP through further oxidation reactions (23).

Most studies of MMOs have been focused on enzymes from *Methyloccus capsulatus* Bath (*M. c.* Bath) and *Methylosinus trichorium* OB3b (*M. t.* OB3b) (25). There are two types of MMOs employed by methanotrophs, soluble MMO (sMMO) (17) and membrane-bound or particulate MMO (pMMO) (26). All but one genus of methanotrophic bacteria express pMMO, and a small subset produces both MMOs (24). In methanotrophs expressing both MMO forms, sMMO is expressed when less than 0.8 μM copper is present in the growth medium, whereas

with $\sim 4 \mu\text{M}$ copper present, pMMO is expressed along with the developments of extensive, intracytoplasmic membranes (27 – 28).

B.2. *pMMO*

Particulate MMOs are integral membrane metalloenzymes produced in nearly all methanotrophs and are composed of the three subunits pmoA, pmoB, and pmoC (26). The three protomers are arranged in an $\alpha_3\beta_3\gamma_3$ trimeric complex, Figure 1.1 (25). The soluble region of the enzyme complex extends $\sim 45 \text{ \AA}$ from the membrane and is composed of six β -barrels. A significant opening spans the length of the pMMO trimer at its center; this pore is $\sim 11 \text{ \AA}$ wide in the soluble portion and expands to $\sim 22 \text{ \AA}$ within the membrane. Despite decades of research and the availability of two crystal structures (29), only recently has the location of the copper active site been identified (30). Balasubramanian et al. demonstrated that expression of only the soluble domain of pMMO, pmoB, from *M.t.*OB3b (31) was sufficient for methane oxidation (30). This study conclusively identified the active site to be a dicopper center with a Cu-Cu distance of $2.5 - 2.7 \text{ \AA}$ coordinated by three highly conserved His residues (32). In light of this discovery, it is puzzling why Nature chose such a large enzyme complex for this reaction, when a soluble sub-domain of pMMO is fully capable of the transformation. One theory forwarded by the authors suggests that the membrane portions may play an important role in increasing the local methane concentration as methane preferentially partitions between the aqueous solution and the membrane (30).

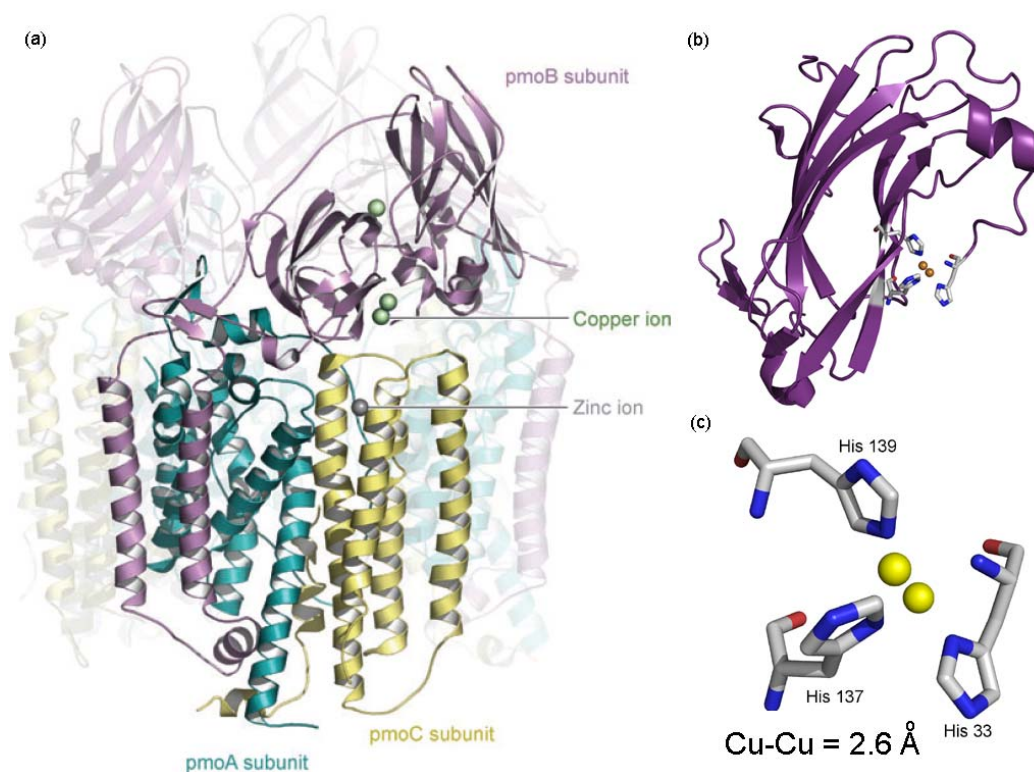


Figure 1.1: The pMMO (*M. c. Bath*) structure (pdb: 1YEW); (a) the full structure with one protomer highlighted, reproduced from ref 29; (b) the soluble domain pmoB; (c) the first coordination sphere of the dicopper metal center

Although pMMO is much more prevalent than sMMO in methanotrophs, difficulties in its characterization due to the fact that it is an integral membrane enzyme have resulted in far less understanding of its biochemistry as compared to sMMO. In fact, the conditions for isolating catalytically active pMMO have been the subject of extensive research, and the optimal conditions still remain unclear. Copper concentration in the growth medium, anaerobicity of the growth condition, and the detergent-protein ratio are among the many conditions that have been shown to affect the measured enzyme activity (33 – 34). The *in vitro* characterization of pMMO is further complicated by the absence of a known physiological reductant. Typically, purified pMMO is assayed for propylene oxidation activity using either NADH or duroquinol as the reductant (35). Activities ranging from 0.002 to 0.126 U/mg (1 U = 1 μmol propylene oxidized per min) have been reported from various preparations (32 – 33, 36 – 37).

pMMO has been shown to oxidize only alkanes and alkenes up to five carbons in length (38 – 39). Interestingly, for these multi-carbon substrates, sub-terminal oxidation at the C-2 position is preferred (40). Studies using chiral alkanes have given evidence to suggest the pMMO mechanism for oxygen insertion occurs in a concerted fashion rather than involving radical or cationic intermediates, as with sMMO or cytochrome P450s (41 – 42). In addition, an absence of a carbon kinetic isotope effect in the oxidation of propane also suggests little or no structural rearrangement occurs at the carbon center during the rate-limiting step (43). Unfortunately, attempts to determine the pMMO mechanism have been sparser compared to similar efforts with sMMO, and much of the mechanism is still not well understood.

B.3. sMMO

Due to both its unique ability to oxidize methane as well as its high substrate promiscuity, i.e., the ability to hydroxylate more than 50 different compounds including aromatics (17, 35), sMMO has been a favored target for research (39). sMMO has been purified from *M. t.* OB3b (44), *M. c.* Bath (45), and several other strains of methanotrophs (46). It belongs to the family of bacterial multi-component monooxygenases (BMMs) (EC.1.14.13.25), which includes toluene monooxygenase, phenol hydroxylase, and alkene monooxygenase (47), that enable their hosts to utilize a variety of hydrocarbons as their sole carbon and energy source (47 – 48). Using a common carboxylate-bridged di-iron center in their hydroxylase, BMMs are able to activate oxygen for formal insertion into the substrate C-H bond, which initiates the metabolism of these hydrocarbons.

Typical of BMM family members, sMMO is comprised of three components; a hydroxylase (MMOH), which houses the di-iron active site, a reductase (MMOR), which contains a flavin adenine dinucleotide (FAD), and a [2Fe-2S]-ferredoxin (Fd) cofactor that

shuttles electrons from the NADH cofactor to the MMOH active site, and a regulatory protein (MMOB), which is required for methane oxidation (17). The MMOH subunit consists of three polypeptides arranged as an $\alpha_2\beta_2\gamma_2$ dimer, Figure 1.2 (a). The di-iron active site is embedded in a four-helix bundle and coordinated by four carboxylates and two imidazoles from two E(D/H)XXH binding motifs.

The resting state of the hydroxylase (H_{ox}) active site is a di(μ -hydroxo)-(μ -carboxylato)diiron (III) species. The catalytic cycle (Figure 1.2 (b)) is initiated by a two-electron reduction to the di-iron (II) form (H_{red}). The reduction occurs simultaneously with a carboxylate shift of the terminally coordinated glutamate (E243), which results in protonation and displacement of both bridging hydroxyl ligands. Rapid reaction of H_{red} with O_2 in the presence of MMOB results in a peroxodiiron (III) intermediate (H_{peroxo}). In the absence of electron-rich substrates, H_{peroxo} rapidly decays into the intermediate Q, a diiron(IV) oxo intermediate with a short Fe-Fe distance of 2.5 Å (49). The Q intermediate has been shown to be responsible for the oxidation of a variety of substrates (50 – 54) including methane. In the absence of substrate, Q decays slowly to H_{ox} by acquiring two electrons and two protons through a still unknown process. Both H_{peroxo} and Q intermediates have well-defined Mossbauer and optical spectroscopic properties (53, 55). The conversion of H_{peroxo} to Q has been shown to be both pH-dependent and exhibiting a solvent kinetic isotope effect (KIE), which indicates that the O-O bond cleavage occurs heterolytically via a proton-promoted mechanism (56 – 57).

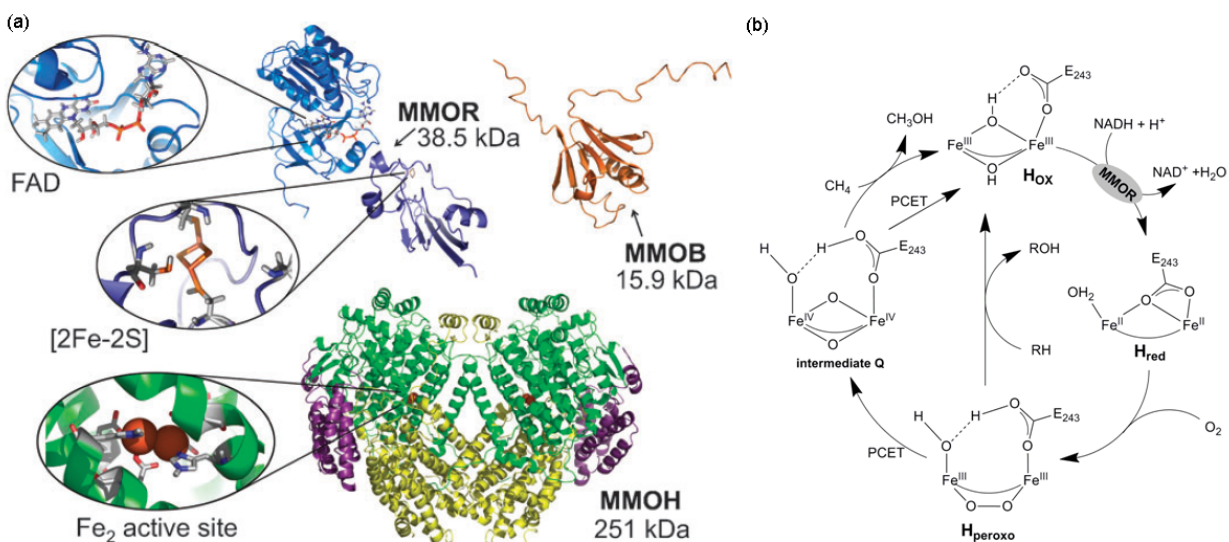


Figure 1.2: The sMMO structure and mechanism. (a) The structure of MMOH (pdb: 1MTY), MMOB (pdb: 1CKV), and MMOR (pdb: 1JQ4) with the cofactors highlighted, reproduced from ref 17. (b) The sMMO catalytic cycle, see text for details, (PCET: proton coupled electron transfer)

Based on density functional theory (DFT) calculation with ~ 100 atoms (58 – 60), methane initially approaches the Q intermediate in the $\sim 185 \text{ \AA}^3$ hydrophobic binding site distal to the histidine ligands. The bridging oxygen atom abstracts a hydrogen atom from methane in an outer-sphere, proton-coupled electron transfer reaction, during which one of the iron atoms is reduced to Fe (III). The electron is taken from a C-H σ -orbital, leaving behind a bound methyl radical. The C-O bond formation along with a second electron transfer to the other iron center from the methyl radical occurs either through a rebound mechanism with very short distances (H-O---C of $1.97 \text{ \AA}$) or a concerted mechanism with the methyl fragment tightly bound to the hydroxyl group (61). These two proposed pathways have comparable activation barriers from DFT calculations, therefore the reaction most likely has a mixed character. The catalytic cycle is then completed with the release of the methanol product, returning the enzyme to its di-iron (III) resting state.

MMOH is only active in the presence of a protein cofactor, MMOB, which when complexed with MMOH changes its structure and reactivity. For example, MMOH from *M. t.* OB3b oxidizes alkanes and nitrobenzene to form secondary alcohols and *m*-nitrophenol products in the absence of MMOB (62). Upon MMOB addition, the product ratios shift such that mostly primary alcohols and *p*-nitrophenol are formed. In addition, MMOB must be present for efficient generation of MMOH intermediates in the reaction cycle, which suggests that binding of MMOB initiates the electron transfer (ET) and O₂ binding steps (63 – 64). The presence of MMOB has been generally reported to enhance ET between MMOH and MMOR (63), but when chemically reduced MMOR was added to premixed solutions of MMOH and MMOB the same ET between MMOH and MMOR was inhibited (65). These apparently conflicting results have led investigators to suggest that slow structural changes associated with MMOB and MMOR binding to MMOH may result in hysteresis in MMOH activity (62). A current hypothesis is that the interaction of one hydroxylase component of MMOH with MMOR or MMOB could be dependent on the presence of MMOR or MMOB bound to the other component of MMOH (66). As a consequence of this dependence, the oxidative phase of the catalytic cycle may only occur at one of the two active sites at a time. This hypothesis has been experimentally verified by observing a ~ 50% maximal conversion of the initial di-iron (II) protein during reactions of MMOH with oxygen (53).

The complexity of the interactions between these three enzyme components could be necessary to facilitate and coordinate the transport of the four substrates, hydrocarbon, oxygen, electrons, and protons of the sMMO reaction. The selective trafficking of these substrates to the diiron active site of the hydroxylase is also aided by the presence of biologically well-engineered substrate tunnels and pockets (67). Co-crystallization of MMOH with halogenated alkanes, Xe

(68), and ω -halogenated primary alcohols (69) has revealed the presence of multiple hydrophobic substrate binding pockets that trace a contiguous pathway from the protein surface to the di-iron center. The entry of the substrate appears to pass through several such cavities in its path from aqueous solution to the enzyme's active site (69). Finally, as many as eleven binding sites have been identified with Xe, which has similar polarity, water solubility, and van der Waals radius as methane. The binding of these surrogate substrates of methane did not induce significant side-chain displacement in the enzyme; therefore it appears that methane and other sMMO substrates are bound in pre-formed hydrophobic pockets.

Kinetic studies of the oxidation of hydrocarbon substrates by intermediate Q monitored through stopped-flow spectroscopy have shown three distinct substrate classes. The first class of substrates, including ethane, methanol, ethanol, and some ethers, displays a linear dependence of reaction rate on substrate concentration. In addition, a kinetic isotope effect (KIE) of near unity was observed, which suggests that the breaking of the substrate C-H bond is not the rate-determining step. The second class of substrates, including methane and diethyl ether, also displays a linear dependence of reaction rate with substrate concentration but display a $\text{KIE} > 1$, suggesting that C-H bond activation reaction is rate-determining. Finally, the last class of substrates includes nitromethane, acetonitrile, and acetaldehyde, and displays normal Michaelis-Menten kinetics with hyperbolic dependence of reaction rate with substrate concentration and a $\text{KIE} > 1$. For many of the hydrocarbon substrates discussed above, with the exception of methane, the H_{peroxo} intermediate is also a viable oxidant. However, when the H_{peroxo} intermediate is used as the oxidant rather than Q, only class II and III kinetic behavior is observed. This has led some investigators to conclude that reactions with H_{peroxo} proceed through a classical

hydrogen atom transfer mechanism, whereas those of Q are extensively non-classical and involve hydrogen atom tunneling.

This difference could be particularly important for methane oxidation, as methane is kinetically stable with a large barrier height for its oxidation. For the reaction with the Q intermediate, tunneling across this barrier could lead to progression along the reaction coordinate, whereas the reaction with the H_{peroxo} intermediate may not proceed due to absence of tunneling. While this explanation could resolve why sMMO homologs cannot activate methane while possessing nearly the same di-iron active site, unfortunately, KIE studies for the sMMO methane reaction which would determine if tunnel effects were present have yielded varied results. Under single-turnover conditions, KIE values of 23 to 50 have been reported (50, 70), which indicates proton tunneling in the transition state. However, under steady-state conditions, a KIE of only 1.7 was observed, when comparing V_{max} (or k_{cat}) values (70 – 71), which suggests an absence of tunneling.

Further complicating the sMMO reaction mechanism is the fact that, while the rate-determining step is thought to be the hydrogen atom transfer, multiple studies have revealed that there is no correlation between the reaction rate of a given substrate with the Q intermediate and its homolytic bond dissociation energy (BDE). For example, the oxidation rates of sMMO for methane and ethane are nearly identical despite a BDE difference of ~ 4 kcal/mol. Another example would be a comparison between acetonitrile and nitromethane, which have similar homolytic and heterolytic BDEs, but display a 62-fold difference in reaction rates at 4 °C (72). Reconciliation of the KIE results that indicate the hydrogen abstraction to be rate limiting and the lack of correlation between substrate BDE and oxidation rate remain a challenge.

B.4. Using methanotrophs/MMOs for methanol synthesis

While methanotrophs and MMOs have been focus of extensive research over the past decades, successful attempts to use either the organisms or enzymes for methanol synthesis have been sparse. The inability to express either pMMO or sMMO in a heterologous host severely limits their utilization in industrially relevant organisms as well as the ability to use standard molecular biology methods to engineer desired protein properties. In addition, the multi-component nature of MMOs is also a hindrance to evolving more active or more stable variants. One successful strategy for methanol biosynthesis using methanotrophs is to inhibit the downstream enzyme in methanol metabolism, methanol dehydrogenase (MDH). Using NaCl as a MDH inhibitor, 7.7 mM of methanol were accumulated in *M. t.* OB3b cultures after 20 hours (73). Optimization of the growth conditions as well as the addition of ethylene diamine tetraacetic acid to further inhibit MDH resulted in 13.2 mM methanol accumulation after 12 h batch fermentations with an overall activity of 0.036 U/mg cell mass (1 U = 1 μ mol methanol/min). While this strategy is successful in producing methanol, significant yield improvements and reduction of the product loss to the natural methanol metabolism of the methanotroph host are hard to envision.

Studies of the sMMO mechanism as well as its crystal structure have also inspired researchers to make biomimetic catalysts replicating the same carboxylate bridged di-iron core as sMMO stabilized with a variety of ligands (19, 74). While advances in ligand design have led to catalysts which can reach the equivalent H_{peroxo} and Q intermediate states in the sMMO catalytic cycle, the obtained reactivity with alkane substrates has been modest, with no reported methane activity (19, 74). A key obstacle in reaching methane oxidation activity for these biomimetics could be an intrinsic inaccuracy in the structural model they are attempting to emulate. As all

available crystal structures of MMOH have been solved in the absence of MMOB, which modulates the MMOH tertiary structure directly affecting both substrate access and the first coordination sphere of the diiron center. It is therefore questionable if the observed active site configurations reflect that of the active configuration during methane oxidation.

B.5. *AlkB and non-heme di-iron alkane monooxygenases*

Expanding the search for potential methane biocatalysts beyond MMOs, two other class of enzymes, non-heme di-iron alkane monooxygenases and cytochrome P450s, are also able to activate oxygen and perform O-atom insertion into inert alkane C-H bonds. The family of non-heme di-iron alkane hydroxylases has been identified in bacteria and fungi utilizing C₅ – C₁₆ *n*-alkanes as their sole carbon source (75). Exemplified by the most studied alkane hydroxylase isolated from *Pseudomonas putida* GPo1, the non-heme di-iron alkane hydroxylase is a three-component system consisting of (1) a soluble NADH-rubredoxin reductase (AlkT) (76), (2) a soluble rubredoxin (AlkG) (77), and (3) the integral membrane oxygenase (AlkB) (78 – 79). Although AlkB can be functionally expressed in *Escherichia coli* as lipoprotein vesicles, purification and maintenance of activity in the purified state is difficult, which has limited its mechanistic and structural analysis (80).

Through alanine scanning mutagenesis, an eight-histidine motif has been shown to be necessary for AlkB function and presumably is responsible for coordination the di-iron core (81). This motif represents a class of di-iron centers that is shared with desaturases, epoxidases, decarbonylases, and methyl oxidases, and differs from the carboxylate bridged di-iron center of sMMO (81). However, Mossbauer studies of the AlkB metal center revealed similar features as sMMO, with characteristics of an antiferromagnetically coupled pair of Fe (III) ions in its resting state (82). The di-iron cluster also becomes high-spin diferrous following reduction and can be

quantitatively oxidized back to its resting state by enzymatic turnover in the presence of substrate and oxygen (82). Further evidence for the similarities between the AlkB and sMMO mechanisms has been provided through studies with the use of norcarane as a chemical probe (83). From these studies, the AlkB reaction has been shown to be consistent with an oxygen-rebound mechanism via a substrate-centered radical, analogous to the proposed P450 and sMMO mechanisms, exhibiting limited rearranged products (83).

The ability to functionally express AlkB heterologously in *E. coli* certainly makes it a potentially better industrial biocatalyst compared to MMOs and also more amenable to enzyme engineering. However, the integral membrane nature of AlkB limits the enzyme's expression to the available membrane surface area. In addition, the lack of a crystal structure and knowledge of both the second coordination sphere of the diiron center and the component interactions are significant hindrances to directed evolution efforts to shift the AlkB substrate range from C₅-C₁₆ alkanes to methane.

B.6. Cytochrome P450s

Cytochrome P450s, which utilize a thiolate-ligated heme (iron protoporphyrin IX) prosthetic group in their active sites (84), represent an entirely different solution to diiron centers for catalytic oxygen insertion into C-H bonds. Unlike MMOs and non-heme diiron alkane hydroxylases, which are only found in methanotrophs and alkanotrophs, the superfamily of cytochrome P450s is one of the most prevalent enzyme families found across all three domains of life. To date, over 10,000 P450 enzymes have been identified (data source: <http://drnelson.utmem.edu/CytochromeP450.html>). P450s are involved in the metabolism of xenobiotics and the biosynthesis of signaling molecules. In the first role, P450s serve as a protective mechanism for the degradation of exogenous compounds by introducing polar

functional groups to facilitate further metabolism or excretion. This defense mechanism is particularly prominent in plants, which require P450s to break down herbicides due to their immobile nature (85 – 86). This is exemplified by the presence of over 400 P450 genes in rice (87). In their other role, P450s are responsible for synthesis of a variety of steroid hormones and the conversion of polyunsaturated fatty acids to biologically active molecules implicated in development and homeostasis.

The defining reaction P450s is the reductive activation of molecular oxygen as it is one of the few oxygenases possessing the requisite “ $\text{Fe}^{\text{IV}}=\text{O}^+$ ” state for alkane C-H bond activation. In this reaction, one oxygen atom is inserted into the substrate while the other is reduced to water. The overall equation for the reaction is $\text{RH} + \text{NAD(P)H} + \text{O}_2 + \text{H}^+ \rightarrow \text{ROH} + \text{NAD(P)}^+ + \text{H}_2\text{O}$, where RH is the substrate. In addition to this canonical reactivity, due to the existence of multiple oxidants in the P450 catalytic cycle, P450s can also catalyze epoxidation, dealkylation, sulfoxidations, desaturation, carbon-carbon bond scission, and carbon-carbon bond formation among other known reactivities (88 – 89).

Most P450s are membrane bound just as MMOs and alkane hydroxylases and thus are relatively difficult to manipulate. Fortunately, many bacterial P450s are soluble, monomeric proteins, and as a result, they have been the focus of early research. In particular, the prototypical enzymes CYP101 (P450_{cam}) from *Pseudomonas putida* (90 – 91) and CYP102A1 (BM3), a natural fusion enzyme from *Bacillus megaterium* in which the flavoproteins required for electron transfer and the heme protein are on a single polypeptide chain (92), provided much of the structural and mechanistic information of P450s. Recent interest in developing industrially useful P450 catalysts has also focused on enzymes from thermophilic organisms, including CYP119

(93), CYP174A1 (94), and CYP231A2 (95) as well as BM3 for its unique self-sufficiency and high catalytic rates (96 – 100).

B.7. P450 structure

The overall P450 fold (Figure 1.3 (a)) is retained across the enzyme superfamily even though members can share less than 20% sequence identity (101). The core four-helix bundle composed of three parallel helices (D, L, and I) and the antiparallel E helix are conserved in all P450s (102). The prosthetic heme group is ligated to the absolutely conserved cysteine located on a loop containing a highly conserved FxxFx(H/R)xCxG binding motif. This thiolate ligation gives rise to the 450 nm Soret absorbance maximum for the ferrous-CO complex for which P450s were named (103). The other common feature among P450s is a kink at the center of the I helix, which contains the amino acid sequence (A/G)Gx(E/D)T that has been implicated in oxygen binding and protonation (104 – 105).

Although the P450 fold is highly conserved, there is sufficient structural diversity to accommodate the binding of significantly different substrates ranging from ethanol in CYP2E1 (106) to large peptide antibiotics in CYP165C1 (107). In addition, since as few as one mutation can alter enzyme reactivity and selectivity, P450 family members (sharing at least 60% sequence identity) can have very different reactivities (108). P450 substrate binding occurs in an induced-fit mechanism accompanied with large (~ 10 Å) shifts in the flexible protein regions (109). As the substrate is embedded in the protein core, it interacts with various protein regions, which results in a large set of substrate recognition sites (SRS). Six SRSs have been found to be common to P450s (110): the B' helix region (SRS1), parts of the F and G helices (SRS2 and SRS3), a part of the I helix (SRS4), the K helix β 2 connecting region (SRS6), and the β 4 hairpin (SRS5).

B.8. P450 catalytic mechanism

The P450 hydroxylation mechanism is well understood and can be described as depicted in Figure 1.3 (b). The P450 catalytic cycle is initiated by substrate binding, which displaces the distal water ligand of the resting low-spin (LS) state of the Fe (III) heme (**1**) resulting in a high-spin (HS) substrate bound complex (**2**). The HS Fe (III) has a more positive reduction potential, which triggers electron transfer from the P450 reductase producing a ferrous intermediate (**3**) (111). Oxygen readily binds to the ferrous iron center leading to the formation of an oxy-P450 complex (**4**), which is the last stable intermediate in this cycle. A second electron transfer, usually the rate-limiting step of the catalytic cycle, results in a ferric hydroperoxo anion (**5**), which after protonation yields a ferric hydroperoxo complex (**6**). A second protonation at the distal oxygen followed by heterolytic cleavage of the O-O bond leads to the release of water and the formation of the oxo-ferryl porphyrin radical intermediate referred to as “Compound I” (CMP I) (**7**). CMP I then transfers an oxygen atom to the substrate, following a hydrogen abstraction-radical rebound mechanism (112) generating the alcohol product and returning to the Fe (III) resting state. The intermediates of this catalytic cycle have common features with peroxidases, cytochrome oxidases, and non-heme di-iron oxidases.

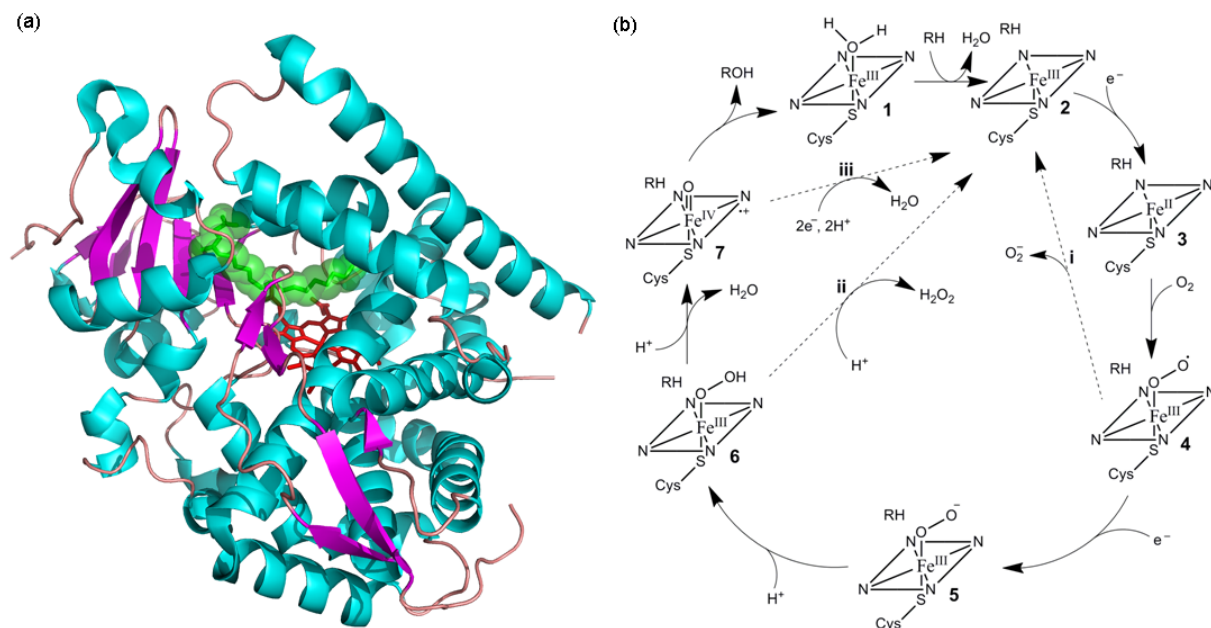


Figure 1.3: Cytochrome P450 structure and catalytic cycle; (a) the structure of the hydroxylase domain of CYP102A1 (BM3) with the heme shown in red and the substrate N-palmitoyl glycine substrate shown in green (pdb: 1JPZ); (b) the P450 catalytic cycle (see text for details)

In addition to having multiple distinct intermediates, many of which are also viable electrophilic and nucleophilic oxidants (113), the P450 catalytic cycle contains three branch points (114). These three abortive reactions are (i) autooxidation of the oxy-ferrous intermediate (4) with the release of a superoxide anion and returning the enzyme to its resting state (2), (ii) a peroxide shunt, where the coordinated hydroperoxide anion (6) dissociates, completing an unproductive two-electron reduction of oxygen, and (iii) oxidase uncoupling, where the CMP I (7) is oxidized to water instead of product formation, which results in a four-electron reduction of oxygen with the formation of two water molecules. These processes are generally referred to as uncoupling, which often occurs in reaction with non-natural substrates that are bound insufficiently to properly regulate solvent/proton access to the active site (89 – 90). These pathways are also prominent in eukaryotic P450s involved in host defense responses to xenobiotics through reactive oxygen species generation.

The electrons for the reduction step of the P450 catalytic cycle are provided by either (a) cytochrome P450 reductase (CPR), a soluble flavoprotein with FAD and FMN prosthetic groups, or (b) an iron-sulfur protein that shuttles electrons from a flavoprotein with a single FMN prosthetic group, or (c) a P450 reductase-like domain fused to the P450 heme domain. In each case, the electron donor uncouples the two electrons provided by NAD(P)H and transfers them singly to the P450 enzyme. Since the final reducing agent for the catalytic cycle is NAD(P)H, which has a midpoint potential of -320 mV (115), the resting state of the heme iron with a midpoint potential of ca. -300 mV (116 – 117) is reduced slowly in the absence of substrate. The substrate binding event triggers a change in the spin state of the heme iron from LS to HS, which induces a positive shift of 100 to 300 mV in the heme reduction potential allowing for rapid electron transfer (118). This mechanism clearly acts as a safeguard against the unproductive consumption of NAD(P)H and the formation of superoxide and peroxides. This substrate-induced initiation of electron transfer represents a specific P450 regulatory mechanism and is a clear departure from the initiation of the MMO catalytic cycle through binding of MMOB.

The P450 proton relay mechanism composed of several water molecules stabilized in the P450 active site as well as an acid-alcohol pair of amino acids (CYP101:Thr252, Glu366 CYP102: T268, Glu409) is equally important to P450 catalysis. This relay along with the electron transfer mechanism regulates the production of reactive intermediates and controls the flux of species into the branching points between productive and nonproductive pathways (119). For example, mutation of the conserved threonine in P450_{cam} to a hydrophobic residue resulted in near normal rates of cofactor oxidation, but was accompanied only by the release of hydrogen peroxide as the mutant could not effectively cleave the O-O bond without proper protonation (120). Coupling of product formation with cofactor consumption was restored by mutating this

position to amino acids capable of hydrogen-bonding interactions. The function of this proton delivery network is also dependent on substrate binding. For non-natural, poorly fitting substrates, their binding is insufficient to expel excess water from the active site, and protonation of the hydroperoxide anion (**6**) can occur at the proximal position, resulting in peroxide release. In fact, the uncoupling of proton and electron transfer does not even require a poorly fitting substrate; simply blocking the site of hydroxylation with fluoro-groups is sufficient to result in normal cofactor consumption with only water or peroxide production (121).

B.9. P450 substrate binding and substrate specificity

As mentioned previously, P450 substrate binding occurs by an “induced fit” model as proposed by Koshland (122) in which the enzyme accommodates different substrates in its active site by virtue of having a high level of flexibility to undergo appropriate conformational changes. Comparison of the X-ray structures of cytochrome P450s crystallized in substrate-free and substrate-bound forms (109) shows large structural rearrangements induced by substrate binding, which suggests that the SRSs are quite flexible and can provide a variety of substrates access to the heme. The absence of charged and hydrogen-bonding groups in the typical P450 substrate, as well as in the active sites of most P450 enzymes, requires such binding mechanisms as an alternative means to stabilize the substrate-enzyme complex. In many cases, different substrate analogues bind tightly to P450 enzymes simply due to their poor solubility in water rather than the presence of specific interactions with active site residues (123).

Given this general mode of substrate binding, it is unsurprising to find that the P450 specificity for substrate hydroxylation can be readily determined by three factors: (a) the affinity of the substrate for the P450 active site, which is largely determined by the substrate lipophilicity, (b) the intrinsic reactivity of the individual C-H bond in the substrate as determined

by the C-H bond strength, and (c) steric constraints imposed by the active site geometry. While the compatibility of a substrate within a P450 active site and steric constraints of binding modes are case specific, lipophilicity has been shown to be directly correlating to K_M or K_d for sets of similarity structured compounds (124 – 125). The general preference of P450 oxidation occurs with the following order of C-H bonds: tertiary>secondary>primary, which was determined using several small molecular probes that minimized the effect of the P450 active site structure in controlling the site of oxidation (126). This preference is reinforced by DFT calculations for the activation barriers for hydrogen abstraction, which predict a similar reactivity preference: benzylic or allylic>tertiary >secondary>primary (127 – 128).

B.10. P450 reactions using terminal oxidants

In addition to the normal P450 “turnover” conditions utilizing oxygen and NAD(P)H, the P450 catalytic cycle can also be accessed through the branching/shunt pathways using a variety of terminal oxidants including hydrogen peroxide, alkyl peroxides, acyl peroxides, and iodosobenzene. Early studies with alkylperoxides provided evidence for the formation of a ferric alkylperoxo complex ($\text{Fe}^{\text{III}}\text{-OOR}$) (129) as well as a compound II-like ferryl ($\text{Fe}^{\text{IV}}\text{=O}$) species, which is one oxidation equivalent higher than the resting ferric state (130 – 131) but little evidence for the formation of a CMP I-like ferryl porphyrin radical. However, recent works have confirmed the formation of both a compound-II – like and a compound-I – like species as transient intermediates (132 – 133).

The obvious advantage between reactions utilizing $\text{O}_2/\text{NAD(P)H}$ vs. peroxide is oxygen binding to a Fe^{2+} heme center vs. peroxide binding to Fe^{3+} heme center. The difference in the redox state between oxygen and peroxide eliminates the need for two reduction steps in the peroxide-driven pathway. However, the efficiency of this mode of reaction is generally poor due

to the intrinsically destructive nature of peroxides as well as the lack of acid-base catalytic residues in the P450 structure, with the exception of P450s which naturally utilize peroxides as their oxidant (134). In natural peroxidases, such as chloroperoxidase, the formation of the ferryl-oxo intermediate involves proton transfer from the proximal to the distal oxygen atom of the bound hydrogen peroxide, which is aided by a conserved His-Arg or His-Asp amino acid pair (135). P450s have highly hydrophobic active sites that lack these acid-base catalytic residues in close proximity to the oxygen binding pockets.

The peroxide-driven P450 reactions proceed through the formation of CMP 0, which after protonation and heterolytic O-O cleavage generates CMP I. In contrast, P450 reactions driven with iodosobenzene (PhIO) produce only CMP I as an oxidant without any potential involvement of peroxo-iron species, since PhIO is a single oxygen donor (136). The initial finding of solvent oxygen incorporation through experiments with ^{18}O -labeled water in reactions supported by PhIO led researchers to question if the oxidation proceeded via a ferryl intermediate (137 – 138). However, subsequent work has shown that the oxygen of PhIO readily exchanges with the medium through a porphyrin-oxidant complex, $[(\text{Porp})\text{Fe}^{\text{III}}\text{-OIPh}]^+$ (139). Nevertheless, whether PhIO-mediated reaction is a faithful mimic of the P450 reaction remains contentious due to differences observed in regio- and chemoselectivities (136) and kinetic isotope effects (140) between reactions supported by PhIO and NAD(P)H/O_2 (141).

B.11. H-abstraction and mechanistic comparisons with MMOs

Similarities between the mechanisms of P450s, di-iron non-heme alkane hydroxylases, and sMMO have long been recognized (142 – 144), as the active oxidants of these enzymes, a μ -oxo-diiron (IV) intermediate called compound Q for sMMO and an oxo-iron (IV) porphyrin π -cation-radical called CMP I for P450s, share the same net oxidation state. This is unsurprising

considering the energy requirements for breaking the inert alkane C-H bonds. In the consensus radical rebound mechanism for the O-atom insertion step, the ferryl oxygen initially abstracts a hydrogen from the substrate, leaving a carbon radical, which in turn recombines with the oxo radical coordinated to the iron atom (145). This mechanism is supported by observed large intramolecular isotope effects as well as the partial loss of stereo-chemistry at the carbon center for reactions with chemical probes (146 – 148).

Complexities in the radical rebound mechanisms arising from the mixed-spin nature of the transition state during the H-atom abstraction by the ferryl-oxo intermediate have been explained by DFT calculations (149). For the P450 mechanism, the reaction can proceed through a the low (doublet)-spin state, where the unpaired electron residing on the substrate after H-atom abstraction has an opposite spin to the electron in the iron-hydroxyl orbital. In contrast, the oxidant can also be in a high (quartet)-spin state, such that the substrate-based radical has the same spin as the P450 iron-hydroxyl species. As the collapse of the low-spin pathway lacks the spin-inversion barrier of the high-spin state, it proceeds without a barrier, and H-atom abstraction and the radical rebound can be considered to proceed in a concerted fashion. This mixed-spin transition state model with two distinct pathways for the H-atom abstraction-rebound mechanism has been able to reconcile seemingly contradictory radical lifetime experiments as well as differences in reaction KIEs (149). In addition to having a LS triplet state and a HS quintet state, the reaction with diiron metal centers (i.e., sMMO, AlkB) is further complicated by two possible angles of approach of the substrate C-H bond. In contrast to P450s, where the presence of the porphyrin prevents side-on or equatorial approaches, both end-on and side-on approaches are possible with di-iron ferryl-oxo species. As a consequence, both linear and bent Fe-O-H geometries are possible for the transition state (so that electrons can be transferred to both σ and

π orbitals), which results in four distinct reaction pathways with intermediates: $^3\text{TS}_\pi$, $^3\text{TS}_\sigma$, $^5\text{TS}_\pi$, and $^5\text{TS}_\sigma$.

Regardless of the spin-state of the reaction pathway, the transition state for the H-abstraction step presents the largest barrier in the reactions involving CMP I, or Q (58, 127). For methane, this barrier height is 26.7 kcal/mol for P450 CMP I, which is significantly higher than the ca.19 kcal/mol barrier for known P450 substrate camphor. The barrier heights for other small gaseous alkanes, ethane (21.6 – 21.8 kcal/mol) and propane (terminal: 21.6 kcal/mol, subterminal: 19 kcal/mol) are also much lower than methane (127, 149 – 151). As a comparison, this transition state barrier for H-abstraction from a methane C-H bond has been calculated to be as low as 13.8 kcal/mol (60) and as high as 23.2 kcal/mol (152) for sMMO. While these calculations show the barrier heights for P450 hydroxylation of known substrates and sMMO hydroxylation of methane are comparable, it is difficult to draw meaningful conclusions for the potential of P450s to oxidize methane.

C. Cytochrome P450 Enzyme Engineering

Due to their involvement in drug metabolism and their ability for regio- and stereoselective hydroxylation on a variety of substrates, numerous potential applications have been suggested for P450s (153 – 155). While, undoubtedly many industrial whole-organism biosyntheses involve P450s in their pathway (156), direct/explicit use of P450s for biotechnology applications has been scarce. The most publicized example of incorporation of P450s into practical products is the generation of transgenic “blue” carnations from petunias (157) and for enhanced production of “blue” roses (158). Examples for the use of P450s in the synthesis of high value pharmaceutical therapeutics include the production of hydrocortisone (159), cortisone (160), Pravastatin (161), and Artemisinin (162).

The limiting factors toward broader utilization of wild-type P450s as biocatalysts include poor expression, being membrane-bound, and lacking known redox partner proteins. For these reasons, protein engineering efforts have been focused on expanding the substrate range of well-expressed bacterial P450s with known redox partners to accept desired target compounds. Much of this work has been focused on P450_{cam} and BM3 (163). P450_{cam} is a type I P450 requiring a putidaredoxin containing a [2Fe-2S] cluster and an FAD-containing putidaredoxin reductase for the highly stereoselective hydroxylation of its physiological substrate (1R)-camphor to (1R)-5-*exo*-hydroxycamphor. BM3, as mentioned previously, is a type II P450 with its FAD and FMN containing reductase fused to its heme domain as a single polypeptide chain (92). BM3's native function is believed to be the detoxification of polyunsaturated fatty acids (164 – 165). Its unique domain architecture has been credited for its high hydroxylation and epoxidation rates ($\sim 17,000 \text{ min}^{-1}$ (166)) on long-chain fatty acid substrates (92, 166). In addition, these two proteins were also the first P450s of which crystal structures in the presence (167 – 168) and absence of substrates (169 – 173) were solved, which has also aided protein engineering efforts.

C.1. P450_{cam}

Protein engineering of P450_{cam} has been successful in switching its substrate specificity (174 – 179) through the introduction of point mutations to the active site. This strategy has been particularly successful because the enzyme backbone remains relatively fixed during catalysis (119). With just one to three active site mutations, P450_{cam} variants have been generated to hydroxylate (+)-R-pinene, a structural relative to (+)-camphor (174), aromatic compounds such as ethylbenzene (179), diphenylmethane, phenylcyclohexane, naphthalene, pyrenes (175, 178, 180 – 182), phenanthrene, fluoranthene, (183), polychlorinated benzenes (184), and indole to form indigo (185). Although improving the promiscuous activity of P450_{cam} for non-natural

substrates has proved to be relatively easy, i.e., requiring just a few mutations, the resulting variants generally exhibited poor coupling between cofactor consumption and product formation, typically ranging from 5% to 32%.

Far fewer examples exist for P450_{cam} variants with nearly wild-type coupling efficiency for non-native substrates, as these variants require multiple rounds of mutagenesis. For example, simply introducing a Y96F mutation will increase P450_{cam}'s ability to epoxidize styrene 25-fold, with 32% coupling compared to only 7% coupling for the wild-type (186). Placing an additional V247L mutation in the active site increases the coupling efficiency to 60% (175). The best example for the ability of P450_{cam} to be engineered to accept new substrates with high coupling efficiency was provided by Xu et al. (187) with the engineering of P450_{cam} to hydroxylate short-chain alkanes. Over the course of multiple studies (176, 187 – 188), the authors gradually decreased the active site volume by incrementally introducing bulky, hydrophobic residues. A P450_{cam} quadruple mutant, F87W/Y96F/T101L/V247L, was able to oxidize butane at 750 min⁻¹ with 95% coupling compared to 4% for wild type (176). To achieve 85% coupling of product formation with NADH consumption for propane oxidation, five additional mutations, L244M/L294M/T185M/L1358P/G248A, were required (187). This variant with nine active site mutations was also able to oxidize ethane at 78.2 min⁻¹ with 10.5 % coupling (187).

Finally, multiple attempts have been made to fuse the electron transfer components of P450_{cam} to its hydroxylase domain to increase enzyme activity (189 – 190). In addition, fusion of the P450_{cam} hydroxylase domain with reductases from naturally occurring self-sufficient P450s such as P450RhF isolated from *Rhodococcus* sp. NCIMPB 9784 (190) has also resulted in self-sufficient enzymes. However, while these constructs are often superior to free enzymes at a 1:1:1 ratio (hydroxylase: putidaredoxin reductase: putidaredoxin) in rates of electron transfer and

product formation, they fall short of the optimal free enzyme activity obtained at higher ratios of electron transfer components, i.e., 1: ≥ 4 : ≥ 12 (121).

C.2. CYP102A1 (P450BM3)

BM3 is one of the most studied and frequently engineered P450s, as it has the fastest known catalytic rate for a P450 and is a natural fusion protein with a type II reductase and its hydroxylase found on a single polypeptide (134). Its high rate of catalysis has been shown to be the result of BM3 domain architecture: when the heme and reductase domains are expressed independently and mixed together, the resulting activity is severely diminished (191). In addition to the self-sufficiency, BM3 can also be easily manipulated genetically and expressed at levels up to 1 g/L in laboratory strains of *Escherichia coli* (192). In contrast to P450_{cam}, the BM3 protein backbone undergoes large structural changes (> 10 Å) during catalysis as revealed by differences between the substrate-free crystal structure (168) and structures of BM3 complexed with known substrates (168 – 169). The large active site volume reflects the poor regioselectivity with which BM3 hydroxylates its preferred C₁₂-C₂₀ fatty acids substrates (193). All these factors make BM3 well suited for directed evolution experiments and potential use as a biocatalyst.

Much like P450_{cam}, BM3 has been engineered to accept a variety of substrates. Most rational engineering efforts of BM3 targeted hydrophobic residues lining the substrate-binding channel as well as active site residues immediate to the heme center. By mutating the two residues (R47, Y51) located at the opening of the substrate pocket that stabilizes the carboxylic acid moiety of the preferred fatty acid substrate, BM3 variants were isolated with increased activity for oxidation of alkyltrimethyl ammonium compounds (194), hydroxylation of shorter chain fatty acids (195), and epoxidation of the anti-malarial drug-precursor amorphadiene (196). Introducing additional active site mutations to these variants yielded a variant (R47L/Y51F

/A264G) with activity toward fluoranthrene (177) and alkoxyresorufins (197) and a variant (R47L/Y51F/F87A/A264G) with activity toward pyrene (177). Screening with a simple NADPH consumption assay to monitor cofactor consumption in the presence of a substrate, directed evolution of BM3 yielded a highly promiscuous variant F87V/L188Q/A74G with enhanced activity for a variety of substrates such as indole, alkanes, arene, and polycyclic, aromatic hydrocarbons (198 – 200). Similar engineering efforts generated variants with activity for β -ionone, a carotenoid intermediate (201), and valencene for (+)-nootkatone production (202).

From these rational engineering efforts, general structural function relationships have emerged, which have aided further engineering efforts. For example, active site residue F87, which is positioned directly between the bound substrate and the heme center, has been shown to affect both the substrate specificity and regioselectivity of fatty acid hydroxylation (203). Introduction of the F87V mutation converted BM3 into a regio- and stereoselective arachidonic acid epoxidase (203). This mutation along with F87A also increases the oxidation activity for a variety of aromatic compounds (204). This improved affinity for aromatic compounds is consistent with the removal of the F87 phenyl side chain directly adjacent to the heme center. Building on these studies, Pleiss and coworkers constructed a focused library targeting residues F87 and A328, allowing for a restricted set of non-polar amino acids (A, V, F, L, I). From this library, variants with activity for the oxidation of linear terpenes, cyclic monoterpenes, cyclic sesquiterpenes (99), cyclo-octane, cyclodecane, and cyclododecane (205) were found.

Work in our group has been focused on evolving BM3 to accept small alkanes as substrates with the ultimate target of methane hydroxylation. Starting from wild-type BM3, we enhanced its promiscuous activity for octane hydroxylation by applying a colorimetric screen for the hydroxylation of *p*-nitrophenoxy octane to evaluate enzyme libraries generated by random

mutagenesis (206). After multiple rounds of random mutagenesis, variant 139-3 was obtained, supporting 1,000 turnover number (TON) on octane, a 6.7-fold increase compared to wild type and measurable activity for propane oxidation (500 TON) (207). Selection pressure was then shifted toward propane hydroxylation through the use of a colorimetric screen based on dimethyl ether demethylation (DME) (208). Subsequent rounds of mutagenesis yielded variant 35E11, with 17 total amino acid mutations, supporting 6,000 propane TON and 250 ethane TON (209). In addition to small alkane hydroxylation activity, other BM3 variants generated in the 35E11 lineage were found with (1) regioselectivity for terminal hydroxylation of octane (210), (2) stereoselective secondary hydroxylation of linear alkanes (208), and (3) stereoselective epoxidation of alkenes (211 – 212).

D. References

1. Arakawa, H., Aresta, M., Armor, J. N., Barteau, M. A., Beckman, E. J., Bell, A. T., Bercaw, J. E., Creutz, C., Dinjus, E., Dixon, D. A., Domen, K., DuBois, D. L., Eckert, J., Fujita, E., Gibson, D. H., Goddard, W. A., Goodman, D. W., Keller, J., Kubas, G. J., Kung, H. H., Lyons, J. E., Manzer, L. E., Marks, T. J., Morokuma, K., Nicholas, K. M., Periana, R., Que, L., Rostrup-Nielsen, J., Sachtler, W. M. H., Schmidt, L. D., Sen, A., Somorjai, G. A., Stair, P. C., Stults, B. R., and Tumas, W. (2001) Catalysis research of relevance to carbon management: Progress, challenges, and opportunities, *Chem. Rev.* **101**, 953-996.
2. Bentley, R. W. (2002) Global oil & gas depletion: an overview, *Energy Policy* **30**, 189-205.
3. Chynoweth, D. P., Owens, J. M., and Legrand, R. (2001) Renewable methane from anaerobic digestion of biomass, *Renewable Energy* **22**, 1-8.
4. O. R. N. Laboratory (2003) *Basic Research Needs To Assure A Secure Energy Future*, Oak Ridge.
5. Hermans, I., Spier, E. S., Neuenschwander, U., Turra, N., and Baiker, A. (2009) Selective oxidation catalysis: opportunities and challenges, *Top. Catal.* **52**, 1162-1174.
6. (2003) *CRC Handbook of Chemistry and Physics*, 84th ed., CRC Press, Boca Raton, FL.
7. Lunsford, J. H. (2000) Catalytic conversion of methane to more useful chemicals and fuels: a challenge for the 21st century, *Catal. Today* **63**, 165-174.
8. Krylov, O. V. (1993) Catalytic reactions of partial methane oxidation, *Catal. Today* **18**, 209-302.
9. Forlani, O., and Rossini, S. (1992) Rare-earths as catalysts for the oxidative coupling of methane to ethylene, *Mater. Chem. Phys.* **31**, 155-158.
10. Maitra, A. M. (1993) Critical performance evaluation of catalysts and mechanistic implications for oxidative coupling of methane, *Appl. Catal. A-Gen.* **104**, 11-59.
11. Kushch, L. A., Lavrushko, V. V., Misharin, Y. S., Moravsky, A. P., and Shilov, A. E. (1983) Kinetics and mechanism of methane oxidation in aqueous-solutions of platinum complexes - direct evidence for a methylplatinum intermediate, *Nouveau Journal De Chimie-New Journal of Chemistry* **7**, 729-733.
12. Kao, L. C., Hutson, A. C., and Sen, A. (1991) Low-temperature, palladium(ii)-catalyzed, solution-phase oxidation of methane to a methanol derivative, *J. Am. Chem. Soc.* **113**, 700-701.
13. Lin, M., and Sen, A. (1994) Direct catalytic conversion of methane to acetic-acid in an aqueous-medium, *Nature* **368**, 613-615.
14. Periana, R. A., Taube, D. J., Evitt, E. R., Loffler, D. G., Wentrcek, P. R., Voss, G., and Masuda, T. (1993) A mercury-catalyzed, high-yield system for the oxidation of methane to methanol, *Science* **259**, 340-343.

15. Periana, R. A., Taube, D. J., Gamble, S., Taube, H., Satoh, T., and Fujii, H. (1998) Platinum catalysts for the high-yield oxidation of methane to a methanol derivative, *Science* 280, 560-564.
16. Labinger, J. A., and Bercaw, J. E. (2002) Understanding and exploiting C-H bond activation, *Nature* 417, 507-514.
17. Merckx, M., Kopp, D. A., Sazinsky, M. H., Blazyk, J. L., Muller, J., and Lippard, S. J. (2001) Dioxygen activation and methane hydroxylation by soluble methane monooxygenase: A tale of two irons and three proteins, *Angewandte Chemie-International Edition* 40, 2782-2807.
18. Que, L., and Tolman, W. B. (2008) Biologically inspired oxidation catalysis, *Nature* 455, 333-340.
19. Friedle, S., Reisner, E., and Lippard, S. J. (2010) Current challenges of modeling diiron enzyme active sites for dioxygen activation by biomimetic synthetic complexes, *Chem. Soc. Rev.* 39, 2768-2779.
20. Ghosh, A., de Oliveira, F. T., Yano, T., Nishioka, T., Beach, E. S., Kinoshita, I., Munck, E., Ryabov, A. D., Horwitz, C. P., and Collins, T. J. (2005) Catalytically active mu-oxodiiron(IV) oxidants from iron(III) and dioxygen, *J. Am. Chem. Soc.* 127, 2505-2513.
21. Wang, D., Farquhar, E. R., Stubna, A., Munck, E., and Que, L. (2009) A diiron(IV) complex that cleaves strong C-H and O-H bonds, *Nat. Chem.* 1, 145-150.
22. Xue, G. Q., Wang, D., De Hont, R., Fiedler, A. T., Shan, X. P., Munck, E., and Que, L. (2007) A synthetic precedent for the Fe-2(IV)(mu-O)(2) diamond core proposed for methane monooxygenase intermediate Q, *Proceedings of the National Academy of Sciences of the United States of America* 104, 20713-20718.
23. Hanson, R. S., and Hanson, T. E. (1996) Methanotrophic bacteria, *Microbiol. Rev.* 60, 439-471.
24. Dumont, M. G., and Murrell, J. C. (2005) Community-level analysis: Key genes of aerobic methane oxidation, In *Environmental Microbiology*, Elsevier Academic Press, Inc, San Diego, 413-427.
25. Hakemian, A. S., and Rosenzweig, A. C. (2007) The biochemistry of methane oxidation, *Annu. Rev. Biochem.* 76, 223-241.
26. Lieberman, R. L., and Rosenzweig, A. C. (2004) Biological methane oxidation: Regulation, biochemistry, and active site structure of particulate methane monooxygenase, *Crit. Rev. Biochem. Mol. Biol.* 39, 147-164.
27. Prior, S. D., and Dalton, H. (1985) The effect of copper ions on membrane content and methane monooxygenase activity in methanol-grown cells of *Methylococcus capsulatus* (bath), *J. Gen. Microbiol.* 131, 155-163.
28. Stanley, S. H., Prior, S. D., Leak, D. J., and Dalton, H. (1983) Copper stress underlies the fundamental change in intracellular location of methane monooxygenase in methane-oxidizing organisms - studies in batch and continuous cultures, *Biotechnol. Lett.* 5, 487-492.

29. Lieberman, R. L., and Rosenzweig, A. C. (2005) Crystal structure of a membrane-bound metalloenzyme that catalyses the biological oxidation of methane, *Nature* 434, 177-182.
30. Balasubramanian, R., Smith, S. M., Rawat, S., Yatsunyk, L. A., Stemmler, T. L., and Rosenzweig, A. C. (2010) Oxidation of methane by a biological dicopper centre, *Nature* 465, 115-U131.
31. Hakemian, A. S., Kondapalli, K. C., Telser, J., Hoffman, B. M., Stemmler, T. L., and Rosenzweig, A. C. (2008) The metal centers of particulate methane monooxygenase from *Methylosinus trichosporium* OB3b, *Biochemistry* 47, 6793-6801.
32. Lieberman, R. L., Shrestha, D. B., Doan, P. E., Hoffman, B. M., Stemmler, T. L., and Rosenzweig, A. C. (2003) Purified particulate methane monooxygenase from *Methylococcus capsulatus* (Bath) is a dimer with both mononuclear copper and a copper-containing cluster, *Proceedings of the National Academy of Sciences of the United States of America* 100, 3820-3825.
33. Choi, D. W., Kunz, R. C., Boyd, E. S., Semrau, J. D., Antholine, W. E., Han, J. I., Zahn, J. A., Boyd, J. M., de la Mora, A. M., and DiSpirito, A. A. (2003) The membrane-associated methane monooxygenase (pMMO) and pMMO-NADH : quinone oxidoreductase complex from *Methylococcus capsulatus* (Bath), *Journal of Bacteriology* 185, 5755-5764.
34. Zahn, J. A., and DiSpirito, A. A. (1996) Membrane-associated methane monooxygenase from *Methylococcus capsulatus* (Bath), *Journal of Bacteriology* 178, 1018-1029.
35. Colby, J., Stirling, D. I., and Dalton, H. (1977) Soluble methane mono-oxygenase of *Methylococcus capsulatus* (Bath) - ability to oxygenate normal-alkanes, normal-alkenes, ethers, and alicyclic, aromatic and heterocyclic-compounds, *Biochem. J.* 165, 395-402.
36. Basu, P., Katterle, B., Andersson, K. K., and Dalton, H. (2003) The membrane-associated form of methane mono-oxygenase from *Methylococcus capsulatus* (Bath) is a copper/iron protein, *Biochem. J.* 369, 417-427.
37. Nguyen, H. H. T., Elliott, S. J., Yip, J. H. K., and Chan, S. I. (1998) The particulate methane monooxygenase from *Methylococcus capsulatus* (Bath) is a novel copper-containing three-subunit enzyme - Isolation and characterization, *Journal of Biological Chemistry* 273, 7957-7966.
38. Burrows, K. J., Cornish, A., Scott, D., and Higgins I. J. (1984) Substrate specificities of the soluble and particulate methane mono-oxygenases of *Methylosinus trichosporium* OB3b, *Microbiology*, 3327-3333.
39. Sullivan, J. P., Dickinson, D., and Chase, H. A. (1998) Methanotrophs, *Methylosinus trichosporium* OB3b, sMMO, and their application to bioremediation, *Crit. Rev. Microbiol.* 24, 335-373.
40. Elliott, S. J., Zhu, M., Tso, L., Nguyen, H. H. T., Yip, J. H. K., and Chan, S. I. (1997) Regio- and stereoselectivity of particulate methane monooxygenase from *Methylococcus capsulatus* (Bath), *J. Am. Chem. Soc.* 119, 9949-9955.
41. Wilkinson, B., Zhu, M., Priestley, N. D., Nguyen, H. H. T., Morimoto, H., Williams, P. G., Chan, S. I., and Floss, H. G. (1996) A concerted mechanism for ethane hydroxylation

- by the particulate methane monooxygenase from *Methylococcus capsulatus* (Bath), *J. Am. Chem. Soc.* 118, 921-922.
42. Yu, S. S. F., Wu, L. Y., Chen, K. H. C., Luo, W. I., Huang, D. S., and Chan, S. I. (2003) The stereospecific hydroxylation of 2,2-H-2(2) butane and chiral dideuteriobutanes by the particulate methane monooxygenase from *Methylococcus capsulatus* (Bath), *Journal of Biological Chemistry* 278, 40658-40669.
 43. Huang, D. S., Wu, S. H., Wang, Y. S., Yu, S. S. F., and Chan, S. I. (2002) Determination of the carbon kinetic isotope effects on propane hydroxylation mediated by the methane monooxygenases from *Methylococcus capsulatus* (Bath) by using stable carbon isotopic analysis, *Chembiochem* 3, 760-765.
 44. Dalton, H. (1980) Oxidation of hydrocarbons by methane monooxygenases from a variety of microbes, In *Advances in Applied Microbiology* (Perlman, D., Ed.), Academic Press, 71-87.
 45. Nakajima, T., Uchiyama, H., Yagi, O., and Nakahara, T. (1992) Purification and properties of a soluble methane monooxygenase from *Methylocystis* sp., *Biosci. Biotechnol. Biochem.* 56, 736-740.
 46. Wallar, B. J., and Lipscomb, J. D. (1996) Dioxygen activation by enzymes containing binuclear non-heme iron clusters, *Chem. Rev.* 96, 2625-2657.
 47. Notomista, E., Lahm, A., Di Donato, A., and Tramontano, A. (2003) Evolution of bacterial and archaeal multicomponent monooxygenases, *J. Mol. Evol.* 56, 435-445.
 48. Leahy, J. G., Batchelor, P. J., and Morcomb, S. M. (2003) Evolution of the soluble diiron monooxygenases, *Fems Microbiol. Rev.* 27, 449-479.
 49. Shu, L. J., Nesheim, J. C., Kauffmann, K., Munck, E., Lipscomb, J. D., and Que, L. (1997) An (Fe₂O₂)-O-IV diamond core structure for the key intermediate Q of methane monooxygenase, *Science* 275, 515-518.
 50. Ambundo, E. A., Friesner, R. A., and Lippard, S. J. (2002) Reactions of methane monooxygenase intermediate Q with derivatized methanes, *J. Am. Chem. Soc.* 124, 8770-8771.
 51. Beauvais, L. G., and Lippard, S. J. (2005) Reactions of the peroxo intermediate of soluble methane monooxygenase hydroxylase with ethers, *J. Am. Chem. Soc.* 127, 7370-7378.
 52. Brazeau, B. J., and Lipscomb, J. D. (2000) Kinetics and activation thermodynamics of methane monooxygenase compound Q formation and reaction with substrates, *Biochemistry* 39, 13503-13515.
 53. Liu, K. E., Valentine, A. M., Wang, D. L., Huynh, B. H., Edmondson, D. E., Salifoglou, A., and Lippard, S. J. (1995) Kinetic and spectroscopic characterization of intermediates and component interactions in reactions of methane monooxygenase from *Methylococcus capsulatus* (Bath), *J. Am. Chem. Soc.* 117, 10174-10185.
 54. Valentine, A. M., Stahl, S. S., and Lippard, S. J. (1999) Mechanistic studies of the reaction of reduced methane monooxygenase hydroxylase with dioxygen and substrates, *J. Am. Chem. Soc.* 121, 3876-3887.

55. Liu, K. E., Valentine, A. M., Qiu, D., Edmondson, D. E., Appelman, E. H., Spiro, T. G., and Lippard, S. J. (1995) Characterization of a diiron(iii) peroxo intermediate in the reaction cycle of methane monooxygenase hydroxylase from *Methylococcus capsulatus* (Bath), *J. Am. Chem. Soc.* 117, 4997-4998.
56. Lee, S. Y., and Lipscomb, J. D. (1999) Oxygen activation catalyzed by methane monooxygenase hydroxylase component: Proton delivery during the O-O bond cleavage steps, *Biochemistry* 38, 4423-4432.
57. Tinberg, C. E., and Lippard, S. J. (2009) Revisiting the Mechanism of Dioxygen Activation in Soluble Methane Monooxygenase from *M. capsulatus* (Bath): Evidence for a Multi-Step, Proton-Dependent Reaction Pathway, *Biochemistry* 48, 12145-12158.
58. Gherman, B. F., Dunietz, B. D., Whittington, D. A., Lippard, S. J., and Friesner, R. A. (2001) Activation of the C-H bond of methane by intermediate Q of methane monooxygenase: A theoretical study, *J. Am. Chem. Soc.* 123, 3836-3837.
59. Musaev, D. G., Basch, H., and Morokuma, K. (2002) Theoretical study of the mechanism of alkane hydroxylation and ethylene epoxidation reactions catalyzed by diiron bis-oxo complexes. The effect of substrate molecules, *J. Am. Chem. Soc.* 124, 4135-4148.
60. Siegbahn, P. E. M. (2001) O-O bond cleavage and alkane hydroxylation in methane monooxygenase, *J. Biol. Inorg. Chem.* 6, 27-45.
61. Baik, M. H., Newcomb, M., Friesner, R. A., and Lippard, S. J. (2003) Mechanistic studies on the hydroxylation of methane by methane monooxygenase, *Chem. Rev.* 103, 2385-2419.
62. Froland, W. A., Andersson, K. K., Lee, S. K., Liu, Y., and Lipscomb, J. D. (1992) Methane monooxygenase component-b and reductase alter the regioselectivity of the hydroxylase component-catalyzed reactions - a novel role for protein-protein interactions in an oxygenase mechanism, *Journal of Biological Chemistry* 267, 17588-17597.
63. Gassner, G. T., and Lippard, S. J. (1999) Component interactions in the soluble methane monooxygenase system from *Methylococcus capsulatus* (Bath), *Biochemistry* 38, 12768-12785.
64. Liu, Y., Nesheim, J. C., Lee, S. K., and Lipscomb, J. D. (1995) Gating effects of component-b on oxygen activation by the methane monooxygenase hydroxylase component, *Journal of Biological Chemistry* 270, 24662-24665.
65. Blazyk, J. L., Gassner, G. T., and Lippard, S. J. (2005) Intermolecular electron-transfer reactions in soluble methane monooxygenase: A role for hysteresis in protein function, *J. Am. Chem. Soc.* 127, 17364-17376.
66. Sazinsky, M. H., and Lippard, S. J. (2006) Correlating structure with function in bacterial multicomponent monooxygenases and related diiron proteins, *Accounts Chem. Res.* 39, 558-566.
67. Murray, L. J., and Lippard, S. J. (2007) Substrate trafficking and dioxygen activation in bacterial multicomponent monooxygenases, *Accounts Chem. Res.* 40, 466-474.

68. Whittington, D. A., Rosenzweig, A. C., Frederick, C. A., and Lippard, S. J. (2001) Xenon and halogenated alkanes track putative substrate binding cavities in the soluble methane monooxygenase hydroxylase, *Biochemistry* 40, 3476-3482.
69. Sazinsky, M. H., and Lippard, S. J. (2005) Product bound structures of the soluble methane monooxygenase hydroxylase from *Methylococcus capsulatus* (Bath): Protein motion in the alpha-subunit, *J. Am. Chem. Soc.* 127, 5814-5825.
70. Wilkins, P. C., Dalton, H., Samuel, C. J., and Green, J. (1994) Further evidence for multiple pathways in soluble methane-monooxygenase-catalyzed oxidations from the measurement of deuterium kinetic isotope effects, *Eur. J. Biochem.* 226, 555-560.
71. Rataj, M. J., Kauth, J. E., and Donnelly, M. I. (1991) Oxidation of deuterated compounds by high specific activity methane monooxygenase from *Methylosinus trichosporium* - mechanistic implications, *Journal of Biological Chemistry* 266, 18684-18690.
72. Tinberg, C. E., and Lippard, S. J. (2010) Oxidation reactions performed by soluble methane monooxygenase hydroxylase intermediates H-peroxo and Q proceed by distinct mechanisms, *Biochemistry* 49, 7902-7912.
73. Lee, S. G., Goo, J. H., Kim, H. G., Oh, J. I., Kim, Y. M., and Kim, S. W. (2004) Optimization of methanol biosynthesis from methane using *Methylosinus trichosporium* OB3b, *Biotechnol. Lett.* 26, 947-950.
74. Gunay, A., and Theopold, K. H. (2010) C-H Bond activations by metal oxo compounds, *Chem. Rev.* 110, 1060-1081.
75. Smits, T. H. M., Witholt, B., and van Beilen, J. B. (2003) Functional characterization of genes involved in alkane oxidation by *Pseudomonas aeruginosa*, *Antonie Van Leeuwenhoek* 84, 193-200.
76. Ueda, T., and Coon, M. J. (1972) Enzymatic omega-oxidation .7. Reduced diphosphopyridine nucleotide-rubredoxin reductase - properties and function as an electron carrier in omega hydroxylation, *Journal of Biological Chemistry* 247, 5010-5027.
77. Peterson, J. A., Kusunose, M., Kusunose, E., and Coon, M. J. (1967) Enzymatic omega-oxidation .2. Function of rubredoxin as electron carrier in omega-hydroxylation, *Journal of Biological Chemistry* 242, 4334-4353.
78. McKenna, E. J., and Coon, M. J. (1970) Enzymatic omega-oxidation .4. Purification and properties of omega-hydroxylase of *Pseudomonas oleovorans*, *Journal of Biological Chemistry* 245, 3882-3897.
79. Ruettinger, R. T., Griffith, G. R., and Coon, M. J. (1977) Characterization of omega-hydroxylase of *Pseudomonas oleovorans* as a nonheme iron protein, *Arch. Biochem. Biophys.* 183, 528-537.
80. Nieboer, M., Kingma, J., and Witholt, B. (1993) The alkane oxidation system of *Pseudomonas oleovorans* - induction of the alk genes in *Escherichia coli* w3110(pgec47) affects membrane biogenesis and results in overexpression of alkane hydroxylase in a distinct cytoplasmic membrane subfraction, *Mol. Microbiol.* 8, 1039-1051.

81. Shanklin, J., and Whittle, E. (2003) Evidence linking the *Pseudomonas oleovorans* alkane omega-hydroxylase, an integral membrane diiron enzyme, and the fatty acid desaturase family, *FEBS Lett.* 545, 188-192.
82. Shanklin, J., Achim, C., Schmidt, H., Fox, B. G., and Munck, E. (1997) Mossbauer studies of alkane omega-hydroxylase: Evidence for a diiron cluster in an integral-membrane enzyme, *Proceedings of the National Academy of Sciences of the United States of America* 94, 2981-2986.
83. Rozhkova-Novosad, E. A., Chae, J. C., Zylstra, G. J., Bertrand, E. M., Alexander-Ozinskas, M., Deng, D. Y., Moe, L. A., van Beilen, J. B., Danahy, M., Groves, J. T., and Austin, R. N. (2007) Profiling mechanisms of alkane hydroxylase activity *in vivo* using the diagnostic substrate norcarane, *Chem. Biol.* 14, 165-172.
84. Dawson, J. H., and Sono, M. (1987) Cytochrome P450 and chloroperoxidase - thiolate-ligated heme enzymes - spectroscopic determination of their active-site structures and mechanistic implications of thiolate ligation, *Chem. Rev.* 87, 1255-1276.
85. Morant, M., Bak, S., Moller, B. L., and Werck-Reichhart, D. (2003) Plant cytochromes P450: tools for pharmacology, plant protection and phytoremediation, *Current Opinion in Biotechnology* 14, 151-162.
86. Persans, M. W., Wang, J., and Schuler, M. A. (2001) Characterization of maize cytochrome P450 monooxygenases induced in response to safeners and bacterial pathogens, *Plant Physiol.* 125, 1126-1138.
87. Schuler, M. A., and Werck-Reichhart, D. (2003) Functional genomics of P450s, *Annu. Rev. Plant Biol.* 54, 629-667.
88. de Montellano, P. R. O. (1986) *Cytochrome P450*, 1st ed., Plenum Publishing Corp., New York.
89. Isin, E. M., and Guengerich, F. P. (2007) Complex reactions catalyzed by cytochrome P450 enzymes, *Biochim. Biophys. Acta-Gen. Subj.* 1770, 314-329.
90. Denisov, I. G., Makris, T. M., Sligar, S. G., and Schlichting, I. (2005) Structure and chemistry of cytochrome P450, *Chem. Rev.* 105, 2253-2277.
91. Poulos, T. L., and Raag, R. (1992) Cytochrome P450cam - crystallography, oxygen activation, and electron-transfer, *Faseb J.* 6, 674-679.
92. Narhi, L. O., and Fulco, A. J. (1986) Characterization of a catalytically self-sufficient 119,000-dalton cytochrome P450 monooxygenase induced by barbiturates in *Bacillus megaterium*, *Journal of Biological Chemistry* 261, 7160-7169.
93. Nishida, C. R., and de Montellano, P. R. O. (2005) Thermophilic cytochrome P450 enzymes, *Biochem. Biophys. Res. Commun.* 338, 437-445.
94. Yano, J. K., Blasco, F., Li, H. Y., Schmid, R. D., Henne, A., and Poulos, T. L. (2003) Preliminary characterization and crystal structure of a thermostable cytochrome P450 from *Thermus thermophilus*, *Journal of Biological Chemistry* 278, 608-616.

95. Ho, W. W., Li, H., Nishida, C. R., de Montellano, P. R. O., and Poulos, T. L. (2008) Crystal structure and properties of CYP231A2 from the thermoacidophilic *Archaeon* *Picrophilus torridus*, *Biochemistry* 47, 2071-2079.
96. Cirino, P. C., and Arnold, F. H. (2002) Protein engineering of oxygenases for biocatalysis, *Curr. Opin. Chem. Biol.* 6, 130-135.
97. Fasan, R., Chen, M. M., Crook, N. C., and Arnold, F. H. (2007) Engineered alkane-hydroxylating cytochrome P450(BM3) exhibiting natively like catalytic properties, *Angewandte Chemie-International Edition* 46, 8414-8418.
98. Nazor, J., Dannenmann, S., Adjei, R. O., Fordjour, Y. B., Ghampson, I. T., Blanusa, M., Roccatano, D., and Schwaneberg, U. (2008) Laboratory evolution of P450BM3 for mediated electron transfer yielding an activity-improved and reductase-independent variant, *Protein Eng. Des. Sel.* 21, 29-35.
99. Seifert, A., Vomund, S., Grohmann, K., Kriening, S., Urlacher, V. B., Laschat, S., and Pleiss, J. (2009) Rational design of a minimal and highly enriched CYP102A1 mutant library with improved regio-, stereo- and chemoselectivity, *Chembiochem* 10, 853-861.
100. van Vugt-Lussenburg, B. M. A., Stjernschantz, E., Lastdrager, J., Oostenbrink, C., Vermeulen, N. P. E., and Commandeur, J. N. M. (2007) Identification of critical residues in novel drug metabolizing mutants of cytochrome P450BM3 using random mutagenesis, *J. Med. Chem.* 50, 455-461.
101. Hasemann, C. A., Kurumbail, R. G., Boddupalli, S. S., Peterson, J. A., and Deisenhofer, J. (1995) Structure and function of cytochromes P450 - a comparative-analysis of 3 crystal-structures, *Structure* 3, 41-62.
102. Presnell, S. R., and Cohen, F. E. (1989) Topological distribution of 4-alpha-helix bundles, *Proceedings of the National Academy of Sciences of the United States of America* 86, 6592-6596.
103. Dawson, J. H., Holm, R. H., Trudell, J. R., Barth, G., Linder, R. E., Bunnenberg, E., Djerassi, C., and Tang, S. C. (1976) Oxidized cytochrome P450 magnetic circular dichroism evidence for thiolate ligation in substrate-bound form implications for catalytic mechanism, *J. Am. Chem. Soc.* 98, 3707-3709.
104. Imai, M., Shimada, H., Watanabe, Y., Matsushimahibiya, Y., Makino, R., Koga, H., Horiuchi, T., and Ishimura, Y. (1989) Uncoupling of the cytochrome P450cam monooxygenase reaction by a single mutation, threonine-252 to alanine or valine - a possible role of the hydroxy amino-acid in oxygen activation, *Proceedings of the National Academy of Sciences of the United States of America* 86, 7823-7827.
105. Kimata, Y., Shimada, H., Hirose, T., and Ishimura, Y. (1995) Role of thr-252 in cytochrome P450cam - a study with unnatural amino-acid mutagenesis, *Biochem. Biophys. Res. Commun.* 208, 96-102.
106. Porubsky, P. R., Meneely, K. M., and Scott, E. E. (2008) Structures of human cytochrome P450 2E1 insights into the binding of inhibitors and both small molecular weight and fatty acid substrates, *Journal of Biological Chemistry* 283, 33698-33707.

107. Pylypenko, O., Vitali, F., Zerbe, K., Robinson, J. A., and Schlichting, I. (2003) Crystal structure of OxyC, a cytochrome P450 implicated in an oxidative C-C coupling reaction during vancomycin biosynthesis, *Journal of Biological Chemistry* 278, 46727-46733.
108. Kelly, S. L., Lamb, D. C., and Kelly, D. E. (2006) Cytochrome P450 biodiversity and biotechnology, *Biochem. Soc. Trans.* 34, 1159-1160.
109. Pylypenko, O., and Schlichting, I. (2004) Structural aspects of ligand binding to and electron transfer in bacterial and fungal p450s, *Annu. Rev. Biochem.* 73, 991-1018.
110. Gotoh, O. (1992) Substrate recognition sites in cytochrome-P450 family-2 (cyp2) proteins inferred from comparative analyses of amino-acid and coding nucleotide-sequences, *Journal of Biological Chemistry* 267, 83-90.
111. Sligar, S. G. (1976) Coupling of spin, substrate, and redox equilibria in cytochrome P450, *Biochemistry* 15, 5399-5406.
112. Groves, J. T. (1985) Key elements of the chemistry of cytochrome P450 - the oxygen rebound mechanism, *J. Chem. Educ.* 62, 928-931.
113. Coon, M. J., Vaz, A. D. N., McGinnity, D. F., and Peng, H. M. (1998) Multiple activated oxygen species in P450 catalysis - Contributions to specificity in drug metabolism, *Drug Metab. Dispos.* 26, 1190-1193.
114. Bernhardt, R. (1996) Cytochrome P450: Structure, function, and generation of reactive oxygen species, In *Reviews of Physiology Biochemistry and Pharmacology, Vol 127*, Springer-Verlag Berlin, Berlin 33, 137-221.
115. Avila, L., Wirtz, M., Bunce, R. A., and Rivera, M. (1999) An electrochemical study of the factors responsible for modulating the reduction potential of putidaredoxin, *J. Biol. Inorg. Chem.* 4, 664-674.
116. Daff, S. N., Chapman, S. K., Turner, K. L., Holt, R. A., Govindaraj, S., Poulos, T. L., and Munro, A. W. (1997) Redox control of the catalytic cycle of flavocytochrome P-450 BM3, *Biochemistry* 36, 13816-13823.
117. Guengerich, F. P., Ballou, D. P., and Coon, M. J. (1975) Purified liver microsomal cytochrome P450 - electron-accepting properties and oxidation-reduction potential, *Journal of Biological Chemistry* 250, 7405-7414.
118. Ost, T. W. B., Clark, J., Mowat, C. G., Miles, C. S., Walkinshaw, M. D., Reid, G. A., Chapman, S. K., and Daff, S. (2003) Oxygen activation and electron transfer in flavocytochrome P450BM3, *J. Am. Chem. Soc.* 125, 15010-15020.
119. Schlichting, I., Berendzen, J., Chu, K., Stock, A. M., Maves, S. A., Benson, D. E., Sweet, B. M., Ringe, D., Petsko, G. A., and Sligar, S. G. (2000) The catalytic pathway of cytochrome P450cam at atomic resolution, *Science* 287, 1615-1622.
120. Imai, M., Shimada, H., Watanabe, Y., Matsushimahibiya, Y., Makino, R., Koga, H., Horiuchi, T., and Ishimura, Y. (1989) Uncoupling of the cytochrome P450cam monooxygenase reaction by a single mutation, threonine-252 to alanine or valine - a possible role of the hydroxy amino-acid in oxygen activation, *Proceedings of the National Academy of Sciences of the United States of America* 86, 7823-7827.

121. Kadkhodayan, S., Coulter, E. D., Maryniak, D. M., Bryson, T. A., and Dawson, J. H. (1995) Uncoupling oxygen-transfer and electron-transfer in the oxygenation of camphor analogs by cytochrome P450cam - direct observation of an intermolecular isotope effect for substrate C-H activation, *Journal of Biological Chemistry* 270, 28042-28048.
122. Koshland, D. E. (1994) The key-lock theory and the induced fit theory, *Angewandte Chemie-International Edition* 33, 2375-2378.
123. Szklarz, G. D., and Paulsen, M. D. (2002) Molecular modeling of cytochrome P450 1A1: Enzyme-substrate interactions and substrate binding affinities, *J. Biomol. Struct. Dyn.* 20, 155-162.
124. Hansch, C., and Zhang, L. T. (1993) Quantitative structure-activity-relationships of cytochrome P450, *Drug Metab. Rev.* 25, 1-48.
125. Lewis, D. F. V., Jacobs, M. N., and Dickins, M. (2004) Compound lipophilicity for substrate binding to human P450s in drug metabolism, *Drug Discov. Today* 9, 530-537.
126. Frommer, U., Ullrich, V., and Stauding, H. (1970) Hydroxylation of aliphatic compounds by liver microsomes .1. Distribution pattern of isomeric alcohols, *Hoppe-Seylers Zeitschrift Fur Physiologische Chemie* 351, 903-912.
127. de Visser, S. P., Kumar, D., Cohen, S., Shacham, R., and Shaik, S. (2004) A predictive pattern of computed barriers for C-H hydroxylation by compound I of cytochrome P450, *J. Am. Chem. Soc.* 126, 8362-8363.
128. Olsen, L., Rydberg, P., Rod, T. H., and Ryde, U. (2006) Prediction of activation energies for hydrogen abstraction by cytochrome P450, *J. Med. Chem.* 49, 6489-6499.
129. Tajima, K., Edo, T., Ishizu, K., Imaoka, S., Funae, Y., Oka, S., and Sakurai, H. (1993) Cytochrome P450-butyl peroxide complex detected by ESR, *Biochem. Biophys. Res. Commun.* 191, 157-164.
130. Blake, R. C., and Coon, M. J. (1981) On the mechanism of action of cytochrome P450 - role of peroxy spectral intermediates in substrate hydroxylation, *Journal of Biological Chemistry* 256, 5755-5763.
131. Jung, C., Schunemann, V., and Lendzian, F. (2005) Freeze-quenched iron-oxo intermediates in cytochromes P450, *Biochem. Biophys. Res. Commun.* 338, 355-364.
132. Rittle, J., and Green, M. T. (2010) Cytochrome P450 Compound I: Capture, Characterization, and C-H Bond Activation Kinetics, *Science* 330, 933-937.
133. Spolitak, T., Dawson, J. H., and Ballou, D. P. (2005) Reaction of ferric cytochrome P450cam with peracids - Kinetic characterization of intermediates on the reaction pathway, *Journal of Biological Chemistry* 280, 20300-20309.
134. Munro, A. W., Girvan, H. M., and McLean, K. J. (2007) Variations on a (t)heme - novel mechanisms, redox partners and catalytic functions in the cytochrome P450 superfamily, *Natural Product Reports* 24, 585-609.
135. Loew, G. H., and Harris, D. L. (2000) Role of the heme active site and protein environment in structure, spectra, and function of the cytochrome p450s, *Chem. Rev.* 100, 407-419.

136. Gustafsson, J. A., Rondahl, L., and Bergman, J. (1979) Iodosylbenzene derivatives as oxygen donors in cytochrome P450 catalyzed steroid hydroxylations, *Biochemistry* 18, 865-870.
137. Heimbrook, D. C., and Sligar, S. G. (1981) Multiple mechanisms of cytochrome P450-catalyzed substrate hydroxylations, *Biochem. Biophys. Res. Commun.* 99, 530-535.
138. Macdonald, T. L., Burka, L. T., Wright, S. T., and Guengerich, F. P. (1982) Mechanisms of hydroxylation by cytochrome P450 - exchange of iron-oxygen intermediates with water, *Biochem. Biophys. Res. Commun.* 104, 620-625.
139. Song, W. J., Sun, Y. J., Choi, S. K., and Nam, W. (2006) Mechanistic insights into the reversible formation of iodosylarene-iron porphyrin complexes in the reactions of oxoiron(IV) porphyrin pi-cation radicals and iodoarenes: Equilibrium, epoxidizing intermediate, and oxygen exchange, *Chemistry-a European Journal* 12, 130-137.
140. Guengerich, F. P., Yun, C. H., and Macdonald, T. L. (1996) Evidence for a 1-electron oxidation mechanism in N-dealkylation of N,N-dialkylanilines by cytochrome P450 2B1 - Kinetic hydrogen isotope effects, linear free energy relationships, comparisons with horseradish peroxidase, and studies with oxygen surrogates, *Journal of Biological Chemistry* 271, 27321-27329.
141. Bhakta, M. N., Hollenberg, P. F., and Wimalasena, K. (2005) P-450/NADPH/O-2- and P-450/PhIO-catalyzed N-dealkylations are mechanistically distinct, *J. Am. Chem. Soc.* 127, 1376-1377.
142. Brazeau, B. J., Austin, R. N., Tarr, C., Groves, J. T., and Lipscomb, J. D. (2001) Intermediate Q from soluble methane monooxygenase hydroxylates the mechanistic substrate probe norcarane: Evidence for a stepwise reaction, *J. Am. Chem. Soc.* 123, 11831-11837.
143. Kopp, D. A., and Lippard, S. J. (2002) Soluble methane monooxygenase: activation of dioxygen and methane, *Curr. Opin. Chem. Biol.* 6, 568-576.
144. Newcomb, M., Shen, R. N., Lu, Y., Coon, M. J., Hollenberg, P. F., Kopp, D. A., and Lippard, S. J. (2002) Evaluation of norcarane as a probe for radicals in cytochrome P450- and soluble methane monooxygenase-catalyzed hydroxylation reactions, *J. Am. Chem. Soc.* 124, 6879-6886.
145. Groves, J. T. (2003) The bioinorganic chemistry of iron in oxygenases and supramolecular assemblies, *Proceedings of the National Academy of Sciences of the United States of America* 100, 3569-3574.
146. Groves, J. T., McClusky, G. A., White, R. E., and Coon, M. J. (1978) Aliphatic hydroxylation by highly purified liver microsomal cytochrome P450 - evidence for a carbon radical intermediate, *Biochem. Biophys. Res. Commun.* 81, 154-160.
147. Krauser, J. A., and Guengerich, F. P. (2005) Cytochrome P450 3A4-catalyzed testosterone 6 beta-hydroxylation stereochemistry, kinetic deuterium isotope effects, and rate-limiting steps, *Journal of Biological Chemistry* 280, 19496-19506.

148. White, R. E., Miller, J. P., Favreau, L. V., and Bhattacharyya, A. (1986) Stereochemical dynamics of aliphatic hydroxylation by cytochrome P450, *J. Am. Chem. Soc.* **108**, 6024-6031.
149. Shaik, S., Kumar, D., de Visser, S. P., Altun, A., and Thiel, W. (2005) Theoretical perspective on the structure and mechanism of cytochrome P450 enzymes, *Chem. Rev.* **105**, 2279-2328.
150. de Visser, S. P., Ogliaro, F., Sharma, P. K., and Shaik, S. (2002) What factors affect the regioselectivity of oxidation by cytochrome P450? A DFT study of allylic hydroxylation and double bond epoxidation in a model reaction, *J. Am. Chem. Soc.* **124**, 11809-11826.
151. Ogliaro, F., Harris, N., Cohen, S., Filatov, M., de Visser, S. P., and Shaik, S. (2000) A model "rebound" mechanism of hydroxylation by cytochrome P450: Stepwise and effectively concerted pathways, and their reactivity patterns, *J. Am. Chem. Soc.* **122**, 8977-8989.
152. Basch, H., Musaev, D. G., Mogi, K., and Morokuma, K. (2001) Theoretical studies on the mechanism of the methane $\rightarrow$ methanol conversion reaction catalyzed by methane monooxygenase: O-side vs N-side mechanisms, *J. Phys. Chem. A* **105**, 3615-3622.
153. Bernhardt, R. (2006) Cytochromes P450 as versatile biocatalysts, *Journal of Biotechnology* **124**, 128-145.
154. Gillam, E. M. (2005) Exploring the potential of xenobiotic-metabolising enzymes as biocatalysts: Evolving designer catalysts from polyfunctional cytochrome P450 enzymes, *Clin. Exp. Pharmacol. Physiol.* **32**, 147-152.
155. Guengerich, F. P. (2002) Cytochrome P450 enzymes in the generation of commercial products, *Nat. Rev. Drug Discov.* **1**, 359-366.
156. Duetz, W. A., van Beilen, J. B., and Witholt, B. (2001) Using proteins in their natural environment: potential and limitations of microbial whole-cell hydroxylations in applied biocatalysis, *Current Opinion in Biotechnology* **12**, 419-425.
157. Fukui, Y., Tanaka, Y., Kusumi, T., Iwashita, T., and Nomoto, K. (2003) A rationale for the shift in colour towards blue in transgenic carnation flowers expressing the flavonoid 3',5'-hydroxylase gene, *Phytochemistry* **63**, 15-23.
158. Holton, T. A., Brugliera, F., Lester, D. R., Tanaka, Y., Hyland, C. D., Menting, J. G. T., Lu, C. Y., Farcy, E., Stevenson, T. W., and Cornish, E. C. (1993) Cloning and expression of cytochrome P450 genes-controlling flower color, *Nature* **366**, 276-279.
159. Petzoldt, K. E. A. *Process for the preparation of 11-beta-hydroxy steroids*, Schering Aktiengesellschaft, Germany.
160. Peterson, D. H., Murray, H. C., Eppstein, S. H., Reineke, L. M., Weintraub, A., Meister, P. D., and Leigh, H. M. (1952) Microbiological transformations of steroids .1. Introduction of oxygen at carbon-11 of progesterone, *J. Am. Chem. Soc.* **74**, 5933-5936.
161. van Beilen, J. B., Duetz, W. A., Schmid, A., and Witholt, B. (2003) Practical issues in the application of oxygenases, *Trends Biotechnol.* **21**, 170-177.

162. Ro, D.-K., Paradise, E. M., Ouellet, M., Fisher, K. J., Newman, K. L., Ndungu, J. M., Ho, K. A., Eachus, R. A., Ham, T. S., Kirby, J., Chang, M. C. Y., Withers, S. T., Shiba, Y., Sarpong, R., and Keasling, J. D. (2006) Production of the antimalarial drug precursor artemisinic acid in engineered yeast, *Nature* 440, 940-943.
163. Rabe, K. S., Gandubert, V. J., Spengler, M., Erkelenz, M., and Niemeyer, C. M. (2008) Engineering and assaying of cytochrome P450 biocatalysts, *Anal. Bioanal. Chem.* 392, 1059-1073.
164. Palmer, C. N. A., Axen, E., Hughes, V., and Wolf, C. R. (1998) The repressor protein, Bm3R1, mediates an adaptive response to toxic fatty acids in *Bacillus megaterium*, *Journal of Biological Chemistry* 273, 18109-18116.
165. Palmer, C. N. A., Causevic, M., and Wolf, C. R. (1997) Modulation of fatty acid signalling by cytochrome P-450-mediated hydroxylation, *Biochem. Soc. Trans.* 25, 1160-1165.
166. Noble, M. A., Miles, C. S., Chapman, S. K., Lysek, D. A., Mackay, A. C., Reid, G. A., Hanzlik, R. P., and Munro, A. W. (1999) Roles of key active-site residues in flavocytochrome P450 BM3, *Biochem. J.* 339, 371-379.
167. Poulos, T. L., Finzel, B. C., Gunsalus, I. C., Wagner, G. C., and Kraut, J. (1985) The 2.6-Å crystal-structure of *Pseudomonas putida* cytochrome P450, *Journal of Biological Chemistry* 260, 6122-6130.
168. Ravichandran, K. G., Boddupalli, S. S., Hasemann, C. A., Peterson, J. A., and Deisenhofer, J. (1993) Crystal-structure of hemoprotein domain of P450BM-3, a prototype for microsomal P450's, *Science* 261, 731-736.
169. Haines, D. C., Tomchick, D. R., Machius, M., and Peterson, J. A. (2001) Pivotal role of water in the mechanism of P450BM-3, *Biochemistry* 40, 13456-13465.
170. Lee, D. S., Park, S. Y., Yamane, K., Obayashi, E., Hori, H., and Shiro, Y. (2001) Structural characterization of n-butyl-isocyanide complexes of cytochromes P450nor and P450cam, *Biochemistry* 40, 2669-2677.
171. Li, H. Y., and Poulos, T. L. (1997) The structure of the cytochrome p450BM-3 haem domain complexed with the fatty acid substrate, palmitoleic acid, *Nat. Struct. Biol.* 4, 140-146.
172. Raag, R., and Poulos, T. L. (1991) Crystal-structures of cytochrome P450cam complexed with camphane, thiocamphor, and adamantane - factors controlling P450 substrate hydroxylation, *Biochemistry* 30, 2674-2684.
173. Schlichting, I., Jung, C., and Schulze, H. (1997) Crystal structure of cytochrome P-450cam complexed with the (1S)-camphor enantiomer, *FEBS Lett.* 415, 253-257.
174. Bell, S. G., Chen, X. H., Sowden, R. J., Xu, F., Williams, J. N., Wong, L. L., and Rao, Z. H. (2003) Molecular recognition in (+)-alpha-pinene oxidation by cytochrome P450(cam), *J. Am. Chem. Soc.* 125, 705-714.
175. Bell, S. G., Harford-Cross, C. F., and Wong, L. L. (2001) Engineering the CYP101 system for in vivo oxidation of unnatural substrates, *Protein Eng.* 14, 797-802.

176. Bell, S. G., Stevenson, J. A., Boyd, H. D., Campbell, S., Riddle, A. D., Orton, E. L., and Wong, L. L. (2002) Butane and propane oxidation by engineered cytochrome P450(cam), *Chemical Communications*, 490-491.
177. Carmichael, A. B., and Wong, L. L. (2001) Protein engineering of *Bacillus megaterium* CYP102 - The oxidation of polycyclic aromatic hydrocarbons, *Eur. J. Biochem.* 268, 3117-3125.
178. England, P. A., Harford-Cross, C. F., Stevenson, J. A., Rouch, D. A., and Wong, L. L. (1998) The oxidation of naphthalene and pyrene by cytochrome P450(cam), *FEBS Lett.* 424, 271-274.
179. Loida, P. J., and Sligar, S. G. (1993) Engineering cytochrome P450cam to increase the stereospecificity and coupling of aliphatic hydroxylation, *Protein Eng.* 6, 207-212.
180. Bell, S. G., Rouch, D. A., and Wong, L. L. (1997) Selective aliphatic and aromatic carbon-hydrogen bond activation catalysed by mutants of cytochrome P450(cam), *J. Mol. Catal. B-Enzym.* 3, 293-302.
181. England, P. A., Rouch, D. A., Westlake, A. C. G., Bell, S. G., Nickerson, D. P., Webberley, M., Flitsch, S. L., and Wong, L. L. (1996) Aliphatic vs aromatic C-H bond activation of phenylcyclohexane catalysed by cytochrome P450cam, *Chemical Communications*, 357-358.
182. Fowler, S. M., England, P. A., Westlake, A. C. G., Rouch, D. R., Nickerson, D. P., Blunt, C., Braybrook, D., West, S., Wong, L. L., and Flitsch, S. L. (1994) Cytochrome P450cam monooxygenase can be redesigned to catalyze the regioselective aromatic hydroxylation of diphenylmethane, *J. Chem. Soc.-Chem. Commun.*, 2761-2762.
183. Harford-Cross, C. F., Carmichael, A. B., Allan, F. K., England, P. A., Rouch, D. A., and Wong, L. L. (2000) Protein engineering of cytochrome P450(cam) (CYP101) for the oxidation of polycyclic aromatic hydrocarbons, *Protein Eng.* 13, 121-128.
184. Jones, J. P., O'Hare, E. J., and Wong, L. L. (2000) The oxidation of polychlorinated benzenes by genetically engineered cytochrome P450(cam): potential applications in bioremediation, *Chemical Communications*, 247-248.
185. Manna, S. K., and Mazumdar, S. (2010) Tuning the substrate specificity by engineering the active site of cytochrome P450cam: A rational approach, *Dalton Trans.* 39, 3115-3123.
186. Nickerson, D. P., HarfordCross, C. F., Fulcher, S. R., and Wong, L. L. (1997) The catalytic activity of cytochrome P450(cam) towards styrene oxidation is increased by site-specific mutagenesis, *FEBS Lett.* 405, 153-156.
187. Xu, F., Bell, S. G., Lednik, J., Insley, A., Rao, Z. H., and Wong, L. L. (2005) The heme monooxygenase cytochrome P450(cam) can be engineered to oxidize ethane to ethanol, *Angewandte Chemie-International Edition* 44, 4029-4032.
188. Stevenson, J. A., Westlake, A. C. G., Whittock, C., and Wong, L. L. (1996) The catalytic oxidation of linear and branched alkanes by cytochrome P450(cam), *J. Am. Chem. Soc.* 118, 12846-12847.

189. Hirakawa, H., and Nagamune, T. (2010) Molecular assembly of P450 with ferredoxin and ferredoxin reductase by fusion to PCNA, *Chembiochem* 11, 1517-1520.
190. Sabbadin, F., Hyde, R., Robin, A., Hilgarth, E. M., Delenne, M., Flitsch, S., Turner, N., Grogan, G., and Bruce, N. C. (2010) LICRED: A versatile drop-in vector for rapid generation of redox-self-sufficient cytochrome P450s, *Chembiochem* 11, 987-994.
191. Boddupalli, S. S., Oster, T., Estabrook, R. W., and Peterson, J. A. (1992) Reconstitution of the fatty-acid hydroxylation function of cytochrome P450BM3 utilizing its individual recombinant hemoprotein and flavoprotein domains, *Journal of Biological Chemistry* 267, 10375-10380.
192. Eiben, S., Kaysser, L., Maurer, S., Kuhnelt, K., Urlacher, V. B., and Schmid, R. D. (2006) Preparative use of isolated CYP102 monooxygenases - A critical appraisal, *Journal of Biotechnology* 124, 662-669.
193. Boddupalli, S. S., Pramanik, B. C., Slaughter, C. A., Estabrook, R. W., and Peterson, J. A. (1992) Fatty-acid monooxygenation by P450BM3 - product identification and proposed mechanisms for the sequential hydroxylation reactions, *Arch. Biochem. Biophys.* 292, 20-28.
194. Oliver, C. F., Modi, S., Primrose, W. U., Lian, L. Y., and Roberts, G. C. K. (1997) Engineering the substrate specificity of *Bacillus megaterium* cytochrome P-450 BM3: hydroxylation of alkyl trimethylammonium compounds, *Biochem. J.* 327, 537-544.
195. Ost, T. W. B., Miles, C. S., Murdoch, J., Cheung, Y. F., Reid, G. A., Chapman, S. K., and Munro, A. W. (2000) Rational re-design of the substrate binding site of flavocytochrome P450BM3, *FEBS Lett.* 486, 173-177.
196. Dietrich, J. A., Yoshikuni, Y., Fisher, K. J., Woolard, F. X., Ockey, D., McPhee, D. J., Renninger, N. S., Chang, M. C. Y., Baker, D., and Keasling, J. D. (2009) A Novel Semi-biosynthetic Route for Artemisinin Production Using Engineered Substrate-Promiscuous P450(BM3), *ACS Chem. Biol.* 4, 261-267.
197. Lussenburg, B. M. A., Babel, L. C., Vermeulen, N. P. E., and Commandeur, J. N. M. (2005) Evaluation of alkoxyresorufins as fluorescent substrates for cytochrome P450BM3 and site-directed mutants, *Anal. Biochem.* 341, 148-155.
198. Appel, D., Lutz-Wahl, S., Fischer, P., Schwaneberg, U., and Schmid, R. D. (2001) A P450BM-3 mutant hydroxylates alkanes, cycloalkanes, arenes and heteroarenes, *Journal of Biotechnology* 88, 167-171.
199. Li, Q. S., Ogawa, J., Schmid, R. D., and Shimizu, S. (2001) Engineering cytochrome P450BM-3 for oxidation of polycyclic aromatic hydrocarbons, *Applied and Environmental Microbiology* 67, 5735-5739.
200. Li, Q. S., Schwaneberg, U., Fischer, P., and Schmid, R. D. (2000) Directed evolution of the fatty-acid hydroxylase P450BM-3 into an indole-hydroxylating catalyst, *Chemistry-a European Journal* 6, 1531-1536.
201. Urlacher, V. B., Makhsumkhanov, A., and Schmid, R. D. (2006) Biotransformation of beta-ionone by engineered cytochrome P450BM-3, *Applied Microbiology and Biotechnology* 70, 53-59.

202. Sowden, R. J., Yasmin, S., Rees, N. H., Bell, S. G., and Wong, L. L. (2005) Biotransformation of the sesquiterpene (+)-valencene by cytochrome P450(cam) and P450(BM-3), *Org. Biomol. Chem.* 3, 57-64.
203. Graham-Lorence, S., Truan, G., Peterson, J. A., Falck, J. R., Wei, S. Z., Helvig, C., and Capdevila, J. H. (1997) An active site substitution, F87V, converts cytochrome p450 BM-3 into a regio- and stereoselective (14S,15R)-arachidonic acid epoxxygenase, *Journal of Biological Chemistry* 272, 1127-1135.
204. Sulistyaningdyah, W. T., Ogawa, J., Li, Q. S., Maeda, C., Yano, Y., Schmid, R. D., and Shimizu, S. (2005) Hydroxylation activity of P450BM-3 mutant F87V towards aromatic compounds and its application to the synthesis of hydroquinone derivatives from phenolic compounds, *Applied Microbiology and Biotechnology* 67, 556-562.
205. Weber, E., Seifert, A., Antonovici, M., Geinitz, C., Pleiss, J., and Urlacher, V. B. (2011) Screening of a minimal enriched P450 BM3 mutant library for hydroxylation of cyclic and acyclic alkanes, *Chemical Communications* 47, 944-946.
206. Farinas, E. T., Schwaneberg, U., Glieder, A., and Arnold, F. H. (2001) Directed evolution of a cytochrome P450 monooxygenase for alkane oxidation, *Advanced Synthesis & Catalysis* 343, 601-606.
207. Glieder, A., Farinas, E. T., and Arnold, F. H. (2002) Laboratory evolution of a soluble, self-sufficient, highly active alkane hydroxylase, *Nat. Biotechnol.* 20, 1135-1139.
208. Peters, M. W., Meinhold, P., Glieder, A., and Arnold, F. H. (2003) Regio- and enantioselective alkane hydroxylation with engineered cytochromes P450 BM-3, *J. Am. Chem. Soc.* 125, 13442-13450.
209. Meinhold, P., Peters, M. W., Chen, M. M. Y., Takahashi, K., and Arnold, F. H. (2005) Direct conversion of ethane to ethanol by engineered cytochrome P450BM3, *Chembiochem* 6, 1765-1768.
210. Meinhold, P., Peters, M. W., Hartwick, A., Hernandez, A. R., and Arnold, F. H. (2006) Engineering cytochrome P450BM3 for terminal alkane hydroxylation, *Advanced Synthesis & Catalysis* 348, 763-772.
211. Graham-Lorence, S., Truan, G., Peterson, J. A., Falck, J. R., Wei, S. Z., Helvig, C., and Capdevila, J. H. (1997) An active site substitution, F87V, converts cytochrome p450 BM-3 into a regio- and stereoselective (14S,15R)-arachidonic acid epoxxygenase, *Journal of Biological Chemistry* 272, 1127-1135.
212. Kubo, T., Peters, M. W., Meinhold, P., and Arnold, F. H. (2006) Enantioselective epoxidation of terminal alkenes to (R)- and (S)-epoxides by engineered cytochromes P450BM-3, *Chemistry-a European Journal* 12, 1216-1220.