Redirecting P450 Ferryl-Oxo Intermediates from Oxygenation to Decarboxylation

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REDIRECTING P450 FERRYL-OXO INTERMEDIATES FROM OXYGENATION TO DECARBOXYLATION

by

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DEDICATION

I would like to dedicate this work to my family, particularly to my parents and my maternal grandparents. They have always emphasized the importance scholarship to me and I only got this far because of them.

Additionally, I would like to thank all my teachers from over the years, in particular Mrs. Seaman-Huynh, Mrs. Brown, Mrs. Bockman, Dr. Wagner.
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I should also thank my undergraduate minions, Megan Mitchell and Julia Bian. You guys were awesome and a tremendous help.
ABSTRACT

A recently discovered cytochrome P450 has garnered much interest for its ability to oxidatively decarboxylate n length fatty acids to n-1 terminal olefins using hydrogen peroxide as an oxidant. This enzyme (P450 OleT) is mechanistically peculiar, as it seemingly abrogates the oxygen insertion chemistry that typifies the P450 superfamily. In this work we explore the origin of this deviant catalysis.

Using stopped-flow and transient kinetic methodologies the ferryl-oxo pication radical and ferryl-hydroxo intermediates, known as compound-I (Cpd-I) and compound-II (Cpd-II) respectively, were isolated in high yield. This is the first time either species has been observed in a substrate bound P450 using a native oxidant. The accumulation of these intermediates has enabled direct observation of the decarboxylation mechanism of P450 OleT. We have found that decarboxylation is initiated with substrate hydrogen atom abstraction by Cpd-I, similar to oxygen insertion chemistries. The branch point in the reaction coordinate occurs at Cpd-II where, through a combination of substrate positioning and stabilization of ferryl-hydroxo by active site hydrogen bonding, oxygen rebound is inhibited allowing for a proton coupled one electron oxidation of the substrate to occur. This one electron oxidation liberates the carboxylate as CO₂ through either a carbocation or biradical mechanism. Additionally, the isolation of Cpd-I in the absence of a bound substrate has allowed for an examination of how the peptide
backbone is protected from a highly oxidizing intermediate, crucial for preventing non-productive self-oxidation.
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CHAPTER 1
ISOLATION OF P450 COMPOUND-I IN FATTY ACID DECARBOXYLATION\textsuperscript{1}

Abstract

OleT, a cytochrome P450, catalyzes the conversion of fatty acids to terminal alkenes using hydrogen peroxide as a co-substrate. Analytical studies with an eicosanoic acid substrate show that the enzyme has exclusive chemoselectivity for alkene formation and that carbon dioxide is the one carbon co-product of the reaction. The addition of hydrogen peroxide to a deuterated substrate-enzyme E-S complex results in the transient formation of an iron (IV) oxo π-cation radical (Compound I) intermediate which is spectroscopically indistinguishable from those that perform oxygen insertion chemistries. A kinetic isotope effect for Compound I decay suggests that it abstracts a substrate hydrogen atom to initiate fatty acid decarboxylation. Together, these results indicate that the initial mechanism for alkene formation, which does not result from oxygen rebound, is similar to that widely suggested for P450 monooxygenation chemistries.

\textsuperscript{1}Grant, J. L., Hseih C. H., Makris T. M., Decarboxylation of Fatty Acids to Terminal Alkenes by Cytochrome P450 Compound I, J. Am. Chem. Soc., 2015, DOI: 10.1021/jacs.5b01965
Introduction

The generation of hydrocarbons from fatty acid metabolites has received intensive interest for the sustainable production of fuels that are compatible within the existing energy infrastructure. 1-2 Several biosynthetic strategies for alkane and alkene production have been recently identified. 3-6 A common mechanistic feature of each pathway is the cleavage of the terminal carbon from a fatty acid (or aldehyde) 7 of chain length n to produce an n-1 alkane (or alkene) respectively. Intriguingly, the enzymes involved in this carbon scission reaction each utilize an iron containing cofactor, non-heme mono- 6 or di-nuclear 8-9 iron, or cytochrome P450 4-5, that typically activate O2 or H2O2 for substrate oxygenations. The elucidation of the mechanism of these carbon-carbon scission reactions, often cryptic, may offer the opportunity for the efficient production of liquid transportation fuels in a recombinant organism.

OleT, a cytochrome P450 from Jeotgalicoccus bacteria, metabolizes Cn chain length fatty acids to produce Cn-1 alkenes, utilizing hydrogen peroxide (H2O2) as a co-substrate in the reaction. 4 The overall reaction (Fig. 1.2.1A) involves the net loss of a hydride (from the fatty acid beta carbon (Cβ)) and production of a one carbon co-product, which has yet to be identified. Notably, cytochrome P450 enzymes are not known to catalyze hydride abstraction nor decarboxylation reactions. The canonical mechanism for the P450 catalyzed monooxygenation of unactivated hydrocarbons is depicted in figure 1.1B for comparison. The prototypical reaction coordinate involves abstraction of a substrate hydrogen atom by a highly reactive iron (IV)-oxo heme π-cation radical intermediate termed
Compound I, recently characterized by Green and colleagues. Subsequent recombination with a substrate radical, in a process termed oxygen rebound, produces an alcohol and regenerates the ferric resting state of the enzyme.

Figure 1.1 Comparison of OleTJE alkene production and prototypical P450 hydroxylation reactions.

The recent X-ray crystal structure of OleT bound to an eicosanoic acid substrate has confirmed the remarkable similarity of its active-site and substrate binding mode to P450 peroxxygenases BSβ and SPα. BSβ and SPα do not competently produce olefin products, but instead hydroxylate fatty acids at the Cα or Cβ positions. While providing a rationale for the enzyme’s ability to efficiently utilize hydrogen peroxide, in which the substrate carboxylate serves a general acid
that is obligatory for the heterolytic cleavage of H$_2$O$_2$, it does not immediately clarify the origin of its capacity for C-C scission.

In order to elucidate OleT’s divergence from P450 monooxygenation chemistry, we have characterized its reaction with eicosanoic acid, a chain length which likely approximates that of the native substrate. Analytical studies have determined an exclusive chemoselectivity of the enzyme for alkene formation, and that the one-carbon co-product of this reaction is carbon dioxide. Transient kinetic studies show that the decarboxylation reaction is initiated by Compound I, mechanistically linking OleT$_{JE}$ catalysis to other P450 oxidations.

**Methods**

**Reagents**

All buffers used in this study were purchased from Research Products International. Eicosanoic acid (C$_{20}$H$_{40}$O$_2$) was purchased from Sigma. The terminal alkene standards, 1-nonadecene and 1-hexadecene, were purchased from TCI Chemicals. Perdeuterated eicosanoic acid (C$_{20}$D$_{40}$O$_2$H) was from CDN isotopes.

**Cloning and heterologous expression of OleT**

An *Escherichia coli* codon-optimized gene for OleT from *Jeotgalicoccus* sp. ATCC 8456 (NP_895059) was synthesized by DNA2.0 (Menlo Park, CA). In order to optimize heterologous overexpression and purification, the gene was amplified by PCR using the following primers with restriction sites underlined:

5’-CGATGTGTCATATGGCAACATTAAAAAGAGATAA
5’-CGATGTGAATTCCGTACGGTCAACCCTC
Following restriction digestion with NdeI and EcoRI, the constructs were ligated into similarly digested pet21b to produce the C-terminal hexahistidine tagged OleT_{JE}. The resulting constructs were verified by sequencing at Engencore (Columbia, SC).

Heterologous expression was performed in *Escherichia coli* BL21 (DE3) containing the pG-Tf2 plasmid (Takara), which overexpresses the GroES and GroEL chaperones. Cells were grown in Luria broth containing 50 mg/L ampicillin and 20 mg/L chloramphenicol at 37°C until reaching an OD of 0.4 at 600 nm. The cultures were subsequently cooled to 20°C and induced at an OD ~1 with 10 μg/L tetracycline (for chaperone induction), 50 μM IPTG (for OleT_{JE} induction), and 25 μM δ-aminolevulinic acid (for heme production). Cells were grown for an additional 15 hours after induction, harvested by centrifugation, and stored at −70°C until further use.

**Purification of OleT**

Frozen cells from 12 x 1 L cultures were resuspended in 250 mL of 50 mM NaH$_2$PO$_4$, 300 mM NaCl, 10 mM imidazole, pH 8.0 (buffer A). Cells were lysed using a Branson Sonifier and centrifuged at 37,500 g for 45 min. The supernatant was then loaded by gravity onto a 20 mL nickel nitrilotriacetic acid (NTA) column (GE healthcare) equilibrated in buffer A. The column was subsequently washed with 200 mL of buffer A containing 20 mM imidazole, followed by 100 mL of a low salt buffer containing 50 mM NaH$_2$PO$_4$ pH 8.0 (buffer B). The protein was eluted with buffer B containing 500 mM imidazole. P450 containing fractions were pooled
and loaded by gravity onto a 50 mL DEAE Sepharose fast flow column (GE Healthcare) equilibrated with buffer B. The column was washed with 5 column volumes of buffer B containing 100 mM NaCl and eluted using a linear gradient from 100 to 500 mM NaCl over 10 column volumes. Fractions containing pure OleTJE, judged by SDS-PAGE and absorbance ratios (Abs417/Abs280), were pooled and concentrated to 5-20 mg/mL using Amicon ultra 30 kDa centrifugal filters, flash frozen, and stored at -70°C until further use. Figure 1.2 shows an SDS-PAGE gel of the final purified protein. Protein concentrations were determined using a calculated extinction coefficient $\epsilon_{417} = 120$ mM$^{-1}$ cm$^{-1}$ following complete substrate removal.

**Figure 1.2** SDS-Page gel of purified OleT.

**Preparation of Substrate Free OleT**

Purified OleT was treated with a five-fold excess of hydrogen peroxide. The protein was subsequently desalted on a PD-10 column (GE Healthcare) equilibrated with 200 mM KH$_2$PO$_4$ pH 7.6.
Preparation of stoichiometric enzyme-substrate complexes

A ten-fold molar excess of eicosanoic acid (prepared as a 25 mM stock in 30% Triton X-100, 70% Ethanol) was added to the substrate free enzyme and incubated for several hours at 4°C. Precipitated fatty acid was removed by centrifugation at 16,000 rpm for 1 minute in a microcentrifuge. Excess, unbound substrate was removed by desalting as described above.

Single-Turnover Alkene Product Determination

A three-fold molar excess of H$_2$O$_2$ was added to the enzyme-eicosanoic acid E-S complex (1 mL, 20 μM). 1-hexadecene (100 nmol) was subsequently added as an internal standard and the reaction mixture was quenched with 1M NaOH and the extracted with 3 mL of chloroform. The organic phase was removed and concentrated under a stream of N$_2$. Gas chromatography mass spectrometry (GC-MS) was performed at the University of South Carolina Mass Spectrometry facility with a Hewlett Packard HP5890 GC and a 30 meter Rbx-5 column. Mass spectra were recorded on a Waters VG 705 magnetic sector mass spectrometer using 70 eV electron impact energy.

Optical Spectroscopy

Optical spectra were obtained using an HP 8453 spectrophotometer. For substrate titration experiments, 4 μM OleT$_{JE}$ in 100 mM potassium phosphate (KH$_2$PO$_4$) pH 7.6 was titrated with sequential additions of a 10 mM eicosanoic acid stock dissolved in 70% ethanol: 30% Triton X-100 (v:v) with a Hamilton gas-tight syringe. The amount of ethanol added never exceeded 5% of the total volume.
Fitting of the substrate induced absorption changes, $A_{\text{obs}}$, (at 417 nm and 392 nm) were done with Origin software using a quadratic function (Morrison equation) (1.1) for tight binding ligands where $A_{\text{max}}$ is the maximal absorbance change at ligand saturation, $S$ is the concentration of eicosanoic acid, $E_i$ is the concentration of OleTJE, and $K_d$ is the dissociation constant:

$$1.1 \quad A_{\text{obs}} = \left(\frac{A_{\text{max}}}{2E_t}\right)(S + E_t + K_d) - \left(((S + E_t + K_d)^2 - (4SE_t))^{0.5}\right)$$

**Stopped-Flow Absorption**

Stopped-flow absorption experiments were performed on an Applied Photophysics Ltd. SX20 stopped-flow spectrophotometer. Eicosanoic acid (protiated or perdeuterated) bound OleTJE (~20 μM) was rapidly mixed with 5 mM H$_2$O$_2$ in 100 mM KH$_2$PO$_4$ (pH 7.6) at 5°C. Single wavelength traces were taken using a photomultiplier tube and full spectrum data were collected by photodiode array. The single wavelength data at 370 nm were fit to summed exponential expressions using Pro Data Viewer version 4.2.18.

**Results**

Partial high-spin content of the enzyme following purification indicated the presence of adventitiously bound compounds in the active-site. These contaminating small molecules, most likely *E. coli* derived fatty acids, were removed by treating the enzyme with H$_2$O$_2$ to initiate turnover and subsequent
desalting. This treatment resulted in a complete conversion of the enzyme to the low-spin state (Fig. 1.3), and restored its ability to bind exogenously added eicosanoic acid. The titration of substrate to the peroxide treated enzyme indicated a near complete conversion (~90%) of the enzyme to the ferric high-spin state upon saturation, with a measured dissociation constant $K_d \sim 0.8 \, \mu\text{M}$ (Fig. 1.4). As a result of this high affinity, a stoichiometric enzyme-substrate (E-S) complex could be generated by poising the enzyme at concentrations well above (typically $\geq 10 \, \mu\text{M}$) the observed $K_d$.

**Figure 1.3** Optical spectrum of the enzyme in “as-purified” (dashed), hydrogen peroxide treated (solid black), and eicosanoic acid bound (solid gray) forms.
Figure 1.4  Binding of eicosanoic acid to peroxide treated OleT_{JE}. Aliquots of eicosanoic acid were added to substrate free OleT_{JE} (inset, black trace) to produce the high spin enzyme (inset, blue trace). The spectral changes at 417 and 392 nm upon binding of eicosanoic acid were fit to a Morrison expression for tight binding ligands for K_{d} determination as described in Methods.

Previous multiple turnover studies performed in vitro and in vivo have established that the reaction of OleT_{JE} with eicosanoic acid and H_{2}O_{2} generates nonadecene.\textsuperscript{4,12} Single turnover studies were utilized to directly evaluate the enzyme’s chemoselectivity for the alkene forming reaction. An E-S complex was mixed with a 3 fold molar excess of H_{2}O_{2} and the hydrocarbon products of the reaction were extracted. Gas chromatography mass spectroscopy (GC-MS) (Fig 1.5) of the organic phase confirmed formation of nonadecene at a yield of
Figure 1.5. Identification of the Alkene Product from the OleTJE: Eicosanoic Acid Single Turnover Reaction. GC chromatogram and MS fragmentation pattern (inset) of the nonadecene product formed from the reaction of a 20 μM OleTJE E-S complex and 60 μM H₂O₂. The yield of deuterated nonadecene produced was determined through peak integration and comparison to an internal hexadecene standard.

0.95 ± 0.1 equivalents of alkene produced per OleTJE, indicating an exclusive preference for alkene formation. The reaction of an E-S complex, prepared with a perdeuterated C₂₀ substrate (CD₃(CD₂)₁₈COOH), produced deuterated nonadecene with comparable yields (Fig. 1.6). No appreciable metabolic switching occurs upon substrate isotopic substitution, substantiating the use of deuterated eicosanoic acid for the mechanistic studies described below.
**Figure 1.6** GC chromatogram and MS fragmentation pattern (inset) of the deuterated nonadecene product formed from the reaction of a 20 μM OleT,JE E-S complex, prepared with perdeuterated eicosanoic acid, and 60 μM H₂O₂. The yield of deuterated nonadecene produced was determined through peak integration and comparison to an internal hexadecene standard. The yields of deuterated alkene produced were similar (within 10%) to those from reactions of the E-S complex prepared with protiated eicosanoic acid.

The oxidant responsible for initiating the OleT,JE decarboxylation reaction is currently unknown. Based upon the fact that the substrate is a fatty acid (rather than aldehyde), and absence of formate production, a mechanism involving nucleophilic attack by a ferric peroxide intermediate, such as that postulated for P450 deformylation and alkane synthesis by aldehyde deformylating
oxygenase, would seem highly unlikely. Rather, the loss of hydrogen from a relatively unactivated Cβ position, and structural similarity of OleTJE to hydroxylases that presumably utilize a Compound I oxidant, suggest that a high-valent intermediate may be involved. Having established a competent single-turnover system with protiated and deuterated fatty acids, we tested whether such an intermediate could be isolated. A 20 μM OleTJE-perdeuterated eicosanoic acid (E-C20D) ternary complex was rapidly mixed with excess H2O2 at 5°C in stopped flow absorption studies. Within 10 msec., the high-spin E-S complex (λmax at 392 nm) had completely decayed, and a new intermediate with decreased absorptivity, a blue-shifted Soret maximum with a Soret maximum of 370 nm, and an additional absorption band at 690 nm, had appeared (Figure 2A red trace, and inset). The absorption characteristics of this species are diagnostic of an iron (IV)-oxo pi cation radical intermediate (Compound I) and are nearly identical to those observed in rapid mixing studies of a thermostable P450(CYP119) with mCPBA and similarly prepared species in other thiolate-ligated heme enzymes (ex. Aae-APO, CPO). Spectral deconvolution procedures, and comparison to these other Compound I species, indicated that this intermediate had accumulated to a high level (> 70 %) at 15 msec. Within 1 second, the intermediate completely decayed to a species that is indistinguishable from the ferric-low spin substrate free form of the enzyme (Figure 2A, blue trace). The time course for its decay at 370 nm (Figure 2c) required two summed exponentials for adequate fitting, suggesting a complex decomposition process that may involve multiple steps. The faster of these two phases, which comprises ~ 90 % of the spectral amplitude at 370 nm,
Figure 1.7 Spectrum of OleT:perdeuterated eicosanoic acid complex reacting with H$_2$O$_2$. The initial spectrum is in magenta and the end spectrum is in blue.

indicates a decay rate of 80 sec$^{-1}$. This decay rate of OleT Compound I (which we designate hereafter as Ole-I) is invariant to H$_2$O$_2$ across high concentrations consistent with the fact that it is formed as a result of O-O heterolysis, an irreversible process.

Results from rapid mixing studies of an E-S complex, prepared with a protiated substrate, and H$_2$O$_2$ are shown in Figure 2b for comparison. Rapid decay of the E-S complex was again observed within 10 msec (green trace).
However, no appreciable accumulation of Ole-I could be detected prior to formation to the ferric low-spin state, suggesting that its previous stabilization was solely attributable to a $^2$H kinetic isotope effect (KIE). This apparent KIE for Ole-I decay strongly favors a mechanism in which alkene formation is initiated by hydrogen abstraction, most likely originating from the Cβ position. This rules out a previous proposal that OleTJE’s atypical reaction may be attributed to initial abstraction of an electron from the fatty acid carboxylate by Compound I.

The kinetics studies and observed reaction products in this study support a proposed catalytic mechanism for alkene synthesis that is shown in Figure 1.8. The mechanistic strategy for carbon-carbon bond scission appears to be identical, in its first steps, to the bulk of P450 oxidation reactions by Compound I. Based on the widely accepted mechanism for P450 hydroxylation of inert hydrocarbons, and the demonstrated incapacity of iron-oxo porphyrin pi-cation radical intermediates to perform hydride transfer in model systems, Ole-I most likely abstracts a substrate hydrogen atom. This would result in the formation of a substrate radical and the Fe (IV)-hydroxide Compound II. The subsequent steps for OleTJE decarboxylation necessitate a divergence from the monooxygenation reaction coordinate, particularly as oxygen rebound is abrogated. One logical route for alkene formation could involve single electron transfer, to Compound II or another oxidant, to produce an unstable substrate carbocation. Subsequent loss of the CO$_2$ leaving group would generate the n-1 alkene. Similar mechanisms involving the generation of substrate carbocations have been invoked to rationalize the ability for some P450s to catalyze desaturation reactions, generate cationic
rearrangement minor products from radical clock substrates, and promote C-C bond cleavage during the third step of androgen formation by P450 aromatase.

Figure 1.8 Proposed catalytic cycle for OleT alkene formation based on identification of the carbon dioxide co-product and stopped-flow absorption spectroscopy.

The apparent conservation of decarboxylase and hydroxylase mechanisms hints to an elegant adaptation that enables OleTJE to efficiently sidestep the monooxygenation reaction coordinate. Does the inability to finalize oxygen rebound stem from an alteration in Ole-I structure or reactivity? It is perhaps noteworthy in this regard that Ole-I (unlike CYP119-I or AaeAPO-I) can be generated in high yields in a reaction with a pre-bound deuterated substrate and H₂O₂, suggesting that it may be more sluggish in its hydrogen abstraction proficiency than hydroxylating Compound I species. Unfortunately, this difference in how Ole-I is prepared undermines a pairwise evaluation of its reactivity to other
metal-oxo intermediates, particularly as pseudo first-order decay rate constants with exogenously added substrates cannot be determined. Assuming that the formation rate of Ole-I is insensitive to fatty acid isotopic substitution, and that no traces of the intermediate can be observed at 10 msec at 690 nm, we place a lower estimate for its reaction rate with a protiated substrate at $k \sim 300 \text{ sec}^{-1}$. Semi-classical KIE limits ($k_{\text{H}}/k_{\text{D}} \sim 7$) may position this value even higher. In either case, this estimated rate is within the range projected by Green and colleagues $^{10}$ for the reaction of CYP119-I and a pre-bound substrate with similar bond dissociation energy, suggesting that Ole-I is similarly reactive. Instead, the capacity for Ole$^{\text{TE}}$ to produce minor aliphatic hydroxylated products $^{4,26}$ in reactions with shorter chain length substrates suggests that there may be additional structural factors that are important in steering the enzyme towards alkene production. An evaluation of each of these potential contributions is currently under investigation.

The highly reactive nature of P450-I has limited a direct interrogation of its chemical reactivity to an extremely small subset of substrates. Here, stopped flow and analytical studies provide evidence that such an intermediate is formed, and can catalyze a decarboxylation reaction that does not result from oxygen insertion.

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8. Aukema, K. G.; Makris, T. M.; Stoian, S. A.; Richman, J. E.; Munck, E.; Lipscomb, J. D.; Wackett, L. P., Cyanobacterial Aldehyde Deformylase


CHAPTER 2
REACTIVITY OF OLET COMPOUND-I AND ISOLATION OF COMPOUND-II¹

Abstract

OleT is a cytochrome P450 which catalyzes the hydrogen peroxide dependent metabolism of Cₙ chain length fatty acids to synthesize Cₙ₋₁ 1-alkenes. The decarboxylation reaction provides a route for the production of drop-in hydrocarbon fuels from a renewable and abundant natural resource. It is also a highly unusual transformation for a P450, which typically utilize a Fe⁴⁺-oxo intermediate known as Compound I for the insertion of oxygen into organic substrates. OleT, previously shown to form Compound I, catalyzes a different reaction. A large substrate kinetic isotope effect (≥8) for OleT Compound I decay confirms that, like monooxygenation, alkene formation is initiated by substrate C-H bond abstraction. Rather than finalizing the reaction through rapid oxygen rebound, alkene synthesis proceeds through the formation of a reaction cycle intermediate with kinetics, optical properties, and reactivity indicative of an Fe⁴⁺-OH species, Compound II. The direct observation of this intermediate, normally

fleeting in hydroxylases, provides a rationale for the carbon-carbon scission reaction catalyzed by OleT.

**Introduction**

Cytochrome P450 (CYP) enzymes catalyze an extraordinary breadth of physiologically important oxidations for xenobiotic detoxification and specialized biosynthetic pathways \(^1\)-\(^2\). The metabolic diversity of CYP enzymes originates from a sophisticated interplay of substrate molecular recognition with precise tuning of metal oxygen species formed at the enzyme active-site. CYPs utilize a thiolate ligated heme-iron cofactor to activate molecular oxygen and produce short-lived ferric-superoxo \(^3\)-\(^5\), ferric-(hydro)peroxo \(^6\)-\(^8\) and ferryl \(^9\)-\(^10\) intermediates. Coordinated efforts over several decades have resulted in isolation of each intermediate, including recent characterization of the principal oxidant thought to be responsible for the vast majority of P450 oxidations, the Fe\(^{4+}\)-oxo pi-cation radical species commonly referred to as Compound I (or P450-I) \(^10\).

The archetypal P450 hydroxylation involves C-H bond abstraction by P450-I and ensuing rapid oxygen insertion through a recombination process termed oxygen rebound \(^11\)-\(^12\). The hydrogen abstraction/rebound mechanism describes the strategy employed for most P450 transformations, and is thought to describe catalysis by numerous metal-dependent oxygenases and synthetic bio-inspired metal-oxo complexes (ex. \(^13\)-\(^17\)). Despite the ubiquity of this mechanism, in some metalloenzymes, a metal-oxo species is formed that is not ultimately destined for incorporation into a substrate. Some characterized examples include the non-heme di-nuclear iron ribonucleotide reductase (RNR R2) \(^18\)-\(^19\) and mono-nuclear
iron halogenase SyrB2 \textsuperscript{20-22}. The mechanisms for RNR and SyrB2 highlight the importance of quaternary structural elements, an extensive 35 Angstrom (Å) proton coupled electron transfer pathway in RNR \textsuperscript{23}, and extremely subtle (sub Å) substrate positional tuning (SyrB2) \textsuperscript{21-22}, to enable efficient circumvention from the monooxygenation reaction coordinate.

P450-I has also been linked to a number of important transformations in which an oxygen rebound step is not readily observed, including the desaturation of pharmaceuticals by liver P450s to afford hepatotoxic metabolites \textsuperscript{24} and the C-C lyase activity of human P450 aromatase that is critical for estrogen biosynthesis \textsuperscript{25}, among others \textsuperscript{26-27}. However, unambiguous determination of the oxidant responsible and operant mechanism for these aberrant P450 transformations has met significant challenges. Compound I has not been isolatable from an O\textsubscript{2} dependent reaction due to rate limiting electron transfer processes, and the products resulting from these atypical reactions can sometimes represent only minor channels of the enzyme/substrate(s) involved. Attempts to resolve the mechanisms for these reactions have therefore largely relied on indirect methods: isotopic labeling strategies (oxygen incorporation \textsuperscript{25,28}, steady state substrate \textsuperscript{24,27} and solvent \textsuperscript{29} kinetic isotope effects (KIE)), inference from the spectroscopic characterization of preceding reaction cycle intermediates \textsuperscript{30}, or computational methods \textsuperscript{31-32}. Although P450-I species have been widely hypothesized to carry out many reactions in addition to monooxygenation, direct visualization has remained elusive due to its fleeting nature.
OleT is a recently discovered bacterial P450 that catalyzes an unusual carbon-carbon scission reaction, converting a Cn chain length fatty acid to a C_{n-1} 1-alkene\textsuperscript{33-34} and carbon dioxide co-product\textsuperscript{35} (Fig. 2.1). In addition to the peculiar chemical nature of this reaction, OleT catalysis has garnered significant biotechnological interest as it involves the conversion of a bio-available and abundant feedstock into a petrochemical and valuable synthetic precursor\textsuperscript{36-38}. OleT differs from most P450s in two fundamental aspects. Belonging to the CYP152 family\textsuperscript{39-40}, catalysis is efficiently initiated by hydrogen peroxide, rather than O$_2$ and reducing equivalents delivered through a redox chain. Moreover, single\textsuperscript{35} and multiple turnover\textsuperscript{36,38} studies of what is generally believed to approximate the chain length of the physiological substrate, eicosanoic acid (EA), have indicated that oxidative decarboxylation, affording nonadecene, is the largely dominant reaction route\textsuperscript{34}. Combined $^{13}$C substrate and H$_2^{18}$O$_2$ isotopic labeling by our laboratory has shown that cleavage of the terminal fatty acid carboxylate...
does not lead to recovery of $^{18}$O in the $^{13}$CO$_2$ product, ruling out a multi-step oxygenolytic mechanism involving successive hydroxylations.

Benefitting from the high chemoselectivity of OleT and a facile means to rapidly trigger catalysis, we recently reported that a highly accumulating Compound I intermediate (Ole-I) could be isolated in reactions of OleT using the native H$_2$O$_2$ terminal oxidant and a bound deuterated substrate analog. The observation of a Compound I species in OleT, optically indistinguishable from those that promote hydroxylations, intimated that the same P450 intermediate can be re-tuned to catalyze a fundamentally different reaction. In this work, we directly demonstrate that P450 Compound I species can promote reactions that do not involve oxygen rebound. A mechanistic basis for the functional divergence of OleT is provided by identification of the subsequent intermediate in the decarboxylation reaction sequence. Single turnover kinetic studies show that alkene formation is initiated by substrate C-H bond abstraction by Ole-I, forming an Fe$^{4+}$-OH species, Compound II. The remarkable stability of this intermediate provides direct insight into how metal-oxo reprogramming is achieved by P450 cytochromes.

Methods

Reagents

All microbiology reagents, yeast extract, tryptone, potassium phosphate, antibiotics, were purchased from Research Products International (RPI). Eicosanoic acid, phenol, 4-chlorophenol, 4-methoxyphenol, 4-methylphenol, and 30 % w/v hydrogen peroxide were purchased from Sigma Aldrich. Perdeuterated
Eicosanoic acid (99.5 atom % D) was purchased from CDN isotopes. 3-chlorophenol was purchased from Acros Organics.

**Heterologous Expression and Purification of OleT**

The cloning, heterologous expression, purification and removal of adventitiously bound fatty acids from OleT are described in 35.

**Stopped flow Methods**

Stopped flow experiments were performed using an Applied Photophysics Ltd. (APP) SX.20 stopped-flow spectrophotometer with photomultiplier tube (PMT) or photodiode array (PDA) detection as indicated. Eicosanoic acid bound OleT (E-S) was prepared by incubation of H2O2 pretreated and desalted substrate free enzyme (typically ~ 10 – 40 mM) in 200 mM K2HPO4 pH 7.4 with a three-fold molar excess of eicosanoic acid (H39 or D39-EA) for 15 hours at 4°C. Eicosanoic acid was prepared as a 10 mM stock in 70% ethanol:30% Triton X-100 (v/v). After incubation, undissolved fatty acid was removed by centrifugation for 10 minutes at 6,000 rpm before loading into a stopped flow syringe. The enzyme was mixed at 4 °C with H2O2, similarly prepared in a 200 mM K2HPO4 pH 7.4 buffer.

**Fitting of Transient Kinetic Reactions**

Single wavelength time courses at 370 nm and 440 nm were fit to the following two summed exponential equation where At,obs is the observed
absorbance, $1/t_i$ is the reciprocal relaxation time (RRT, s$^{-1}$), $a_i$ is the amplitude of phase $i$, $t$ is time (s) and $A_\infty$ is the final absorbance.

$$2.1 A_{t,obs} = A_\infty + \sum_{i=1}^{n} a_i \ e^{-t/t_i}$$

The two phases observed at these wavelengths indicate that there are at least two steps in the reaction. The decay time course of Ole-I at 690 nm was adequately fit to a single exponential expression. Representative fits and residuals obtained from these procedures are provided in Fig. S1. The amplitudes of these phases, RRTs, and errors with multiple preparations of the enzyme are provided in Table 2.1.

**Peroxide Dependence of Int-2 formation**

The fast phase associated with intermediate 2 (Int2) formation at 440 nm was determined using the methods described above. For experiments using D39-EA, the hyperbolic dependence of the observed RRT on H2O2 was fit using the following kinetic model, where Fe$^{3+}$(HS) represents the ferric high-spin state of the enzyme and the other species have their typical designations.

$$2.2 Fe^{3+}(HS) + H_2O_2 \rightleftharpoons Compound \ I \rightleftharpoons Int\ 2$$

The RRT ($1/\tau_{obs}$) versus [H2O2] plot was fit using the following hyperbolic expression where the apparent $k_D^{H_2O_2}$ is provided by $\left(\frac{k_{-1}}{k_1}\right)$.

$$2.3 \frac{1}{\tau_{obs}} = \frac{k_2[H_2O_2]}{\left(\frac{k_{-1}}{k_1}\right)+[H_2O_2]} + k_{-2}$$
Non-linear fitting indicated that $k_2 = 0$, indicating an irreversible process. In reactions with a H39-EA, the linear dependence of the fast RRT versus $[H_2O_2]$ demonstrated a clear change in at least one of the rate constants. It is not anticipated that steps leading to Compound I formation would be substrate isotopically sensitive, while its decay rate constant ($k_2$) is accelerated. In the linear plots of RRT versus $[H_2O_2]$, the rate constant for the $H_2O_2$ association step is provided by the slope of the plot ($k_1$), and the off rate ($k_{-1}$) is provided by the intercept. Similar methods were used to analyze the decay rate of Ole-I at 370 nm for the D39-EA reaction.

**Preparation of Fe$^{3+}$-OH OleT**

The spectrum of the OleT ferric-hydroxide was obtained by rapid mixing of the 20 µM substrate free enzyme, prepared in 25mM K$_2$HPO$_4$ pH 7.4, with 250 mM K$_2$HPO$_4$ and 250 mM arginine pH 12.0. The species is stable for several minutes at 4 °C.

**Global analysis and Singular Value Decomposition (SVD)**

Singular-value decomposition of photodiode array data was performed using Pro-KIV global analysis software (Applied Photophysics). Initial SVD fitting for both substrates showed three non-zero singular values, indicating three transitions between four spectrally distinguishable species. The following kinetic model was used where $k_{1'}$ represents a pseudo first-order rate constant at $H_2O_2$ concentrations in large excess to the enzyme used.

\[
2.4 \text{Fe}^{3+}(\text{HS}) + H_2O_2 \xrightarrow{k_{1'}} \text{Compound I} \xrightarrow{k_2} \text{Int2} \xrightarrow{k_3} \text{Fe}^{3+}(\text{LS})
\]
Authentic Fe$^{3+}$(HS) and Fe$^{3+}$(LS) spectra are readily produced without rapid mixing, and were input into Pro-KIV to improve the accuracy of resolving intermediate spectra. The initial components of the system at $t = 0$ were determined by mixing an E-S complex against buffer with no H2O2. The sample typically comprised ~80-90% Fe$^{3+}$(HS) and a minor contribution from the Fe$^{3+}$(LS) species. Initial rates for the kinetic model were input from those determined by single wavelength kinetics. The final rate constants determined by global analysis showed good agreement with these values.

**Derivation of the Int-2 Spectrum by Linear Combination Methods**

The kinetic model was simulated using the rates for $k1'$, $k2$, and $k3$ determined by the methods described above. From the speciation plots shown in Fig. S4, a time regime ($t$) when the reaction only consisted of Int-2 and Fe3+(LS) was readily identified ($t \geq 20$ and 80 ms, for H$_{39}$-EA and D$_{39}$-EA respectively). From data at several defined time points, a weighted Fe$^{3+}$(LS) spectrum, taken from the endpoint of the reaction, was subtracted to produce a calculated spectrum of Int-2.

**Double mixing reactivity studies**

Double mixing studies were performed using the stopped flow configured in sequential mixing mode. A 40 µM E-S complex was mixed 1:1 (v/v) with 2 mM H$_2$O$_2$, aged for 20 ms, and subsequently mixed 1:1 with phenolic derivatives. Phenol stocks were buffered in 200 mM K$_2$HPO$_4$. The final pH of phenol stocks was adjusted to 7.4.
Results

Alkene Formation Proceeds by Fatty Acid C-H Abstraction by Compound I

Stopped flow absorption studies have shown that first detectable intermediate in the single turnover reaction of perdeuterated eicosanoic acid (D_{39}-EA) bound high-spin ferric OleT and H_{2}O_{2} is Ole-I, which has absorption maxima at 370 and 690 nm, as reported for other thiolate-ligated Compound I species. Ole-I is a chemically competent species as its decay rate constant, determined directly below, exceeds the turnover number of OleT (~0.2 s^{-1} at 25^\circ C). Single-turnover reactions of EA-OleT and H_{2}O_{2} produce nearly one equivalent of alkene per enzyme. Fitting the time course for Ole-I decay at 370 nm using regression methods (Fig. 2.2, red trace) required a two-summed exponential expression with reciprocal relaxation times (RRTs) 1/\tau_1 = 77 \pm 2 \text{ s}^{-1} and 1/\tau_2 = 8.1 \pm 0.4 \text{ s}^{-1} (Table 2.1, Fig. 2.3). The complex biphasic kinetic behavior for Ole-I decay may be attributed to multiple populations of the intermediate that react at different rates, or may alternatively hint towards the presence of an additional intermediate that absorbs at this wavelength.

In order to delineate between these possibilities, the decay of Ole-I was alternatively monitored at 690 nm (Fig. 2.4). The data, which could be accurately fit with a single exponential expression, indicates a homogeneously reactive Ole-I species. Consequently, the measured RRT measured at this wavelength (1/\tau = 83 \pm 2 \text{ s}^{-1}) correlates well with the fast phase from the 370 nm data.
Figure 2.2  Stopped flow absorption evidence for a substrate $^2$H kinetic isotope effect in the OleT reaction. Representative single wavelength time course for the reaction of 20 μM OleT-D$_{39}$EA (red) or OleT-H$_{39}$EA (blue, and inset) with 10 mM H$_2$O$_2$ monitored at 370 nm. The superimposed white traces represent two summed exponential fits to the data.

We $^{35}$ and others $^{34}$ have postulated that alkene formation may proceed via initial substrate H atom abstraction based on the accepted mechanism for aliphatic hydroxylations by P450-I, loss of a hydrogen from the C$\beta$ position during conversion of a C$n$ fatty acid to a C$n$-1 alkene, and structural similarity of OleT $^{33}$ with fatty acid hydroxylases $^{42-43}$ that exhibit significantly large substrate $^2$H steady-state kinetic isotope effects (KIEs) $^{39}$. Appreciable accumulation of Ole-I was not previously detected in the OleT single turnover reaction with protiated EA (H$_{39}$-EA), suggesting that the rate of reaction was too fast to be captured in our earlier photodiode array studies $^{35}$. 

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Figure 2.3 Representative time courses (black) superimposed fits (red) and residuals for the 20 μM OleT-EA + 10 mM H₂O₂ time course using single wavelength detection at 370 nm (A, B) and 440 nm (C, D). The substrate utilized is indicated in each panel.
Table 2.1 Reciprocal relaxation times and amplitudes from fitting single wavelength data

<table>
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<tr>
<th>Reciprocal relaxation time number</th>
<th>wavelength</th>
<th>690 nm</th>
<th>370 nm</th>
<th>370 nm</th>
<th>440 nm</th>
<th>440 nm</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>substrate</td>
<td>D$_{39}$-EA</td>
<td>s$^{-1}$ (amp)</td>
<td>D$_{39}$-EA</td>
<td>s$^{-1}$ (amp)</td>
<td>H$_{39}$-EA</td>
</tr>
<tr>
<td>1</td>
<td></td>
<td>83 ± 2$^a$ (0.06)$^c$</td>
<td>77 ± 2 (0.19)</td>
<td>630 ± 60 (0.14)</td>
<td>82 ± 3 (0.36)</td>
<td>670 ± 60 (0.25)</td>
</tr>
<tr>
<td>2</td>
<td></td>
<td>8.1 ± 0.4 (0.06)</td>
<td>6.9 ± 0.4 (0.05)</td>
<td>10.8 ± 0.1 (0.24)</td>
<td>9.0 ± 0.2 (0.2)</td>
<td></td>
</tr>
</tbody>
</table>

$^a$With the exception of the 690 nm data, fitting was to a 2 summed exponential expression by nonlinear regression using ProData viewer software. A 20 μM OleT-EA complex was mixed with 10 mM H$_2$O$_2$ at 4°C at pH 7.5

$^b$ Error represents multiple experimental runs using different preparations of OleT

$^c$ The sign of an amplitude is opposite that of the observed change in absorbance

Figure 2.4 Time course for the 20 μM OleT-D$_{39}$EA + 10 mM H$_2$O$_2$ reaction (black) using single wavelength detection at 690 nm, single exponential fit (red), and residuals
In order to further probe the reactivity of Ole-I, the H$_{39}$-EA single turnover system was monitored at 370 nm with a photomultiplier tube, providing greater sensitivity. Biphasic characteristics were again observed, although the initial decay phase was significantly more rapid than for D$_{39}$-EA, reaching completion within 10 ms (Fig. 2.2, blue trace, Fig. 2.3B). Despite this rapid disappearance, relatively accurate RRTs (within 10% error) were obtained from a two summed exponential fit of the data, with $1/\tau_1 = 630 \pm 60$ s$^{-1}$ and $1/\tau_2 = 6.9 \pm 0.4$ s$^{-1}$. Only the fast RRT demonstrated appreciable isotopic sensitivity. This indicates that if the slower phase indeed derives from a second intermediate species that contributes to the absorbance at 370 nm, it does not directly abstract an H atom. An observed $^2$H KIE for Ole-I decay at this H$_2$O$_2$ concentration, which represents a lower limit for the unmasked value, is provided by a ratio of the fast RRTs, $(^4k_{\text{app}}/^2k_{\text{app}} = \text{RRT}_1 (H_{39}\text{-EA}) / \text{RRT}_1 (D_{39}\text{-EA}))$, giving a KIE $\geq 8.1 \pm 1.1$. The large KIE, which has not been previously measured for a P450-I generated with a bound substrate, is in accord with unmasked KIEs for steady state P450 hydroxylations, rapid mixing studies of P450-I$^{10}$ and similar thiolate ligated heme iron-oxo species$^{41}$, and theoretical considerations$^{44}$. This confirms that the distinctive C-C$\alpha$ cleavage catalyzed by OleT most likely commences in a very similar way as P450 monooxygenations.
A Second Intermediate in the Decarboxylation Reaction

Kinetic evidence for an additional chromophoric species in OleT decarboxylation prompted a close examination of the photodiode array data for the EA single turnover reaction. In spectra obtained from mixing EA-D$_{39}$ bound OleT with a large excess of H$_2$O$_2$, the loss of Ole-I (Fig. 2.5, green spectrum) over 40 ms is accompanied with the formation of a new transient species (Fig. 2.5 orange spectra). We refer to this second OleT intermediate as Int-2.

![Absorbance vs Wavelength](image)

**Figure 2.5** Identification of an additional intermediate (Int-2) that forms concomitant with OleT Compound I (Ole-I) decay. Photodiode array spectra of a single turnover reaction of 20 μM OleT-D$_{39}$EA with 10 mM H$_2$O$_2$, 2 to 40 ms timeframe.

Int-2 has a Soret maximum that appeared to be red shifted and significantly less intense than the ferric water ligated low-spin ($\lambda_{\text{max}} = 417$ nm) product state of the
enzyme, which formed more slowly over the course of 500 ms (Fig. 2.6, blue spectrum). The optical features of this presumptive new intermediate are partially revealed by subtracting the final Fe$^{3+}$-H$_2$O species from the 40 ms spectrum (Fig. 2.6, inset), which shows a prominent positive feature at 440 nm and a secondary peak at 370 nm. A similar species was detected in parallel studies of OleT-H$_3$EA (Fig. 2.7), although its kinetics are clearly altered, instead maximizing within 10 ms. As a result, the difference spectrum (10 ms - 500 ms) shares the same positive optical features at 370 and 440 nm, but is approximately two-fold more intense (Fig. 2.7, inset). This implies that one or both rate constants for Int-2 formation and decay are altered upon substrate isotopic substitution.

Figure 2.6 Photodiode array spectra of a single turnover reaction of 20 μM OleT-D$_{39}$EA with 10 mM H$_2$O$_2$, 40 ms to 500 ms timeframe. Inset is the difference spectrum between the start (orange) spectrum and the end (blue) spectrum.
Figure 2.7  Photodiode array spectra of a single turnover reaction of 20 μM OleT-H<sub>39</sub>EA with 10 mM H<sub>2</sub>O<sub>2</sub>, 10 ms to 500 ms timeframe. Inset is the difference spectrum between the start (orange) spectrum and the end (blue) spectrum.

Int-2 is the Decay Product of Compound I

The kinetic behavior of Int-2 was monitored at 440 nm using single wavelength stopped flow. The time courses for both substrates reveal rapid development of the chromophore associated with Int-2 and a subsequent slower decay phase upon conversion to the final ferric low-spin product form (Fe<sup>3+</sup>-OH<sub>2</sub>) of the enzyme (Fig. 2.8, Fig. 2.3 C, D). Each of the single wavelength time courses
could be appropriately fit to a sum of two exponential functions with well resolved RRTs (Table 2.1). Intriguingly, the fast phase RRT exhibited a large dependence on the isotopic composition of the substrate and, for both types of substrate, matched the decay rate constant for Ole-I. Both kinetic features suggest that Int-2 results from the decomposition of Ole-I, and therefore represents the next intermediate in the reaction sequence.

In order to delineate the kinetics of Int-2 more fully, the dependence of both RRTs at various H₂O₂ concentrations was examined. For both H₃⁹-EA and D₃⁹-
EA substrates, the slow RRT associated with Int-2 decay was independent of $\text{H}_2\text{O}_2$ concentration, and demonstrated only minor isotopic sensitivity (Fig. 2.9). This is consistent with an assignment of Int-2 as an intervening intermediate between Ole-I and Fe$^{3+}$-OH$_2$. For the D$_{39}$-EA plot (Fig. 2.10, and inset), the fastest RRT shows a hyperbolic dependence on $\text{H}_2\text{O}_2$ concentration, indicating that there are at least two reaction steps that lead to the generation of Int-2. Provided that this phase corresponds to an irreversible step in the reaction sequence, then the measured RRT corresponds to the rate constant for Int-2 formation. Results from a hyperbolic fit of the $\text{H}_2\text{O}_2$ concentration dependence shows that this is the case. The y-intercept of the plot, zero, corresponds to the reverse rate constant for the

Figure 2.9 Peroxide independence and isotopic insensitivity of the slower RRT associated with Int-2 decay at 440 nm.
Figure 2.10 Plots of the dependence of the larger reciprocal relaxation time at 440 nm on H$_2$O$_2$ concentration in respective colors. The solid lines represent non-linear fitting to a hyperbolic expression (for the D$_{39}$-EA reaction) and linear fitting (H$_{39}$-EA). Inset is data (for the D$_{39}$-EA reaction) at 370 and 440 nm superimposed.

The hyperbolic fit of the H$_2$O$_2$ concentration dependence shows that this is the case. The y-intercept of the plot, zero, corresponds to the reverse rate constant for the Int-2 formation step ($k_2 = 0$). The asymptote of the plot (equal to $k_2 + k_{-2}$) represents the formation rate constant for Int-2 ($k_2 = 86 \pm 4$ s$^{-1}$) and gives an apparent $K_{D_{H_2O_2}} \approx 100$ μM. These results support a scheme in which Int-2 is produced as a result of an irreversible process, which would be expected for
substrate C-D bond cleavage, and that its rate of formation is identical to the decay rate constant of Ole-I.

The Ole-I $\rightarrow$ Int-2 $\rightarrow$ Fe$^{3+}$-OH$_2$ catalytic sequence is further verified by examination of the fast RRT versus H$_2$O$_2$ for the H$_{39}$-EA reaction (Fig. 2.10). In this case, the plot reveals a linear dependence of the RRT and H$_2$O$_2$, with no evidence of saturation within the accessible range of H$_2$O$_2$ concentrations in which rates could be reliably measured. This altered kinetic behavior can be interpreted as arising from more facile cleavage of the target substrate C-H bond (relative to C-D), such that Int-2 formation is now partially dominated by the rate constant for H$_2$O$_2$ binding. Accordingly, the forward and reverse rate constants for the peroxide binding step are provided by the slope ($k_1 = 5.4 \times 10^5$ M$^{-1}$ s$^{-1}$), and intercept of the plot ($k_{-1} = 27 \pm 3$ s$^{-1}$), respectively. A kinetic model that accounts for all of the observed kinetic data is shown in Figure 2.11.

![Figure 2.11](image)

**Figure 2.11** Summary of the kinetic parameters for OleT Compound I formation, and subsequent decay to Int-2 and the low-spin ferric enzyme. The decay rate constants for Ole-I with D$_{39}$-EA and H$_{39}$-EA are indicated as $k_D$ and $k_H$ respectively. The formation of nonadecene and carbon dioxide was demonstrated in a previous study $^{35}$. 
Assignment of Int-2 as an iron(IV)-hydroxide species

If Int-2 derives directly from hydrogen atom abstraction by Compound I, with no intervening reaction steps, it should resemble a Fe$^{4+}$-OH species similar to Compound II. Rapid radical recombination by oxygen rebound prohibits the observation of Compound II in P450 hydroxylations. However, the progressive decrease in decay rate constants for Ole-I and Int-2 allows for the latter to appreciably accumulate in OleT, most significantly for the H$_{39}$-EA reaction (Fig. 2.12 and 2.13).

![Figure 2.12 Speciation plots for OleT reaction cycle intermediates computed using rate constants determined in single wavelength studies with D$_{39}$-EA](image)

Figure 2.12 Speciation plots for OleT reaction cycle intermediates computed using rate constants determined in single wavelength studies with D$_{39}$-EA
Using global analysis methods, we extracted the pure optical spectra for Ole-I and Int-2. A comparison of the optical spectra of these species with ferric low-spin Fe$^{3+}$-OH$_2$ OleT is shown in Fig. 2.14. The optical spectrum of Int-2 has characteristics that are highly similar to those reported for protonated thiolate Fe$^{4+}$ hydroxide complexes prepared in P450 45 and the thiolate ligated heme peroxygenase Aae-APO 46. Notably, Int-2 is characterized by a Soret maximum at $\lambda_{\text{max}} = 426$ nm that is clearly red-shifted and has a lower molar extinction coefficient relative to the Fe$^{3+}$-OH$_2$ form of the enzyme. Int-2 also exhibits a split Soret band, with an additional absorption maximum at 370 nm. This feature contributes to the slower, isotopically insensitive kinetic phase described earlier.
In order to rule out the possibility that Int-2 could be more appropriately assigned as a deprotonated ferric-hydroxide species (Fe$^{3+}$-OH), which in principle could arise from hydrogen abstraction and ensuing rapid electron transfer from the substrate or a neighboring redox active amino acid, this form of the enzyme was generated for direct comparison. The addition of strong base to the substrate free Fe$^{3+}$-OH$_2$ enzyme results in generation of a transiently stable Fe$^{3+}$-OH (Fig. 2.15). Notably, the Fe$^{3+}$-OH species did not exhibit any of the principle optical spectroscopic features (Soret maximum, extinction coefficient, hyperporphyrin) that clearly define the spectrum of Int-2.
A model-independent approach was also used to verify the optical features of Int-2 obtained from global analysis. Following Ole-I depletion, the reaction consists of only two principle absorbing species, Int-2 and Fe$^{3+}$-H$_2$O. As a result, the spectrum of Int-2 can be simply derived at any timeframe from a linear combination of two spectra, one known. No additional input of kinetic parameters is necessary. This subtraction method consistently reproduced the same Int-2 spectrum determined from global analysis (Fig. 2.16). Moreover, the relative composition of each species is in excellent agreement with those computed using rate constants from the kinetic model.

Additional support for the assignment of Int-2 as having an oxidation state higher than Fe$^{3+}$ is provided through an examination of its reactivity in double-mixing studies. Thiolate ligated Compound II species, including that of chloroperoxidase (CPO-II) $^{47}$ and AaeAPO (APO-II) $^{46}$ are able to oxidize phenols, albeit with variable proficiency. Int-2 was first prepared by mixing the EA-H$_{39}$ bound enzyme with H$_2$O$_2$ to favor the rapid depletion of Ole-I and formation of Int-2. After a 20 ms aging time, various concentrations of pH buffered phenols were added in a second push. The decay of Int-2 was measured at 440 nm and fit to a single exponential decay process ($k_{obs}$) (Fig. 2.17). The addition of phenols significantly accelerated the decay of Int-2 in a concentration dependent manner.

This, along with the optical characterization of Int-2, rules out possible interpretation a Fe$^{3+}$-OH species, which is inert to phenols. Representative plots for the reactivity of phenol and 3-Cl phenol with Int-2 are shown in Fig. 2.18 with
Figure 2.15 (A) Comparison of the optical absorption spectra of OleT Int-2 (red), ferric-H₂O (blue), and ferric-OH (black) forms of the enzyme. The ferric-hydroxide form was generated by a rapid pH jump to 12, and is stable for several seconds prior to decomposition. (B) Difference spectra of the ferric-hydroxide (black) and Int-2 minus ferric-H₂O (red) forms show that the two species are readily distinguishable.
Figure 2.16  Kinetics independent computation of the Int-2 optical spectrum by the linear combination of two spectra. The $H_{39}$-EA (A-C) or $D_{39}$-EA (D-I) reaction was analyzed at the time points indicated above each column. The Int-2 spectrum was reproduced by subtracting varying amounts of a ferric –$H_2$O component. A comparison of the Int-2 spectra produced by linear subtraction and from SVD is shown in the middle panel (B, E, H). Speciation plots for Int-2 and the ferric low-spin state based on rates obtained from global analysis are shown in the bottom panel (C, F, I). A dashed line shows the predicted fraction of each species at the time point of interest.

apparent second order rate constants, calculated from the slopes in the inset.

Additional data for a series of substituted phenols is provided in Table 2.2.
Fig 2.17 Double mixing studies to probe the reactivity of Int-2 towards phenols. Int-2 was generated by mixing 40 μM OleT:H₃⁹-EA with 4 mM H₂O₂. After aging for 20 ms, the resulting solution was mixed 1:1 with buffered phenols at various concentrations. Representative time traces at 440 nm and superimposed single exponential fits (red) are shown for a reaction with no phenol (black) and with 10 mM 4-Cl-phenol (gray). The observed rates for these reactions are $k_{obs} = 7.3 \text{ s}^{-1}$ and $26.7 \text{ s}^{-1}$ respectively.

Figure 2.18 Plot of the apparent Int-2 decay rate constant with phenol (circle) and 3-chlorophenol (square). OleT-H₃⁹EA (40 μM) was mixed with 2 mM H₂O₂, aged 20 ms and then mixed 1:1 with substituted phenols. The concentrations shown are after mixing. Error bars represent one SD.
Table 2.2  Summary of reactivity of Int-2 with various substituted phenols

<table>
<thead>
<tr>
<th>substituent</th>
<th>$k$ (mM$^{-1}$ s$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>none</td>
<td>1.00 ± 0.07</td>
</tr>
<tr>
<td>3-Cl</td>
<td>4.69 ± 0.29</td>
</tr>
<tr>
<td>4-Cl</td>
<td>1.72 ± 0.42</td>
</tr>
<tr>
<td>4-OMe</td>
<td>0.94 ± 0.08</td>
</tr>
<tr>
<td>4-Me</td>
<td>1.06 ± 0.03</td>
</tr>
</tbody>
</table>

Discussion

Transient kinetics directly demonstrate that a P450 Compound I oxidant can carry out reactions that do not get finalized by an oxygen rebound step. Although alternative reactions for CYP-I species have been proposed for decades, prompting considerable discussion, the highly fleeting nature of Compound I prevented prior observation of such a reaction taking place. The ability to rapidly trigger catalysis with the native oxidant utilized by the enzyme in the presence of a tightly bound substrate, and fortuitous rate constants for intermediate interconversions, provides direct insight into the origins for the impressive metabolic versatility of CYP oxidations. As a result of the visualization of two ferryl intermediates during OleT catalysis, C-C bond cleavage can be added to the diverse repertoire of transformations catalyzed by metal-oxo species.

The mechanistic strategy adopted by OleT contrasts with those hypothesized for other metalloenzymes involved in hydrocarbon biosynthesis. In alkane formation by dinuclear iron aldehyde deformylating oxygenases (ADOs), cleavage of the terminal aldehyde carbon, which is eventually lost as formate$^{48}$, is
thought to be mediated by formation of a nucleophilic iron-peroxide intermediate.

This species is different from the highly oxidizing dinuclear Fe$^{4+}$ cluster more commonly utilized by structurally related monooxygenases. Recently, a family of unrelated non-heme Fe$^{2+}$ dependent enzymes (UndA) has also been shown to catalyze the conversion of medium chain length Cn fatty acids to Cn-1 alkenes. Although the active oxidant involved in this O$_2$ dependent reaction has yet to be clarified, a Fe$^{3+}$ superoxide intermediate has been proposed on the basis of the enzyme having no strict requirement for a reductant to facilitate O-O heterolysis. The $^2$H substrate KIE for Ole-I decay demonstrates that alkene synthesis is not accommodated by the use of an alternative active-site oxidant that precedes Compound I, nor from the rearrangement of an oxidized product. Instead, the OleT reaction coordinate diverges after C-H abstraction, and results from an apparent suppression of radical recombination.

The remarkable stability of Ole-II provides a framework to compare OleT catalysis with hallmark CYP monooxygenations. Although thiolate ligated Compound II species have recently been prepared in both CYP and Aae-APO enzymes, these species are produced from the deleterious oxidation of nearby aromatic amino acids from the protein framework (CYP), or by the addition of suitable poised reductants to rapidly quench Compound I (Aae-APO), rendering them quasi stable. The catalytic efficiency of the H$_2$O$_2$ single turnover system and kinetic behavior of the Fe$^{4+}$-OH species observed in OleT demonstrates that it is a competent reaction intermediate, and that it is directly produced from substrate C-H abstraction. To our knowledge, an analogous “rebound”
intermediate has not been observed in any metal containing oxygenase to date. Although the optical data presented here do not permit a finite measure of the lifetime of the substrate radical, nor a precise localization of this species, a comparison of the rate of Ole-II decomposition to rates of radical recombination estimated for CYPs using radical clock substrates is informative. Although the rates computed from these approaches can vary with the CYP and substrate tested, typical radical lifetimes generally reside at the nano- to picosecond time scale. Ole-II, by comparison, persists for hundreds of milliseconds and does not elicit oxygen rebound.

A number of factors may contribute to the recalcitrance of Ole-II •OH rebound. Intriguingly, the reactivity of Ole-II towards phenols more closely resembles that of chloroperoxidase CPO-II than the high proficiency observed for AaeAPO-II. However, the limited reactivity of Ole-II towards these substrates may not necessarily immediately signal a change in electronic structure, but is more likely to stem from restricted access of molecules to the heme-iron when a fatty acid is bound. Consequently, the apparent pseudo first-order decay rate constants across the series exhibit poor correlation with phenol OH bond strength (Table 2.2).

The predisposition of OleT to generate a substantial proportion of fatty alcohol products with shorter, non-physiological substrates highlights that the enzyme is able to simultaneously function as an oxygenase. We propose, based on analogy to mechanisms purported for desaturases, which can also exhibit dual functionality (reviewed in 52), that bifurcation of the two pathways results from a
competition of •OH rebound and abstraction of an additional substrate electron by Ole-II. The latter pathway, coupled to recruitment of a proton to restore Fe$^{3+}$-OH$_2$ (Fig. 2.19), would lead to generation of a carbocation that would be poised for C-C$_\text{α}$ cleavage to liberate CO$_2$. Although the intrinsic reactivity of Ole-II is most likely undervalued here, largely due to its method of preparation, the estimated reduction potential$^{53}$ and oxidative prowess of APO-II$^{46}$ serves as a useful guide. As both olefin and alcohols result from the metabolism of chemically similar substrates, both rigid.
and precise positioning of the carbon centered radical would seem necessary to inhibit oxygen rebound. While regiospecific Cβ-H atom abstraction would most certainly need to be primarily satisfied, it does not fully ensure subsequent over-oxidation to furnish a hydrocarbon by OleT and related CYP152 orthologs. 43, 54. Interesting comparisons are found with the functional divergence observed for non-heme iron enzymes and synthetic complexes, which effectively negotiate •OH bond insertion, rebound of alternative radical species, and substrate desaturation. 52, 55. We anticipate that further elucidation of the factors that promote alkene formation will provide important parallels with these systems in a different structural framework, and provide opportunities to better leverage OleT activity with a broader substrate scope.

References


29. Khatri, Y.; Luthra, A.; Duggal, R.; Sligar, S. G., Kinetic solvent isotope effect in steady-state turnover by CYP19A1 suggests involvement of Compound 1 for


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CHAPTER 3

REACTIVITY OF COMPOUND-II AND SECONDARY OXIDATION OF THE SUBTRATE

Abstract

The fatty acid decarboxylase decarboxylase P450 OleT has been the focus of much interest due its potential applications for biofuel synthesis and for its curious mechanism, which reroutes normal oxygen insertion chemistry. Based on orthologues it has been suggested that the active site histidine (H85) may play a role in directing the reaction coordinate. In this work we show that H85 is not necessary for decarboxylation, but that H-bonding to position 85 does partially contribute to directing the reaction coordinate, along with substrate mobility, toward decarboxylation. We have characterized the secondary oxidation of the substrate by following the kinetics of the Fe$^{4+}$-OH intermediate and through site-directed mutagenesis. Kinetic solvent isotope studies show that the process is proton coupled and involves a single proton in the rate limiting step. The deuterium fractionation factor of this proton varies from 0.2 in the wild-type enzyme to 0.5 for the Gln85 variant. Additionally, we have found a very high activation energy (18 kcal/mol) for this oxidation step with pre-exponential factors that are consistent with electron tunneling distance of approximately 2-4 Å. The general conclusion drawn from these data is that the decarboxylation pathway relies on physical separation of substrate β-carbon radical from the activated oxygen species to
prevent the rebound process, allowing for the Fe$^{4+}$-OH species to slowly oxidize
the substrate to promote decarboxylation.

**Introduction**

The growing need for liquid automotive fuels in the face of concerns over
dwindling petroleum reserves and global carbon emissions has spurred research
in to biofuels as a viable alternative$^{1-2}$. One of the most successful methods for
producing biofuels today is the synthesis fatty acid methylesters (FAMEs) from
common biologically abundant and energy rich fatty acids. FAMEs, however, are
not an ideal fuel substitute for fossil fuels; they are labile to hydrolysis under
ambient conditions$^{3}$ and are only fungible with heavy fuels such as diesel. This has
generated great interest in identifying a means to convert fatty acids* in vivo* to high
value fuels like alkenes and alkanes. The search for enzymes capable of these
transformations has yielded wild selection of metalo-enzymes from across multiple
domains of life including; cyanobacterial aldehyde-deformylating oxygenases
(cADOs) found in *Nostoc punctiforme*$^{4}$, the fatty acid decarboxylases OleT from
*Jeotgalicoccus ATTC* 8456$^{5}$ and UndA from *Pseudomonas fluorescens*$^{6}$, fatty
aldehyde decarboxylyase CYP4G1 from *Drosophila melanogaster*$^{7}$. This diverse
selection of enzymes utilizes non-heme mono-iron, di-iron actives and heme active
sites, but share a common feature in that they eliminate the carbonyl carbon from
the substrate to produce C$_{n-1}$ hydrocarbons. Additionally, these active sites are far
more commonly associated with performing oxygen insertion chemistry than
carbon-carbon bond cleavage$^{8-11}$. 

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The most well-studied of these fuel producing enzymes is cADO\textsuperscript{4-5, 12-17}, which produces alkanes by inserting one oxygen atom onto the carbonyl carbon of a fatty aldehyde through a reactive peroxyanion species, ultimately generating a formate side product. CYP4G1 is similar to cADO in that it acts on fatty aldehydes and inserts oxygen at the carbonyl, however it differs by oxidizing the one carbon co-product by two electrons to carbon dioxide\textsuperscript{18}. These enzymes are mechanistically fascinating, and in the case of cADO, have been successfully used to create bio-propane\textsuperscript{19}, but utilize altered oxygen insertion chemistries. OleT and UndA completely circumvent oxygen insertion, instead performing net hydride abstractions from fatty acids to create terminal alkenes.

Similar to cADO, OleT has received a great deal of attention from biotechnologists for fuel applications and from mechanistic biochemists for its rerouting of P450 chemistry away from oxygen insertion\textsuperscript{20-27}. In our previous work with OleT, we demonstrated that fatty acid decarboxylation is initiated by the ferryl-oxo porphyrin centered cation radical species (Fe\textsuperscript{4+}=O (por\cdot+)), known as Compound-I\textsuperscript{26}, performing hydrogen atom transfer (HAT). The products of this abstraction are a stable ferryl-hydroxo species (Fe\textsuperscript{4+}-OH) known as Compound-II\textsuperscript{27} and, presumably, a substrate radical which persists for several hundred milliseconds before decaying to the Fe\textsuperscript{3+}-H\textsubscript{2}O resting state of the enzyme. The final step of the mechanism is still enigmatic, radical C-C cleavage to form carbon dioxide requires a further one electron oxidation. The nature of this oxidation of the fatty acid is unknown, but is presumably performed by the Fe\textsuperscript{4+}-OH intermediate, which in other thiolate-ligated heme enzymes has been measured to have a high
oxidizing potential of ~800 mV\textsuperscript{28-29}. The currently understood mechanism of OleT is shown in (Fig. 3.1) and highlights importance of OleT abrogating of oxygen insertion.

![Reaction scheme for decarboxylation and \(\beta\)-hydroxylation P450 OleT](image)

Figure 3.1 Reaction scheme for decarboxylation and \(\beta\)-hydroxylation P450 OleT. The two mechanisms diverge after hydrogen atom abstraction (HAT).

Using chemical probes (i.e. radical clocks) the rebound of the Fe\textsuperscript{4+}-OH to substrate radicals has been clocked at rates \(\geq 10^9 \text{ s}^{-1}\) \textsuperscript{30-34} in P450s that perform substrate hydroxylations. However, stopped flow studies of OleT indicate a lifetime of the Fe\textsuperscript{4+}-OH species as \(\sim 10 \text{ s}^{-1}\) \textsuperscript{27}. This suggests that the determinant feature of the OleT mechanism is the stabilization of the Fe\textsuperscript{4+}-OH species. The stabilization of Fe\textsuperscript{4+}-OH and its relationship with determining the reaction coordinate has additional interest outside of OleT and biofuels. Small amounts of desaturation side products have been noted in otherwise hydroxylating P450s for years, and can represent highly-toxic drug metabolites \textsuperscript{32,35-37}. This underscores the need to better understand secondary one electron oxidations by P450s.
As aforementioned, the reduction of Fe$^{4+}$-OH in OleT is accompanied by protonation of intermediate to Fe$^{3+}$-H$_2$O. Proton transfers accompanying electron transfers serve to conserve the net charge of the process, are common in biology, and are collectively known as proton coupled electron transfers (PCET). It is possible that the oxidation of the substrate by Fe$^{4+}$-OH is mediated by PCET. Many have suggested, based on the structure of OleT to its closest structurally elucidated ortholog (P450 BSβ)$^{38}$ which hydroxylates fatty acids at both C$\alpha$ and C$\beta$ positions, that histidine 85 (H85) in the active site may serve as a proton donor. In P450 BSβ, the corresponding residue is a glutamine and represents the only major difference in the proximal pocket between the two enzymatic active sites. Mutagenesis studies have been conducted on both OleT and BSβ converting the 85 position to glutamine and histidine respectively, and have showed only minor effects on reaction outcome, suggesting that the position has little importance$^{5,22,39}$. Theoretical work by deVisser et al. has proposed that the principle importance of the position is as an anchor for a hydrogen bonding network of waters in the active site$^{11}$ and may not necessarily have to act as a direct proton donor. A full panel of mutants of the 85 position may be required to elucidate the function of H85 and H-bonds in OleT.

To this end we prepared glutamine (Q), tyrosine (Y), phenylalanine (F) and alanine (A) mutants of H85. The series creates two mutants that are capable of H-bonding but potentially changes the geometry of the active site water (Q, Y), and two which are incapable of H-bonding but have different steric (F, A). We show that hydrogen bonding to H85 affects the activation of H$_2$O$_2$ by OleT and efficiency.
of spin-state conversion upon binding fatty acid substrates. We show through solvent isotope effects (SIE) and pH studies that reduction of the Fe$^{4+}$-OH species in OleT involves a single proton. Through proton inventories studies, we demonstrate that this proton does not originate from H85 nor from bulk solvent, but rather from a single active water molecule that is likely anchored by H85. Proton delivery from this water was additionally characterized by thermal dependence studies which, together with the proton inventory, indicate a concerted electron proton transfer (CEPT) process. The step is versatile against active site changes so long as the ability to hydrogen bond to position 85 is maintained. However, the positioning of this water is not the sole determinant of the reaction outcome. Substrate mobility is also a key factor governing the regio-selectivity of HAT by Fe$^{4+}$=O (por$^+\cdot$), and for influencing hydroxylation versus decarboxylation chemo-selectivity.

**Methods**

**Reagents**

Antibiotics, buffers and general molecular biology reagents were purchased from Research Products International (Mount Prospect, IL). δ-aminolevulinic was purchased from AOKBIO (Shanghai, China). Hydrogen peroxide, protiated fatty acids and BSTFA:TCMI were from Sigma Aldrich (St. Louis, MO). Perdeuterated fatty acids came from CDN isotopes (Pointe-Claire, QC). D$_2$O was from Cambridge Isotope Laboratories, Incorporated (Andover, MA).
Site-Directed Mutagenesis

The H85A, H85Y, H85F and H85Q mutants were prepared using standard methods. The mutant primers were designed based on the version of the P450 OleTJE gene optimized for expression in *E. coli* used in previous work [26-27]. The primers were designed with predicted annealing temps between 68 °C and 76 °C and were synthesized by ACGT, inc. The primer sequences are in (Table 3.1). The mutagenesis was performed using standard techniques.

<table>
<thead>
<tr>
<th>Mutagenesis primers</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>H85F</strong> Forward:</td>
<td>5’ CTGTTTGGTAAAGGCGCAATTITTTCGGTTGACGGTAAGAAACAC</td>
</tr>
<tr>
<td>Reverse:</td>
<td>5’ GTTTTTCTTACCGTGCAAACGTAAATTCGCGCTTTACAAACAG</td>
</tr>
<tr>
<td><strong>H85A</strong> Forward:</td>
<td>5’ CTGTTTGGTAAAGGCGCAATTTACGTTGACGGTAAGAAACAC</td>
</tr>
<tr>
<td>Reverse:</td>
<td>5’ GTTTTTCTTACCGTGCAAACGTCGCAAT</td>
</tr>
<tr>
<td><strong>H85Q</strong> Forward:</td>
<td>5’ GTTTGGTAAGGCGCAATTTGAAACGTTGACGGTAAGAAAC</td>
</tr>
<tr>
<td>Reverse:</td>
<td>5’ GTTTCTTACCGTCAACCGTTITCAATTCGCGCCTTTACAAAC</td>
</tr>
<tr>
<td><strong>H85Y</strong> Forward:</td>
<td>5’ CTGTTTGGTAAGGCGCAATTATACGTTGACGGTAAGAAACAC</td>
</tr>
<tr>
<td>Reverse:</td>
<td>5’ GTTTTTCTTACCGTCAACCGTATAAATTTCGCGCCTTTACAAACAG</td>
</tr>
</tbody>
</table>
**Expression Purification**

Expression and purification of WT OleT and mutants from *E. coli* was performed using previously published methods.26

**Fatty Acid Dissociation Constant**

The H85 series of mutants were titrated with eicosanoic acid (C20:0) from a 1 mM stock in 200 mM HK2PO4 pH 7.4. The 1 mM stock was prepared by dilution from a 20 mM stock of fatty acid (FA) in Triton X-100/ethanol 30:70 v/v. The enzyme was prepared at a concentration of ~3 µM based on the 418 nm Soret band extinction (110 mM⁻¹ cm⁻¹) of the low spin form in 200 mM HK2PO4 pH 7.4. The spectra were collected on an Agilent 8453 spectrophotometer. The fraction high spin in each trace was calculated with (equation 3.1) below:

\[
\text{Fraction HS} = \frac{(A_{\text{417 nm}} - A_{\text{394 nm}}) \cdot \varepsilon_{\text{405 nm}}}{A_{\text{405 nm}} \cdot (\varepsilon_{\text{417 nm}} \cdot \varepsilon_{\text{394 nm}} - \varepsilon_{\text{417 nm}} \cdot \varepsilon_{\text{394 nm}})} - \frac{\varepsilon_{\text{417 nm}} \cdot \varepsilon_{\text{394 nm}}}{\varepsilon_{\text{417 nm}} \cdot \varepsilon_{\text{394 nm}} - \varepsilon_{\text{417 nm}} \cdot \varepsilon_{\text{394 nm}}}
\]

\[
\varepsilon_{\text{405 nm}} = 82 \text{ mM}^{-1} \text{cm}^{-1} \quad \varepsilon_{\text{417 nm}}^{\text{LS}} = 114 \text{ mM}^{-1} \text{cm}^{-1} \quad \varepsilon_{\text{417 nm}}^{\text{HS}} = 58 \text{ mM}^{-1} \text{cm}^{-1}
\]

\[
\varepsilon_{\text{394 nm}}^{\text{LS}} = 50 \text{ mM}^{-1} \text{cm}^{-1} \quad \varepsilon_{\text{394 nm}}^{\text{HS}} = 102 \text{ mM}^{-1} \text{cm}^{-1}
\]

A is the absorbance for the given wavelength, and ε is the molar extinction coefficient for the given wavelength and species (HS = ferric high-spin, and LS = ferric low-spin respectively). For H85Y, 424 nm was substituted for 417 nm, 400 nm for 394 nm and 411 nm for 405 nm due the overall red shift of the Soret in the mutant, but the extinction values were similar. The dissociation constant (K_d) was
determined by fitting a plot of the spin conversion against concentration of total FA. Due to the tight binding of FA by OleT the Morrison quadratic equation was used.

**Stopped Flow**

WT OleT and the H85 mutants were prepared at 10-20 µM in 200 mM K₂HPO₄ at pH 7.4. Three equivalents of perdeuterated eicosanoic acid (C20:0-D), or relevant FA, were then added from a stock of 20 mM FA dissolved in Triton X-100/Ethanol 30:70 v/v and incubated overnight at 4 °C. Aggregated FA was removed by centrifugation at 14000 r.c.f. for 10 minutes at 8 °C and loaded into the sample handing unit (SHU) of an Applied Photophysics Ltd. (APP) SX 20 stopped flow photospectrometer. The samples were then rapidly mixed 1:1 with various concentrations of H₂O₂ prepared in a matched buffer. Full spectra were collected with a photodiode array (PDA) detector and high-resolution single wavelength data were collected using a photomultiplier tube (PMT) accessory.

**Kinetic Fitting**

All experiments were conducted under pseudo-first order conditions to eliminate the need for quadratic fitting. The single wavelength data were fit in APP Pro-Data Viewer to a summed exponential expression using the smallest number of terms with (equation 3.2).

\[ 3.2A_{t,obs} = A_{\infty} + \sum_{i=1}^{n} a_i e^{-t/\tau_i} \]
where $A_{t,\text{obs}}$ is the absorbance amplitude at time $t$ (s), $\tau_i$ is the relaxation time constant (s$^{-1}$) of phase $i$, $a_i$ is the amplitude of the absorbance change of phase $i$, and $A_\infty$ is the final absorbance. The reciprocal of $\tau_i$ (RTC) is the decay rate of phase $i$. Kinetics studies of $\text{Fe}^{4+}=\text{O (por}^\cdot+)\text{)}$ were performed at 370 nm and 690 nm, the Soret maximum and porphyrin cation radical signal respectively. Observations of $\text{Fe}^{4+}\text{-OH}$ were made at 440 nm, the maximum difference between $\text{Fe}^{4+}\text{-OH}$ and low-spin $\text{Fe}^{3+}\text{-H}_2\text{O}$ (LS).

**Singular Value Decomposition (SVD)**

Spectra/kinetic data from 10 mM $\text{H}_2\text{O}_2$ experiments conducted on the stopped flow with the PDA were analyzed with APP Pro-K IV SVD-Global Analysis software. Prior to analysis, the data below 300 nm was truncated from the spectra, due to saturation of UV portion of the spectrum which generates high amplitude noise that obstructs fitting. The spectra/kinetic data were approximated with 8 matrices. The number of real non-zero singular values was then determined by whether or not the kinetic component of the approximating matrix showed an exponential decay or growth. This showed at most 3 non-zero values that indicates that there are only four optically distinguishable species are in the data. The reaction of $\text{OleT}$ with $\text{H}_2\text{O}_2$ proceeds in four steps, a reversible binding of $\text{H}_2\text{O}_2$ by the ES complex, and three irreversible steps: O-O bond cleavage in the activation of $\text{H}_2\text{O}_2$, HAT by $\text{Fe}^{4+}=\text{O (por}^\cdot+)\text{)}$, and reduction of $\text{Fe}^{4+}\text{-OH}$. 


The high concentration of H$_2$O$_2$ drives $k_1 >> k_{-1}$ and the lack of any intermediate species accumulating between ES(HS) and Fe$^{4+}$=O (por$^\cdot$+) indicates that $k_2>>k_1$. As a result, the formation of Fe$^{4+}$=O (por$^\cdot$+) is governed primarily by the H$_2$O$_2$ on-rate ($k_1$). For the kinetic model this is approximated by a single combined term ($k_1'$) which represents a pseudo-first order rate constant. The UV-Vis absorbance of H$_2$O$_2$ and the alkene are treated as being negligible, simplifying the number of optical species to the scheme shown below, which restricts number of phases to the observed number of singular values.

\[
3.4 \text{ES(HS)} \xrightarrow{k_1'} \text{Fe}^{4+} = O(\text{por}^\cdot+) \xrightarrow{k_3} \text{Fe}^{4+} - OH \xrightarrow{k_4} \text{E (LS)}
\]

This reaction scheme was input to Pro-K IV SVD-Global Analysis software and used to fit full spectra/kinetic data from the stopped flow. The spectra/kinetic data were reconstructed using only the first 3 matrices and then fit using global analysis to the above scheme. The initial rates of $k_3$ and $k_4$ were estimated from single wavelength fitting at 370 nm, 440 nm and 690 nm and $k_1'$ was estimated to be 2000 s$^{-1}$. The rates were allowed to float. Additionally, pure reference spectra of Fe$^{3+}$ (HS) and Fe$^{3+}$ (LS) were supplied to the program and the initial mix of optical species set based on amount of HS and LS species observed, taken from mixing of the ES complex with a buffer not containing H$_2$O$_2$. In the mutants, the kinetic model was simplified based on the number of singular values required to reconstruct the spectra/kinetic data and observable intermediate features.
H$_2$O$_2$ Dependence

Measuring the efficiency of H$_2$O$_2$ activation by OleT WT with different chain-length fatty acids and the H85 series of mutants was conducted using established stopped flow methodology. The H85 mutant series and WT were initially complexed with C20:0-D. Initial 20 mM H$_2$O$_2$ stocks were prepared fresh in 200 mM HK$_2$PO$_4$ at pH 7.4 and the concentration determined using the extinction of H$_2$O$_2$ at 240 nm (43.6 cm$^{-1}$ M$^{-1}$). Final H$_2$O$_2$ solutions used in the stopped flow were prepared by serial dilution of the 20mM stocks with matched buffer. The rate of peroxide activation was measured at 440nm. The observed relaxation rate for all observed phases were plotted against the post-mix H$_2$O$_2$ concentration. The phases which showed no response to peroxide were omitted from further analysis.

The H$_2$O$_2$ dependent phases were hyperbolic and fit to (equation 3.5). The rate limiting step leading to the accumulation of Fe$^{4+}$-OH is $k_{limit}$. In WT, H85Q and H85F $k_{limit} = k_3$, HAT by Fe$^{4+}$=O (por$^{-}$), and in H85Y and H85A this shifts to $k_2$ the activation of peroxide.

$$3.5 \frac{1}{\tau_i} = \frac{k_{limit}[H_2O_2]}{K_{1/2}+[H_2O_2]}$$

$$K_{1/2} = \frac{k_{-1}+k_{limit}}{k_1} \quad k_{limit} = k_2 \text{ or } k_3$$

In addition to C20:0, WT OleT was complexed with a series of FAs, stearic (C18:0), palmic (16:0), myristic (14:0), both normal and perdeuterated. The reciprocal relaxation times (RRTs) observed with protiated fatty acids do not show any hyperbolic saturation behavior and are linear across the range of H$_2$O$_2$ that can be
accurately measured with the stopped flow. Instead of being fit to equation 5 these were fit to simple linear regressions and the $K_d$ of $\text{H}_2\text{O}_2$ determined by (equation 3.6) below:

$$3.6 K_d = \frac{k_{-1 \text{ (intercept)}}}{k_{1 \text{ (slope)}}}$$

**GC Product Determination**

WT OleT and the H85 mutants were reacted with a panel of fatty acids and slowly turned-over by infusion of $\text{H}_2\text{O}_2$ from a syringe pump. The reaction conditions were 2 mL of 5 µM enzyme in 200 mM HK$_2$PO$_4$ at pH 7.6 with 500 µM of FA added from 10 mM stock. C20:0, C16:0 stocks were prepared in 70% ethanol/ 30% Triton X-100 (v/v). Lauric acid (C12:0) was prepared in DMSO. Enzyme concentrations were determined by Soret absorption at 418 nm (110 cm$^{-1}$ mM$^{-1}$). Each reaction was gently stirred and 2 mL of 5 mM $\text{H}_2\text{O}_2$ in paired buffer was slowly added over an hour to final volume of 4 mL. Immediately after the reaction was completed the enzyme was inactivated with concentrated HCl and internal standards were added. 50 nmol of hexadecene was added to each reaction from a 10 mM DMSO stock as an internal standard for alkene products. For C20:0 and C16:0 reactions 50 nmol C18:0 acid was added from a DMSO stock, and for C12:0 reactions 50 nmol C14:0 acid was added as an internal control for unreacted fatty acid and hydroxylated fatty acid products. Each reaction was extracted with 8 mL of chloroform and vortexed for 1 minute. The organic and aqueous layers were then separated by centrifugation at 1,200 rcf for 5 minutes. The aqueous layer was then removed by pipetting and the organic layer decanted.
into clean vials and evaporated under dry nitrogen to less than 100 µL. 100 µL of BSTFA:TCMI 99:1% v/v was added to each sample and the vials sealed and heated to 60 °C for 30 minutes to derivatize the fatty acids. The derivatized samples were then transferred to GC autosampler vials.

All samples were run on a HP 5890 II gas chromatograph equipped with DB-5ms column (30m x 0.5 micron) and a flame ion detector (FID). Both the detector and the inlet were heated to 300 °C. Samples with C20:0H and C16:0 were run on program that starts with a 3 minutes hold at 170, then heats at 10 °C/min to 220 °C, then 5 °C/min to 320 °C and holds for a final 3 minutes at 320 °C. Samples with C12:0 start at 100 °C for 3 minutes, then heat at 5 °C/min to 250 °C and holds for 3 minutes at 250 °C. Retention times for alkenes, and derivatized FA products under these conditions were determined in previous work40.

**pH Dependence**

Samples of 10-20 µM WT and H85Q were first prepared in a buffer of 25 mM HK2PO4 200 mM NaCl at pH 7.4. These were then mixed 50:50 v/v with a buffer of 500 mM HK2PO4 at the experimental pH and allowed to incubate overnight with C20:0 FA. They were mixed 1:1 with 5 mM H2O2 (post-mix) on the stopped flow and the kinetics at 440 nm monitored. The H2O2 was prepared in the same buffers as the enzyme. The final pH of these solutions was checked on a HACH HQ11d pH meter. Kinetic rates were taken from the decay of Fe4+-OH observed at 440 nm. Studies in D2O were prepared by evaporation of a K2HPO4 200 mM buffer at the target pH in H2O and reuspended in an equal volume of D2O. 200 µM WT
or H85Q were then mixed 1:10 v/v with the D2O buffers. The RTCs of Fe4+-OH decay were plotted against pL (H or D) and the pKa determined by the midpoint of the sigmoid curve. The pKa shift in D2O can be used to measure of fraction factor (φ) of the exchangeable site (HA) using (equation 3.7) 41.

\[ 3.7 \Delta pK_a = \log \frac{\phi_{HA}}{\phi_{L3O^+}(\phi_{L3O^+})^2} \]

\[ \phi_{HA} = 1 \quad \phi_{L3O^+} = .69 \]

Equation 3.7 pertains to exchangeable sites where the pKa is perturbed both by acid/base equilibrium with L3O+ (φi) and secondary sphere interactions from two solvating L3O+ (φe) ions. That active site of OleT is highly hydrophobic, however, and there would be no solvating L3O+ giving (equation 3.8), which was used in this work.

\[ 3.8 \Delta pK_a = \log \frac{\phi_{HA}}{.69} \]

**Proton Inventory**

The D2O buffers were prepared in same manner as in the pH studies. For the D2O gradient, H2O/D2O the stocks were mixed v/v to achieve the desired fraction D2O. Stocks of OleT WT or H85Q complexed with FA in 50 mM K2PO4 200 mM NaCl at pH 7.4 were mixed with these buffers 1:10 v/v. The final D2O concentrations reported account for this dilution. All samples were incubated overnight at 4 °C to ensure complete exchange with the solvent. These were mixed on the stopped flow against 5 mM H2O2 prepared in an analogous manner to the protein and the kinetics monitored at 440nm. The deuterium fraction factor (φ) of
The decay of the Fe$^{4+}$-OH was determined plotting the RTC Fe$^{4+}$-OH against fraction D$_2$O and fitting it using (equation 3.10), a simplification of Gross-Bulter (equation 3.9).

\[
\frac{v_n}{v_0} = \frac{\prod_i^{T}(1 - n + n\phi_i^T)}{\prod_j^{R}(1 - n + n\phi_j^R)}
\]

Equation 10 assumes that all the reactant state $\phi^R$ are 1, and all the transition state $\phi^T$ are equivalent. This is necessary limit the number of independent variables in the fit.

**Reaction Thermodynamics**

10 µM WT and H85Q were shot on the stopped flow against 5 mM H$_2$O$_2$ in a means identical to previous experiments, with the exception of temperature. While all other kinetics were performed at 4 °C, the thermodynamics experiments were conducted from 4 to 24 °C. The temperature was taken from the internal thermometer in the stopped-flow sample handling unit, and samples were equilibrated for a minimum of 5 minutes before being shot. The RTCs were from 440nm traces. The ln(1/ $\tau$) was plotted against 1/T and the linear plot fit using the Arrhenius equation (3.11) to determine the thermodynamic values.

\[
\ln(k) = \frac{-\Delta G^\dagger}{R} \times \frac{1}{T} + \ln(A)
\]
**Results**

**Substrate Binding by H85 mutants**

OleT has been shown to undergo a type-I spectral shift upon binding of fatty acid substrates (FA), resulting in a low- to high- spin (LS to HS) transition due to displacement of the water ligand. Although binding of the native eicosanoic acid (C20:0) substrate results in efficient spin-state conversion (≥ 90 %), the binding of shorter chain-length substrates to WT OleT \(^{20, 26}\) and the binding of C20:0 to distal pocket variants \(^{22}\) has been shown to result in variable levels of HS state conversion. The binding of C20:0 to the H85 series was probed by optical absorption spectroscopy. The H85Y mutant showed a slightly reduced efficiency (75 %) for spin state conversion versus WT. H85Q had a greatly diminished maximum high spin conversion (40%), consistent with prior observations,\(^{22}\) and the H85F and H85A mutants underwent almost no spin shift upon the addition C20:0 (Fig. 3.2). The loss of spin conversion in response to binding C20:0 indicates that in the mutants, particularly H85A and H85F, the water ligand is better retained by the heme in the presence of substrate. The binding affinity of the mutants for C20:0 is unperturbed from WT, however (≈ 300 nM) \(^{20, 26}\) (Fig 4.3 and Table 3.2). In addition to reduced spin conversion, H85Y also has a perturbed Soret maximum. The HS Soret is shifted to 401 nm (relative to 392 nm in WT) and the LS to 421 nm (from 418nm) (Fig. 3.4). This suggests an alteration to the active site of this mutant that is not present in the others mutants nor in the WT enzyme. This is possibly due to a steric clash of the heme with tyrosine, which models approximately 3.3 Å above the heme plane (Fig. 3.5). While it is possible that
Figure 3.2 UV-Vis absorption spectra of H85Q, H85Y, H85F and H85A titrated with C20:0 fatty acid. All four mutants start as Fe$^{3+}$ low-spin (LS) (red spectrum) and transition to Fe$^{3+}$ high-spin (HS) (blue spectrum) to some degree upon addition of C20:0 fatty acid.

the shifted LS spectrum is caused by direct ligation of the tyrosine to the iron center, it does not explain the alteration of the HS spectrum. Shifts in the Soret band that are caused by alterations in heme planarity have been demonstrated in a number of enzymes, $^{42}$ and pronounced red shifts similar to those in observed in H85Y have been found in P450cam under increased hydrostatic pressure $^{43}$. 
Figure 3.3 Plot of fraction Fe\(^{3+}\) high-spin (HS) verses substrate C20:0 concentration. Each point represents three replicate measurements and the fit lines are to a Morrison tight binding equation. H85Y shows the greatest spin shift followed by H85Q. H85A and H85F show minimal transitions. The $K_d$ for substrate for all of the mutants is approximately equivalent.

Table 3.2 Summary of C20:0 binding parameters

<table>
<thead>
<tr>
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<th>$K_d$ (nM)</th>
<th>Maximal spin shift (fraction)</th>
</tr>
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<tbody>
<tr>
<td>WT</td>
<td>200 ± 100</td>
<td>0.85 ± 0.03</td>
</tr>
<tr>
<td>H85Q</td>
<td>200 ± 100</td>
<td>0.38 ± 0.01</td>
</tr>
<tr>
<td>H85Y</td>
<td>100 ± 60</td>
<td>0.75 ± 0.02</td>
</tr>
<tr>
<td>H85F</td>
<td>600 ± 500</td>
<td>0.11 ± 0.02</td>
</tr>
<tr>
<td>H85A</td>
<td>300 ± 100</td>
<td>0.16 ± 0.02</td>
</tr>
</tbody>
</table>
Figure 3.4 Overlay of the UV-Vis spectrum of Fe$^{3+}$ low-spin (LS) (red) the observed maximum Fe$^{3+}$ high-spin (HS) (black) and pure HS calculated by linear subtraction of the pure LS spectrum from observed HS spectrum in H85Y.

Figure 3.5 X-ray crystal structure of OleT (4L40) (green) and a model of the H85Y mutation (magenta). The red disks represent steric clash between the mutation and the porphyrin.
In the crystal structure of the OleT C20:0 ES complex (PDB:4L40) the carboxylate head of the fatty acid forms two salt bridges to R242 and hydrophobically interacts with an extensive number of residues that include F291, P246, I170, F79 and L78 (Fig. 3.6). H85 is not in a distance to directly interact with the substrate, consistent with the observation that the mutants do not alter the affinity for the substrate. In previous work with P450cam, it was found that introducing greater hydrophobicity to the distal pocket favored the formation of HS due to greater desolvation of the active site. This is the reverse of what is observed in OleT with a greater hydrophobicity of the position favoring the LS state. The key difference may be the highly isolated nature of the OleT active site. The water ligand displaced by substrate binding from the heme may not be able to exit the active site past the hydrophobic tail of the fatty acid. H85 thus acts as an H-bonding anchor, which may facilitate the displacement of water from the heme iron.

**Influence of the substrate on peroxide activation and intermediate kinetics**

To probe the effects of the chain length of aliphatic tail on H2O2 activation, WT OleT was complexed with an array of protiated fatty acids, C14:0 – C20:0 and perdeuterated fatty acids C14:0-D – C20:0-D and rapidly mixed with against H2O2 using a stopped flow. Previous work has shown that WT rapidly activates H2O2 when complexed with C20:0-D, generating the Fe4+≡O por•+ species and the subsequent Fe4+-OH species. The accumulation of these multiple intermediates
results in complicated kinetics with multiple summed exponential phases. At 440 nm there are growth and decay phases that can be easily monitored and correspond to the accumulation and decay of the Fe$^{4+}$-OH intermediate. In reactions of the WT enzyme and C20:0-D, the formation phase can be used to measure the reactivity of the preceding Fe$^{4+}$=O por$^+$ intermediate, and under H$_2$O$_2$ limiting conditions, to characterize the efficiency of H$_2$O$_2$ activation. Titrations with all of the tested perdeuterated fatty acid substrates show hyperbolic saturation curves indicating that at high H$_2$O$_2$ C-D bond cleavage remains rate limiting (Fig 3.7). In contrast, titrations with protiated fatty acids show only a linear dependence on H$_2$O$_2$ (Fig 3.8), which as we have argued previously is likely attributed to more facile C-H bond cleavage, which never becomes rate limiting in the single turnover
Figure 3.7  Plot of rate of the growth phase observed at 440nm (accumulation of Fe$^{4+}$-OH) against H$_2$O$_2$ concentration in WT OleT perdeuterated fatty acids (FA) of various chain lengths C14:0-D to C20:0-D. While the various FAs show varied saturation rates they similar H$_2$O$_2$ concentrations are required achieve saturation.

reaction. For both isotopic substrates, the decay phase at 440 nm showed no H2O2 dependence.

The observed saturation rate in the deuterated traces ($k_{limit}$), varies approximately four fold across the series (Table 3.3). The rates initially drop dramatically in going from C20:0 to C18:0, but then increase as the fatty acids get shorter. The origin of this variation is unclear, but may represent slight variability in how the fatty acid rests in the active site altering the distance between the Fe$^{4+}$=O por$^+$ and the C-D bond that gets attacked. This is potentially important
Figure 3.8  Plot of rate of the growth phase observed at 440nm (accumulation of Fe<sup>4+</sup>-OH) against H<sub>2</sub>O<sub>2</sub> concentration in WT OleT fatty acids of various chain lengths C14:0 to C20:0. None of the FAs show saturation of the 440nm Fe<sup>4+</sup>-OH accumulation rate and have similar H<sub>2</sub>O<sub>2</sub> dependent slopes.

as sub Å variances in the active site of SyrB2 have been found to redirect the final reaction outcome from oxygen rebound to halogenation<sup>45-47</sup>.

Table 3.3  H<sub>2</sub>O<sub>2</sub> binding and activation parameters of WT OleT with normal and perdeuterated fatty acids 14:0 to 20:0

<table>
<thead>
<tr>
<th>Deuterated Fatty Acids</th>
<th>Protiated Fatty Acids</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>K&lt;sub&gt;1/2&lt;/sub&gt; (μM)</td>
</tr>
<tr>
<td>C20:0-D</td>
<td>92 ± 8</td>
</tr>
<tr>
<td>C18:0-D</td>
<td>19 ± 2</td>
</tr>
<tr>
<td>C16:0-D</td>
<td>153 ± 19</td>
</tr>
<tr>
<td>C14:0-D</td>
<td>135 ± 27</td>
</tr>
</tbody>
</table>
Fitting the rates of Fe\textsuperscript{4+}-OH formation to equation 3.5 gives the term $K_{1/2}$, which empirically is the concentration of $\text{H}_2\text{O}_2$ that corresponds to a rate half that of $k_{\text{limit}}$. $K_{1/2}$ varies with the chain length of the fatty acid, but shows a constant ratio with the observed $k_{\text{limit}}$. Under the assumption that $k_{\text{limit}} >> k_{-1}$, then $k_{\text{limit}}/K_{1/2} \approx k_1$, the on-rate for $\text{H}_2\text{O}_2$. The constancy of this ratio indicates that the $\text{H}_2\text{O}_2$ on rate is largely unaffected by FA chain-length. The affinity for $\text{H}_2\text{O}_2$ can also be directly measured with a simple $K_d$, using the slope/intercept method by the linear kinetics plots of the protiated fatty acids, as C-H bond cleavage by Fe\textsuperscript{4+}=O por$^+$ is never becomes rate limiting. $K_d$ measurements also showed the chain length of the FA has no effect on $\text{H}_2\text{O}_2$ affinity (Table 3.3). Due to the extremely small value of $k_{-1}$, $K_d$ measurements can be highly error prone. The $k_{\text{limit}}/K_{1/2}$ method shows comparable accuracy for monitoring $\text{H}_2\text{O}_2$ activation and allows simultaneous collection of reactivity of Fe\textsuperscript{4+}=O por$^+$ from the same experiment. The lack of any observable effect from fatty acid chain-length confirms prior assertions that only the carboxylate head of the FA is required for efficient $\text{H}_2\text{O}_2$ activation \cite{21, 25, 48}. 

Influence of H85 on ferryl intermediate accumulation and kinetics

Of key interest is whether the critical Fe\textsuperscript{4+}=O por$^+$ and Fe\textsuperscript{4+}-OH intermediates, observable in the WT\cite{26, 27}, still accumulate in the mutants. To probe this, stopped-flow spectroscopy was combined with singular value decomposition (SVD) and global analysis fitting. The full series of mutants were incubated with C20:0-D fatty acid and then rapidly mixed on a stopped-flow with 10 mM $\text{H}_2\text{O}_2$, several orders of magnitude higher than what is required to achieve saturating rates of C-D bond cleavage in WT. The reaction progress was monitored with a
photodiode array (PDA). Figure 3.9 shows representative raw PDA data for all of the mutants. All the spectra show an overall red-shift of the Soret band from $\approx 390$ nm to $\approx 420$ nm, indicating a loss of HS and the eventual formation of LS. H85Q and H85Y both show a small peak at 650nm, which decays less than a second, also indicating the loss of HS. This small peak is not observable in H85A and H85F due to a small $\sim 10\%$ HS fraction in the reactant species. An important feature is the 690nm peak seen in the H85Q and H85F spectra, which corresponds to the porphyrin centered radical cation$^{26,49-50}$ and can be used to fingerprint $\text{Fe}^{4+}=\text{O por}^+$, as there are no additional heme spectral features that contribute in this regime. Fitting the decay of the 690nm gives a rate of $6.7\pm0.5$ s$^{-1}$ in H85F and $57\pm3$ s$^{-1}$ in H85Q. The rate observed in H85Q is comparable to that of WT ($80\pm3$ s$^{-1}$)$^{26}$, but that of H85F is an order of magnitude slower, indicating that the species is far less reactive. The Soret of the $\text{Fe}^{4+}$-OH intermediate spectrum ($\lambda_{\text{max}} \sim 426$ nm) overlaps with other species, but its accumulation can be observed by monitoring its maximum difference with LS at 440nm$^{27}$. Fig. 3.10 shows the time courses monitored at 440 nm. H85Q and H85A both show biphasic kinetics with a rapid growth phase and a slow decay, similar to WT OleT and indicating that $\text{Fe}^{4+}$-OH still accumulates in these mutants. H85Y and H85F only show a single growth phase, indicating no accumulation of a $\text{Fe}^{4+}$-OH intermediate.

Deconvolution of the spectra into the individual component species also confirmed the lack of $\text{Fe}^{4+}$-OH accumulation. SVD found three non-zero singular values for H85Q, identical to deconvolution of WT. Using kinetic traces at 370 nm,
Figure 3.9  UV-Vis spectra of enzyme substrate (ES) complexes of OleT H85 mutants with C20:0-D reacting with 5 mM H$_2$O$_2$ over 1 second collected with the photodiode array attachment on the stopped flow at 4 °C. H85Q and H85F show minor 690 nm peaks indicating the accumulation of Fe$^{4+}$=O por•+ in these mutants.

440 nm and 690 nm the rate of decay for Fe$^{4+}$=O por•+ and Fe$^{4+}$-OH were estimated as approximately 60 s$^{-1}$ and 10 s$^{-1}$ respectively and were input as starting parameters for global analysis fitting. The pure spectrum of Fe$^{4+}$-OH was easily separated and resembles that of WT, however the solved Fe$^{4+}$=O por•+ spectrum showed an add-mixing with the HS. The speciation plots, deconvoluted spectra, and fit residuals are in Fig. 3.11 & 3.12. H85A and H85F both showed simpler
Figure 3.10 The absorbance at 440nm of the ES complexes of OleT H85 mutants reacting with 5 mM H$_2$O$_2$ collected with a photomultiplier tube on stopped flow at 4 °C. The data is black and the fit in red. The residuals the fit are plotted below each trace. H85Q and H85A both show biphasic kinetics indicating the accumulation of Fe$^{4+}$-OH.
spectra with only two non-zero singular values. Since Fe$^{4+}$-OH was suspected to accumulate in H85A, an initial rate of 10 s$^{-1}$, obtained from single wavelength fitting, was used in the global analysis. For H85F the slow decay rate observed at 690 nm, 7 s$^{-1}$, was used. Global analysis was able to isolate a small amount of the Fe$^{4+}$-OH intermediate from the H85A data, and the Fe$^{4+}$=O por$^{-}$ intermediate species for H85F. SVD of H85Y showed one non-zero singular value and global analysis solved neatly for only the starting and final species with no intervening intermediates. The full speciation plots, solved spectra, and fitting residuals for H85Y, H85F and H85A are in Fig. 3.11 and 3.12. The relative accumulations and the decay rates solved for by global analysis are in (Table 3.4). While the accumulations of the intermediate species are compromised, they remain observable in several of the mutants. H85Q most closely resembles WT and the decay rate of Fe$^{4+}$-OH in H85A and H85Q matches that previously observed in WT$^{27}$. The lack of accumulation of Fe$^{4+}$=O por$^{+}$ in H85A is likely indicative of poor H$_2$O$_2$ activation by the mutant, and that it does not generate the intermediate faster than it decays. H85F appears to be opposite, the reduced activity of its Fe$^{4+}$=O por$^{+}$ intermediate enables it to accumulate, with the expense of observing the subsequent Fe$^{4+}$-OH species. However, the absence of any observable intermediates in H85Y cannot be rationalized with simple kinetic arguments. The simplest explanation is that Fe$^{4+}$=O por$^{+}$ is generated but quickly reacts in a non-productive manner through oxidation of the nearby tyrosine. Quenching of Fe$^{4+}$=O por$^{+}$ by tyrosine has been observed in attempts to trap Compound I in other P450s$^{51}$. 
Figure 3.11  SVD and global analysis of H85Q and H85F mutants of OleT. The top is the raw spectra. The second row are simulated speciation profiles the third row are deconvoluted spectra of the pure species determined by global analysis fitting. The residuals are difference between simulation of the spectra based on the global analysis solution and the original data.
Figure 3.12  SVD and global analysis of H85Y and H85A mutants of OleT. The top is the raw spectra. The second row are simulated speciation profiles the third row are deconvoluted spectra of the pure species determined by global analysis fitting. The residuals are difference between simulation of the spectra based on the global analysis solution and the original data.
The impaired accumulation of the intermediates in some mutants suggests that H85 may play a role in H$_2$O$_2$ activation. To probe this, C20:0-D was added to each mutant and titrations with H$_2$O$_2$ were performed in a manner comparable to the WT fatty acid chain-length experiments. Studies were limited to C20:0-D, since most of the mutants show a reduced spin shift which precludes characterization using shorter chain length FAs. Since H85Q and H85A accumulate Fe$^{4+}$-OH, the 440 nm timecourse could be fit with two summed exponentials (Fig. 3.10) as with the WT. Without a second phase, H85Y and H85F were instead fit using single exponential growth curves at 440 nm. Figure 3.13 shows the response at 440 nm against H$_2$O$_2$ and the fitting parameters to a hyperbolic expression (equation 5) are in (Table 3.5). The saturating rate $k_{\text{limit}}$ and $K_{1/2}$ values of H85Q are within the range observed with WT and varied fatty acids. All of the other mutants show significantly decreased $k_{\text{limit}}$ values and much larger $K_{1/2}$ values. In WT, H85Q and H85F the observed $k_{\text{limit}}$ correlates to the known decay rate of their Fe$^{4+}$=O por$^+$ intermediates from global analysis, indicating that for these variants C-D bond cleavage sets the $k_{\text{limit}}$. For H85Y and H85A the $k_{\text{limit}}$ rates do not correlate any
Figure 3.13  Plot of the growth phase at observed at 440nm for WT OleT and the series of H85 mutants. Each point represents three independent measurements of the respective C20:0-D ES complex rapidly mixing in a stopped flow with H2O2 at 4 °C. Traces were collected using photomultiplier tube attachment.

observable intermediate and likely represents a shift to an earlier rate limiting step such as the O-O bond cleavage of H2O2 to make Fe4+=O por++. This limits the buildup of ferryl intermediates and suggests that H2O2 activation is particularly impaired in these mutants. This may also be the case for H85F, but the poor activation is also masked by the low reactivity of Fe4+=O por++ in that particular mutant. The \( \frac{k_{\text{limit}}}{K_{1/2}} \) values for the mutants (Table 3.5) indicate that in the H85Y, H85F and the H85A mutants the H2O2 on-rate \( (k_1) \) is two to three orders of
Table 3.5 H$_2$O$_2$ activation parameters WT OleT and H85 Mutants

<table>
<thead>
<tr>
<th></th>
<th>$K_{1/2}$ (μM)</th>
<th>$k_{limit}$ (s$^{-1}$)</th>
<th>$k_{limit} / K_{1/2}$ (mM$^{-1}$ s$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>92 ± 8</td>
<td>82 ± 2</td>
<td>890 ± 90</td>
</tr>
<tr>
<td>H85Q</td>
<td>82 ± 5</td>
<td>63 ± 1</td>
<td>710 ± 50</td>
</tr>
<tr>
<td>H85Y</td>
<td>4400 ± 900</td>
<td>30 ± 4</td>
<td>7 ± 2</td>
</tr>
<tr>
<td>H85F</td>
<td>250 ± 90</td>
<td>4 ± 1</td>
<td>8 ± 7</td>
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<tr>
<td>H85A</td>
<td>400 ± 70</td>
<td>19 ± 1</td>
<td>38 ± 9</td>
</tr>
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magnitude smaller than in both H85Q or the WT, resulting in a drastically impaired ability of these mutants to activate peroxide.

The functional equivalence of H85Q and WT indicates that position 85 does not require an exchangeable proton to facilitate H$_2$O$_2$ activation, only that it need be able to contribute H-bonding. Modeling the OleT active site with an end on bound peroxo (Fig. 3.14), however, indicates that direct H-bonding from H85 is unlikely as the (NH-O) bond distance would be minimally 4.1 Å. Ordered water networks have been implicated in H$_2$O$_2$ activation by QM/MM studies on the CYP152 P450spa$^{25}$. For OleT, H85 may anchor such a network, potentially through the water molecule that is displaced upon substrate binding.

**Chain length and position 85 effects on chemoselectivity**

WT OleT and H85 mutants were incubated with 100 equivalents of C20:0, C16:0, or C12:0 fatty acids and then driven by slowly introducing 1000 equivalents of peroxide over an hour. They were then extracted and derivatized to volatilize
Figure 3.14 Model of the ferric-peroxo (Fe$^{3+}$-O-O$^{2-}$) intermediate of OleT based on crystal structure 4L40. Fe-O bond is rotated to minimize the distance between the distal oxygen and H85. The yellow dashed line are the distance between the centers of the electronegative atoms in potential H-bonds. H85 is too far away to effectively H-bond directly.

unreacted fatty acids and hydroxylation products, and analyzed by gas chromatography. The product yields are shown in (Fig. 3.15) and detailed in (Table 3.6). When C20:0 is the substrate WT and all the mutants nearly exclusively produce the corresponding n-1 alkene, nondecene. The H85Y mutant exhibits significantly impaired catalytic efficiency, showing only a third of the activity of the other mutants with C20:0 and no detectable products with shorter chain lengths. This is consistent with the proposed non-productive pathway for H85Y inferred
Figure 3.15 A stacked bar graph of Reaction products of WT OleT and the H85 series of mutants with C20:0, C16:0 and C12:0 FAs. Each reaction contained 100 equivalents of the respective FA and had 1000 equivalents of H₂O₂ introduced slowly over an hour. Black corresponds to the n-1 alkene product, red are the β-hydroxylated FAs and blue are α,γ,δ FAs.

from the transient kinetics data. For shorter chain length fatty acids, the amount of n-1 alkene product drops off across the mutant series and is accompanied by an increase in levels of hydroxylated fatty acids. However, it is notable that this feature does not scale uniformly across the series of mutants. Only in the H85F and H85A mutants does fatty acid chain length directly correlate with overall hydroxylation products. In WT and H85Q the most hydroxylated products are made with C16:0 fatty acid and not C12:0.
Table 3.6  Reaction products of WT OleT and H85 series of mutants

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th>H85Q</th>
<th>H85Y</th>
<th>H85F</th>
<th>H85A</th>
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<td></td>
<td>TON</td>
<td></td>
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<tr>
<td>C20:0</td>
<td>68 ± 4</td>
<td>77 ± 21</td>
<td>33 ± 1</td>
<td>99 ± 0</td>
<td>74 ± 14</td>
</tr>
<tr>
<td>% Alkene</td>
<td>99 ± 0</td>
<td>99 ± 0</td>
<td>99 ± 0</td>
<td>96 ± 5</td>
<td>77 ± 11</td>
</tr>
<tr>
<td>% β-Hydroxy</td>
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<td>0 ± 0</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
<td>16 ± 3</td>
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<tr>
<td>% α-Hydroxy</td>
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<td>0 ± 0</td>
<td>0 ± 0</td>
<td>3 ± 6</td>
<td>7 ± 9</td>
</tr>
<tr>
<td>% γδ-Hydroxy</td>
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<td>0 ± 0</td>
<td>0 ± 0</td>
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<table>
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<th>C20:0</th>
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<tr>
<td>% Alkene</td>
<td>92 ± 7</td>
<td>90 ± 16</td>
<td>0 ± 0</td>
<td>66 ± 29</td>
<td>55 ± 32</td>
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<td>% β-Hydroxy</td>
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<td>20 ± 8</td>
<td>0 ± 0</td>
<td>36 ± 11</td>
<td>35 ± 9</td>
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<tr>
<td>% α-Hydroxy</td>
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<td>0 ± 0</td>
<td>18 ± 3</td>
<td>24 ± 16</td>
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<tr>
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<td>0 ± 0</td>
<td>21 ± 19</td>
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<table>
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<td>98 ± 1</td>
<td>63 ± 22</td>
<td>0 ± 0</td>
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<td>83 ± 9</td>
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<td>% β-Hydroxy</td>
<td>61 ± 7</td>
<td>74 ± 4</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
<td>31 ± 11</td>
</tr>
<tr>
<td>% α-Hydroxy</td>
<td>27 ± 5</td>
<td>23 ± 1</td>
<td>0 ± 0</td>
<td>77 ± 4</td>
<td>57 ± 13</td>
</tr>
<tr>
<td>% γδ-Hydroxy</td>
<td>3 ± 1</td>
<td>3 ± 2</td>
<td>0 ± 0</td>
<td>28 ± 3</td>
<td>12 ± 3</td>
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</tbody>
</table>

The proportional composition of the individual regioselective hydroxylation products (α,β,γ) varies across the mutant series too. In WT with C16:0, the majority of the hydroxylated products are not at the β position. Whereas, H85A and H85F both make threefold more β-OH products than non-β-OH products. This has mechanistic relevance as the decarboxylation deviates from β-hydroxylation at a different point in the reaction coordinate than hydroxylations at other positions on the acyl chain. The site of the initial hydrogen atom abstraction (HAT) by Fe⁴⁺=O por⁺⁺ determines the regiospecificity of the OH product and can occur at any position on the fatty acid. While hydroxylation chemistry can occur at any position, C-C bond cleavage must be initiated by abstraction of the Cβ-H bond. This means that regioselectivity is conserved between β-hydroxylation and decarboxylation.
and that ratio of these two products is particularly informative about the mechanism following Fe$^{4+}$=O por$^{++}$ abstraction, while other hydroxylolation products are more telling of steps prior.

Grouping the reaction products by region of the initial Fe$^{4+}$=O por$^{++}$ attack (table 3.7) shows there is little variation across the mutant series. Within error, the sum of β-hydroxylation products and alkenes is a constant fraction of the total reaction products across the mutant series. There is some variation in the hydrogen abstraction based on the length of the aliphatic tail of the fatty acid. The H85 residue has little effect on the regioselectivity of hydrogen abstraction by the Fe$^{4+}$=O por$^{++}$, which is instead driven by the identity of the substrate. The decreased regioselectivity with shorter chain length suggests that shortening the aliphatic tail may increase the mobility of the substrate in the active site, which is otherwise locked with the C20:0 fatty acid.

**Table 3.7** WT OleT and H85 mutants reactions % β abstraction products and (% n-1 alkene products)

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th>H85Q</th>
<th>H85Y</th>
<th>H85F</th>
<th>H85A</th>
</tr>
</thead>
<tbody>
<tr>
<td>C20:0</td>
<td>100 ± 0</td>
<td>99 ± 1</td>
<td>99 ± 1</td>
<td>96 ± 5</td>
<td>92 ± 11</td>
</tr>
<tr>
<td></td>
<td>(98 ± 1)</td>
<td>(99 ± 1)</td>
<td>(99 ± 1)</td>
<td>(99 ± 1)</td>
<td>(83 ± 5)</td>
</tr>
<tr>
<td>C16:0</td>
<td>59 ± 12</td>
<td>85 ± 5</td>
<td>97 ± 3</td>
<td>76 ± 4</td>
<td>88 ± 10</td>
</tr>
<tr>
<td></td>
<td>(85 ± 8)</td>
<td>(77 ± 9)</td>
<td>n.p.</td>
<td>(40 ± 16)</td>
<td>(57 ± 15)</td>
</tr>
<tr>
<td>C12:0</td>
<td>89 ± 6</td>
<td>97 ± 3</td>
<td>76 ± 4</td>
<td>61 ± 9</td>
<td>81 ± 13</td>
</tr>
<tr>
<td></td>
<td>(69 ± 7)</td>
<td>(76 ± 2)</td>
<td>n.p.</td>
<td>(40 ± 16)</td>
<td>(57 ± 15)</td>
</tr>
</tbody>
</table>

The 85 position does however effect the bifurcation of the reaction coordinate after hydrogen abstraction (table 3.7). In the mutants where the ability to H-bond has been eliminated (H85A and H85F) there is precipitous drop off in the amount n-1 alkenes being made at fatty acid chain lengths shorter than C20:0.
Suggesting that hydrogen bonding to the H85 residue plays a role in stabilizing Fe$^{4+}$-OH, preventing rebound and directing catalysis toward decarboxylation. The possible of importance of H-bonding to Fe$^{4+}$-OH has being suggested before\textsuperscript{11}. Substrate mobility also seems to play an important role in this chemoselectivity. In the hydrophobic mutants near total hydroxylation products is only observed with short highly mobile substrates, the predominate product with the highly constrained C20:0 fatty acid is still n-1 alkene. This suggests that redirecting from hydroxylation to decarboxylation is mostly driven by the relative positioning of Fe$^{4+}$-OH and a substrate radical after HAT and that the latter in particular must be immobilized to prevent oxygen rebound.

**Mechanism of Fe$^{4+}$-OH Decay (pH and kinetic solvent isotope effect studies)**

WT OleT and H85Q were titrated against pL (pH or pD) in H$_2$O and 90% D$_2$O to better characterize the involvement of protons in the decay of the Fe$^{4+}$-OH intermediate. Fe$^{4+}$-OH decays to a Fe$^{3+}$-H$_2$O LS species, which in the decarboxylation pathway, requires one electron from the substrate and a proton from an unknown source. Further interrogation of this step could elucidate the second electron transfer in the decarboxylation pathway, determine whether the process is proton regulated and potentially identify the source of this proton. H85A was excluded from these studies, because its minimal accumulation of Fe$^{4+}$-OH precluded highly accurate determination of its decay kinetics.

In previous work on Fe$^{4+}$-OH in OleT \textsuperscript{27} it was observed that its decay is pH dependent, however it is unknown whether this is reflective of regulation of the
reaction by a “proton gate” or if a titratable group may be involved in switching the mechanism. The H85Q mutant allows us to probe this, since H85 is most likely cause for a pH dependence near pH 7. The pH dependence of Fe$^{4+}$-OH found in this work is shown in (Fig. 3.16). While H85Q and WT start at a similar basal rates (~10 s$^{-1}$) WT shows a five-fold increase the rate of Fe$^{4+}$-OH decay at pH 6 whereas H85Q only shows a modest 50% increase. In addition, WT requires two exponentials to fit the decay at low pH, suggesting that there may be two different populations that decay at different rates under these conditions, a property that is not shared by H85Q (Fig. 3.17). For simplicity, Fig 3.16 shows the WT decay rates fit to one exponential. The fitting of both decay phases is shown in Fig. 3.18 and 3.20). The relative amplitudes of the two phases in also show a pH dependence, with the slow phase predominating at the higher end of accessible pH range (≥ 7.5) and decreasing as the pH drops (Fig. 3.20). The transition in the magnitude of the two phases fits to a sigmoidal curve indicating a single titratable group with a $pK_a$ ~6.4 (Fig. 3.20). This suggests that difference between the two populations involves a protonation event. Given that this behavior is absent in H85Q it can be concluded that this protonation likely occurs at H85. The individual phases also show a pH dependence and have titratable $pK_a$ values at 7.0 (Fig. 3.18 and 3.20). The change in rates observed in these two phases is relatively minor and comparable in magnitude to the change observed in the single phase in H85Q. The single phase observed in H85Q has $pK_a$ of 6.4. Since most of the pH dependence is lost in H85Q, we conclude that the decay of Fe$^{4+}$-OH is not likely
Figure 3.16  Plot of decay rate of Fe$^{4+}$-OH of WT OleT (black) and H85Q (red) in H$_2$O (solid points) and 91% D$_2$O (open points) as function of pH or pD ($pL$). WT shows significant variance in the decay of Fe$^{4+}$-OH as function of pH, whereas H85Q does not. WT also shows an apparent negative pKa shift in D$_2$O, the reverse of what is normally observed.

to be gated by protons from bulk solvent. However, this does not preclude protons being involved in process. Proton coupled electron transfers (PCET)s can take three different forms: sequential proton transfer electron transfer (PTET) or proton gated, electron transfer proton transfer (ETPT) or electron gated, or a concerted electron proton transfer (CEPT). PTET scales linearly with proton concentration since it represents the sole component of the rate limiting step. In contrast, EPT exhibits smaller pH dependences since proton transfer is only part of the driving force at the rate limiting step.
Figure 3.17  Representative plots of 440nm WT OleT and H85Q at pH 7.6 and pH 6.0. Data in black, fits with a single decay exponential in red and two decay exponentials in blue. The residuals of each fit is plotted below. Most data is adequately fit a single decay exponentials except WT at pH 6.0 which requires two.

To further investigate whether Fe$^{4+}$-OH decay is proton dependent, WT and H85Q OleT were equilibrated with D$_2$O, which showed a peculiar effect on the observed pKa values. D$_2$O normally shifts the pKa of titratable sites +0.45 - 0.52
Figure 3.18 Plot of the two decay phases of Fe$^{4+}$-OH observed in WT OleT in H$_2$O as function of pH. The fast rate is only observable at low pH and both show a titration point at pH 7.

Figure 3.19 Plot of the two decay phases of Fe$^{4+}$-OH observed in WT OleT in D$_2$O as function of pH. The fast rate is only observable at low pH and both show a titration point at pH 6.5.
Figure 3.20 Plot of the relative amplitude of the fast decay phase of Fe$^{4+}$-OH observed in WT OleT in H$_2$O and D$_2$O as function of pH. The amplitude of the fast phase approaches 0 above pH 7 and 1 at lower pH in H$_2$O. In D$_2$O similar behavior observed, but in shifted negatively with regards to pL by about 0.5.

pH units, however neither protein shows this effect. In D$_2$O H85Q has a relatively pH independent kinetic solvent isotope effect (KSIE) of ~1.5 and minor negative perturbation of the observed intrinsic p$K_a$. WT shows a larger and varying KSIE of 2.5 - 4.0 and negative perturbations to both the p$K_a$ of the histidine and the intrinsic p$K_a$. (Fig. 3.18-20). The normally observed +0.45 - 0.52 p$K_a$ shift in bulk D$_2$O is caused by the deuterium fraction factor ($\phi$) of three lyonium ions (L$_3$O$^+$), one from direct exchange with the site and two from hydration sphere $^{41}$. In the highly desolvated active site of OleT the hydration sphere contribution may be eliminated and would cause a smaller positive shift than normal. However, this does not explain why a negative $\Delta$p$K_a$ is observed, which signals a significant perturbation.
of the \( \phi \) of the titratable group. Based on the \( pK_a \) shifts observed, the calculated \( \phi \) values for the titratable site are in (Table 3.8). The small \( \phi \) values indicate a tendency to preclude deuterium for both WT and the H85Q mutant. WT in particular has values below what are expected for conventional H-bonds between electronegative atoms. The large KSIEs clearly demonstrate that at least one proton is involved in the rate limiting step of Fe\(^{4+}\)-OH decay. The \( \phi \) predicted by the pH shifts are typical of proton transfer intermediates \(^{52}^{51},^{53}\), and would be expected for CEPT, but may also indicate a multi-proton process involving several more normal \( \phi^{54} \), as SIE effects are multiplicative. Intriguingly, Low-Barrier Hydrogen Bonds (LBHB) have also been demonstrated to have \( \phi \) values on this scale \(^{55-57}\).

### Table 3.8 \( pK_a \) and fraction factors of Fe\(^{4+}\)-OH decay in WT OleT and H85Q

<table>
<thead>
<tr>
<th></th>
<th>( pK_a ) (H(_2)O)</th>
<th>( \Delta pK_a )</th>
<th>( \phi )</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>( pK_a )</td>
<td>-0.42 ± 0.05</td>
<td>0.26 ± 0.03</td>
</tr>
<tr>
<td></td>
<td>( pK_a ) His</td>
<td>-0.29 ± 0.21</td>
<td>0.31 ± 0.13</td>
</tr>
<tr>
<td>H85Q</td>
<td>( pK_a )</td>
<td>-0.1 ± 0.2</td>
<td>0.54 ± 0.32</td>
</tr>
</tbody>
</table>

Proton Inventories

To further investigate the \( \phi \) values and number of protons involved in the decay of Compound II, the full proton inventories of WT and H85Q were measured at \( pL = 7.6 \). In the proton inventory technique, the kinetic solvent isotope effect (KSIE) is measured in variable fractions of deuterated solvent. The response of the KSIE to the deuterium fraction can in principle be used to determine the number of protons involved in a process and the \( \phi \) values associated with each using the
Gross-Butler equation (eq. 9). In practice, this treatment is generally limited to discriminating between single proton (linear plots) and multiple proton transfer processes (bowed plots)\textsuperscript{52} using a simplified version of the equation (eq. 10). This is useful with the WT, since the extremely small $\phi$ observed overall could result from multiple protons in flight during the transition state, each with a prototypical $\phi$ values for an H-bond. The inventories of WT and H85Q at pL 7.6 (Fig. 3.21) both show linear plots and the $\phi$ values solved for agree with those found using the p$\text{Ka}$ data. This suggests only one proton is involved in the process, however the data are not formally accurate enough to discriminate between a one and two proton process. H85Q could function through

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure321.png}
\caption{Plot of the rate of decay of Fe$^{4+}$-OH in n molar fraction D$_2$O over the rate in pure H$_2$O. WT OleT data and fit in black and H85Q data and fit in red. These linear plots indicate a single proton is involved in the decay in both WT and H85Q.}
\end{figure}
a one proton process from an active site water and WT utilizes a two proton process causing the lower apparent $\phi$. To probe this WT was additionally proton inventoried at pH 6, 7 and 8 to alter the protonation state of the histidine. All the inventories were linear (Fig. 3.22), and the $\phi$ values for pH 7 and pH 8 are the same.

**Figure 3.22** Plot of the rate of decay of Fe$^{4+}$-OH in n molar fraction D$_2$O over the rate in pure H$_2$O at various pHs. All the plots are linear indicating that there is no mechanistic switch from a one proton to a multiple proton process behind the faster decay observed in WT OleT.
as at pL 7.6, but the $\phi$ for pL 6 (table 3.9) is significantly higher, resembling that of H85Q. If a second proton originated from the histidine one would expect the smaller apparent $\phi$ in the low pL inventory from an increased contribution from two proton process, the reverse of what is observed. Additionally, the pL 6 and pL 7 proton inventories could be measured for both the fast and slows phase observed in WT. The $\phi$ did not change in-between these two phases, indicating that titratable site on H85 cannot contribute a proton to Fe$^{4+}$-OH. The proton delivered to Fe$^{4+}$-OH during decay likely originates from a water/protium ion coordinated in the active site. The variance in the observed $\phi$ between WT and H85Q may originate from differences in the binding modes of the functional water in the active site.

### Table 3.9 Rates in H$_2$O and fraction factors $\phi$ of Fe$^{4+}$-OH decay

<table>
<thead>
<tr>
<th></th>
<th>Fast Phase</th>
<th>Slow Phase</th>
</tr>
</thead>
<tbody>
<tr>
<td>pL</td>
<td>$k_H$ s$^{-1}$</td>
<td>$\phi$</td>
</tr>
<tr>
<td>WT</td>
<td>6</td>
<td>74.1 ± 0.8</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>24.0 ± 1.0</td>
</tr>
<tr>
<td></td>
<td>7.6</td>
<td>na</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>na</td>
</tr>
<tr>
<td>H85Q</td>
<td>7.6</td>
<td>na</td>
</tr>
</tbody>
</table>

The change in the WT $\phi$ as a function of pH may stem from a reorientation of the histidine residue upon protonation. The crystal structure of WT OleT (4L40) shows that the histidine is likely deprotonated and that the lone pair on the nitrogen is participating in a hydrogen bond to an amide on peptide backbone (Fig. 3.23). Protonating the histidine would break this bond and cause the histidine to rotate to minimize steric clash and changing how it positions the water. This would explain the low $\phi$ observed in H85 as bridging H-bonds are known to strongly exclude deuterium.
Figure 3.23 Model of the active site of OleT Fe$^{4+}$-OH intermediate based on PDB 4L40. The n1 nitrogen on the imidazole ring poised for H-bonding the peptide backbone. An active site water could bridge H85 to –OH group stabilizing it and preventing rebound.

**Thermodynamic Energy Barriers of Fe$^{4+}$=O por$^+$ and Fe$^{4+}$-OH**

The proton inventory data confirm that single proton with a small $\phi$ is involved. The simplest explanation for this is that proton delivered to Fe$^{4+}$-OH comes from strong H-bond. The stronger an H-bond is the more likely it is to preclude deuterium. During the decay of Fe$^{4+}$-OH this H-bond should be broken and its strength measurable by the Arrhenius dependence of the Fe$^{4+}$-OH decay rate. To assay this, WT and H85Q were complexed with C20:0-D mixed with 10mM peroxide at a series of temperatures from 4°C to 24°C and the rates at 440 nm fit to the Arrhenius equation. From the plot (Fig. 3.24) there is no radical change between WT and H85Q. There is only a minor difference in the activation energy of WT OleT 19.2 ± 0.4 kcal/mol and H85Q 16.9 ± 0.4 kcal/mol. There is
approximately an order of magnitude difference in their pre-exponential terms. WT is faster at $7.1 \pm 3.6 \times 10^{13}$ s$^{-1}$ and H85Q is $1.3 \pm 0.6 \times 10^{12}$ s$^{-1}$. These rates are in good agreement with rate of electron transfer predicted for $2 - 4$ Å jump by the Moser-Dutton ruler $5.7 \times 10^{12}$ to $9.1 \times 10^{13}$ s$^{-1}$. This agreement gives credence to our assignment of Fe$^{4+}$-OH decay as being an electron transfer process from the substrate in addition to a proton dependent process. While $\sim 18$ kcal/mol is far stronger than most H-bonds is not outside the realm of those observed in enzymes and very similar activation energies have been observed in model EPT systems. Although the breaking the H-bond likely not the only contributor to
activation energy barrier it is likely the largest component, which is curious since it implies the proton couple retards the rate of electron transfer rather than enhances it. The oxidation potential of secondary carbon radical is approximately 0 mV\textsuperscript{62} which a Fe\textsuperscript{4+} species should be able to oxidize to carbocation radical without assistance of a proton couple unless the activation energy of the pure electron transfer is even more prohibitive.

**Discussion**

In this work we have shown that H85 affects OleT throughout most stages of the catalytic cycle (Fig. 3.25). Binding studies show that H85 affects the degree spin conversion associated with Type-I substrate binding, although it has no influence on affinity for fatty acids (FA) by OleT. H85 likely does so by H-bonding the water ligand displaced during substrate binding and stabilizing the pentacordinate Fe\textsuperscript{3+} high spin (HS) species. Stabilizing this water then enhances the rate of activation H\textsubscript{2}O\textsubscript{2} by freeing up a site on the heme for the H\textsubscript{2}O\textsubscript{2} to bind and stabilizing the Fe\textsuperscript{3+}-OOH adduct through H-bonds, evidenced by two orders of magnitude reduction in H\textsubscript{2}O\textsubscript{2} on-rate observed in the mutants incapable of H-bonding. There is no influence on hydrogen atom transfer (HAT) by Fe\textsuperscript{4+}=O(por●\textsuperscript{+}) by H85. The rate variances seen H85Q and H85F with C20:0 FA are within the same range observed in WT with various different chain length FAs. HAT is understood to be a concerted electron-proton tunneling process and is extremely sensitive to the tunneling distance, which means sub-Ångström changes in the C-H to Fe=O distance can significantly alter the transfer rate. While not observable by transient kinetics, this work does show that H85 has a role in determining the
Figure 3.25  Overview influence of H85 on the OleT catalytic cycle. Starting in the top left. H85 stabilizes the water ligand displaced by fatty acid binding by H-bonding. This enables the binding of H₂O₂ to the heme and the generation of primary oxidant Fe⁴⁺=O(por•⁺). H85 then stabilizes the Fe⁴⁺-OH species preventing rebound and allowing for proton coupled electron transfer to oxidize the substrate β carbon radical to carbocation. The carboxylate then leaves as CO₂ and the alkene product is formed.

final reaction outcome through thorough accounting of all products via gas chromatography with a variety of FAs. Shorter FAs seem to have greater mobility in the active site and impair alkene production by changing the position of the initial HAT by Fe⁴⁺=O(por•⁺), as decarboxylation to the alkene can only occur following HAT from the β carbon. Following β HAT, the mutant series shows H-bonding to the 85 position, but not necessarily histidine, is essential to efficient decarboxylation. This likely due to the formation of a water bridge between Fe⁴⁺-OH and position 85, which immobilizes the hydroxo. The normal P450 oxygen rebound process is a heavy atom rearrangement and necessarily collisional,
whereas the substrate oxidation for decarboxylation is an electron transfer process and can occur at distance by tunneling. With this understanding the decarboxylation pathway is likely selected for in OleT purely by physically separating the substrate radical from hydroxo. This relies both highly immobilized substrate and hydroxo.

From our previous work\textsuperscript{27} we showed that the decay product of Fe$^{4+}$-OH is the low spin Fe$^{3+}$-OH$_2$ species, normally thought of as the resting state for P450s. In WT OleT this process demonstrated a pH dependence implying a proton coupled mechanism either “proton gated” proton transfer electron transfer (PTET) or electron-proton transfer (EPT). However, in H85Q this pH dependence is largely gone, raising the possibility the process may switch to “electron gated” electron transfer proton transfer (ETPT). Using solvent isotope effect (SIE) and proton inventory techniques we ultimately demonstrate that a proton is involved in the rate limiting step in the decay of Fe$^{4+}$-OH in both WT and H85Q, precluding the ETPT mechanism. In addition, the measured thermodynamic parameters of Fe$^{4+}$-OH decays in WT and H85Q are completely fungible, suggesting they are the same mechanism. The pH dependence of the Fe$^{4+}$-OH on closer inspection is sigmoidal indicating a change at titratable site and not a collisional process, eliminating a PTET mechanism. The loss of this pH effect indicates this titratable site is the H85 residue itself. Based on the proton inventory results, protonating H85 perturbs the stabilizing H-bonding to hydroxo (Fig. 3.26), but no more so than H85Q mutation. The H85Q mutation, however, does not cause the same increase in decay rate.
While this data makes it clear that this decay process is EPT, it is somewhat enigmatically versatile against active site alterations, perhaps suggesting that is it not a finely tuned process. The thermodynamic studies indicate a large energy barrier, $\sim 18$ kcal/mol, to this decay. Effectively this could be thought of as a 780mV overpotential on a one electron oxidation that is energetic favorable by $\sim 800$mV$^{28-29,62}$.

This high barrier highlights the importance of maintaining separation between carbon radical and the hydroxo in the decarboxylation pathway (Fig. 3.27). Given the Fe$^{4+}$-OH decay is EPT the driving force is bond dissociation energy (BDE) of the formed O-H bond. The strength of which is given by the formula $\text{BDE} = 23.06 \text{ kcal/mol} \times E^0 + 1.37 \text{ kcal/mol} \times pK_a + 57 \pm 2 \text{ kcal/mol}^{63}$. Given an $E^0$ of 800mV for Fe$^{4+}$-OH by Groves$^{28-29}$ and $pK_a$ of $\sim 10$ from our previous work$^{27}$ this BDE is $\sim 89$ kcal/mol. Oxidation of carbon radical to carbocation takes $\sim 57$ kcal/mol$^{62}$. Though this energetically favorable by 32kcal/mol, the high barrier makes this process non-competitive against oxygen rebound, which radical clock

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**Figure 3.26** Schemes of the water bridged hydrogen bonding between position 85 and the Fe$^{4+}$-OH. Left is deprotonated histidine with a hydrogen bond to the peptide backbone. Center is the protonated histidine, which has rotated due to steric clash. Right is the H85Q mutation.
Figure 3.27 Energy diagram of the two competing decay pathways for Fe⁴⁺-OH, rebound (solid line), and proton coupled electron transfer, dotted line. Rebound is highly favorable.

studies in OleT have suggested it has a negligible energy barrier. There is no way to rationalize OleT favoring decarboxylation if the face of clear kinetically and thermodynamically favoring hydroxylation unless it is simply not possible due to the substrate and the hydroxo being unable to collide. In the case of C20:0 FA the stability of the substrate itself is sufficient and the mobility of the hydroxo irrelevant. There is nothing about the active site itself that determines the chemistry, but rather just the substrate positioning. The substrate must be positioned to favor β-
HAT, but be far enough way that none of the Fe$^{4+}$-OH vibrational modes can touch it. Given this must also be close enough for HAT to occur it is likely a very narrow range of functional distances (Fig. 3.28).

![Scheme for substrate and H85 driven mechanistic switch between β-hydroxylation and decarboxylation.](image)

**Figure 3.28** Scheme for substrate and H85 driven mechanistic switch between β-hydroxylation and decarboxylation. As long as the β carbon radical is outside the rebound frontier, shown at 2 Å from the oxygen, the slower electron transfer process will drive the reaction toward decarboxylation. Initial HAT however, requires the β carbon to be close enough to tunnel the Hydrogen off, shown in the figure as 4Å.

### References


15. Eser, B. E.; Das, D.; Han, J.; Jones, P. R.; Marsh, E. N., Correction to oxygen-independent alkane formation by non-heme iron-dependent


CHAPTER 4

CHARACTERIZATION OF OLET SELF-OXIDATION PATHWAY:
GENERATION OF SUBSTRATE FREE COMPOUND-I

Abstract

The ability to accumulate high yields of the ferryl-oxo pi-cation radical species, known as Compound-I (Cpd-I), in P450 OleT should not be possible. There are two redox-active amino acids, tyrosine 59 (Y59) and tryptophan 149 (W149), within 6 Å of the heme, which should rapidly quench Compound-I to the ferryl-hydroxo Compound-II (Cpd-II). In this work, we demonstrate a new method to generate Compound-I in OleT in a substrate-independent manner using hypochlorite (NaClO), and we leverage this to study the self-oxidation of the OleT peptide. Through pH, solvent isotope and mutagenesis studies, we have found that OleT preferentially oxidizes Y59 but is also capable of oxidizing W149. Our studies suggest that such oxidations are unfavorable due to high activation energy barriers of 10-15 kcal/mol compared to the measured 6 kcal/mol barrier of hydrogen atom transfer (HAT) from the substrate. This provides evidence that non-productive self-oxidation of the peptide is selected against by some mechanism, which may be one reason for the stability of Cpd-I in OleT. The simplest explanation from Marcus theory suggests that ΔG and λ
are optimized near zero for HAT, such that any off-path with will result in prohibitive thermodynamic barriers.

Introduction

The ferryl-oxo pi-cation radical intermediate in the P450 catalytic cycle, known as Compound-I (Cpd-I), has been nicknamed “nature’s blowtorch” due to its ubiquitous capacity to oxidize a variety of substrates and functionalize extremely inert C-H bonds\(^1\). The ability of Cpd-I to perform these chemistries in peptide frameworks poses a variety of intriguing questions. C-H bonds have dissociation energies (BDE)s of \(\sim 100\ \text{kcal/mol}\), and based on multiple measurements in comparable ferryl-oxo species\(^2-4\), Cpd-I has been estimated to have an oxidizing potential of only \(\sim 1.4\ \text{V}\), which should not be strong enough to cleave a C-H bond. The canonical explanation is that Cpd-I breaks C-H bonds by hydrogen atom transfer (HAT),\(^5\) a specific case of proton-coupled electron transfer (PCET) in which the electron and proton originate from and terminate in the same orbitals\(^6\). In PCET, additional driving force stems from the acid/base equilibrium of the oxidant. For Cpd-I in hydrogen transfer reactions, this correlates to BDE of the formed O-H bond, given by the (equation 4.1)\(^7\) in units of kcal/mol. \(E_{Cpd-I}^0\) is the oxidation potential of Cpd-I in volts, and \(pK_{a_{Cpd-II}}\) is the equilibrium constant of the one-electron reduced species, the ferryl-hydroxo (Cpd-II). The final term is a correction factor for the difference between the zero-point energy and the
arbitrary zero (standard hydrogen electrode) used in electrochemistry measurements.

\[ D(O - H) = 23.06 \times E_{cpd-I}^{0} + 1.37 \times pK_{cpd-II} + 57 \pm 2 \]

In the case of Cpd-I of thiolate-ligated hemes, such as P450s, the high basicity of the corresponding Cpd-II species\(^3\)\(^8\) increases its effective oxidation potential \(\sim 700\) mV for PCET reactions. Even with the assistance of the proton couple, it has been theorized that C-H bond cleavage by Cpd-I is endergonic\(^9\) as the BDE of the formed O-H bond may still be lower than that of the C-H bond that is cleaved. PCET has also been invoked in explaining an even greater conundrum involving Cpd-I. How is it that “nature’s blowtorch” does not simply incinerate the peptide scaffold of the enzyme? PCET sequesters part of the driving force for the catalytic reaction coordinate, allowing Cpd-I to perform its chemistry with a weaker oxidant than it would otherwise be necessary. In the absence of the proton couple, Cpd-I would have to have a potential of \(\sim 2.2\) V to break unactivated C-H bonds. To extend the blowtorch metaphor, PCET in Cpd-I serves a similar role as a pure oxygen stream used by a cutting torch. Blowtorch flames are not hot enough to melt steel as that would damage the torch. Instead, the metal is actually cut by the combination of heat from flame and the excess oxygen rapidly rusting metal into slag.

In addition of PCET, it has been widely theorized that chains of redox active amino acids, particularly tryptophan and tyrosine, may serve a protective role in oxygenase enzymes\(^10\) such as P450s. In the event that the oxidant is
generated without substrate present, these redox chains can channel oxidizing equivalents from the active site to the outside of the enzyme to dissipate the oxidizing species. This useful in nature to protect the peptide, but stymies the accumulation Cpd-I high yield *in vitro*. There have been decades of failed attempts to isolate Compound I in rapid mixing studies of substrate free CYPs with oxidants such as meta-chloroperoxybenzoic acid (mCPBA), which often result in isolation of a ferryl intermediate analogous to Compound II and a protein radical (e.g. tyrosine)\textsuperscript{8, 11-12}. In our previous work with P450 OleT, however, we have demonstrated the ability to generate high yields of Cpd-I\textsuperscript{13} despite multiple adjacent redox active residues to the heme. Figure 4.1, below, shows the distribution of tryptophan and tyrosine residues in P450 OleT, color coded for distance to the heme.
Figure 4.1 The structure of P450 OleT (PDB ID: 4L40) with the tryptophan and tyrosine residues highlighted and color coded based on proximity to conjugated portions of the heme. Warm colors are closer, cool colors are further away.

Table 4.1 details each residues distance to the heme based on crystal structure 4L40\textsuperscript{14}. Using the Moser-Dutton ruler (equation 4.2)\textsuperscript{15-16} and the assumption that $\Delta G^0 = -\lambda$ (that the free energy of the reaction matches the reorganizational energy) and $\beta = 1.4$ (a common dielectric constant for proteins), the electron transfer rate can be simply predicted based on the donor-acceptor distance $R$. 

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Due to the proximity of these residues, particularly Y59 and W149, the predicted half-life of Cpd-I in OleT should be in the nsec to fsec range. These rates are based on the assumption that the system is perfectly tuned for electron transfer ($\Delta G = -\lambda$), and hence reflect an upper limit on the rate of protein self-oxidation. Indeed, high extremely high electron transfer rates are commonplace in many proteins\textsuperscript{15-16} including P450s\textsuperscript{8, 11-12}, as well as bio-inspired model complexes\textsuperscript{17}. The observed reaction half-life of Cpd-I in OleT with inert C-D bonds is \textasciitilde40 msec\textsuperscript{13}, which is orders of magnitude than these predictions. OleT Cpd-I is approximately one million-fold more stable than it should be based on the experimental literature and about ten billion-fold more stable than it should be in theory. Simply, OleT should not be able to metabolize substrates given these considerations alone. There is something perturbed about the Cpd-I in OleT, and perhaps other CYPS, which enhances its stability in presence of redox active amino acids.

In this work, we determine a method to generate Cpd-I in OleT without a prebound substrate, overcoming the limitations of substrate dependence of our

<table>
<thead>
<tr>
<th>Residue</th>
<th>R (Angstroms)</th>
<th>$k_{ET}$ at $\Delta G = -\lambda$</th>
<th>$t_{1/2}$ at $\Delta G = -\lambda$</th>
</tr>
</thead>
<tbody>
<tr>
<td>TRP 149</td>
<td>4.4</td>
<td>2.29E+12</td>
<td>440 fsec</td>
</tr>
<tr>
<td>TYR 59</td>
<td>5.5</td>
<td>5.01E+11</td>
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<td>TYR359</td>
<td>8.3</td>
<td>1.05E+10</td>
<td>95 psec</td>
</tr>
<tr>
<td>TYR 289</td>
<td>10</td>
<td>1.00E+09</td>
<td>1 nsec</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>----</td>
<td>----</td>
<td>----</td>
<td>----</td>
</tr>
<tr>
<td>TRP 369</td>
<td>10.3</td>
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<td>2 nsec</td>
</tr>
<tr>
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<td>2.19E+08</td>
<td>5 nsec</td>
</tr>
<tr>
<td>TYR 135</td>
<td>11.1</td>
<td>2.19E+08</td>
<td>5 nsec</td>
</tr>
<tr>
<td>TYR 405</td>
<td>11.4</td>
<td>1.45E+08</td>
<td>7 nsec</td>
</tr>
<tr>
<td>TYR 321</td>
<td>12.1</td>
<td>5.50E+07</td>
<td>18 nsec</td>
</tr>
<tr>
<td>TYR 110</td>
<td>12.2</td>
<td>4.79E+07</td>
<td>20 nsec</td>
</tr>
<tr>
<td>TRP 223</td>
<td>12.9</td>
<td>1.82E+07</td>
<td>55 nsec</td>
</tr>
<tr>
<td>TRP 360</td>
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<td>1.20E+07</td>
<td>83 nsec</td>
</tr>
<tr>
<td>TYR 288</td>
<td>13.2</td>
<td>1.20E+07</td>
<td>83 nsec</td>
</tr>
<tr>
<td>TRP 119</td>
<td>13.9</td>
<td>4.57E+06</td>
<td>220 nsec</td>
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<td>TRP 344</td>
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<td>1.00E+06</td>
<td>1 μsec</td>
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<tr>
<td>TRP 330</td>
<td>15.6</td>
<td>4.37E+05</td>
<td>2 μsec</td>
</tr>
<tr>
<td>TRP 196</td>
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<td>1.91E+05</td>
<td>5 μsec</td>
</tr>
<tr>
<td>TYR 279</td>
<td>16.3</td>
<td>1.66E+05</td>
<td>6 μsec</td>
</tr>
<tr>
<td>TYR 23</td>
<td>16.9</td>
<td>7.24E+04</td>
<td>14 μsec</td>
</tr>
<tr>
<td>TYR 226</td>
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<td>14 μsec</td>
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<tr>
<td>TYR 218</td>
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<td>TYR 381</td>
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<td>2.63E+03</td>
<td>380 μsec</td>
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<tr>
<td>TYR 183</td>
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<tr>
<td>TYR 388</td>
<td>20.3</td>
<td>6.61E+02</td>
<td>2 msec</td>
</tr>
<tr>
<td>TYR 277</td>
<td>20.8</td>
<td>3.31E+02</td>
<td>3 msec</td>
</tr>
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</table>

previous $\text{H}_2\text{O}_2$-based method and allowing for direct observation of self-oxidation of the peptide by Cpd-I. Using site-directed mutagenesis, transient kinetics and thermodynamics measures, the amino acid juxtaposed to the heme that is the main target of self-oxidation has been identified. Additionally, pH and solvent isotope studies have probed the importance of protons to the oxidation process. We find, unsurprisingly, that OleT can competitively oxidize several positions of the peptide and that the process has a considerable thermodynamic barrier.
compared to HAT from the substrate. We hypothesize that Cpd-I is inhibited from the non-productive reaction coordinate through large solvent reorganizational energies.

**Methods**

**Reagents**

Molecular biology reagents were purchased from Research Products International (Mount Prospect, IL). Potassium phosphate dibasic (K$_2$HPO$_4$) was purchased from BDH Chemicals. 30% W/V hydrogen peroxide (H$_2$O$_2$) and 10-15% a.c. sodium hypochlorite (NaClO) were sourced from Sigma-Aldrich (St. Louis, MO).

The concentrations of the oxidants were determined by dilution in deionized water and measurement of the absorbance with an Agilent 8453 Spectrometer. The molar extinction coefficients used were 350 M$^{-1}$cm$^{-1}$ at 292 nm for NaClO and 43.6 M$^{-1}$cm$^{-1}$ at 240 nm for H$_2$O$_2$. NaClO stocks were used immediately after dilution and characterization.

Meta-chloroperoxybenzoic acid 77% w/w was purchased from Sigma Aldrich and purified to 99% purity by recrystallization.$^{18}$ Peracetic acid (PAA; CH$_3$COOOH) was synthesized from H$_2$O$_2$ and acetic acid (CH$_3$COOH) from Sigma reagents by preparing a solution of ~10% w/v H$_2$O$_2$, ~30% v/v CH$_3$COOH, 1% v/v hydrochloric acid (HCl) and refluxing at 50 °C for four hours, followed by stored at 4 °C. The yield of PAA was determined by iodometric titration.
Perdeutrated eicosanoic acid \((\text{C}20:0-\text{D})(\text{C}19\text{D}_{39}\text{COOH})\) 99.5\% D was purchased from CDN Isotopes (Pointe-Claire, QC). Deuterium oxide (D\(_2\)O) 99.9\% D was purchased from Cambridge Isotope Laboratories (Andover, MA).

**Expression and Purification of OleT**

OleT was overexpressed in *E. coli* and purified using the same methodology as in our previous work\(^{13}\) with one alteration to the procedure. Following the elution from the Ni-NTA column, the protein was salt cut to 30\% saturated ammonium sulfate ((NH\(_4\))\(_2\)SO\(_4\)) and bound to a butyl-sepharose column. OleT was then eluted from the butyl-sepharose column with a gradient from 30\% saturated (NH\(_4\))\(_2\)SO\(_4\) + 200 mM K\(_2\)HPO\(_4\) pH 7.6 to 10\% v/v glycerol (C\(_3\)H\(_5\)(OH)\(_3\)) + 200 mM K\(_2\)HPO\(_4\) pH 7.6. Purity and yield were determined spectroscopically using the absorbance ratio of 280 nm to 418 nm. High purity fractions were pooled and dialyzed against 200 mM K\(_2\)HPO\(_4\) pH 7.6 overnight at 8 °C then concentrated, flash frozen with liquid nitrogen and stored at -70 °C.

**Mutagenesis**

The Y59H and W149F mutants were prepared by standard site-directed mutagenesis techniques in the expression-optimized version of the P450 OleT gene from DNA 2.0 (Newark, California). Primers used in mutagenesis are below:
Table 4.2 Mutagenesis Primers

<table>
<thead>
<tr>
<th>Prime</th>
<th>Forward</th>
<th>Reverse</th>
</tr>
</thead>
<tbody>
<tr>
<td>Y59H</td>
<td>5' GAGATGTTCCATAAACGATGTCGTTCAACGCGAGGGGTATG 3'</td>
<td>5' GAGATGTTCCATAAACGATGTCGTTCAACGCGAGGGGTATG 3'</td>
</tr>
<tr>
<td>W149F</td>
<td>5' CCCCTCACGCACCACCCAGCCGAAAGCTATCG 3'</td>
<td>5' GTGCGTGGAAGGCTATAAGGCGAAAGAAGCGC3'</td>
</tr>
</tbody>
</table>

**Electron Transfer Rate Calculations**

The rates of electron transfer in were calculated by first measuring the distance between every tryptophan and tyrosine in OleT to the heme in the crystal structure 4L40 \(^\text{14}\). The distances taken correspond to the shortest heavy atom to heavy atom distance in the conjugated portion of each molecule, i.e. the heme macrocycle and vinyl groups were included, but not the carboxylates on the propionate arms. The distances were then input to the Moser-Dutton equation (equation 4.2) \(^\text{15-16}\). The assumption \(\Delta G = -\lambda\) (that the free energy of the electron transfer matches the reorganizational energy) was used, which corresponds to a perfectly tuned electron transfer (i.e. zero activation energy). The \(\beta\) term was set to 1.4, a commonly accepted value for proteins, which corresponds to the dielectric composition of the gap between the electron donor and acceptor.

\[
\log_{10} k_{ET} = 13 - \frac{\beta}{2.303} \times (R - 3.6) - \gamma \frac{(\Delta G + \lambda)^2}{\lambda}
\]
Stopped-Flow

All kinetic measurements were collected on an Applied Photophysics (APP) (Surry, UK) SX20 stopped-flow spectrophotometer. The same methodology was used as in previous studies of OleT\textsuperscript{13,19} (Chapter 4). In general, OleT and an oxidant were prepared in the same buffers and loaded into separate syringes in the SX20 sample handling unit (SHU). The SX20 used in this work is equipped with a 20 μL cuvette and 150 μL stop-syringe and can achieve mixing times of approximately 1 msec. Measurements were collected with both a photodiode array (PDA) and photomultiplier tube (PMT) attachments. Unless noted otherwise all measurements were collected at 4 °C in 200 mM K\textsubscript{2}HPO\textsubscript{4} at pH 7.6.

Kinetic Fitting and Analysis

Single wavelength kinetic traces collected with PMT were fit in APP Pro-Viewer to a summed series of exponential decays, using the smallest number of terms possible of adequate fitting. Full spectrum kinetic data collected with the PDA were truncated at 300 nm, to eliminate high amplitude noise from the saturated UV region. The truncated data were analyzed via singular value decomposition (SVD) and global analysis in APP Pro-KIV. To deconvolute the spectra of the pure species, a kinetic model based on the single wavelength kinetics was built in APP Pro-KIV. Full detail of this methodology can be found in our previous work (Chapter 4). The model used in this work, (equation 4.3) adds
a rate to bleaching of enzyme (BE) to account for the slow destruction of the enzyme by NaClO.

\[ Cpd - I \overset{k_1}{\rightarrow} Cpd - II \overset{k_2}{\rightarrow} LS \rightarrow BE \]

**pH and D\textsubscript{2}O Experiments**

To study the effects of solution pH and D\textsubscript{2}O on peptide oxidation by Cpd-I, substrate-free enzyme was reacted with 10 mM (post-mix) NaClO on the stopped-flow. For both pH and D\textsubscript{2}O studies the enzyme was equilibrated overnight in the target buffer. These samples were prepared from ~200 μM stocks of enzyme diluted ten-fold into 200 mM K\textsubscript{2}HPO\textsubscript{4} buffers at the target pH in H\textsubscript{2}O or D\textsubscript{2}O (for D\textsubscript{2}O samples this makes the isotopic substitution 91%). All the samples were then sealed in 1.5-ml centrifugal tubes, placed on a rocker at 8 °C overnight and then centrifuged at 14,000 r.c.f. for 5 minutes at 8 °C. Oxidants were prepared fresh the same day as data collection in a paired fashion with 10x stocks diluted into the target condition.

Determination of reaction order of H\textsuperscript{+} in Cpd-I decay was performed by taking \(\log_{10}\) of equation 4.4 and substituting –pH for \(\log_{10}[H^+]\) (equation 4.5). The inverse of the slope of a plot of \(\log_{10}(k)\) vs. pH is the order of the reaction.

\[ k = K[H^+]^n \]

\[ \log_{10}(k) = \log_{10}(K) + -n \ast pH \]
Thermodynamic Experiments

For studies of substrate hydrogen atom transfer (HAT), an enzyme substrate complex (ES) was formed with OleT and perdeuterated eicosanoic acid (C20:0-D) using established methodology\textsuperscript{13} and rapidly mixed on the stopped-flow with 5 mM (post-mix) H\textsubscript{2}O\textsubscript{2}. This reaction was repeated at a series of temperatures from 2 to 28 °C. For the peptide oxidation studies, substrate-free enzyme (E) was mixed on the stopped-flow with 10 mM (post-mix) NaClO and sampled across the same temperature regime. Both oxidants were prepared at the target pH by same method as above. The activation energy (G\textsuperscript{‡}) and the barrierless reaction rate (A) were determined empirically using the Arrhenius equation (equation 4.6) by plotting ln(k) of the growth phase at 440 nm against 1/T and fitting the data using simple linear regression (equation 4.7).

\begin{equation}
4.6 \quad k = A \times e^{G^\ddagger \over RT}
\end{equation}

\begin{equation}
4.7 \quad \ln(k) = \frac{G^\ddagger}{R} \times \frac{1}{T} + \ln(A)
\end{equation}

Results

Substrate Free Compound-I

The activation of H\textsubscript{2}O\textsubscript{2} to form the ferryl-oxo species Compound-I (Cpd-I) is a well-studied reaction in several heme enzymes, from the original Cpd-I isolated in horseradish peroxidase \textsuperscript{18}, chloroperoxidase \textsuperscript{3, 20} and P450\textsubscript{BSβ} \textsuperscript{21}, a fatty acid peroxygenase and close analogue of P450 OleT. This mechanism is understood to require an acid-base pair motif in the active-site, but in the case of
P450 OleT and other CYP152s, e.g. P450BSβ, the acid residue is absent and the carboxylate group on the fatty acid substrate fills this role in catalysis. This mechanism makes generating Cpd-I in substrate-free OleT (E) for characterizing self-oxidation impossible with H₂O₂. Previously, we have attempted to circumvent this through introduction of a carboxylate into the active-site of OleT (P246D) to enable substrate free H₂O₂ activation. This mutant enables fatty acid free catalysis of non-native substrates by OleT (e.g. alkanes), but is not efficient enough to drive the accumulation of Compound I required for detection in transient kinetic studies.

A common tactic to generate Cpd-I in P450s has been to utilize activated alkyl peroxides such as peracetic acid (PAA) and meta-chloroperbenzoic acid (mCPBA). Cpd-I can also reversibly oxidize halide ions to the corresponding hypohalite acids. This reaction has been demonstrated with hypochlorite (NaClO) to generate high yields of Cpd-I in ubiquitous peroxynase (UPO), a thiolate ligated heme that is in some ways (primarily ligation sphere) similar to P450.

In this work, we screened several oxidants (PAA, mCPBA and NaClO) to generate Cpd-I in substrate free OleT. Neither PAA (Fig. 4.2) nor mCPBA (Fig. 4.3) generated Cpd-I. However, the addition of NaClO generated small but readily detectable amounts of Cpd-I. After optimization of the reaction through alteration of the pH, ~50% accumulation of Cpd-I was achieved (Fig. 5.4). This Cpd-I species rapidly decays in under a second, likely due the autoxidation of an active site amino acid.
Figure 4.2 UV-Vis spectra of OleT collected over 1 sec after rapid mixing with PPA on a stopped-flow. There is no spectral change.

Figure 4.3 UV-Vis spectra of OleT collected over 1 sec after rapid mixing with mCPBA on a stopped-flow. There is minor bleaching of the Soret band, but no meaningful spectral change.
Figure 4.4 UV-Vis spectra of OleT collected over 1 sec after rapid mixing with NaClO on a stopped-flow. The initial spectrum, in red, has a Soret peak 370 nm and small peak at 690 nm indicative of Cpd-I. This initial species undergoes complex decay over one second to the ferric resting state of the enzyme.

**Confirmation of Cpd-I by SVD**

The raw stopped-flow spectra obtained from mixing OleT with NaClO (Fig. 4.4) clearly indicate the accumulation of an optical species that resemble P450 Cpd-I with aSoret absorption at 370 nm and low intensity peak at 690 nm\(^{13,23,25}\), indicative of a porphyrin pi-cation radical. The initial spectrum appears to be an add-mix of species, which ultimately decays to the ferric low-spin (LS) resting state of the enzyme over the course of one second. To further confirm the identity of this species, the pure spectrum of was isolated via singular value decomposition (SVD) and global analysis methods.
Single wavelength kinetic fitting of the Soret maximum of Cpd-I (370 nm) in OleT + NaClO shows biphasic decay process with a fast phase (~100 sec\(^{-1}\)) that comprises the majority of the amplitude and a slow phase (~15 sec\(^{-1}\)) with a diminished amplitude (Fig. 4.5). This is markedly similar to the kinetic behavior observed previously in OleT (ES) + H\(_2\)O\(_2\), where the slower phase was ascribed to a contribution from the hyper-porphyrin absorption of the ferryl-hydroxo species (Cpd-II). This suggests that OleT similarly accumulates Cpd-II in the substrate-free reaction. Previously we established the wavelength of the maximum difference in absorption between Cpd-II and LS is at 440 nm. For OleT + NaClO, there is rapid growth phase (~100 sec\(^{-1}\)) and slow decay phase (~15 sec\(^{-1}\)) (Fig. 4.5), again similar to what is observed with OleT (ES) + H\(_2\)O\(_2\). The matched rates of decay at 370 nm and the growth at 440 nm are indicative of the direct decay of Cpd-I into Cpd-II without intervening intermediate species\(^{13, 19}\). The apparent rates measured at these two wavelengths remained matched when the reaction was performed in D\(_2\)O despite a change in overall rate to X s\(^{-1}\), indicating that they did not only coincidentally match (Fig. 4.5).

SVD approximation of OleT + NaClO spectra under various conditions always required three non-zero singular values, indicating a complex decay behavior involving anywhere from 4 to 6 different optical species. Single wavelength fitting indicates a sequential process with at least two intermediates species, which may be assigned Cpd-I and Cpd-II. The simplest explanation for the further kinetic complexity of the spectrum is from the slow bleaching of the heme chromophore by NaClO. For global fitting, equation 4.5 was utilized which
assumes a unidirectional decay of Cpd-I to Cpd-II to the LS resting state, followed by slow bleaching of the enzyme. The decay rate of Cpd-I ($k_1$) was initially set at $100 \text{ sec}^{-1}$ and the Cpd-II decay rate ($k_2$) at $10 \text{ sec}^{-1}$. Rate of the enzyme bleaching ($k_3$) was estimated at $1 \text{ sec}^{-1}$. These rates were allowed to float during global analysis fitting, and the pure spectra of the intermediates were extracted (Fig. 4.6-A). The reconstructed data from SVD (Fig. 4.6-D) accurately recreate the raw data (Fig. 4.6-C).

The pure spectra of the intermediates isolated by SVD (Fig. 4.6-A) generally agree with prior previous isolations of Cpd-I (Fig. 4.7) and Cpd-II (Fig. 4.8). The Cpd-I species has the signature 690 nm band and 370 nm Soret band. There are minor additional bands in OleT + NaClO spectrum that are not in the previous (ES) OleT + H$_2$O$_2$ spectrum. There is small additional absorbance 405 nm which may correlate an add-mix of a tyrosine radical absorbance into the Cpd-I spectrum. A band at ~620 nm is also observed. This band was been observed in substrate free preparations of Cpd-I using CYP152 + mCPBA by Green and colleagues$^{23}$. The short wavelength features of Cpd-I are understood to arise from blue-shifted Q-bands from the resting state$^{26}$, which are known to be sensitive to the local environment of the heme; ~620 nm peak may be a general feature of substrate free Cpd-I.

The overlay of OleT + NaClO Cpd-II and (ES) OleT + H$_2$O$_2$ (Fig 4.8) shows several variations between the spectra. OleT + NaClO Cpd-II retains the hyper-porphyrin peak, however, it is slightly red shifted to 375 nm. The Soret band is also
Figure 4.5. The left traces are at 370 nm the right traces are 440 nm under paired conditions. Residuals from the fitting are plotted below each trace. The kinetic terms of fitting 370 nm and 440 nm are matched in H$_2$O and in D$_2$O.
Figure 4.6 SVD analysis of OleT + NaClO at pH (formally pD) of 6.22. Panel A is the pure spectra of Cpd-I, Cpd-II and LS (green, red and blue throughout, respectively). Panel B is percent composition of each of the individual species over time based on the kinetic model built in from single wavelength fitting. Panel C is the raw data with the highest accumulation of each species highlighted in its respective color. Panel D is the SVD reproduction of the raw data with the highest accumulation of each species highlighted as in Panel C. The residuals between the raw the SVD plotted below.

slightly red shifted to 430 nm. The most distinguishing feature between the preparations is in the Q-bands. The OleT + NaClO Q-bands have far greater amplitude and are slightly blue shifted ($\beta = 532$ nm and $\alpha = 565$ nm). These
variances again resemble spectra of other substrate free preparations of the intermediate. It particularly resembles the protonated Cpd-II, with intense α-β bands and a Soret band significantly more intense than the hyper-porphyrin band, prepared in CYP158. Since Cpd-I is oxidizing the peptide in the absence of substrate, the gaining of a proton to generate Cpd-II implies that the putative oxidation of the peptide is a form of proton coupled electron transfer (PCET). This could mean the additional oxidative force of PCET might not select for hydrogen atom transfer but can also oxidize the peptide.

Figure 4.7 Overlay of Cpd-I in OleT + NaClO (black) and (ES) OleT + H₂O₂ (red). The two spectra are generally in agreement. The additional peak at 620 nm in OleT + NaClO is similar to one observed in other substrate free preparations of Cpd-I.
Determining the Order of the PCET Reaction

The protonation of the observed Cpd-II in the absence of substrate does not necessarily imply that Cpd-I gets additional potential from the proton couple during self-oxidation of the peptide like it does during HAT. There are three different PCET reaction pathways, traditionally shown as a box diagram (Fig. 4.9). The top path is the “proton gated” proton electron transfer (PET), the bottom path is “electron gated” electron proton transfer (EPT), and the middle path is concerted electron proton transfer (CEPT). Only PET and CEPT increase
the effective strength of oxidant for single electron transfers. In PET, the oxidant is protonated first and the protonated form is an inherently stronger oxidant. CEPT is less direct; the driving force is the bond dissociation free energy (BDFE) of the formed –H bond, for which the strength is given by equation 4.1 and which contains a pK_a component. In EPT, the reduced form becomes basic and picks up a proton to equilibrate with solution afterwards. All of the pathways are energetically the same. The difference in the pK_a of the oxidized and reduced forms is related to the difference between in oxidation potential of the protonated and deprotonated forms by equation 4.5, a derivative of the Nernst equation, $\Delta E^0 = n_f \Delta pK_a$.

Pathway the reaction follows is determined by a combination of pH, $\Delta E$ and activation energy ($G^\ddagger$). Experimentally, they can be determined by the pH dependence of the reaction. PET should have a first order kinetic relationship with solvent protons (reaction order $|n|=1$) (Equation 4.4). CEPT may show pH dependence but it should milder than that of PET ($1>|n|>0$). EPT will have no proton dependence ($n=0$).

From the initial optimization of conditions to generate Cpd-I on the stopped flow with NaClO, the self-oxidation reaction was measured under a variety of pH values in either H_2O or in D_2O. The reaction rates were abstracted from the growth phase at 440 nm, formally the accumulation of Cpd-II. The reaction shows pH dependence in H_2O but this dependence is eliminated in D_2O (Fig. 4.10). The self-oxidation reaction of Cpd-I is slower in D_2O and at low pH, and explains why
Figure 4.9 Box diagram of PCET by Cpd-I. Deprotonated Cpd-I (top-left) goes to protonated Cpd-II (bottom-right) through one of three mechanisms. Through a protonated Cpd-I (top-right) (PET), a deprotonated Cpd-II (bottom-left) (EPT) or directly without an intermediate (CEPT).

explains why Cpd-I accumulates to higher levels under these conditions. The lost pH (formally pD) dependence in D₂O may indicate a mechanism switch based on the solvent. The presence this kinetic solvent isotope effect (KSIE) strongly indicates a PCET mechanism. The KSIE k_H/k_D varies from 1.3 to 2.3 over the range of pH values measured. The full effect is likely masked by the mechanism switch that occurs in D₂O, but KSIEs greater than >2 are indicative of the CEPT pathway.
The self-oxidation of OleT by Cpd-I verse pL (H or D) measured at 440 nm (the generation of Cpd-II). The rates in H2O are in black and D2O are in red. The loss of pL dependence in D2O suggests a mechanism switch, and the KSIE indicates H2O involves a CEPT process.

Additional evidence that the decay of Cpd-I in substrate-free OleT is CEPT comes from the mild pH dependence seen in H2O. There is only a ~65% increase in the decay rate over a two order magnitude change in solvent proton concentration. This suggests a value of n less than one. The exact value of n can be found from the fitting slope of a plot log10(k) vs pH (Fig. 4.11) to equation 4.5. In H2O, n = -.09 ± .01 firmly placing it in CEPT range and suggesting the proton couple is only minor component of the driving force in Cpd-I decay. Additionally, the negative sign on n indicates that self-oxidation involves the release of proton
to bulk solvent rather than binding a proton from solvent. In PCET, electron transfer rate increases as the equilibrium driving force from proton couple becomes greater and deprotonation is more favorable under basic conditions. This means the proton couple is portion of the driving force in the self-oxidation process does not come from the protonation of Cpd-I, but derives from the deprotonation of electron donor (i.e. residue that is oxidized).

Figure 4.11 Plot of log$_{10}$(k) verses pL (H or D) of Cpd-I decay in H$_2$O (black solid) and D$_2$O (red dotted). The shallow slope of H$_2$O indicates a CEPT mechanism and the positive slope suggests release of a proton to bulk solvent. The flat plot of D$_2$O indicates EPT in this solution.

The closest redox active amino acids to the heme are Trp149 (4.4 Å) and Tyr59 (5.5 Å). The observation that the amino acid likely releases a proton during
the reaction with Cpd-I hints that Tyr59 is the amino acid that is oxidized. Tyrosine is known to undergo pH dependent PCET, while tryptophan does not \(^7\). This can also explain the observed switch to a pH-independent process in D\(_2\)O. Deuteration of Tyr59 inhibits oxidation of that residue, thus Cpd-I switches to oxidizing Trp149 in a proton-independent manner.

**Thermodynamics of Self-Oxidation**

To determine which active site residue is oxidized, two point mutants W149F and Y59H were made. W149F, however, did not express well (<2mg/L), precluding its study. Y59F was found to generate comparable quantities of Cpd-I with NaClO under the same conditions as wild type (WT). The rate of Cpd-I decay in Y59F of (~80 sec\(^{-1}\)) at pH 6.2 was similar to WT. The rates of the two putative Cpd-I decay mechanisms, self-oxidation of Y59 (H\(_2\)O) and self-oxidation of W149 (D\(_2\)O) are very similar at pH 6.2. CEPT mechanisms are known to have higher thermodynamic barriers relative to EPT, since the sequential mechanism has two smaller barriers instead of one large one. Thus, the two mechanisms could be distinguished based on their activation energy.

To this end, the self-oxidation rates of WT (H\(_2\)O, D\(_2\)O) and Y59H (H\(_2\)O) were measured across a range of temperatures and ln(k) vs 1/T was plotted (Fig. 4.12). The data were then fit using the linear version of the Arrhenius equation (Eq. 4.4). The G\(^\ddagger\) of WT in H\(_2\)O was the highest in the series at 15.6 ± 0.6 kcal/mol. This is consistent with our previous observations of CEPT in OleT (Chapter 4) and in model systems. Both WT in D\(_2\)O and Y59H had smaller
energy barriers, 10.8 ± 0.2 kcal/mol and 11.8 ± 0.9 kcal/mol, respectively. The pre-exponential term (A) for these conditions are also paired (Table 4.3), which indicates they occur via the same process. The fact that Y59H displays the same thermodynamic parameters as the WT enzyme in D₂O confirms our previous conjecture that OleT Cpd-I natively oxidizes Y59 in the absence of substrate.

Table 4.3 Thermodynamic Properties of OleT self-oxidation

<table>
<thead>
<tr>
<th></th>
<th>ΔG‡ (kcal/mol)</th>
<th>A (s⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT H₂O</td>
<td>15.6 ± 0.6</td>
<td>1.7 ± 1.1 x 10¹⁴</td>
</tr>
<tr>
<td>WT D₂O</td>
<td>10.9 ± 0.2</td>
<td>3.4 ± 1.1 x 10¹⁰</td>
</tr>
<tr>
<td>Y59H H₂O</td>
<td>11.8 ± 0.9</td>
<td>1.7 ± 1.4 x 10¹¹</td>
</tr>
</tbody>
</table>

Figure 4.12 Arrhenius plot of the decay of OleT Cpd-I, WT in H₂O (black with squares) and in D₂O (red with circles) and Y59H in H₂O (blue with diamonds). The similarity of WT in D₂O and Y59H suggests that they represent the same
mechanism of EPT and that WT OleT normally self-oxidizes at Y59.

The pre-exponential term A in the Arrhenius equation corresponds to the barrierless rate of reaction and are the same as the rates predicted using the Moser-Dutton ruler (equation 5.1) under the assumption that $\Delta G = -\lambda$ (Table 5.1). The A terms derived for the reaction of Cpd-I for the WT in $D_2O$ (and Y59H) agree with the rate predicted for autoxidation of the W159 and Y59 given their close proximity to the heme-iron. This confirms that the process observed in this experiment is an electron transfer over a 4-5 Å distance. The rate observed with the WT enzyme in $H_2O$, however, is three orders of magnitude faster and more consistent with direct Van der Waals contact between the electron donor and acceptor. H-bonding has been shown to be an excellent conductor of electrons through proteins and Tyr59 is potentially H-bonded directly to one of heme propionate arms in OleT. This could explain the extremely fast A term seen in the Arrhenius data.

**Comparison of the $G^\dagger$ OleT Productive and Nonproductive Pathways**

The decay rates of Cpd-I observed in substrate free OleT (95-160 sec$^{-1}$) are comparable to those observed previously with OleT bound with perdeuterated eicosenoic acid (C20:0-D) ($\sim$80 sec$^{-1}$) $^{13}$. It has been shown that OleT can metabolize C20:0-D to perdeuterated 1-nonadecene. The similar decay rates raise the possibility that the Cpd-I decay observed in C20:0-D bound OleT result from partial oxidation of the peptide, rather than from substrate hydrogen
atom transfer (HAT). Again, these mechanisms again should be distinguishable on the basis of their respective activation energies.

The Arrhenius dependence of Cpd-I decay in C20:0-D bound OleT is clearly different from that of substrate free OleT in H₂O (Fig. 5.13). C20:0-D has a G‡ of 6.2 ± 0.2 kcal/mol and a pre-exponential rate of 2.0 ± 1.2 x 10⁷ sec⁻¹. Both of these terms are far smaller than any observed for peptide oxidation, confirming that the Cpd-I decay observed with a bound substrate does not appear to include oxidation of the protein framework. The pre-exponential rate is several orders of magnitude slower than an electron transfer rate over a similar distance, consistent with assignment as a process involving HAT⁶.

![Arrhenius plot of peptide autoxidation in WT OleT in H₂O (red with circles) and substrate hydrogen atom transfer](image)

Figure 4.13 Arrhenius plot of peptide autoxidation in WT OleT in H₂O (red with circles) and substrate hydrogen atom transfer.
with C20:0-D bound OleT (black with squares). The reaction with perdeuterated substrate around is clearly different from that of peptide autoxidation.

**Discussion**

At the most basic level we have demonstrated that self-oxidation of OleT by Cpd-I is primarily performed on Y59 and involves PCET. The ability of OleT to also oxidize W149 in a non-proton dependent manner additionally indicates that Cpd-I has an oxidizing potential >1.2 V.

Our ability to generate and observe the reaction kinetics of Cpd-I in OleT in both substrate bound and substrate free forms enables us to probe P450 at a level never before possible. The Arrhenius dependences Cpd-I decay under these conditions clearly demonstrate that the reaction in the presence of perdeuterated substrate is different that of self-oxidation observed in substrate free OleT. This is significant because we have previously observed that this Cpd-I irreversibly decays to Cpd-II\textsuperscript{19}, yet HAT by Cpd-I is formally a reversible process, as the hydrogen atom could transfer back to the substrate and regenerate the C-H bond. Quantum mechanical theory calculations suggest Cpd-I breaking the C-H bond is endergonic\textsuperscript{9}, so the reverse rate of HAT back to the substrate ($k_{rev}$) would be faster than Cpd-I taking it off ($k_{for}$). This implies that in the presence of substrate, Cpd-I and Cpd-II should be in equilibrium with each other and favor Cpd-I. The under this model, the reaction coordinate is driven forward by the subsequent and extremely rapid “oxygen rebound” step\textsuperscript{27-28} depleting Cpd-II. Our apparent unidirectional Cpd-I, however, suggests that $k_{for}$
$>> k_{rev}$, and with a conservative estimate that $K_{eq} = 10^3$, this reaction step is exergonic by $\sim 5$ kcal/mol. This accumulation of Cpd-II is expected in self-oxidation since the BDE H-O-Tyr is only 88 kcal/mol, but with clear assignment that the Cpd-I decay, in OleT (ES), is substrate HAT from a 98 kcal/mol bond$^{29}$ means that OleT Cpd-II has a O-H BDE of $\sim 103$ kcal/mol.

The present conclusions about the Cpd-II BDE and direct measurement of the activation energy barriers in this and previous (Chapter 4) works allow us map out the entire free energy reaction coordinate of P450 OleT from the initial generation of Cpd-I (Fig. 4.14). The only unknown term is the $\Delta G$ of oxygen rebound.

The conventional oxygen insertion pathway is in black in Figure 4.14, Cpd-I overcomes a 6 kcal/mol barrier to break a 98 kcal/mol bond and form a new 103 kcal/mol bond in Cpd-II. Oxygen rebound of Cpd-II is a near barrierless process. The decarboxylation reaction pathway bifurcates after HAT by Cpd-I (red dotted line). This process is highly exergonic but has a significant activation energy barrier. The non-productive self-oxidation pathway (green dotted line) similarly is highly exergonic but has a large barrier that results in HAT being kinetically favored in the presence of substrate.

It is apparent that Cpd-I and Cpd-II species are thermodynamically tuned to insert oxygen into substrate and designed to suppress competitive pathways, including self-oxidation or decarboxylation. These alternative pathways both involve solvent proton-coupled oxidations, suggesting that OleT has a
mechanism for discriminating against these reaction pathways. Although formally only describing electron transfers, Marcus theory has been used without modification.

\[
\Delta G^\ddagger = \frac{(G^0 + \lambda)^2}{4\lambda}
\]

Figure 4.14 Reaction energy diagram of P450 OleT post Cpd-I generation. The hydroxylation reaction pathway is in black. The green dotted line is non-productive self-oxidation of the peptide and the red dotted line is the decarboxylation pathway. The hydroxylation pathway, which is the conventional reaction catalyzed by P450s, is the path of least resistance.

for modeling PCET reactions and can be used to understand the thermodynamic barriers to PCET by equation 4.8.
$\lambda$ is the reorganizational energy and is a global term that sums the energy changes in the electron donor/acceptor pair as well as in the surround matrix of peptide and solvent. The energy barrier is minimized under the condition of $G^0 = -\lambda$. Given that $\Delta G$ for substrate HAT is very small, it is rational that $\lambda$ minimization is crucial to catalysis. In normal PCET, solvent contributions to $\lambda$ are largest component; however, HAT in a highly desolvated local environment of the P450 active site would largely abrogate solvent contributions to $\lambda$. Cpd-I is likely tuned that $\Delta G \approx \lambda \approx 0$ for substrate HAT, so that any perturbation from that reaction pathway will rapidly retune the system and generate a large activation energy barrier (Fig. 4.15), even if the off-path is more energetically favorable than the productive path.

Figure 4.15 3D Marcus theory plot of the rate of electron transfer at distance of 5Å. The red band corresponds to the predicted energy for tyrosine oxidation by Cpd-I.
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