JACS
JOURNAL OF THE AMERICAN CHEMICAL SOCIETY
Communication
pubs.acs.org/JACS

Decarboxylation of Fatty Acids to Terminal Alkenes by Cytochrome P450 Compound I

Job L. Grant, Chun H. Hsieh, and Thomas M. Makris*

Department of Chemistry and Biochemistry, University of South Carolina, Columbia, South Carolina 29208, United States

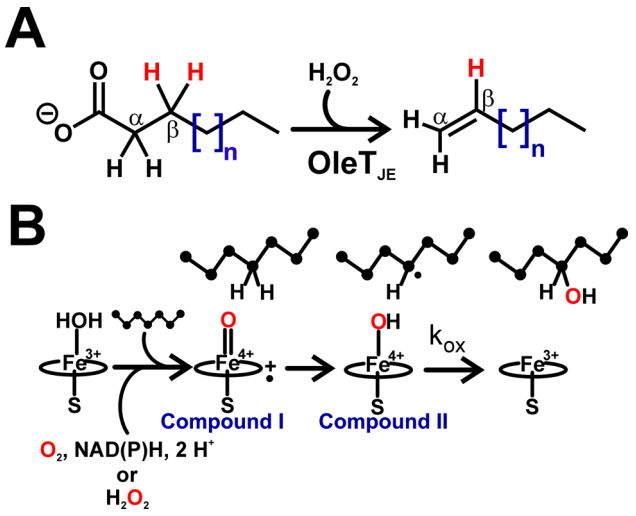
Supporting Information

ABSTRACT: $OleT_{JE}$, a cytochrome P450, catalyzes the conversion of fatty acids to terminal alkenes using hydrogen peroxide as a cosubstrate. Analytical studies with an eicosanoic acid substrate show that the enzyme predominantly generates nonadecene and that carbon dioxide is the one carbon coproduct 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 reactions.

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 many of these pathways 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, nonheme mono-[6] or dinuclear[8,9] iron, or cytochrome P450,[4,5] that typically activates O_2 or H_2O_2 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.

$OleT_{JE}$, a cytochrome P450 from *Jeotgalicoccus* sp. ATCC 8456, metabolizes n chain length fatty acids to produce $n-1$ alkenes, utilizing hydrogen peroxide (H_2O_2) as a cosubstrate in the reaction.[4] The overall reaction, shown in Scheme 1A, involves the loss of a hydride (from the fatty acid beta carbon (C_β)) and production of a one carbon coproduct, which has yet to be identified. Notably, cytochrome P450 enzymes are not typically known to catalyze hydride abstraction chemistry or decarboxylation reactions. Although a few P450s have been implicated in the oxidative decarboxylation of substrates,[10,11] the reactions are often the result of the metabolism of non-native substrates,[12] or are nonenzymatically derived.[13] The canonical mechanism for the P450 catalyzed monooxygenation of unactivated hydrocarbons is depicted in Scheme 1B for comparison. The commonly accepted 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.[14] Subsequent recombination with a substrate radical, in a process termed oxygen rebound,[15] produces an alcohol product and regenerates the ferric resting state of the enzyme.

Scheme 1. Comparison of $OleT_{JE}$ Alkene Production and Prototypical P450 Hydroxylation Reactions



The recent X-ray crystal structure[16] of $OleT_{JE}$ bound to an eicosanoic acid substrate has confirmed the remarkable similarity of its active-site and substrate binding mode to P450 peroxygenases BSβ[17] and SPα.[18] BSβ and SPα do not appreciably produce $n-1$ olefin products, but instead primarily hydroxylate fatty acids at the C_α or C_β position. While providing a rationale for the ability of the enzyme to efficiently utilize hydrogen peroxide, in which the substrate carboxylate serves a general acid that is obligatory for the heterolytic cleavage of H_2O_2,[19] it does not immediately clarify the origin of its capacity for C−C scission.

In order to elucidate the divergence of $OleT_{JE}$ from P450 monooxygenation chemistry, we have characterized its reaction with eicosanoic acid, a chain length which approximates that of

Received: February 23, 2015
Published: April 5, 2015

DOI: 10.1021/jacs.5b01965
J. Am. Chem. Soc. 2015, 137, 4940−4943

the native substrate based on identification of the alkenes found in *Jeotgalicoccus*.[4] Analytical studies have determined that the enzyme generates alkene as the major reaction product and that the one-carbon coproduct of the 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.

The subcloning, heterologous expression, and purification of $OleT_{JE}$ are described in Materials and Methods in the Supporting Information. The 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_2O_2 to initiate turnover and subsequent desalting. This treatment resulted in complete conversion of the enzyme to the low-spin state (Figure S1) and restored its ability to bind exogenously added eicosanoic acid. The titration of the 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 $K_d \approx 0.3$ μM (Figure S2). As a result of this high affinity, a nearly stoichiometric enzyme−substrate (E−S) complex could be generated by poising the enzyme at concentrations well above (typically ≥10 μM) the observed K_d.

Previous multiple turnover studies performed *in vitro* and *in vivo* have established that the reaction of $OleT_{JE}$ with eicosanoic acid and H_2O_2 generates nonadecene.[4,16] Single turnover studies were utilized to directly evaluate the chemoselectivity of the enzyme for alkene formation. An E−S complex was mixed with a 3-fold molar excess of H_2O_2, and the hydrocarbon products of the reaction were extracted. Gas chromatography mass spectrometry (GC-MS) (Figure S3) of the organic phase confirmed formation of nonadecene at a yield of 0.9 ± 0.1 equiv of alkene produced per $OleT_{JE}$, indicating that alkene formation is the major reaction channel. Attempts to detect possible hydroxylated fatty acid minor products through derivatization methods were unsuccessful (Figure S4). The reaction of an E−S complex, prepared instead with a perdeuterated C_{20} substrate ($CD_3(CD_2)_{18}COOH$), produced deuterated nonadecene with comparable yields to the protiated substrate (Figure S5). No appreciable metabolic switching[20] occurs upon substrate isotopic substitution, substantiating the use of deuterated eicosanoic acid for the mechanistic studies described below.

Having demonstrated that the $OleT_{JE}$ reaction with eicosanoic acid produces nonadecene as a major product, we tested for the one-carbon coproduct of the reaction. In analogy to other metalloenzymes, including P450s that perform C−C bond scission, either formate or CO_2 might be anticipated. Headspace FTIR measurements were conducted in an N_2 purged and sealed reaction vial containing $OleT_{JE}$ and excess substrate. The addition of H_2O_2 resulted in the immediate formation of a doublet at 2340 and 2360 cm^{-1} (Figure 1A, blue trace), deriving from the asymmetric stretch of carbon dioxide, which was not observed when the enzyme was omitted from the reaction (Figure 1A, gray trace). In order to more directly assign that the CO_2 produced derived from the cleavage of the fatty acid carboxylate, single turnover reactions were performed with an isotopically labeled substrate in which the terminal carboxylate was ^{13}C labeled ($CH_3(CH_2)_{18}{}^{13}COOH$). GC-MS of the headspace from reactions containing the labeled substrate identified a new signal from $^{13}CO_2$ at m/z = 45 in

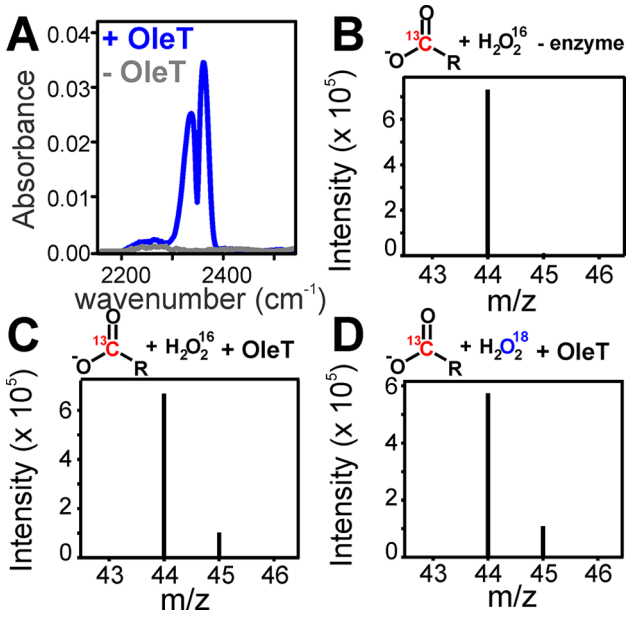


Figure 1. Carbon dioxide production by $OleT_{JE}$. Headspace FTIR spectra of the reaction headspace of an $OleT_{JE}$ multiple turnover reaction with eicosanoic acid and H_2O_2 (A). Mass spectra of the reaction headspace of single turnover reactions with a terminally ^{13}C labeled substrate. A control reaction in which OleT was omitted (B), a reaction containing OleT + $H_2{}^{16}O_2$ (C) and OleT + $H_2{}^{18}O_2$.

addition to a signal at m/z = 44, originating from environmentally derived $^{12}CO_2$ (Figure 1C). The intensity of the $^{13}CO_2$ signal, ~25% of the total (environmental and enzymatically generated CO_2) was significantly larger than that predicted for natural isotopic abundance (1.1%). Significant levels of $^{13}CO_2$ were not observed in reactions in which the enzyme was omitted (Figure 1B), or in reactions containing an unlabeled $^{12}CO_2$ substrate (Figure S6). The amplitude of the $^{13}CO_2$ is unchanged when using $H_2{}^{18}O_2$. Taken together, these results establish that $OleT_{JE}$ performs an oxidative decarboxylation reaction. Oxygen was not inserted into either major reaction product when eicosanoic acid was used as a substrate.

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 that CO_2 is the reaction coproduct, a mechanism involving nucleophilic attack by a ferric peroxide intermediate, such as that postulated for P450 deformylation[21] and alkane synthesis by aldehyde deformylating oxygenase,[9] would seem highly unlikely. Rather, the loss of hydrogen from a relatively unactivated Cβ position and the structural similarity of $OleT_{JE}$ to hydroxylases that presumably utilize a Compound I oxidant suggest that a high-valent intermediate may be involved. Previously advanced mechanistic proposals for $OleT_{JE}$ catalysis have speculated that a Compound I intermediate, if formed, may initiate decarboxylation through hydrogen abstraction from the fatty acid Cβ position,[4] or electron abstraction from the substrate carboxylate.[16] Having established a competent single-turnover system with protiated and deuterated fatty acid substrates, we tested whether such an intermediate could be isolated. A 20 μM $OleT_{JE}$-perdeuterated eicosanoic acid (E-$C_{20}D$) ternary complex was rapidly mixed with excess H_2O_2 at 5 °C in stopped flow absorption studies. Within 15 ms, the high-spin E−S complex (λ_{max} at 392 nm) had completely decayed, and a new intermediate with decreased absorptivity, a

DOI: 10.1021/jacs.5b01965
J. Am. Chem. Soc. 2015, 137, 4940−4943

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

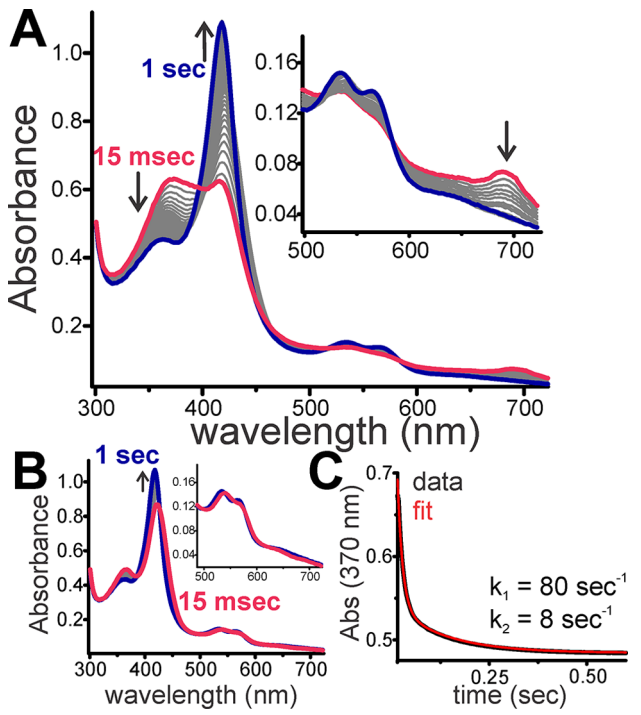


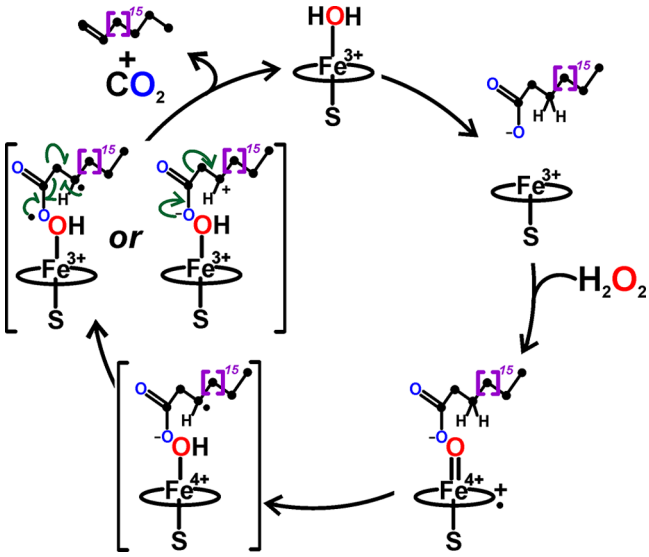
Figure 2. Reaction of $OleT_{JE}$:eicosanoic acid with H_2O_2. A 20 μM E:S complex prepared with a perdeuterated substrate (A) or protiated substrate (B) was rapidly mixed with 5 mM H_2O_2 at 5 °C. (C) Single turnover time course for the decay of Compound I in reactions with perdeuterated eicosanoic acid. A two summed exponential fit (red) is shown superimposed on the data.

of this species are diagnostic of an iron(IV)-oxo π cation radical intermediate (Compound I) and are nearly identical to those observed in rapid mixing studies of a thermostable P450-(CYP119)[14,22] with mCPBA and similarly prepared species in other thiolate-ligated heme enzymes (ex. Aae-APO, CPO).[23,24] The Compound I intermediate could not be formed when the fatty acid was omitted from the reaction (Figure S7). Spectral deconvolution procedures, and comparison to other Compound I species, indicated that this intermediate had accumulated to a high level (>70%) at 15 ms. Within 1 s, 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, indicates a decay rate of 80 s^{-1}. This decay rate of $OleT_{JE}$ Compound I (which we designate hereafter as Ole-I) is invariant to H_2O_2 concentration (Figure S8), 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_2O_2 are shown in Figure 2B for comparison. Rapid decay of the E−S complex was again observed within 15 ms (red 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 attributable to a ^2H 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 Cβ, the position from which it is ultimately lost to generate an alkene. This rules out a previous proposal that the atypical reaction catalyzed by $OleT_{JE}$ may be initiated by abstraction of an electron from the fatty acid carboxylate by Compound I.[16]

The kinetics studies and observed reaction products in this study support a proposed catalytic mechanism for alkene synthesis that is shown in Scheme 2. The mechanistic strategy

Scheme 2. Proposed Catalytic Cycle for $OleT_{JE}$ Alkene Formation Based on Identification of the Carbon Dioxide Coproduct and Stopped-Flow Absorption Spectroscopy



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 π-cation radical intermediates to perform hydride transfer in model systems,[25] 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 $OleT_{JE}$ decarboxylation necessitate a divergence from the monooxygenation reaction coordinate, particularly as oxygen rebound is largely abrogated. One logical route for alkene formation could involve single electron transfer, to Compound II or another oxidant, to produce either an unstable substrate carbocation or a substrate diradical. Subsequent loss of the CO_2 leaving group would generate the $n-1$ alkene. Mechanisms involving the generation of substrate carbocations have been invoked to rationalize the ability for some P450s to catalyze desaturation reactions,[26−28] to generate cationic rearrangement products,[29] including those from radical clock substrates,[30] and to promote C−C bond cleavage during the third step of androgen formation by P450 aromatase.[31]

The apparent conservation of decarboxylase and hydroxylase mechanisms hints toward an elegant adaptation that enables $OleT_{JE}$ 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

DOI: 10.1021/jacs.5b01965
J. Am. Chem. Soc. 2015, 137, 4940−4943

noteworthy in this regard that Ole-I (unlike CYP119-I or AaeAPO-I) can be generated in high yields in a reaction with a prebound deuterated substrate and H_2O_2, 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 ms at 690 nm, we place a lower estimate for its reaction rate with a protiated substrate at $k \sim 300\ s^{-1}$, and a lower bound for an apparent KIE ≈ 3. Semiclassical KIE limits ($k_H/k_D \approx 7$) may position the value for its reaction rate with a protiated substrate higher. In either case, this estimated rate is within the range projected by Green and colleagues[14] for the reaction of CYP119-I and a prebound substrate with similar bond dissociation energy, suggesting that Ole-I is similarly reactive. Instead, the capacity for $OleT_{JE}$ to produce minor aliphatic hydroxylated products[4,32] in reactions with shorter chain length substrates suggests that there may be additional structural factors that are important in steering the enzyme toward alkene production. An evaluation of each of these potential contributions is currently under investigation.

The highly reactive nature of P450-I has limited 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.

■ ASSOCIATED CONTENT

Supporting Information

Description of the cloning, heterologous expression and purification of $OleT_{JE}$, detailed experimental procedures, characterization of alkene products from single turnover reactions, and Figures S1−S8. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*makrist@mailbox.sc.edu

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

We thank M. Walla for assisting with the GC-MS analysis of alkene and gaseous products and M. Myrick for use of the FTIR instrument. This work is supported by startup funds from the University of South Carolina, a Magellan grant to J.L.G., and funding from the U.S.C. Office of Research through the Aspire program to T.M.M.

■ REFERENCES

(1) Kung, Y.; Runguphan, W.; Keasling, J. D. *ACS Synth. Biol.* **2012**, *1*, 498.
(2) Peralta-Yahya, P. P.; Zhang, F. Z.; del Cardayre, S. B.; Keasling, J. D. *Nature* **2012**, *488*, 320.
(3) Schirmer, A.; Rude, M. A.; Li, X.; Popova, E.; del Cardayre, S. B. *Science* **2010**, *329*, 559.
(4) Rude, M. A.; Baron, T. S.; Brubaker, S.; Alibhai, M.; Del Cardayre, S. B.; Schirmer, A. *Appl. Environ. Microbiol.* **2011**, *77*, 1718.
(5) Qiu, Y.; Tittiger, C.; Wicker-Thomas, C.; Le Goff, G.; Young, S.; Wajnberg, E.; Fricaux, T.; Taquet, N.; Blomquist, G. J.; Feyereisen, R. *Proc. Natl. Acad. Sci. U.S.A.* **2012**, *109*, 14858.
(6) Rui, Z.; Li, X.; Zhu, X.; Liu, J.; Domigan, B.; Barr, I.; Cate, J. H.; Zhang, W. *Proc. Natl. Acad. Sci. U.S.A.* **2014**, *111*, 18237.
(7) Warui, D. M.; Li, N.; Norgaard, H.; Krebs, C.; Bollinger, J. M.; Booker, S. J. *J. Am. Chem. Soc.* **2011**, *133*, 3316.
(8) Aukema, K. G.; Makris, T. M.; Stoian, S. A.; Richman, J. E.; Munck, E.; Lipscomb, J. D.; Wackett, L. P. *ACS Catal.* **2013**, *3*, 2228.
(9) Pandelia, M. E.; Li, N.; Norgaard, H.; Warui, D. M.; Rajakovich, L. J.; Chang, W. C.; Booker, S. J.; Krebs, C.; Bollinger, J. M., Jr. *J. Am. Chem. Soc.* **2013**, *135*, 15801.
(10) Fukuda, H.; Fujii, T.; Sukita, E.; Tazaki, M.; Nagahama, S.; Ogawa, T. *Biochem. Biophys. Res. Commun.* **1994**, *201*, 516.
(11) Sanchez, C.; Zhu, L.; Brana, A. F.; Salas, A. P.; Rohr, J.; Mendez, C.; Salas, J. A. *Proc. Natl. Acad. Sci. U.S.A.* **2005**, *102*, 461.
(12) Fukuda, H.; Nakamura, K.; Sukita, E.; Ogawa, T.; Fujii, T. *J. Biochem.* **1996**, *119*, 314.
(13) Howard-Jones, A. R.; Walsh, C. T. *J. Am. Chem. Soc.* **2007**, *129*, 11016.
(14) Rittle, J.; Green, M. T. *Science* **2010**, *330*, 933.
(15) Groves, J. T.; McClusky, G. A. *J. Am. Chem. Soc.* **1976**, *98*, 859.
(16) Belcher, J.; McLean, K. J.; Matthews, S.; Woodward, L. S.; Fisher, K.; Rigby, S. E. J.; Nelson, D. R.; Potts, D.; Baynham, M. T.; Parker, D. A.; Leys, D.; Munro, A. W. *J. Biol. Chem.* **2014**, *289*, 6535.
(17) Lee, D. S.; Yamada, A.; Sugimoto, H.; Matsunaga, I.; Ogura, H.; Ichihara, K.; Adachi, S.; Park, S. Y.; Shiro, Y. *J. Biol. Chem.* **2003**, *278*, 9761.
(18) Fujishiro, T.; Shoji, O.; Nagano, S.; Sugimoto, H.; Shiro, Y.; Watanabe, Y. *J. Biol. Chem.* **2011**, *286*, 29941.
(19) Matsunaga, I.; Sumimoto, T.; Ueda, A.; Kusunose, E.; Ichihara, K. *Lipids* **2000**, *35*, 365.
(20) Atkins, W. M.; Sligar, S. G. *J. Am. Chem. Soc.* **1987**, *109*, 3754.
(21) Vaz, A. D. N.; Pernecky, S. J.; Raner, G. M.; Coon, M. J. *Proc. Natl. Acad. Sci. U.S.A.* **1996**, *93*, 4644.
(22) Kellner, D. G.; Hung, S. C.; Weiss, K. E.; Sligar, S. G. *J. Biol. Chem.* **2002**, *277*, 9641.
(23) Wang, X.; Peter, S.; Kinne, M.; Hofrichter, M.; Groves, J. T. *J. Am. Chem. Soc.* **2012**, *134*, 12897.
(24) Palcic, M. M.; Rutter, R.; Araiso, T.; Hager, L. P.; Dunford, H. B. *Biochem. Biophys. Res. Commun.* **1980**, *94*, 1123.
(25) Jeong, Y. J.; Kang, Y.; Han, A. R.; Lee, Y. M.; Kotani, H.; Fukuzumi, S.; Nam, W. *Angew. Chem.* **2008**, *47*, 7321.
(26) Whitehouse, C. J. C.; Bell, S. G.; Wong, L. L. *Chem.—Eur. J.* **2008**, *14*, 10905.
(27) Skiles, G. L.; Yost, G. S. *Chem. Res. Toxicol.* **1996**, *9*, 291.
(28) Rettie, A. E.; Boberg, M.; Rettenmeier, A. W.; Baillie, T. A. *J. Biol. Chem.* **1988**, *263*, 13733.
(29) Zhu, D. Q.; Seo, M. J.; Ikeda, H.; Cane, D. E. *J. Am. Chem. Soc.* **2011**, *133*, 2128.
(30) Auclair, K.; Hu, Z.; Little, D. M.; Ortiz De Montellano, P. R.; Groves, J. T. *J. Am. Chem. Soc.* **2002**, *124*, 6020.
(31) Yoshimoto, F. K.; Guengerich, F. P. *J. Am. Chem. Soc.* **2014**, *136*, 15016.
(32) Zachos, L.; Gassmeyer, S. K.; Bauer, D.; Sieber, V.; Hollmann, F.; Kourist, R. *Chem. Commun.* **2015**, *51*, 1918.

DOI: 10.1021/jacs.5b01965
J. Am. Chem. Soc. 2015, 137, 4940−4943