Supporting Information

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

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Supporting Information

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EXPERIMENTAL PROCEDURES

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. ^{13}C labeled eicosanoic acid ($CH_3(CH_2)_{18}{}^{13}COOH$) and perdeuterated eicosanoic acid ($C_{20}D_{40}O_2H$) were from CDN isotopes.

Cloning and heterologous expression of $OleT_{JE}$. An *Escherichia coli* codon-optimized gene for $OleT_{JE}$ 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'-CGATGTCATATGGCAACATTAAAAAGAGATAA

5'-CGATGTGAATTCCGTACGGTCAACCACCTC

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 $OleT_{JE}$. Frozen cells from 12 x 1 L cultures were resuspended in 250 mL of 50 mM NaH_2PO_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_2PO_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 $OleT_{JE}$, judged by SDS-PAGE and absorbance ratios (Abs_{417}/Abs_{280}), were pooled and concentrated to 5-20 mg/mL using Amicon ultra 30 kDa centrifugal filters, flash frozen, and stored at -70^{o}C until further use. Protein concentrations were determined using a calculated extinction coefficient ε_{417} = 110 mM^{-1} cm^{-1}, as determined by the pyridine hemochromagen method, [1] following complete substrate removal.

Preparation of Substrate Free $OleT_{JE}$. Purified $OleT_{JE}$ 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_2PO_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^{o}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_2O_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.

Multiple-Turnover Reactions. $OleT_{JE}$ (5 mL, 20 μM) was incubated with 1 mM eicosanoic acid in 200 mM KH_2PO_4 pH 7.6. Reactions were initiated by the addition of 4 mM H_2O_2 and allowed to proceed for 8 hours. 1-hexadecene (100 nmol) was subsequently added as an internal standard and the reaction mixture was quenched with 0.5 mL 12 M HCl . The reactions were extracted twice with 5 mL of chloroform. The organic phases were pooled and concentrated under a stream of N_2 to less than 50μL. Samples were derivatized with 200μL of BSTFA:TMCS (99:1). Samples were sealed and incubated at 75^{o}C for 20 minutes for trimethylsilylation. Following derivatization, samples were concentrated under a stream of N_2, diluted to 200 μl in chloroform, and analyzed by GC-MS as described above.

Gaseous Product Analysis. Fourier transform infrared spectra were recorded in rubber septum sealed 10 mm. pathlength Spectrosil quartz cuvettes (Starna). Spectra were optained using a Thermo-Nicolet Nexus system model 670 FTIR spectrometer. The FTIR spectrometer was purged with carbon dioxide-free gas for 10 minutes prior to each measurement. A nitrogen purged reaction mixture (400 μL) containing 100 μM eicosanoic acid and 10 μM $OleT_{JE}$ were initiated through addition of 150 μM H_2O_2. Control reactions were identically prepared without the addition of enzyme. Measurements were

collected in transmission mode using 10 scans per sample at a resolution of 4 cm^{-1} in the 2200-2600 cm^{-1} region. The spectra were baseline corrected using the automatic baseline correction function in OMNIC software. Headspace gas chromatography MS experiments were performed on ES complexes 50 µM containing either an unlabeled or ^{13}C terminally isotopically labeled ($(CH_3(CH_2)_{18}{}^{13}COOH)$) eicosanoic substrate in sealed 2mL reaction vials. A 15 fold molar excess of H_2O_2 was used to initiate the reaction. After 1 minute, 500 µL of gaseous headspace was introduced to the GC instrument via purge and trap Hamilton syringe needle. The GC chromatography was performed on a Hewlett Packard model 5890 GC with an Rtx-Wax fused silica column (30 m x 0.25 mm I.D., film thickness 0.25 µm) at a constant oven temperature of 25 °C. The column effluent was fed directly into a Waters VG 705 magnetic sector mass spectrometer and ionized by electron impact at 70 eV.

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_2PO_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_{obs}, (at 417 nm and 392 nm) were done with Origin software using a quadratic function (Morrison equation) for tight binding ligands where A_{max} is the maximal absorbance change at ligand saturation, S is the concentration of eicosanoic acid, E_t is the concentration of $OleT_{JE}$, and K_d is the dissociation constant:

$$Aobs = \left(\frac{Amax}{2Et}\right)(S + Et + Kd) - (((S + Et + Kd)^2 - (4SEt))^{0.5})$$

Stopped-Flow Absorption. Stopped-flow absorption experiments were performed on an Applied Photophysics Ltd. SX20 stopped-flow spectrophotometer. Eicosanoic acid (protiated or perdeuterated) bound $OleT_{JE}$ (~20 µM) was rapidly mixed with 5 mM H_2O_2 in 100 mM KH_2PO_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.

SUPPLEMENTARY FIGURES

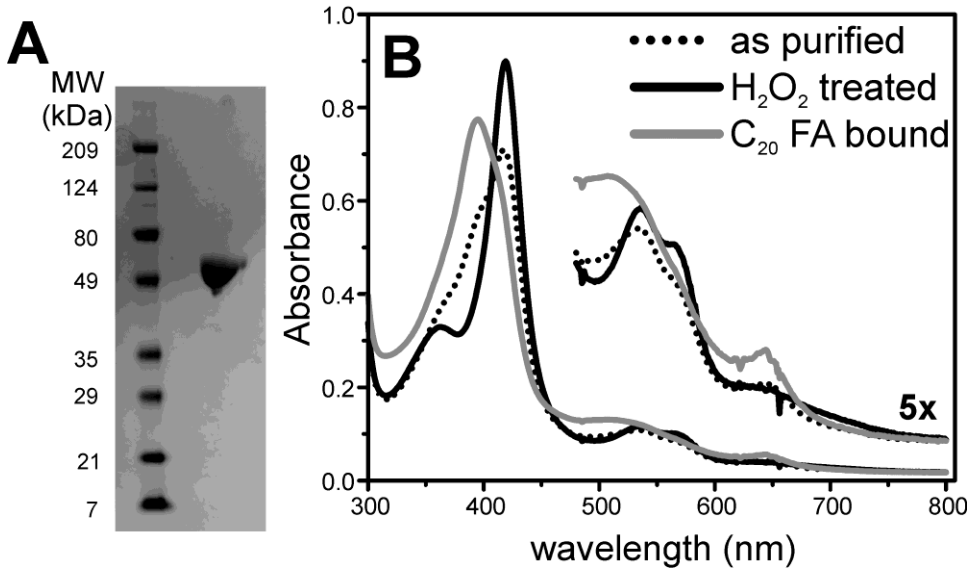

Figure S1: Characterization of Purified $OleT_{JE}$ used in this study. (A) SDS-PAGE of purified $OleT_{JE}$ (B) and optical spectroscopy of the enzyme in "as-purified" (dashed), hydrogen peroxide treated (solid black), and eicosanoic acid bound (solid gray) forms.

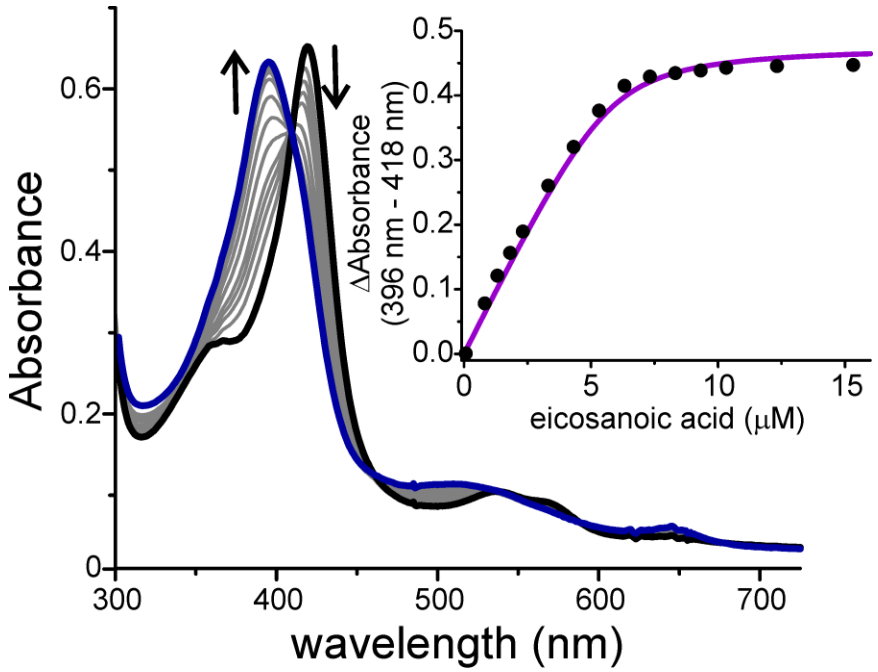

Figure S2. Binding of eicosanoic acid to peroxide treated $OleT_{JE}$. Aliquots of eicosanoic acid were added to substrate free $OleT_{JE}$ (black trace) to produce the high spin enzyme (blue trace). The spectral changes at 417 and 392 nm upon binding of eicosanoic acid (inset) were fit to a Morrison expression for tight binding ligands for K_d determination as described in Methods.

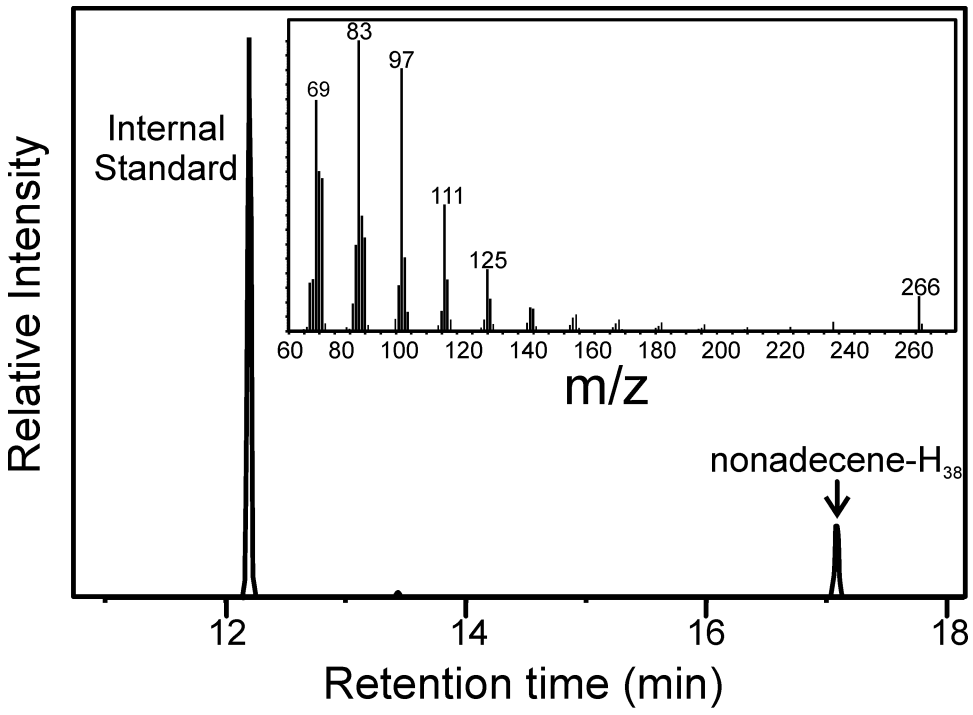

Figure S3. Identification of the Alkene Product from the $OleT_{JE}$:Eicosanoic Acid Single Turnover Reaction. GC chromatogram and MS fragmentation pattern (inset) of the nonadecene product formed from the reaction of a 20 μM $OleT_{JE}$ E-S complex and 60 μM H_2O_2. The yield of deuterated nonadecene produced was determined through peak integration and comparison to an internal hexadecene standard

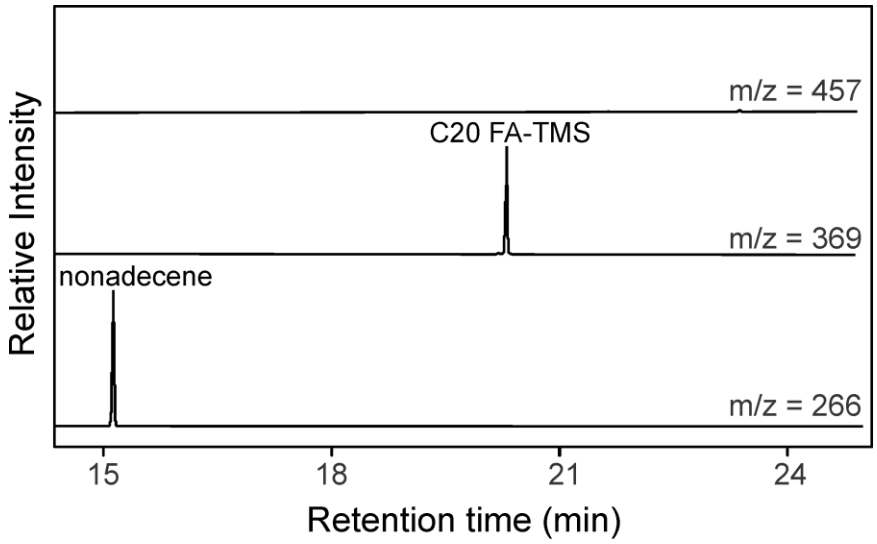

Figure S4. Characterization of the Products from an $OleT_{JE}$:Eicosanoic Acid Multiple Turnover Reaction with Derivatization. A 5 mL reaction containing 20 µM $OleT_{JE}$, 1 mM eicosanoic acid, and 4 mM H_2O_2 was extracted and derivatized by trimethylsilylation (TMS). Products were analyzed by GC-MS as described in Supplementary Methods. The extracted ion chromatograms at m/z = 266 (molecular ion of nonadecene), m/z = 369 (fragment ion of TMS-eicosanoic acid), and m/z = 457 (fragment ion of derivatized hydroxyeicosanoic acid) are shown.

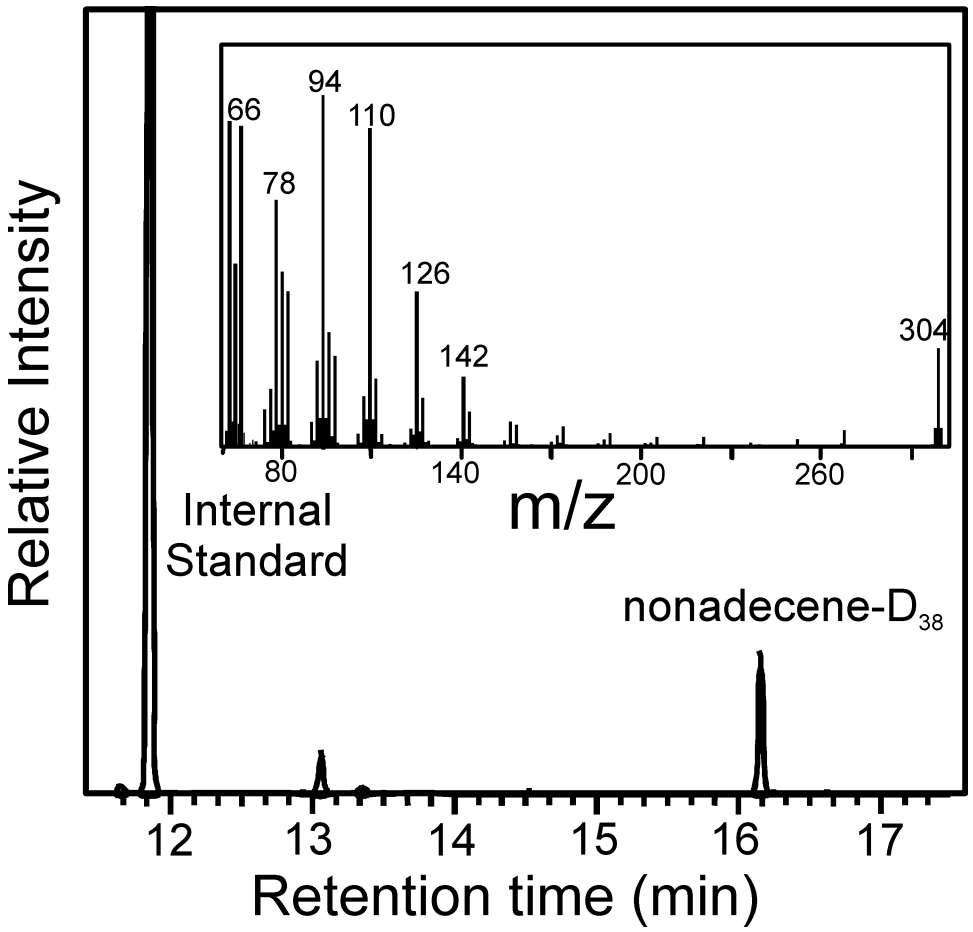

Figure S5: GC chromatogram and MS fragmentation pattern (inset) of the deuterated nonadecene product formed from the reaction of a 20 μM $OleT_{JE}$ E-S complex, prepared with perdeuterated eicosanoic acid, and 60 μM H_2O_2. The yield of deuterated nonadecene produced was determined through peak integration 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.

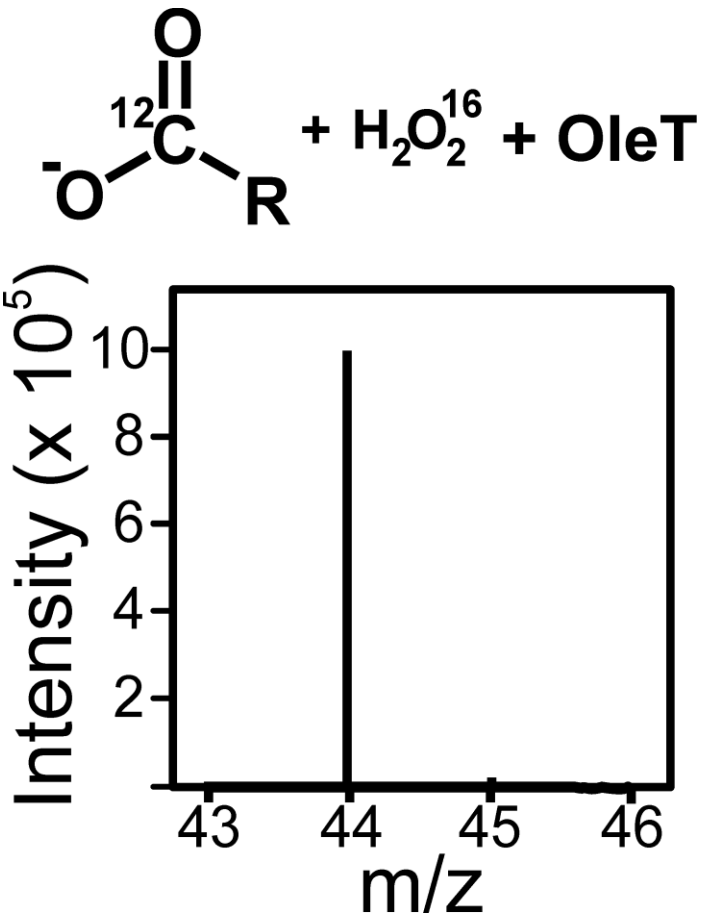

Figure S6. Headspace GC-MS of single turnover reactions of $OleT_{JE}$:^{12}C eicosanoic acid + $H_2{}^{16}O_2$.

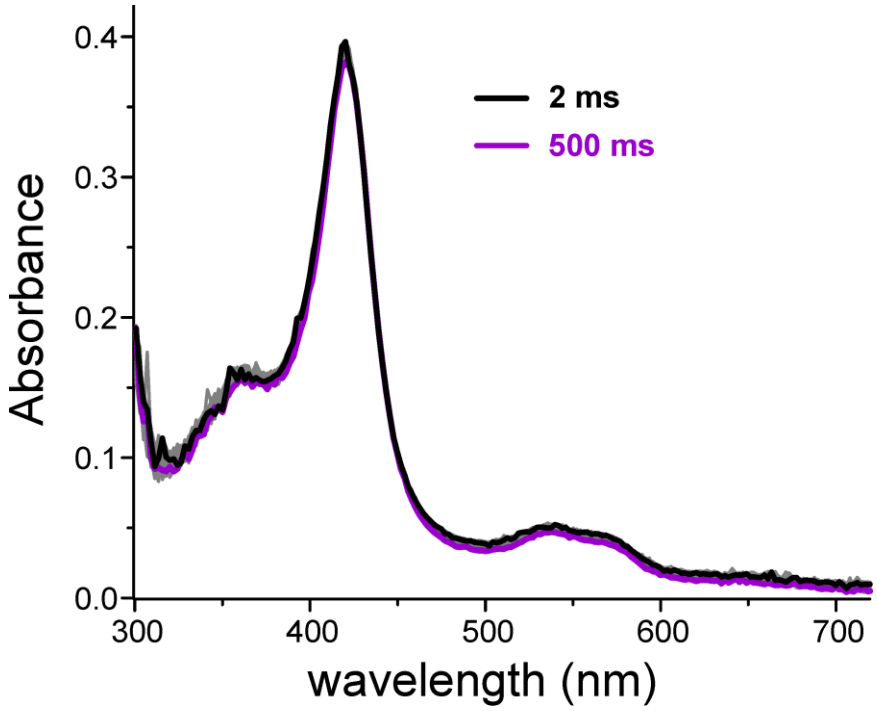

Figure S7: Photodiode array spectra of the reaction of substrate free $OleT_{JE}$ and H_2O_2

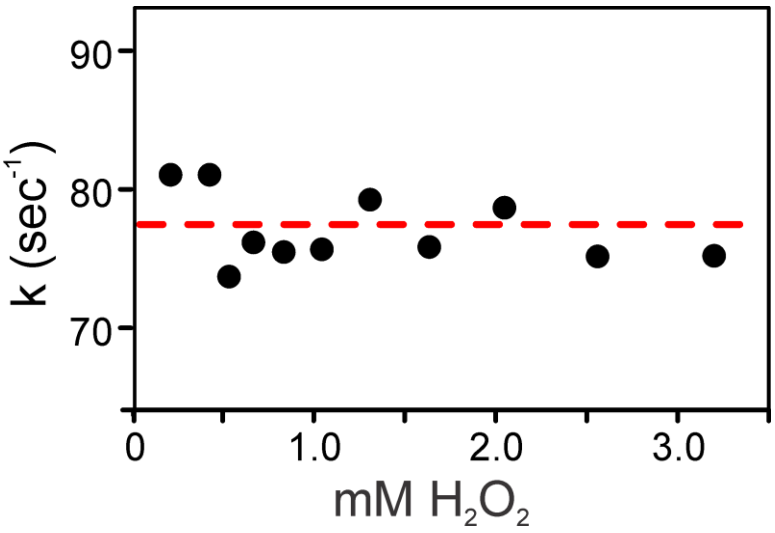

Figure S8: H_2O_2 dependence of Compound I decay from the reaction of $OleT_{JE}$: deuterated eicosanoic acid + H_2O_2 at 5°C. The decay of Ole-I, monitored at 370 nm, was fit with a two summed exponential expression. The faster of the two exponentials phases, comprising more than 90 % of the spectral amplitude, is plotted against the H_2O_2 concentration after mixing.

Supplementary References

(1) Berry, E. A.; Trumpower, B. L. *Anal. Biochem.* 1987, *161*, 1.