Structure and Biochemical Properties of the Alkene Producing Cytochrome P450 OleTJE (CYP152L1) from the Jeotgalicoccus sp. 8456 Bacterium*

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Background: OleTJE oxidatively decarboxylates fatty acids to produce terminal alkenes.

Results: OleTJE is an efficient peroxide-dependent lipid decarboxylase, with high affinity substrate binding and the capacity to be resolubilized from precipitate in an active form.

Conclusion: OleTJE has key differences in active site structure and substrate binding/mechanistic properties from related CYP152 hydroxylases.

Significance: OleTJE is an efficient and robust biocatalyst with applications in biofuel production.

The production of hydrocarbons in nature has been documented for only a limited set of organisms, with many of the molecular components underpinning these processes only recently identified. There is an obvious scope for application of these catalysts and engineered variants thereof in the future production of biofuels. Here we present biochemical characterization and crystal structures of a cytochrome P450 fatty acid peroxygenase: the terminal alkene forming OleTJE (CYP152L1) from Jeotgalicoccus sp. 8456. OleTJE is stabilized at high ionic strength, but aggregation and precipitation of OleTJE in low salt buffer can be turned to advantage for purification, because resolubilized OleTJE is fully active and extensively dissociated from lipids. OleTJE binds avidly to a range of long chain fatty acids, and structures of both ligand-free and arachidic acid-bound OleTJE reveal that the P450 active site is preformed for fatty acid binding. OleTJE heme iron has an unusually positive redox potential (−103 mV versus normal hydrogen electrode), which is not significantly affected by substrate binding, despite extensive conversion of the heme iron to a high spin ferric state. Terminal alkenes are produced from a range of saturated fatty acids (C12–C20), and stopped-flow spectroscopy indicates a rapid reaction between peroxy and fatty acid-bound OleTJE (167 s⁻¹ at 200 μM H₂O₂). Surprisingly, the active site is highly similar in structure to the related P450, which catalyzes hydroxylation of fatty acids as opposed to decarboxylation. Our data provide new insights into structural and mechanistic properties of a robust P450 with potential industrial applications.

The cytochromes P450 (P450s2 or CYPs) are oxidases that catalyze a vast array of oxidative reactions in nature (1). These hemoproteins are found in virtually all organisms, from bacteria and archaea to humans, and are responsible for several chemical transformations that are essential, for instance, in the microbial biosynthesis of antibiotics (e.g. erythromycin in Saccharopolyspora erythraea and vancomycin in Amycolatopsis orientalis) (2, 3) and in the mammalian formation of estrogens (estrone and 17β-estradiol) through the action of the aromatase P450 (CYP19A1) on androgen substrates (androstanediol and testosterone, respectively) (4, 5). The majority of characterized P450s are monoxygenases that interact with one or more redox partners to provide them with the two electrons (typically derived from NAD(P)H) required for oxidative catalysis (6). The first electron reduces the P450 cysteine thiolate-coordinated heme iron from ferric to ferrous, enabling dioxygen binding to the ferrous iron. The second electron reduces the resulting ferric-peroxo complex to the ferric-peroxo state. Two successive productions produce first the ferric-hydroperoxo species (compound 0) and then (following the loss of a water molecule) the ferryl-oxo compound I (7) (Fig. 1). The transient and highly reactive nature of compound I prevented its definitive characterization for many years, until Rittle and Green (8) produced compound I in a large yield following rapid mixing of CYP119 (from the thermoacidophilic crenarchaeon Sulfolobus acidocaldarius) with the oxidant m-chloroperbenzoic acid and confirmed its identity using Mössbauer, EPR, and UV-visible spectroscopy. Compound I is considered to be the major oxidizing species in the P450 catalytic cycle and to be responsible for the bulk of oxidative reactions (e.g. hydroxyla-

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‡The atomic coordinates and structure factors (codes 4L54 and 4L40) have been deposited in the Protein Data Bank (http://wwpdb.org/).

¶The abbreviations used are: P450 or CYP, cytochrome P450; CV, column volume; HS, high spin heme iron; LS, low spin heme iron; OleTJE, Jeotgalicoccus sp. 8456 cytochrome P450 decarboxylase (CYP152L1); P450spB. subtilis fatty acid hydroxylase (CYP152A1); P450spP. paucimobilis fatty acid hydroxylase (CYP152A1); PDA, photodiode array; PDB, Protein Data Bank; NHE, normal hydrogen electrode; Ni-IDA, nickel-iminodiacetic acid.
tion, epoxidation, oxidative demethylation, etc.) observed throughout the P450 superfamily (9, 10).

The vast majority of P450s use NAD(P)H-dependent redox systems consisting of either (i) an FAD-binding reductase that shuttles electrons to the P450 via a ferredoxin (or a flavodoxin in a small number of cases) or (ii) an FAD- and FMN-binding cytochrome P450 reductase, the individual flavin-binding domains of which are evolutionarily related to NAD(P)H-binding ferredoxin oxidoreductases and flavodoxins (5, 11). However, other types of P450 redox partner systems exist (e.g. P450-redox partner fusion enzymes, such as the CYP116B family of P450:phthalate dioxygenase reductase fusions) (12). In addition, other P450s catalyze isomerization (e.g. mammalian thromboxane synthase, CYP5A1) and dehydration (e.g. flax allene oxide synthase, CYP74A1) reactions that do not require an external source of electrons and which are completed entirely within the P450 active site (13, 14). Further, through exploration of in vitro routes to driving P450 catalysis, it is now well established that the addition of hydrogen peroxide (H2O2) or organic peroxides (e.g. cumene hydroperoxide) to P450s can facilitate substrate oxidation by directly producing compound 0, which is then protonated to generate compound I (15) (Fig. 1). This “peroxide shunt” procedure is rarely an efficient means of driving P450s, because the peroxides oxidize heme and protein. However, a small number of P450s that have evolved to exploit the peroxide shunt are now known. Notably, the Bacillus subtilis CYP152A1 (P450BS) and the Sphingomonas paucimobilis CYP152B1 (P450SP) naturally use H2O2 to catalyze long chain fatty acid hydroxylation and are thus referred to as peroxygenases (16, 17). P450SP catalyzes nearly exclusively hydroxylation at the α-position, whereas P450BS catalyzes hydroxylation at α- and β-positions but with the majority at the β-position (~60:40 ratio) (16).

In recent studies, Rude et al. (18) characterized a novel enzyme from the bacterium Jeotgalicoccus sp. ATCC 8456 (OleTJE) that is 41% identical in amino sequence to P450BS and 37% identical to P450SP. OleTJE was identified as a P450 based on this sequence similarity and designated by the authors as a CYP152 P450 family member (18). The Jeotgalicoccus sp. ATCC 8456 host strain was shown to produce a number of C18–C20 linear and branched chain terminal alkenes, and other Jeotgalicoccus strains were shown to generate a similar spectrum of terminal alkenes in the C18–C21 range. A His-tagged version of OleTJE was expressed in Escherichia coli and purified using nickel-nitrilotriacetic acid column chromatography and shown to catalyze formation of n-1 alkenes through H2O2-dependent decarboxylation of C14, C16, C18, and C20 saturated fatty acids (18).

In view of the potential importance of the OleTJE enzyme as a producer of terminal alkenes for exploitation in areas such as biofuels and fine chemical production, we have undertaken a study of the biochemical and biophysical properties of the isolated OleTJE enzyme and have determined its crystal structure in complex with arachidic acid. These data reveal novel properties of this biotechnologically important P450 peroxygenase. These include (i) the high catalytic efficiency of OleTJE and its capacity to be resolubilized from a precipitated form as a fully active enzyme, (ii) the extensive development of high spin (HS) heme iron in OleTJE on binding various long chain fatty acids (distinguishing it from related bacterial peroxygenases), and (iii) its unusually positive heme iron reduction potential, which is also negligibly affected by fatty acid binding despite the substrate inducing extensive HS ferric heme iron formation.

**EXPERIMENTAL PROCEDURES**

**Bioinformatics**—The OleTJE sequence and additional members of the CYP152 family, including all known subfamilies, were BLAST-searched against a set of all the prokaryotic P450 sequences. Members of the highest scoring CYP families from these searches were used to build a tree. Sequence alignments were computed using ClustalW and checked manually for consistent alignment of known CYP motifs. Neighbor-joining trees were generated with the Phylip package (version 3.6; distributed by the author, J. Felsenstein (University of Washington, Seattle)) using ProtDist (a program in Phylip) to compute difference matrices. Trees were drawn and colored with FigTree
Expression and Purification of OleT—The gene encoding OleT_E from Jeotgalicoccus sp. ATCC 8456 was codon-optimized (for expression in E. coli), synthesized, and cloned into the pET47b (Merck Millipore, Madison, WI) vector by GenScript. The E. coli strain C41 (DE3) (Lucigen, Middleton, WI) was used as the expression host. Cells transformed with the pET47b-OleT_E plasmid were grown at 37 °C with shaking at 200 rpm in total volumes of 500 ml to 3 liters of 2YT broth containing kanamycin (30 μg/ml) supplemented with 500 μM δ-aminolevulinic acid. Expression of OleT_E was induced by the addition of 100 μM isopropyl-1-thio-β-D-galactopyranoside when an optical density of 0.5 (at 600 nm) was reached, at which point the incubation temperature was lowered to 25 °C, and the cells were grown for a further 16 h. Cells were harvested by centrifugation at 6000 rpm, 4 °C, using a JLA-8.10000 rotor in an Avanti J-26 XP centrifuge. Pellets were resuspended in a minimal volume of ice-cold buffer A (100 mM potassium phosphate (KPi), pH 8.0), supplemented with 500 μM δ-aminolevulinic acid. The sample was then incubated overnight with 10 ml CompleteTM EDTA-free protease inhibitor mixture tablet (Roche Applied Science) per 50 ml of cell suspension, DNase I (100 μg/ml, bovine pancreas, Sigma-Aldrich), and lysozyme (100 μg/ml, hen egg white, Sigma-Aldrich). The cells were disrupted by two passes through a French press (Thermo Scientific, Hemel Hempstead, UK), and the homogenate was centrifuged at 20,000 rpm, 4 °C, for 90 min using a JA-25.50 rotor. Alternatively, cells were lysed by sonication using a Bandelin Sonopuls sonicator set to 45% amplitude with 30 30-s pulses, at 60-s intervals, with the cell suspension kept on ice throughout.

Cells were thawed at 4 °C and resuspended in 3 volumes of extraction buffer/g of cell pellet. The extraction buffer consisted of buffer A containing 1 M NaCl, 20% glycerol, with a Complete™ EDTA-free protease inhibitor mixture tablet (Roche Applied Science) per 50 ml of cell suspension, DNase I (100 μg/ml, bovine pancreas, Sigma-Aldrich), and lysozyme (100 μg/ml, hen egg white, Sigma-Aldrich). The cells were disrupted by two passes through a French press (Thermo Scientific, Hemel Hempstead, UK), and the homogenate was centrifuged at 20,000 rpm, 4 °C, for 90 min using a JA-25.50 rotor. Alternatively, cells were lysed by sonication using a Bandelin Sonopuls sonicator set to 45% amplitude with 30 30-s pulses, at 60-s intervals, with the cell suspension kept on ice throughout. The homogenate was then centrifuged as previously. The supernatant was removed, and the pH was set to 8.0 as necessary. The sample was then incubated overnight with 10 ml (per 100 g of cell pellet) of nickel-iminodiacetic acid (Ni-IDA) chromatographic medium (Generon, Maidenhead, UK) on a chromatographic medium (Generon, Maidenhead, UK) on a roller table at 4 °C. The mixture was then poured into a column, and the collected bed of OleT_E-bound medium was washed with 10 column volumes (CV) of 100 mM KP, (pH 8.0) containing 750 mM NaCl, 20% glycerol (buffer B), and 50 mM imidazole to remove weakly bound contaminants. The column was then washed with 2 CV of the buffer B containing 125 mM imidazole, followed by 5 CV of buffer B plus 150 mM imidazole, which eluted the bulk of the OleT_E protein. The partially purified OleT_E sample was dialyzed overnight against 15 liters of buffer A at 4 °C, which caused OleT_E to precipitate. Post-dialysis, precipitated protein was isolated by centrifugation at 4000 rpm, 4 °C, using an A-4-62 rotor in an Eppendorf 5810 R centrifuge. The pellet was washed gently with 50 ml of buffer A, and centrifugation was repeated. OleT_E was resuspended in 5 ml of buffer A containing 1 M NaCl and 10% glycerol, which produced OleT_E at high purity (Method 1). For OleT_E destined for crystallographic studies, HRV 3C protease (Merck Millipore) was incubated with OleT_E for ~16 h at 4 °C (50:1 μg of protein/unit of protease) to remove the N-terminal polyhistidine tag. The proteolysed protein was applied to 5 ml of pre-equilibrated nickel-Sepharose resin (GE Healthcare) to bind the cleaved His tag and the tagged HRV 3C. The cleaved OleT_E was eluted from the column by washing with 100 mM KP, (pH 8.0) plus 750 mM NaCl and 10% glycerol (buffer C).

In separate preparations (avoiding the OleT_E precipitation step; Method 2), the dialysis step post-Ni-IDA chromatography was removed, and the OleT_E eluate was instead diluted (5×) in buffer C and concentrated in an Amicon ultrafiltration device. The OleT_E sample was then centrifuged to clarify the sample (16,000 rpm, 4 °C, using the JA-25.50 rotor), and the supernatant was then applied again to a 5-ml Ni-IDA column. The column was washed with 5 CV of buffer C containing 50 mM imidazole and then 10 CV of buffer C plus 100 mM imidazole. The His-tagged OleT_E was then eluted with 150 mM imidazole in the same buffer. All procedures generated highly purified OleT_E protein. In both cases, the pure OleT_E protein was concentrated to >20 mg/ml using a Vivaspin centrifugal concentrator (Generon), snap-frozen in liquid nitrogen, and stored at ~80 °C.

**UV-visible Spectroscopy**—Analysis of the UV-visible spectroscopic properties of OleT_E was done on a Cary 60 UV-visible spectrophotometer (Varian UK). Spectra were recorded using ~4–10 μM OleT_E in 100 mM KP, (pH 8.0) plus 750 mM NaCl (buffer D). Reduction of OleT_E was achieved by the addition of sodium dithionite to enzyme in buffer D made anaerobic by extensive bubbling with oxygen-free nitrogen. The ferrous-CO complex of OleT_E was formed by slow bubbling of gas into anaerobically reduced enzyme until no further absorbance change occurred. The NO complex was formed by the addition of 5–8 bubbles of NO into a sample of ferric OleT_E, in anaerobic buffer.

**Fatty Acid and Inhibitor Binding Titrations with OleT_E**—Spectral binding titrations of OleT_E with saturated fatty acids (C12, C14, C16, C18, and C20) were performed at 25 °C in buffer D. Fatty acids were from Sigma-Aldrich. Substrates (typically 0.25 mg/ml) were dissolved in 70% (v/v) EtOH (for C18 and C20) or 70% methanol (with the sodium salts of C12, C14, and C16 fatty acids) and 30% (v/v) Triton X-100 (Sigma-Aldrich). A parallel set of binding titrations was also performed using fatty acids (1 mg/ml) dissolved in 100% EtOH or methanol without Triton X-100. Prior to titrations, OleT_E samples (~50 μl at >20 mg/ml) in buffer C were passed through a Lipidx column of dimensions 5 × 1 cm (PerkinElmer Life Sciences) in order to remove any residual lipid retained during purification of the protein from E. coli. OleT_E recovered from the column was in an extensively low spin (LS) ferric state and was used directly for titration at a final P450 concentration in the range from 5 to 10 μM. Titrations were performed by stepwise additions of aliquots (0.1–1 μl) of the fatty acids to the OleT_E sample (substrate additions to <1% of total volume). Spectra (800–300 nm) were recorded for the ligand-free OleT_E and following each addition of substrate using a Cary 60 UV-visible spectrophotometer. Difference spectra at each stage in the titration were computed by subtracting the spectrum of ligand-free OleT_E from each successive fatty acid-bound spectrum.
collected during the titration. A pair of wavelengths were identified that defined the absorbance maximum ($A_{\text{peak}}$) and minimum ($A_{\text{rough}}$) in the difference spectra from each titration set. The overall absorbance change ($A_{\text{max}}$) at each substrate concentration point was calculated as $A_{\text{peak}}$ minus $A_{\text{rough}}$, and $A_{\text{max}}$ was plotted versus [substrate]. These data were fitted using either a hyperbolic (Michaelis-Menten) function, the Morrison equation for tight binding ligands, or the Hill function (where sigmoidal behavior was observed) in order to determine dissociation constants ($K_d$ values), as described previously (19, 20). Titrations and data fitting for OleT$_{JE}$ with dithiothreitol (DTT), imidazole, and cyanide (sodium salt) inhibitors were done in the same way as for the fatty acids, with ligands dissolved in buffer D.

**Stopped-flow Analysis of Substrate Turnover**—Stopped-flow absorption measurements were made using an Applied Photophysics SX18 MR stopped-flow spectrophotometer (Leatherhead, UK). Stopped-flow spectral accumulation was done using a photodiode array (PDA) detector on the same instrument. Fatty acid substrate-bound OleT$_{JE}$ was mixed versus different concentrations of H$_2$O$_2$ in 100 mM KP (pH 8.0) containing 750 mM NaCl at 25 °C. OleT$_{JE}$ (9.2 μM) was converted to an extensively HS heme iron form by mixing with arachidic acid (12 μM) from a concentrated stock prepared in 80% EtOH, 20% Triton X-100. Reactions were initiated by mixing the arachidic acid bound OleT$_{JE}$ (4.6 μM final concentration) with H$_2$O$_2$ (3.29–200 μM final concentration). Stopped-flow traces at single wavelengths reporting on the conversion of HS OleT$_{JE}$ heme iron toward LS were collected over periods of up to 30 s to follow depletion of HS (390 nm) and formation of LS heme iron (418 nm). Data were analyzed and fitted using a single exponential function with the Pro-Data SX software suite (Applied Photophysics). The observed reaction rate constants ($k_{\text{obs}}$ values) were plotted versus the relevant H$_2$O$_2$ concentrations, and the resultant data plot was fitted using a linear function to obtain the second order rate constant reporting on H$_2$O$_2$-dependent decarboxylation of substrate and the consequent heme iron spin state conversion. Entire spectral acquisition (750–280 nm) was also done using the PDA detector for the same set of titration. A pair of wavelengths were identified that defined the absorbance maximum ($A_{\text{peak}}$) and minimum ($A_{\text{rough}}$) in the difference spectra from each titration set. The overall absorbance change ($A_{\text{max}}$) at each substrate concentration point was calculated as $A_{\text{peak}}$ minus $A_{\text{rough}}$, and $A_{\text{max}}$ was plotted versus [substrate]. These data were fitted using either a hyperbolic (Michaelis-Menten) function, the Morrison equation for tight binding ligands, or the Hill function (where sigmoidal behavior was observed) in order to determine dissociation constants ($K_d$ values), as described previously (19, 20). Titrations and data fitting for OleT$_{JE}$ with dithiothreitol (DTT), imidazole, and cyanide (sodium salt) inhibitors were done in the same way as for the fatty acids, with ligands dissolved in buffer D.

**Redox Potentiometry**—To determine the midpoint potential for the OleT$_{JE}$ Fe$^{3+}$/Fe$^{2+}$ couple, redox titrations were performed at 25 °C in an anaerobic glove box (Belle Technology, Weymouth, UK) under a nitrogen atmosphere with O$_2$ levels maintained at less than 2 ppm. All solutions were deoxygenated by sparging with nitrogen gas. For substrate-free OleT$_{JE}$, the titration was done using 9.3 μM OleT$_{JE}$ in 100 mM KP (pH 7.0) plus 10% glycerol. For substrate-bound OleT$_{JE}$, the titration was done under the same conditions, following the addition of arachidic acid (from a 32 mM stock in 80% EtOH, 20% Triton X-100) until no further conversion of the heme iron to the HS heme state was observed (~12 μM arachidic acid). Mediators were added to expedite electronic equilibration in the system (2 μM phenazine methosulfate, 7 μM 2-hydroxy-1,4-naphthoquinone, 0.3 μM methyl viologen, and 1 μM benzyli viologen to mediate in the range from +100 to −480 mV versus normal hydrogen electrode (NHE)) and data fitting (using the Nernst equation), and analysis was done as described previously (21–23).

**EPR Analysis of OleT$_{JE}$**—Continuous wave X-band electron paramagnetic resonance EPR spectra of OleT$_{JE}$ were obtained at 10 K using a Bruker ELEXYS E500 EPR spectrometer equipped with an ER 4122SHQ Super High Q cavity. Temperature control was effected using an Oxford Instruments ESR900 cryostat connected to an ITC 503 temperature controller. Microwave power was 0.5 milliwatt, modulation frequency was 100 kHz, and the modulation amplitude was 5 G. EPR spectra were collected for OleT$_{JE}$ (305 μM) in the substrate-free form and for OleT$_{JE}$ (205 μM) bound to arachidic (C20:0) acid (at a saturating concentration).

**Crystallography of OleT$_{JE}$**—Crystallization trials for OleT$_{JE}$ were performed using 400 nl (200 nl of protein plus 200 nl of precipitant) sitting drops in Art Robbins 96-well plates, using Molecular Dimensions 96-deep well crystallization screens (Clear Strategy Screen I (CSS1), Clear Strategy Screen II, PACT premier, JCSG-plus and Morpheus) and a Mosquito nanoliter pipetting robot (TTP Labtech, Melbourne, UK). Crystals formed between 2 days and 1 month at 4 °C in several conditions. The crystals giving best diffraction were formed under the following conditions: 35 mg/ml OleT$_{JE}$ in 0.1 M Tris (pH 8.5) containing 0.2 M MgCl$_2$ and 25% (w/v) polyethylene glycol 2000 mono-methyl ether (substrate-free OleT$_{JE}$) and 43 mg/ml OleT$_{JE}$ incubated with 235 μM arachidic acid in 0.1 M Tris (pH 8.5) containing 0.2 M MgCl$_2$, 15% (w/v) polyethylene glycol 20,000 and 15% (w/v) polyethylene glycol 550 mono-methyl ether (substrate-bound OleT$_{JE}$).

For preparation of substrate-bound OleT$_{JE}$, P450 samples were concentrated by ultrafiltration, and a stock solution of arachidic acid (32 mM) dissolved in 100% EtOH was added to a final concentration of 235 μM. The concentration of EtOH did not exceed 1% of the total volume. The mother liquor was supplemented with 10% PEG 200, where an additional cryoprotectant was required, and crystals were flash-cooled in liquid nitrogen prior to data collection. Data were collected at Diamond synchrotron beamline IO4-1 and reduced and scaled using XDS (24). Structures were solved by molecular replacement with the previously solved P450 BS crystal structure (PDB 2ZQJ) using PHASER (25). Structures were refined using Refmac5 (25) and Coot (26). Final refinement statistics are given in Table 1.

**Analysis of Products Formed by OleT$_{JE}$ in Reactions with H$_2$O$_2$ and Fatty Acids**—OleT$_{JE}$ reactions with long chain saturated fatty acids (C12–C20) were set up as follows. 5-mL reactions were done in buffer D, with 250 μM dodecanedioic acid (sodium salt), palmitic acid or arachidic acid, 500 μM hydrogen peroxide, and 0.6 μM OleT$_{JE}$. The final reaction mixtures were incubated for periods up to 30 min at room temperature. 1 mL of the reaction mixture was then extracted (at different reaction times) with an equal volume of HPLC grade heptane, and the sample was centrifuged at 14,000 rpm for 20 min. The top layer was then analyzed by GC/MS. Analysis was done using a Thermo Fisher D50 II GC/MS instrument with a 30 m × 0.25 mm × 0.25-μm ZB5MS GC column (Phenomenex). Injection was cold on-column. The oven program was set so that an initial temperature of 50 °C was ramped at 10 °C/min to 300 °C.
post-injection. Electronic ionization was used, and ions in the range of 40 – 640 m/z were scanned at two scans/s.

RESULTS

Classification of OleTJE as CYP152L1—There are currently 21,039 named cytochrome P450 sequences (see the Cytochrome P450 Homepage). Approximately 6% are bacterial (1254 sequences), and an additional 48 are from archaea. Initial BLAST searches with OleTJE showed that it was less than 40% identical to most known CYP152 sequences and barely over the 40% recommended cut-off for CYP family membership to two CYP152 sequences (having 41% identity with CYP152A1 from B. subtilis and 40% with CYP152A2 from Clostridium acetobutylicum). The location of the OleTJE sequence in a phylogenetic tree (as CYP152L1) strongly argues for inclusion in the distinct CYP152 clade. The same logic applies to the renamed tree (as CYP152L1) strongly argues for inclusion in the distinct CYP152 clade. This proved to be the case, and it was found that the protein might be stabilized in solution at high ionic strength. This proved to be the case, and it was found that the protein might be stabilized in solution at high ionic strength. This proved to be the case, and it was found that the protein might be stabilized in solution at high ionic strength. This proved to be the case, and it was found that the protein might be stabilized in solution at high ionic strength.

Expression and Purification of OleTJE—The OleTJE gene was codon-optimized for expression in E. coli, and preliminary studies revealed that the enzyme was expressed well in a number of E. coli strains. The C41 (DE3) strain (Lucigen) was selected for protein production with the gene cloned into pET47b via the BamHI and EcoRI restriction sites with a 6-His N-terminal tag and transcribed using the T7-lac RNA polymerase/promoter system. Expression cell extracts were red in color, indicative of the production of a heme protein. However, our initial studies revealed that the OleTJE protein precipitated on dialysis following elution from an Ni-IDA protein in the first chromatographic purification step. Previous studies by Rude et al. (18) used high salt (NaCl) concentration in several purification buffers, and in view of this and the halophilic nature of the host bacterium (Jeotgalicoccus sp. ATCC 8456), we considered that the protein might be stabilized in solution at high ionic strength. This proved to be the case, and it was found that the precipitation of OleTJE could be used to advantage, because resolubilization of the centrifuged protein pellet in buffer A containing 1 M NaCl and 10% glycerol produced an OleTJE sample with a P450-like heme spectrum (Amax at ~418 nm). SDS-PAGE at this stage also indicated the protein to be extensively purified (puriﬁcation Method 1). Specifically for crystallization, the OleTJE His tag was removed by incubation with HRV 3C protease, and the mixture was loaded onto a nickel-Sepharose column. Washing the column in buffer C (100 mM KP4 (pH 8.0) plus 750 mM NaCl and 10% glycerol) resulted in elution of a highly puriﬁed tag-free OleTJE protein (Fig. 3) and the retention of the cleaved His tag and the tagged protease on the column.

Having identiﬁed the issues with the propensity of OleTJE to aggregate at low ionic strength, an alternative strategy was developed to avoid its precipitation, by eluting OleTJE from Ni-IDA in the high salt buffer C, centrifuging the sample, and then reapplying to Ni-IDA resin equilibrated in buffer C. By washing the column with increasing concentrations of imidazole in buffer C, His-tagged OleTJE was eluted at 150 mM imidazole in a highly pure form (puriﬁcation Method 2). A typical yield of puriﬁed OleTJE was ~20 mg/liter of E. coli cell culture using either Method 1 or Method 2 for protein puriﬁcation.

UV-visible Absorption Properties of OleTJE—Rude et al. (18) inferred the cytochrome P450 nature of OleTJE from amino acid sequence similarities to peroxigenase members of the CYP152 family of P450s and demonstrated in vitro that cell extracts of Jeotgalicoccus sp. ATCC 8456 could decarboxylase the saturated fatty acids arachidic acid (C20) and stearic acid (C18) to their respective n-1 terminal alkenes (1-nonadecene and 1-heptadecene, respectively). A His-tagged OleTJE, isolated from E. coli was also shown to catalyze stearic acid decarboxylation in an H2O2-dependent reaction (18). However, UV-visible absorption features typical of a P450 enzyme were not presented in this earlier study.

Fig. 4 shows characteristic absorption spectra for pure OleTJE in its oxidized (ferric, Fe3+) and sodium dithionite-reduced (ferrous, Fe2+) forms and for the ferrous-carbon monoxide (Fe2+-CO) and ferric-nitric oxide (Fe3+-NO) species. The resting (ferric) form of OleTJE shows a heme spectrum typical of a P450 enzyme with its ferric heme iron in an LS state. The major absorption feature (the Soret band) is at 418 nm, with the smaller α- and β-bands in the visible region at ~566 and 535 nm, respectively. These values are similar to those of other LS bacterial P450s (e.g. the Bacillus megaterium P450 BM3 (CYP102A1) heme domain with maxima at 418, 534, and 568 nm and the Mycobacterium tuberculosis CYP121A1 at 416,5, 538, and 568 nm) (27, 28). The two methods of preparing OleTJE (i.e. with or without a protein precipitation step) produced identical oxidized OleTJE spectra. Any residual imida-
zole ligand from nickel column chromatography (in both cases) was extensively depleted by ultrafiltration used to concentrate the proteins and thus did not produce any imidazole-ligated OleTJE heme iron.

Reduction of OleTJE with sodium dithionite produced a ferrous hemoprotein with the Soret band diminished in intensity and shifted to 414 nm. In the visible (heme Q-band) region, a single, slightly asymmetric feature is seen at ~540 nm. The blue shift of the Soret spectrum on reduction indicates substantial retention of cysteine thiolate proximal coordination in the OleTJE ferrous state, and the spectral maxima are similar to those features seen in, for example, the well characterized *Pseudomonas putida* camphor hydroxylase P450cam (CYP101A1, 411 and 540 nm) and in the explosive degrading P450 XplA from *Rhodococcus rhodochrous* strain 11Y (CYP177A1, 408 and 542 nm) (23, 29). The addition of carbon monoxide to anaerobically reduced OleTJE produced a characteristic P450 heme spectrum with the Soret band red-shifted to 449 nm and a Q-band feature at 551 nm. A small shoulder on the Soret feature at ~423 nm probably indicates a minor proportion (~5%)
Structure of an Alkene Producing P450

Analysis of Substrate and Inhibitor Binding to OleTJ\textsubscript{HE}—The binding of substrates to P450s is often associated with alteration of the spin state of their ferric heme iron, usually through displacing its weakly bound sixth ligand water molecule and inducing a shift toward the HS form (e.g. see Refs. 33 and 34). For OleTJ\textsubscript{HE}, we investigated the binding of a series of saturated fatty acids (C12–C20) and found that in all cases, the lipids induced a LS to HS transition, with the Soret band shifting from 418 nm toward 394 nm. Table 2 shows fatty acid binding $K_d$ data for OleTJ\textsubscript{HE} (purified using Method 1) and using fatty acid stocks dissolved in alcohol or in alcohol containing 30% (v/v) Triton X-100 (see “Experimental Procedures”). The $K_d$ values and the extent of HS heme iron developed were improved in all cases in the presence of the detergent, although Triton X-100 alone induces no spin state change (e.g. 0.67 ± 0.03 μM versus 6.20 ± 0.26 μM for palmitic acid). The extent of spin state change induced varied according to chain length, with the longer chain fatty acids (C18:0 and C20:0) inducing a more complete conversion to the HS ferric state than observed for the C12:0 to C16:0 fatty acids). For a titration using an arachidic acid (C20:0) stock, including Triton X-100, the HS conversion was almost complete (estimated at ≥95%), as shown in Fig. 5A. In contrast, lauric acid (C12:0) produced ~52% HS at saturation (Fig. 5B). For studies with non-precipitated OleTJ\textsubscript{HE} (prepared using Method 2) in the presence of Triton X-100, tight binding of fatty acids was again observed (e.g. $K_d$ values of 1.54 ± 0.19 μM for arachidic acid and 12.7 ± 0.3 μM for lauric acid) (Table 2). However, the $K_d$ values increased by approximately an order of magnitude for all fatty acids tested compared with those for OleTJ\textsubscript{HE} prepared by Method 1 (Table 2). Thus, contrary to what may have been expected, the resolubilized OleTJ\textsubscript{HE} shows higher affinity than the non-precipitated form for the panel of fatty acid substrates tested.

Binding of cyanide and imidazole to OleTJ\textsubscript{HE} produced typical type II P450 heme absorption shifts to longer wavelength. Soret shifts to 433 nm ($K_d$ > 10 mM) and 424 nm ($K_d$ = 193 ± 11 μM) were observed for cyanide and imidazole, respectively. The binding of DTT to OleTJ\textsubscript{HE} was also analyzed in view of the report from Rude et al. (18), which indicated that DTT could support OleTJ\textsubscript{HE} fatty acid decarboxylase activity by producing H$_2$O$_2$ under aerobic conditions in the presence of the P450 heme iron (18, 35). However, in previous studies, we showed that DTT coordinated the heme iron in the explosive degrading XplA P450 (23). DTT is known to bind P450 heme iron, and ligation is feasible in both DTT thiol and thiolate forms (36, 37). Fig. 5C shows data from a spectral titration of OleTJ\textsubscript{HE} with DTT in buffer D. The DTT-bound form has three distinct absorption features in the Soret region, with peaks at 372 and 423 nm, and a strong absorbance shoulder at ~460 nm. The central band is the most intense. The 423 nm peak arises from distal ligation of DTT thiol to OleTJ\textsubscript{HE} heme iron, whereas the outer peaks result from a split (hyperporphyrin) Soret spectrum in which DTT thiolate ligates the iron (36, 37). Comparable spectral maxima are at 374, 423.5, and 453.5 nm for XplA (23). In XplA, the intensities of the three absorbance bands are quite similar, but in OleTJ\textsubscript{HE}, the outer bands are much weaker than the 423 nm feature, suggesting that DTT favors heme ligation in the thiol state under the conditions used. Fig. 5C (inset) shows fitting of
TABLE 2

| Fatty acid          | $K_d \mu^M$ | High spin heme (%) | $K_d \mu^M$ | High spin heme (%) | $K_d \mu^M$ | High spin heme (%) |
|---------------------|-------------|--------------------|-------------|--------------------|-------------|--------------------|
| C20:0 (arachidic)   | 0.29 ± 0.05 | 95                 | 2.3 ± 0.2   | 84                 | 1.6 ± 0.2   | 59                 |
| C18:0 (stearic)     | 0.20 ± 0.02 | 67                 | 7.4 ± 0.5   | 51                 | 4.0 ± 0.8   | 36                 |
| C16:0 (palmitic)    | 0.67 ± 0.03 | 27                 | 6.2 ± 0.3   | 27                 | 4.3 ± 0.9   | 23                 |
| C14:0 (myristic)    | 0.66 ± 0.03 | 39                 | 37.7 ± 1.9  | 41                 | 11.4 ± 0.5  | 29                 |
| C12:0 (lauric)      | 0.77 ± 0.02 | 45                 | 59.2 ± 7.7  | 27                 | 12.7 ± 0.3  | 30                 |

DTT-induced heme absorption change for OleTJE, leading to a $K_d$ of $159 \pm 7 \mu^M$. In the study by Rude et al. (18), DTT at 200 $\mu^M$ was used to support OleTJE catalysis. However, our data indicate that substantial inhibition of OleTJE probably occurs under such conditions.

Determination of the Heme Iron Redox Potentials of Substrate-free and Substrate-bound OleTJE—Fatty acid binding to OleTJE induces substantial shifts in heme iron spin state equilibrium toward HS (e.g. see Fig. 5A), and such shifts in spin state equilibrium are often associated with the heme iron developing a more positive potential and becoming easier to reduce (e.g. see Refs. 22 and 33). Spectroelectrochemical titrations were done for both substrate-free and arachidic acid-bound forms of OleTJE to determine the midpoint potentials for the heme iron Fe$^{3+}$/Fe$^{2+}$ couples (versus NHE). Despite the extensive heme content in the arachidic acid-bound OleTJE, its heme potential (~105 ± 6 mV) is not significantly different from that of the substrate-free form (~103 ± 6 mV) (Fig. 6). In both cases, the heme iron potentials are quite positive compared with many bacterial P450s, which rely on NAD(P)H-dependent electron transfer from protein reductx partner systems. Examples include the camphor binding-induced shift in heme iron potential from ~300 to ~170 mV (versus NHE) in P450cam (enabling electron transfer from the ferredoxin partner at ~240 mV) (33, 38) and the arachidonic acid-induced shift in potential from ~429 to ~289 mV (versus NHE) in P450 BM3 (22). However, unlike the aforementioned P450s, OleTJE is evolutionarily adapted to interact directly with H$_2$O$_2$ in order to form reactive iron-oxo species (initially the ferric-hydroperoxo compound 0, which is probably transformed to the ferryl-oxo compound 1), and its positive potential is probably a consequence of the environment of the heme and its cysteine thiolate ligand. The fact that the OleTJE heme potential is effectively unchanged in the HS substrate-bound form may be a consequence of the proximity of a negatively charged substrate carboxylate group to the heme iron in the arachidic acid-bound form.

Another notable feature in the spectra for the reduced forms of substrate-free and arachidic acid-bound OleTJE is that neither form a unique spectral species that could be assigned to a cysteine thiolate-coordinated ferrous P450 heme iron. As shown in Fig. 6, the UV-visible spectrum for OleTJE immediately following reduction has its Soret feature at 414 nm, with a small shoulder at ~423 nm, indicative of a mixture of Cys thiolate-coordinated (major species) and thiol-coordinated (minor species) forms. In the redox titration for substrate-free OleTJE (Fig. 6A), the Soret peak for the reduced P450 is split into two components, with a peak at 406 nm and a shoulder at ~425 nm. The latter probably represents thiolate-coordinated ferrous OleTJE, and the former the thiol-coordinated form (39). A similar phenomenon is seen for the arachidic acid-bound OleTJE (Fig. 6B), although in this case, the main peak is at 420 nm with a shoulder at ~400 nm, suggesting a higher proportion of the thiol-coordinated ferrous form in the substrate-bound OleTJE. For both substrate-free and arachidic acid-bound OleTJE redox titrations, it is evident that there is a single set of isosbestic points throughout the titrations, indicating that the equilibrium between thiol- and thiolate-coordinated ferrous forms remains constant as the concentration of ferrous OleTJE accumulates. The Soret isosbestic point is at 408 nm for the arachidic acid-bound form and at 410 nm for substrate-free OleTJE. Thus, under the same redox titration conditions, arachidic acid substrate binding seems to push the ferrous heme cysteine thiolate/thiol equilibrium slightly further toward the thiol-coordinated state.

Stopped-flow Analysis of OleTJE Turnover Kinetics—In order to determine the kinetics of H$_2$O$_2$-dependent fatty acid oxidation, we exploited the fact that turnover of bound substrate is accompanied by a reconversion of OleTJE heme iron spin state from HS to LS as the substrate is decarboxylated. The two states of the P450 have considerably different heme spectra, and thus we used stopped-flow absorbance spectroscopy to measure the rate constants for LS OleTJE heme formation at 417 nm across a range of H$_2$O$_2$ concentrations up to 200 $\mu^M$. Reaction kinetics are second order with respect to [H$_2$O$_2$], with observed rate constants ($k_{obs}$) for arachidic acid oxidation and concomitant LS heme recovery up to 167 s$^{-1}$ at the highest [H$_2$O$_2$] tested (200 $\mu^M$) (Fig. 7A). The $k_{obs}$ versus [H$_2$O$_2$] data were fitted using a linear equation, giving a second order rate constant ($k_0$) of $(8.0 \pm 0.2) \times 10^{3} M^{-1} s^{-1}$ to describe the catalytic process. The apparent $k_{obs}$ value at the $y$ axis intercept (zero [H$_2$O$_2$]) is $8.32 \pm 1.96$ s$^{-1}$, giving an estimate for the H$_2$O$_2$ on/off rate constant. The $k_{off}/k_{on}$ ratio thus gives an estimate of the apparent $k_d$ for H$_2$O$_2$ as $10.40 \pm 2.71$ $\mu^M$. Fig. 7B shows overlaid spectra captured during the reaction of arachidic acid-bound OleTJE with H$_2$O$_2$ at a final concentration of 7.58 $\mu^M$. The spectral overlay describes a smooth transition from the substrate-
FIGURE 5. Analysis of fatty acid and dithiothreitol binding to OleTJE. A, a spectral titration for OleTJE (9.8 μM) with arachidic acid (C20:0). Arrows indicate the progressive decrease in the ferric LS Soret band (at 418 nm) and the concomitant increase in the ferric HS feature at 394 nm. The development of a small thiolate-to-HS ferric charge transfer band is seen at ~650 nm as the titration progresses. The inset shows a fit (using the Morrison equation) of arachidic acid-induced Soret absorbance change versus fatty acid concentration, yielding a $K_d$ value of $0.29 \pm 0.05 \mu M$ for arachidic acid. B, spectra for an OleTJE (9.8 μM) titration with lauric acid (C12:0). In this case, HS heme development is less extensive than for arachidic acid, and the $K_d$ value (inset) is $0.77 \pm 0.02 \mu M$. C, titration of OleTJE (6.1 μM) with DTT. The binding of DTT is associated with the splitting of the heme signal into two distinct features: a hyperporphyrin (split Soret) spectrum with maxima at 372 and 460 nm and a distinct Soret feature at 423 nm. The former results from distal coordination of substrate, arachidic acid, whereas the latter has a distinct Soret feature at 460 nm and a HS band at 394 nm. The inset shows a plot of DTT-induced Soret absorbance shift versus DTT concentration, fitted using a hyperbolic equation to yield a $K_d$ value of $159 \pm 7 \mu M$.

EPR Analysis of OleTJE—The continuous wave X-band EPR spectrum of substrate-free OleTJE (prepared using Method 1) displays features attributable to the $S = \frac{3}{2}$ LS ferric heme with a thiolate-proximal ligand to the iron and a distal ligation water molecule (Fig. 8A). Several such LS forms with rhombic anisotropy are evident from the multiplicity of lines observed, and the resolvable contributions at $g_x$ show $g$ values ranging from those typical for LS ferric P450s (2.43 and 2.48) (e.g. see Refs. 39–42) to those associated with chloroperoxidases and the fatty acid hydroxylase P450$_{S_p}$ (CYP152B1) (2.55, 2.61, and possibly 2.70) (17, 43). Overall, the EPR spectrum suggests a large, water-filled site with multiple coordination geometries and hydrogen bonding partners available to the distal water ligand. The addition of substrate, arachidic acid, produces a very different EPR spectrum. The high-spin heme is in contrast to P450$_{S_p}$, which shows no spin state change on substrate binding (17) and where x-ray crystallography has shown that the heme retains the water sixth ligand when substrate is bound. Approximately 15% of the protein (as determined by relative integration of the low spin forms, accounting for differences in concentration and subtraction of baselines to account for underlying high spin species) is converted to a new LS species with $g$ values of $g_x = 2.46, g_y = 2.25, and g_z = 1.89$, which is not present in the substrate free enzyme. It is likely that this minor LS species is in equilibrium with the HS form.

OleTJE-catalyzed Substrate Turnover—OleTJE turnover assays were done using H$_2$O$_2$ and with a range of saturated fatty acids (C12–C20), as described under “Experimental Procedures.” As reported by Rude et al. (18), products were identified and characterized as terminal alkenes. Fig. 9 shows formation of 1-alkene products from arachidic acid (forming 1-nonadecene) and from lauric acid (forming 1-undecene). Rude et al. (18) reported a $k_{o bs}$ for differences in concentration and subtraction of baselines to yield a $K_d$ value of 159 ? 7 μM. Data were collected, processed, and fitted as described under “Experimental Procedures.”
reported (from in vivo and/or in vitro analyses) data consistent with the oxidative decarboxylation of fatty acids in the chain length range 14–22, forming the \( n-1 \) terminal alkenes (18).

Here we demonstrate that the chain length selectivity of OleTJE extends to the C12:0 saturated fatty acid lauric acid.

**Crystal Structure of OleTJE**—The structure of the OleTJE P450 was determined to a resolution of 2.3 Å for the substrate-free form (PDB 4L54) and to 2.5 Å for the arachidic acid fatty acid-bound form (PDB 4L40). Despite the fact that both forms were obtained from different crystallization conditions, the crystal packing is identical, with little difference in structure noted between the C20-bound and ligand-free OleTJE P450s (root mean square deviation of 0.125 for 410 C\(_a\) atoms). The overall OleTJE P450 structure closely resembles the related peroxoxygenase P450\(_{BS} \) with a root mean square deviation of 0.99 Å for 379 C\(_a\) atoms (Fig. 10). Areas where significant deviations occur are located at the FG-loop and the adjacent C-terminal loop regions. Both of these regions line the fatty acid binding pocket, more specifically the area involved in binding the fatty acid tail. In OleTJE residue Leu-177 from the FG-loop closes the narrow access channel to the solvent that is present in both P450\(_{BS} \) and the related P450\(_{Prx} \). The OleTJE fatty acid binding pocket is elongated in comparison with P450\(_{BS} \) due to three point mutations in the N-terminal \( \beta \)-sheet region (from...}

![FIGURE 6. Determination of the OleTJE heme iron reduction potential in its substrate-free and arachidic acid-bound forms. A, data from a spectroelectrochemical redox titration of ligand-free OleTJE (8.1 \( \mu \text{M} \)). The spectrum for the oxidized enzyme (solid line) shows the Soret maximum at 419 nm, whereas that for the fully dithionite-reduced P450 (dashed line) has its Soret maximum at 406 nm and shows a single feature in the Q-band region at \(-560 \text{ nm}. Intermediate spectra are shown in dotted lines. Arrows, direction of absorption changes observed during the reductive part of the titration. Inset, plot of absorbance at the Soret peak (417 nm) versus the applied potential corrected for the NHE. Data are fitted using the Nernst equation to give a midpoint potential of \( E^0 = -103 \pm 6 \text{ mV} \). B, redox titration for arachidic acid-bound OleTJE (8.1 \( \mu \text{M} \) thick solid line). The oxidized substrate-bound species has its Soret maximum at 395 nm, and the fully reduced form (thick dotted line) has a maximum at \(-420 \text{ nm}. Intermediate spectra in the titration are shown in dotted lines. Arrows again indicate absorption changes observed during the reductive part of the titration. The inset shows a plot of absorbance at the substrate-bound Soret peak (395 nm) versus the applied potential corrected for the NHE, with data fitted using the Nernst equation to yield \( E^0 = -105 \pm 6 \text{ mV} \).](image)

![FIGURE 7. Stopped-flow kinetics of \( \text{H}_2\text{O}_2 \)-dependent oxidation of substrate-bound OleTJE. A, plot of the observed rate constants (\( k_{\text{obs}} \)) for \( \text{H}_2\text{O}_2 \) binding to OleTJE (and concomitant substrate oxidation) versus the \( \text{H}_2\text{O}_2 \) concentration. Data were measured at 417 nm, reflecting recovery of the LS OleTJE form. The \( k_{\text{obs}} \) versus [\( \text{H}_2\text{O}_2 \)] data were fitted to a linear function to yield the second order rate constant of \( \text{H}_2\text{O}_2 \) binding/substrate (arachidic acid) oxidation of \( k_{\text{on}} = 0.80 \pm 0.02 \text{ M}^{-1} \text{s}^{-1} \), \( k_{\text{off}} = 8.32 \pm 1.96 \text{ s}^{-1} \), and an apparent \( K_d \) value of 10.40 \( \pm 2.71 \text{ M} \) for \( \text{H}_2\text{O}_2 \) binding, derived from \( k_{\text{off}}/k_{\text{on}} \). B, stopped-flow PDA data observed at 7.58 \( \mu \text{M} \text{H}_2\text{O}_2 \), demonstrating the OleTJE spectral conversion from the HS, substrate-bound form (Soret maximum at 394 nm) to the LS reoxidized state (Soret maximum at 417 nm) upon mixing with \( \text{H}_2\text{O}_2 \). The inset shows the corresponding plot of the absorbance data at 417 nm (open circles) against time. The data were fitted using a single exponential function to yield an apparent rate constant of 12.50 \( \pm 1.16 \text{ s}^{-1} \).](image)
Ile-25, Leu-41, and Leu-315 to the corresponding OleTJE Thr-24, Ala-40, and Ala-317) (Fig. 11). This provides additional space to accommodate fatty acids with a chain length up to C20.

The active site of OleTJE is remarkably similar to P450BS/H9252, despite the fact that both enzymes favor distinct catalytic reactions (decarboxylation versus hydroxylation, respectively). The fatty acid carboxylate group is bound by the conserved Arg-245 and placed approximately perpendicular to the heme plane in OleTJE, with the Cα and Cβ carbons closest to the heme iron (at 5.1 and 5.7 Å, respectively; Fig. 12). No other direct polar contacts are made between the carboxylate headgroup and the protein. A water molecule is seen to occupy a position between His-85 and the C20 carboxylate, in close proximity to both moieties (at distances of 3.3 and 2.7 Å, respectively). This water molecule is too distant from the heme iron (3.3 Å) to act as a direct ligand, and the heme is pentacoordinate in the substrate-bound OleTJE P450 structure. In comparison, the substrate-free OleTJE active site contains several water molecules with ill-defined density above the heme plane, probably corresponding to a range of distinct water structures in the absence of the substrate, consistent with conclusions from EPR data.

One of the few key differences between the OleTJE and P450BS/H9252 active sites is the switch from His-85 (OleTJE) to Gln-85 (P450BS/H9252). In OleTJE, the His-85 imidazole side chain points into the active site, directly toward the heme iron (at a distance of 5.8 Å). The imidazole moiety is sandwiched between the heme edge and Phe-79 and makes no polar contacts with other amino acids. In addition, the A and B pyrrole groups of the OleTJE P450 heme are distorted, with the effect of moving these closer to the substrate by ~0.8 Å when compared with P450BS/H9252. This is independent of substrate binding because the same deviation from planarity can be observed for the substrate-free OleTJE P450 heme group. The difference in heme conformation between OleTJE and P450BS/H9252 cannot be attributed directly to mutations in the heme vicinity because most heme binding residues are identical, and it appears instead to be linked to small changes in the position of secondary elements containing heme binding residues.

DISCUSSION

In this paper, we present the first structural and detailed kinetic and biochemical characterization of the Jeotgalicoccus OleTJE enzyme, which catalyzes the production of terminal alkenes from long chain fatty acids, an industrially relevant reaction. The enzyme is one of a growing number of P450 enzymes that has evolved to use hydrogen peroxide (rather than NAD(P)H-dependent redox partners) in order to form reactive iron-oxo species for substrate oxidation (5, 16). Its closest bacterial relatives are the S. paucimobilis P450SP/H9251 and the B. subtilis P450BS/H9252, which catalyze predominantly the α- and β-hydroxylation of long chain fatty acids (44, 45). OleTJE was classified as CYP152L1, in the same family as the peroxygenases P450SP/H9251 (CYP152B1) and P450BS/H9252 (CYP152A1) and the Clostridium acetobutylicum CYP152A2, a further fatty acid α-hydroxylase (46). OleTJE is assigned as the first member of a new CYP152 subfamily (CYP152L1).

Crystal structure data are confirmatory of the typical P450 fold in OleTJE and also of a close evolutionary relationship with the other structurally resolved peroxygenases P450SPα and...
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A

B
FIGURE 10. Comparison between the OleTJE and P450BS fatty acid binding modes. An overlay is shown for the substrate-bound forms of OleTJE (in blue; PDB code 4L40) and P450BS (in gray; PDB code 1H2O) in a schematic representation. The bound substrates and heme groups are represented in sticks, colored in magenta for the OleTJE arachidic acid-bound form and in green for the palmitic acid-bound form of P450BS.

FIGURE 11. Comparison of the OleTJE and P450BS fatty acid binding modes. The image shows a side by side overlay of the OleTJE and P450BS fatty acid-bound active sites, with key residues contacting the substrates shown. For clarity, main chain atoms have been removed. The left panel depicts OleTJE in atom colored sticks, with the corresponding P450BS residues shown in gray lines. The right panel shows the same view but with the P450BS residues in atom colored sticks and OleTJE shown in gray lines. Substrates are arachidic acid (C20:0) for OleTJE and palmitic acid (C16:0) for P450BS.

P450BS, OleTJE does not undergo any major structural change on binding the C20:0 lipid arachidic acid, which nearly completely occupies the entire P450 active site cavity. The OleTJE fatty acid binding mode is very similar to that seen in P450SPA and P450BS. The major differences seen in OleTJE relate to the active site His-85 residue (which is replaced by a glutamine residue in both the hydroxylases P450SPA and P450BS) and the distortion of its heme cofactor (as compared with these hydroxylases). Despite extensive structural identity between OleTJE, P450SPA, and P450BS, and the similarities in their specificity for long chain fatty acids, there is a major difference in reaction mechanism and product formation. OleTJE catalyzes mainly oxidative decarboxylation to form terminal alkenes from fatty acids, whereas P450SPA and P450BS favor hydroxylation at the α- and β-carbons, respectively (16–18). Rude et al. (18) reported that the Q85H mutant of P450BS catalyzes decarboxylation of palmitic acid (to 1-pentadecene) ∼1.5-fold faster than the WT enzyme and also enhances palmitic acid β-hydroxylation at the expense of α-hydroxylation, thus confirming the importance of the residue at this position. The initial reaction with H2O2 in each of these P450s should form the reactive iron-oxo species compound 0 (the ferric hydroperoxo intermediate in the P450 catalytic cycle) using the peroxide “shunt” mechanism (7). However, a fundamental difference in the OleTJE reaction mechanism leads to formation of the terminal alkene as the major product (rather than hydroxylated fatty acids).

To explain this phenomenon, we are drawn to the role of the active site histidine (His-85) in OleTJE. It has been postulated that the high pKₐ of compound II in P450s generally ensures that compound I in these enzymes effectively abstracts a hydrogen atom from the substrate (as opposed to a single electron). However, in OleTJE, His-85 could function to donate a proton to compound I, concomitant with its reduction to compound II by abstraction of an electron from the fatty acid carboxylate moiety. The formation of the carboxylate radical would lead to homolytic scission of the substrate C–C bond, following hydrogen atom abstraction from the Cα position by compound II. In the hydroxylases, such a reaction is unlikely to occur in the absence of a proton donor to form protonated compound II, and these enzymes are thus likely to use compound I to abstract hydrogen from either the Cα or the Cβ position, ultimately leading to monohydroxylation of substrate (Fig. 13) (47).

FIGURE 12. Detailed view of the OleTJE active site. Left, active site region for the ligand-free OleTJE structure (key amino acids shown in atom colored sticks with green carbons), with water molecules close to the iron shown as red spheres. Right, the same region in the same orientation for the ligand-bound OleTJE structure (key amino acids in blue), with the C20 ligand in magenta sticks. The omit map for the ligand is shown as a green mesh contoured at 3 σ.

FIGURE 9. Oxidative decarboxylation of arachidonic acid and lauric acid by OleTJE. A, total ion count from GC separation of the C19 terminal alkene 1-nonadecene in the reaction of OleTJE with arachidic acid (C20:0) (top), with mass spectrometric analysis (blue circles) of the major peak at 6.63 min confirming its identity (bottom; inset highlighting the region of the 1-nonadecene mass ion with m/z = 260). B, total ion count from GC separation of the C11 terminal alkene 1-undecene following reaction of OleTJE with lauric acid (C12:0) (top), with mass spectrometric analysis of the major peak at 6.63 min confirming its identity (bottom; inset highlighting the region of the 1-undecene mass ion with m/z = 154).
In early studies of OleTJE, we noted the propensity of the enzyme to aggregate and precipitate in typical (low salt) buffer conditions. However, we were able to turn this to our advantage through demonstrating that precipitation of the OleTJE P450 (induced through dialysis into low salt buffer) could readily be reversed by resolubilization (in high salt buffer) in a form that had a higher proportion of LS ferric heme iron (probably due to displacement of lipid retained in its active site during isolation from E. coli) than did the enzyme isolated without the precipitation step (Method 2). OleTJE prepared using Method 1 was readily crystallized to produce the structural data shown in Figs. 10–12 for substrate-free and arachidic acid-bound forms.

The binding of fatty acids displaces the axial water ligand from the heme iron of OleTJE, leading to a shift of the ferric heme iron toward the HS state and to accompanying large changes in the heme absorption spectrum. This provides the basis for determination of fatty acid $K_d$ values by optical titration. Many of the fatty acids tested for binding to OleTJE have very limited solubility in water. For this reason, we investigated their suspension in detergent solutions, and found that although there was no evidence for binding of Triton X-100 to OleTJE, the $K_d$ values for fatty acids suspended in 30% Triton X-100 were markedly improved over those suspended in alcohol alone for OleTJE prepared by Method 1. By comparison, the $K_d$ values for Triton X-100-suspended lipids with OleTJE prepared by Method 2 were ~5–20-fold greater, possibly due to the P450 prepared without the precipitation step retaining contaminant short lipids that hinder the binding of the C12 to C20 fatty acids tested (Table 2). The robust nature of OleTJE and its stability to low salt precipitation and resolubilization in high salt is unusual in the P450 superfamily but perhaps not surprising given the halotolerant nature of the Jeotgalicoccus bacterial genus. The cysteine thiolate ligand to the OleTJE heme iron is clearly retained following its resolubilization, as evidenced by retention of catalytic activity and the production of a Fe(III)CO (P450) complex with Soret maximum at 449 nm (Fig. 4). The stability of OleTJE to precipitation and resolubilization thus provides a convenient route to its purification and also bodes well for its application in synthesis of terminal alkenes.

Stopped-flow kinetic analysis of OleTJE indicated that the extensively HS fatty acid-bound form is rapidly reconverted back to the LS ferric form upon mixing with H$_2$O$_2$, with a second order rate constant of 0.80 ± 0.02 mM$^{-1}$ s$^{-1}$ with respect to H$_2$O$_2$ concentration. Previous studies of P450SP$\alpha$ reported a specific activity of 838 min$^{-1}$ for the $\alpha$-hydroxylation of myristic acid using 200 $\mu$M H$_2$O$_2$, whereas P450BS$\beta$ catalyzed the $\alpha$/$\beta$-hydroxylation of myristic acid with a specific activity of 209 min$^{-1}$ at 100 $\mu$M H$_2$O$_2$ (16, 44, 48). The OleTJE rate constants determined here are substantially faster than those for P450SP$\alpha$ and P450BS$\beta$ although they report only on the catalytic steps of substrate decarboxylation, alkene displacement from the active site, and restoration of the water ligand to the OleTJE heme iron. However, in turnover studies with different fatty acids, considerable amounts of alkene products were observed, consistent with efficient decarboxylase activity of OleTJE. Another notable difference between these peroxygenases lies in their optical response to binding fatty acid substrates. For P450SP$\alpha$, there is negligible heme iron spin state and associated Soret absorption change upon binding fatty acids, whereas with P450BS$\beta$ some HS shift does occur, but to a much smaller extent than is observed for OleTJE with its best substrates (49). Because binding of H$_2$O$_2$ requires displacement of the sixth ligand water on the P450 heme iron, this process may be much more efficient for OleTJE compared with P450SP$\alpha$ and P450BS$\beta$.

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In its evolution toward peroxygenase activity, OleTJE has undergone mutations that reinforce its divergence from the typical class I and II P450 enzymes that interact with NAD(P)H.
dependent redox partner enzymes and reductively activate oxygen bound to the heme iron to facilitate substrate oxidation (50). Most notably, it is evident that the acid-alcohol amino acid pair in the P450 I helix that is common to oxidase P450s (e.g. Asp-251 and Thr-252 in P450cam or Glu-267 and Thr-268 in P450 BM3) is not conserved in OleT<sub>E</sub> or in either P450<sub>BG5</sub> or P450<sub>SP</sub> (44, 45, 51, 52). Because protonation reactions on heme iron-bound dioxygen do not feature in the catalytic cycle of the peroxygenases, this motif is dispensed with in these enzymes. Instead, the acid-alcohol pair is replaced in OleT<sub>E</sub> by Arg-245 (which binds the substrate carboxylate) and Pro-246 in a highly conserved I-helix region of the CYP152 family enzymes (7). A conserved phenylalanine residue (Phe-393 in P450 BM3; Phe-350 in P450cam) in the P450 heme binding region is also absent from the bacterial peroxygenases. Phe-393 interacts with the heme thiolate bond in CYP102A1 and was shown to be important in maximizing P450 catalytic efficiency through regulating heme iron potential and thus heme iron reduction rate and ferrous-oxo complex stability in studies of various Phe-393 variants (53, 54). In OleT<sub>E</sub> and the other bacterial peroxygenases, the absence of this extensively conserved phenylalanine and amino acid insertions into this “heme binding loop” consensus region indicate that heme thermodynamic properties are regulated differently (45). Our potentiometric data show that OleT<sub>E</sub> has a very positive heme iron Fe(III)/Fe(II) redox potential (−103 mV versus NHE), which is not significantly altered in the HS form when bound to arachidonic acid (−105 mV) (Fig. 6). Thus, the absence of key residues in OleT<sub>E</sub> BS<sub>UP</sub> and SP<sub>β</sub> is a clear indicator of their distinct evolutionary pathway, such that protonation of iron-oxo intermediates or substrate-dependent regulation of electron transfer from redox partners has been bypassed through direct use of H<sub>2</sub>O<sub>2</sub>. Similarly, the introduction of the conserved arginine-proline pair (Arg-245 and Pro-246 in OleT<sub>E</sub>) in place of the acid/alcohol pair in each of the these structurally characterized peroxygenase P450s is clearly an evolutionary adaptation that facilitates positioning of fatty acid substrate to enable its oxidative decarboxylation or α/β-hydroxylation. However, a more detailed understanding of general structural determinants that might define a peroxygenase P450 “class” may require further structural/mechanistic studies of more divergent enzymes.

In conclusion, we have determined novel structural and biochemical properties of an unusual P450 peroxygenase enzyme (CYP152LI, OleT<sub>E</sub>) that catalyzes oxidative decarboxylation of fatty acids to produce terminal alkenes. OleT<sub>E</sub> is a catalytically efficient and structurally robust P450 with great potential in the synthesis of a variety of alkenes as “drop-in” biofuels (i.e. biofuels that may be used interchangeably with conventional fuels) or chemical reagents. Ongoing research is directed at engineering altered fatty acid chain length selectivity into OleT<sub>E</sub> in order to enable production of short chain, volatile alkenes.

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