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Oxygenation of Monoenoic Fatty Acids by CYP175A1, an Orphan Cytochrome P450 from *Thermus thermophilus* HB27

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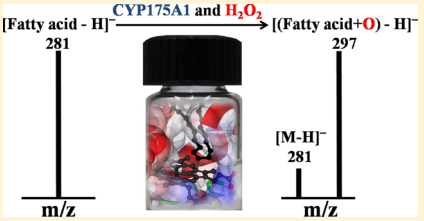
Supporting Information

ABSTRACT: The catalytic activity of CYP175A1 toward monooxygenation of saturated and monounsaturated fatty acids of various chain lengths (C16−C24) has been investigated to assess the enzymatic properties of this orphan thermostable cytochrome P450 enzyme. The results showed that the enzyme could catalyze the reaction of monounsaturated fatty acids but not of saturated fatty acids. The product analyses using ESI-MS and GC-MS revealed an important regioselectivity in the CYP175A1 catalyzed monooxygenation of the monoenoic fatty acids depending on the ethylenic double bond (C═C) configuration. When the double bond was in cis-configuration, an epoxy fatty acid was found to be the major product and two allyl-hydroxy fatty acids were found to be the minor products. But when the double bond was in trans-configuration the product distribution was reversed. The oxygenation efficiency was found to be the highest for palmitoleic acid (chain length C16), but there was no direct correlation of the activity with the chain length or the position of unsaturation of the fatty acid. Molecular docking calculations showed that the "U"-type conformations of the monoenoic fatty acids are particularly responsible for their binding in the enzyme pocket, and that is also consistent with the observed regioselectivity in the oxygenation reaction. The present results provide evidence that CYP175A1 can catalyze the regioselective oxygenation reaction of several monoenoic fatty acids though it cannot catalyze the oxygenation of the corresponding saturated analogues. These studies may provide critical information on the nature of the enzyme pocket and of the possible natural substrate of this orphan enzyme.



Cytochrome P450 forms a superfamily of hemoproteins[1] that catalyze the monooxygenation of various organic compounds[2] including aromatic and aliphatic hydroxylation, N-oxidation, and epoxidation of C═C double bonds.

These enzymes often catalyze regio- and stereospecific oxygenation of nonactivated hydrocarbons under mild conditions, which is a challenging task for chemical catalysis in organic synthesis.[3] Thus, cytochrome P450s have great potential as biocatalysts to perform numerous industrially important reactions such as synthesis of drugs and other fine chemicals.[3] However, low tolerance to various solvents and high temperature have generally limited the usefulness of cytochrome P450 for industrial application. Interestingly, two thermophilic cytochrome P450s, e.g., CYP119 and CYP175A1, have been isolated, purified, and characterized[4−7] in recent years. Both of them possess extreme stability (T_m = 91 °C for CYP119[8] and T_m = 88 °C for CYP175A1[5]) and might be used to overcome the above limitations.

The natural substrates and the biological functions of both of these enzymes (CYP119 and CYP175A1) are unknown,[9] and therefore they are considered as the so-called orphan P450.[10] However, there have been recent advances in using an engineered CYP119 to catalyze the fatty acid hydroxylation[11] and mutant Q67G/Y68I of CYP175A1 to catalyze testosterone hydroxylation.[12] Native CYP175A1 was shown to hydroxylate β-carotene at 3- and 3′-positions.[13]

CYP175A1 shows the highest sequence identity of 26% with cytochrome P450BM-3 (CYP102A1)[5] from *Bacillus megaterium* among all the structurally characterized bacterial cytochrome P450 enzymes. A close inspection of the substrate binding pocket from the crystal structure of CYP175A1 and CYP102A1 shows that the pockets/active sites of these two enzymes are similar and mostly surrounded by hydrophobic residues. Hence, both of them should prefer hydrophobic substrates to show catalytic activity. Long chain saturated fatty acids (lauric acid, myristic acid, palmitic acid, and stearic acid) were found to be the natural substrate of P450BM-3.[14] However, no substrate other than β-carotene[13] has been reported for the native CYP175A1 so far. It is known that *Thermus thermophilus* HB27 produces lipolytic enzymes,[15,16] and it has also its own lipolytic activity.[17] Therefore, we systematically screened several fatty acids (saturated and monounsaturated) for their potential as substrates for CYP175A1.

Here we report that CYP175A1 is able to catalyze the monooxygenation reaction of several monoenoic fatty acids though it cannot catalyze the oxygenation of the corresponding saturated analogues. Along with the substrate specificity, we also investigated the regioselectivity of the monooxygenation reactions of those monoenoic fatty acids both experimentally and theoretically.

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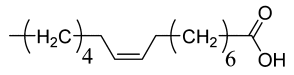
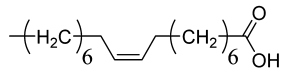
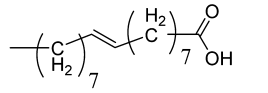
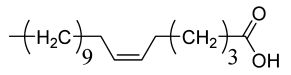
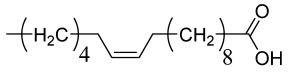
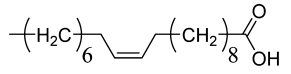
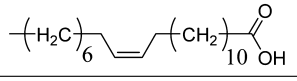
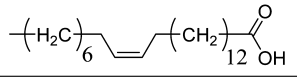
Table 1. Name of the Monoenoic Fatty Acids (Substrates) under Investigations along with Their K_d Values

Trivial name	IUPAC name	Abbreviation used*	Chemical structure	K_d (μM)
Palmitoleic acid	Cis-9-hexadecenoic acid	**16C9**		804 ± 20
Oleic acid	Cis-9-octadecenoic acid	**18C9**		7900 ± 100
Elaidic acid	Trans-9-octadecenoic acid	**18T9**		w.b.†
Petroselinic acid	Cis-6-octadecenoic acid	**18C6**		147 ± 15
Cis-vaccenic acid	Cis-11-octadecenoic acid	**18C11**		205 ± 25
Gondoic acid	Cis-11-eicosenoic acid	**20C11**		w.b.
Erucic acid	Cis-13-docosenoic acid	**22C13**		w.b.
Nervonoic acid	Cis-15-tetracosenoic acid	**24C15**		w.b.

*The first number represents the carbon chain length in fatty acids, C/T represents the cis/trans conformation of the double bond, and the second number represents the positioning of double bond in fatty acids. †w.b.: weak binding.

EXPERIMENTAL SECTION

Materials. The various components of bacteriological culture media were purchased from Himedia, India. Ampicillin sodium salt, chloramphenicol, DNaseI (from bovine pancrease), PMSF, IPTG, sodium cholate, anhydrous sodium sulfate, potassium bicarbonate, hexamethyldisilazane, trimethylchlorosilane, pyridine, 70% aqueous solution of TBHP, (Trimethylsilyl) diazomethane, NADH, and several fatty acids (saturated and monounsaturated) were purchased from Sigma Aldrich. The lysozyme (from hen egg white) and *m*-CPBA was purchased from Fluka. The salt ammonium sulfate, dipotassium hydrogen ortho phosphate and potassium dihydrogen orthophosphate, tris-HCl (2-amino-2-hydroxymethyl-propane-1,3-diol hydrochloric acid) and tris-base were purchased from USB Chemicals. The column material Phenyl Sepharose was purchased from GE Healthcare Bio-Sciences AB, Uppasala. Hydroxyapatite material was purchased from Biorad Laboratories Inc., Hercules, CA. HPLC and/or GC grade methanol, chloroform, diethyl ether, dichloromethane, toluene, cyclohexane, and other required solvents were purchased from Merck Chemicals. EDTA and was purchased from s. d. Fine Chemicals, India. Hydrogen peroxide was purchased from Qualigens. All other buffer components were purchased from Qualigen/s. d. fine chemicals. Millipore water was used for buffer preparation and for all other experimental work wherever required.

The concentration of H_2O_2 was measured spectrophotometrically using the molar extinction coefficient (ε) 39.4 M^{-1} cm^{-1} at 240 nm.[18] *m*-CPBA was recrystallized in a 1:3 diethyl ether/petroleum ether solution using the method described by Davies et al.[19]

Protein Expression and Purification. The *pKK-223* plasmid, encoding the gene for WT CYP175A1, was a kind gift from Prof. V. B. Urlacher (University of Stuttgart, Stuttgart, Germany). The wild type CYP175A1 was expressed and purified by earlier reported method.[5,20,21] In brief, CYP175A1 was grown by taking a single colony of the BL21-DE3 codon plus RP cells, harboring the plasmid encoding the gene for WT protein and inoculating it into the 2 × YT media containing ampicillin (100 μg/mL) and chloramphenicol (50 μg/mL). Expression of protein was induced by addition of IPTG (1 mM final concentration), and the culture was grown at 30 °C for 48 h. After the cell lysis, the protein was precipitated by $(NH_4)_2SO_4$ (35−50% saturation), redissolved in buffer and purified by a hydrophobic column (Phenyl Sepharose), and fractions having R_z (Reinheitszahl, A_{417}/A_{280}) ≥ 1.4 were collected. The protein was further purified on a hydroxyapatite column and fractions having R_z ≥ 1.5 were collected.[20] The purified fractions were concentrated using centriprep concentrators (10 kDa cutoff membrane, Millipore), dialyzed in 50 mM KPi, pH 7.5 buffer and stored at −25 °C in 50% glycerol. The concentration of CYP175A1 was estimated spectrophotometrically from the absorbance at 417 nm (ε_{417} = 104 mM^{-1} cm^{-1}).[5] The purity of the protein was checked by SDS−PAGE, and the ESI-mass spectrum of the purified protein (see Figure S1 in the Supporting Information) confirms the molecular weight of the protein ∼44.87 kDa.

The plasmids encoding wild type putidaredoxin (PdX) and putidaredoxin reductase (PdR) were kindly supplied by Prof. S. G. Sligar (University of Illinois, USA). The wild type putidaredoxin (PdX) and putidaredoxin reductase (PdR) were expressed and purified using a reported procedure,[22] and the concentration of PdX was determined from the absorbance at 455 nm (ε_{455} = 5.9 mM^{-1} cm^{-1})[23] and that of PdR was estimated from the absorbance at 454 nm (ε_{454} = 10.9 mM^{-1} cm^{-1}).[24]

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Enzymatic Oxygenation of Fatty Acids. Fatty acid oxygenation by CYP175A1 was carried out by incubating 500 μL reaction mixture containing 20 μM CYP175A1, 600 μM fatty acid, and 20 mM H_2O_2 in 50 mM KPi buffer (pH = 7.5) at 45 °C unless otherwise mentioned. The controls for the reactions were carried out that contained all the reactants except CYP175A1 or except H_2O_2 in the assay mixture. Each reaction was allowed to proceed for 60 min unless otherwise stated. The reaction mixture was then extracted with chloroform, and the chloroform layer was collected, dried over anhydrous sodium sulfate, filtered through 0.2 μm syringe filter, and then concentrated for mass spectrometric analysis.

The kinetic parameters for the enzymatic reaction were determined by the initial rate method at 45 °C by mixing 20 mM of H_2O_2 to a solution containing different concentrations of the substrate fatty acid (varied between 100 and 500 μM), 20 μM CYP175A1 in 50 mM buffer (pH 7.5). The reaction was stopped by acetic acid (1%) after 1 min, and the unreacted fatty acid along with the products of the enzymatic reaction were extracted in $CHCl_3$ and subjected to ESI-mass spectrometric identification. The concentrations of the reactants and products were determined from the ratio of peak currents, as well as by comparing with the ion currents from standard solutions of the reactants and the products. The initial rates of formation of monooxygenated fatty acid obtained from the mass spectra were estimated at different concentrations of the substrate and analyzed by Lineweaver−Burk method to evaluate the kinetic parameters K_m and k_{cat} of the reaction.

Synthesis of Standard Samples of the Oxygenation Products. *Epoxidation of Monoenoic Fatty Acids.* The epoxidation of the ethylenic double bond present in the monoenoic fatty acid was performed following a slight modification of the literature procedure.[25] 5 mg monoenoic fatty acid (**16C9**, **18C9**, **18T9**, **18C6**, and **18C11**, see Table 1) was reacted with 4 mg *m*-CPBA in 500 μL chloroform at room temperature overnight. The epoxy fatty acid derivative was solvent extracted with diethyl ether after addition of 1 mL of 5% aqueous potassium bicarbonate solution to the reaction mixture. These epoxy derivatives of the monoenoic fatty acids were used as standards in the tandem mass spectrometry for identification of the enzymatic reaction products of these substrates.

Allylic Hydroxylation of Monoenoic Fatty Acids. Allylhydroxy fatty acids were synthesized following a slight modification of the literature procedure.[26] 100 μL of 70% aqueous solution of *tert*-butyl hydroperoxide (TBHP) mixed with ∼1.5 mg selenium dioxide in 200 μL of dichloromethane (DCM) was stirred for 30 min This reaction mixture was added to 200 μL of DCM solution of the monoenoic fatty acid (∼25 mM, **16C9**, **18C9**, **18T9**, **18C6**, and **18C11**, see Table 1) and the solution was kept in a shaker for 24 h. The solution was subsequently mixed with 1 mL of water and solvent extracted with DCM/brine solution. The unreacted fatty acids and the reaction products (allyl hydroxy- derivatives) were separated in the DCM phase and identified by ESI-MS, which showed formation of allylic monohydroxylation products from each of the monoenoic fatty acids (**16C9**, **18C9**, **18T9**, **18C6**, and **18C11**; see Table 1). There were two isomers of the allylic hydroxy- derivatives identified by tandem mass spectrometry, and these allyl hydroxy derivatives were used as standards for identification of the enzymatic reaction products of the monoenoic fatty acids in the present experiment.

Esterification of the Fatty Acids and Their Derivatives for GC-MS Analysis. Esterification of the fatty acids was carried out following a slight modification of the literature procedure.[27] ∼1 mg fatty acid/epoxy fatty acid/hydroxy (allylic) fatty acid was dissolved in 150 μL of toluene and 100 μL of methanol followed by the addition of 2 M trimethylsilyl diazomethane (∼5 μL in ether). The reaction mixture was kept in a shaker for 30 min at room temperature and subsequently concentrated to give the corresponding methyl ester of the fatty acid/epoxy fatty acid/hydroxyl fatty acid, that was used for GC-MS analysis.

Trimethylsilylation of the Hydroxyl Functional Group[28,29] for GC-MS Analysis. The ester of the allyl hydroxy product of the monoenoic fatty acid (as described above) was further converted to trimethylsilyl derivative of the hydroxy group for GC-MS analyses. The hydroxy fatty acid ester was reacted with 15 μL of hexamethyldisilazane and 5 μL of trimethylchlorosilane in the presence of 50 μL of pyridine, and subsequently the trimethylsilyl ether derivative was extracted in cyclohexane and used for GC-MS analysis.

UV−visible Absorption Study. The UV−visible absorption spectra were recorded from 220 to 700 nm (as per needed) in a quartz cuvette having a path length of 1 cm on a double beam Perkin-Elmer (Lambda 750-UV/vis/NIR) spectrophotometer. The wavelength accuracy for the spectrophotometer was ±0.15 nm and it was equipped with a Peltier cell temperature controller with an accuracy of ±0.3−0.5 °C.

Spectral Titration of Binding of the Substrates. The dissociation constants for various fatty acids were determined by titrating the substrate free enzyme (∼5 μM) with fatty acids at room temperature. A decrease in absorbance at Soret (417 nm) and increase in absorbance at 390 nm with the increasing concentration of fatty acids are indicative of binding of the substrate to CYP175A1 and of the conversion of heme iron from low-spin to high-spin state. Data were analyzed by a reported method[30] to obtain the dissociation constant (K_d) values from the intercept and the slope of the plot of $1/\Delta A_{417}$ against 1/[substrate] according to the following equation:

$$1/\Delta A_{417} = (K_d/(P_T \times \Delta\varepsilon_{417})) \times (1/[\text{substrate}]) + (1/(P_T \times \Delta\varepsilon_{417}))$$

where ΔA_{417} is change in absorbance at 417 nm, K_d (slope/intercept) = dissociation constant of enzyme−substrate complex, P_T is the total concentration of the holo enzyme. $\Delta\varepsilon_{417}$ is the difference in extinction coefficient of substrate-free and substrate-bound form at 417 nm, and [substrate] is total concentration of substrate.

Electrospray Ionization Mass Spectrometry (ESI-MS). ESI-MS and collision induced dissociation (ESI-MS/CID) studies were carried out using a Thermo Finnigan LCQ Deca Electrospray quadrupole ion trap mass spectrometer.[31,32] All the experiments were done under identical conditions unless otherwise stated. The flow rate of the analyte solution (in methanol) was maintained at 5 μL/min, and the solution was directly injected through the ion source kept in negative ion mode. Nitrogen was used as sheath and auxiliary gas. The ion source conditions were sheath gas flow rate 25 (arbitrary unit), with no auxiliary gas flow. Capillary temperature (for desolvation) was maintained at approximately 200 °C and capillary voltage was kept at −15 V. The ion-spray voltage and tube lens offset were maintained at −5 kV and −30 V respectively. CID product ion spectra were acquired using an

isolation width of 2 m/z under the normalized collision energy ranged from 40 to 50% with wide band activation turned on. The activation Q (related to the parameter q_z in the Mathieu equation for the precursor ions)[33] of 0.250 with an activation time 30 ms was used for CID-MS/MS (tandem mass spectrometry) studies. Helium was used as buffer as well as collision gas in the collision cell (ion trap). Data acquisition was performed for 1 min using XCalibur software (Thermo Fisher Scientific).

Gas Chromatography–Mass Spectrometry (GC-MS). The fatty acids and their oxidized products were derivatized (esterified and/or silylated) to make them amenable to GC-MS analysis. The GC-MS analysis was performed using a gas chromatograph (Trace GC ultra, Thermo Finnigan Electron Corporation) coupled with an ion trap mass spectrometer (Polaris Q, Thermo Finnigan).[34] Agilent J & W GC capillary column (30 m length, 0.32 mm i.d., 0.50 μm film thickness) was utilized. Helium was used as a carrier gas (99.99% purity) maintained at a constant flow rate of 1.6 mL/min. The GC column temperature program was as follows: initial temperature 150 °C for 2 min, from 150 to 300 °C at a rate of 10 °C/min, finally kept at 300 °C for 2 min. Analyte (in chloroform) injection (1 μL) was performed in a split less mode and the temperature at the injector was 280 °C. The MS conditions were as follows: electron ionization (EI) mode at ionization energy of 70 eV, emission current of 250 μA, ion source and transfer line temperature at 230 and 280 °C, respectively. Standard samples were used to identify the retention times and the products were further verified by matching the fragmentation pattern with a database in the software provided with the instrument.

Molecular Docking Calculations. Docking of various fatty acids with CYP175A1 (PDB ID: 1N97) was performed using GOLD software (version 5.1).[35,36] GOLD is an automated ligand docking program used to calculate the possible conformations of the substrate that binds to the active site of the protein. It uses a genetic algorithm (GA) to check the ligand conformational flexibility and partial flexibility of the protein.[37] It satisfies the fundamental requirement that the ligand must displace loosely bound water on binding.[37] The genetic algorithm parameters used are population size 100, number of islands 5, niche size 2, selection pressure 1.1, migration frequency 10, number of operations 100000, mutation frequency 95, and crossover frequency 95. The default speed selection was used to avoid a potential reduction in docking accuracy. One hundred genetic algorithm (GA) runs were performed without early termination with diverse solution generation. The substrate conformer with the highest binding score was taken into account for prediction of possible mode of binding of the substrate to the enzyme.

RESULTS AND DISCUSSION

Binding of Monoenoic Fatty Acids with CYP175A1. The crystal structure analysis suggested that the active site (enzyme pocket) of CYP175A1 consists predominantly of hydrophobic residues analogous to that in case of cytochrome P450BM-3, which has high structural similarity with CYP175A1. Since fatty acids are known to be the substrates of P450 BM-3,[14] we screened the binding of several saturated and unsaturated (monoenoic) fatty acids in the enzyme pocket of CYP175A1 spectrophotometrically. However, among all the fatty acids tested only some unsaturated monoenoic fatty acids induced changes in the absorption spectra in the Soret region. Titration of CYP175A1 with different monoenoic fatty acids showed an increase in absorbance at 390 nm and decrease in absorbance at 417 nm (see Figure S2 in the Supporting Information). A typical isosbestic point at 405 nm was observed in the difference spectra of substrate-bound versus substrate free CYP175A1 (see Figure S2 in the Supporting Information). This result suggests a typical type I substrate binding.[2] The calculated K_d values are reported in Table 1. The observation of large dissociation constant (K_d) values (Table 1) suggests that the substrate may be weakly bound or binding of the substrate does not efficiently displace the axial water molecule from the heme center of the enzyme.

Oxygenation of Monoenoic Fatty Acids by CYP175A1. The catalytic activity of CYP175A1 was assayed using H_2O_2 as the source of oxidizing equivalents.[2,20,38−44] The high-valent redox intermediate (cpdI) of the cytochrome P450 could be generated by the peroxide shunt pathway using H_2O_2 or analogues (*m*-CPBA, cumenehydroperoxide, peroxyacid, etc.) to catalyze the oxygenation reaction of the substrate.[2,20] A reaction mixture containing CYP175A1, fatty acids, and H_2O_2 (see Experimental Section for details) at 45 °C showed the oxygenation of the monoenoic fatty acids.

The product and the unreacted substrate extracted in organic phase were analyzed in ESI-MS by direct injection in the negative ion mode (see Figure 1). In the negative ion mode of ESI, ionization of the fatty acid or oxygenated fatty acid occurs via deprotonation of the carboxylic acid functional groups.[33,45] The ionization efficiency[45] of both substrate (fatty acid) and

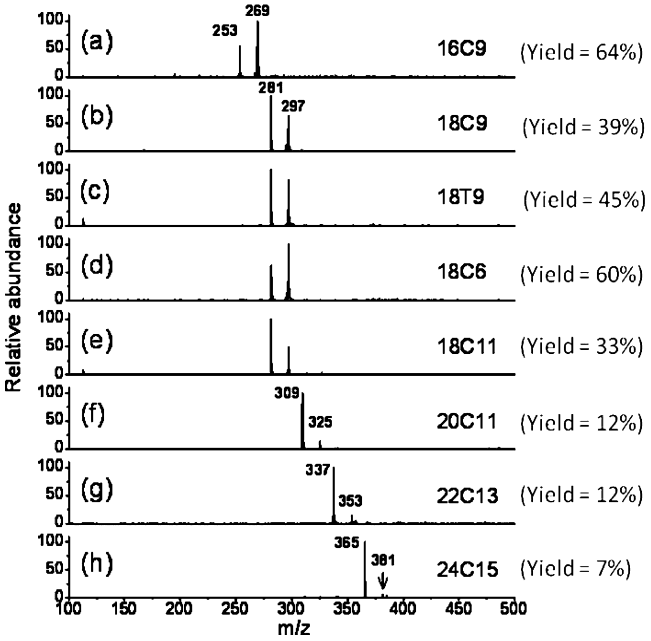

Figure 1. Negative ion ESI-MS profile of CYP175A1 catalyzed oxygenation at 45 °C of (a) **16C9**; palmitoleic acid (m/z 253 for substrate and m/z 269 for oxygenation product), (b) **18C9**; oleic acid (m/z 281 for substrate and m/z 297 for oxygenation product), (c) **18T9**; elaidic acid (m/z 281 for substrate and m/z 297 for oxygenation product), (d) **18C6**; petroselinic acid (m/z 281 for substrate and m/z 297 for oxygenation product), (e) **18C11**; *cis*-vaccenic acid (m/z 281 for substrate and m/z 297 for oxygenation product), (f) **20C11**; gondoic acid (m/z 309 for substrate and m/z 325 for oxygenation product), (g) **22C13**; erucic acid (m/z 337 for substrate and m/z 353 for oxygenation product), and (h) **24C15**; nervonoic acid (m/z 365 for substrate and m/z 381 for oxygenation product).

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product (oxygenated fatty acid) were found to remain essentially the same. Control experiments with known concentrations of the fatty acids and their oxygenated derivatives showed almost the same peak current per μM of the sample (e.g., a control mixture of 500 μM of 16C9 and 500 μM of its standard oxygenated derivative showed an average ion count of 4.92×10^5 at m/z=253.4 for the fatty acid, and an average ion count of 4.3×10^5 at m/z = 269.4 for the oxygenated derivative, under identical instrument conditions). Thus, the ratio of the concentration of the unreacted substrate to the concentration of product in the enzymatic reaction could be calculated from the ratio of the peak current (ion signal) of the substrate (unreacted fatty acid) to that of the product (oxygenated fatty acid) recorded in ESI-MS (Figure 1).

It is evident from Figure 1 that the monooxygenation has taken place (an increase by 16 Da in the molecular weight of the products relative to that of the corresponding substrates) in all the substrates under investigation (see Table 1). Moreover, no ion signals corresponding to addition of two or more oxygen atoms to the fatty acid were detected in the present studies, indicating that only single monooxygenation of the fatty acids is catalyzed by CYP175A1. The percentage yields for monooxygenation of each fatty acid are shown in Figure 1. Figure 1 shows that the catalytic activity of CYP175A1 is maximum for **16C9** (64%) and **18C6** (60%) and minimum for **20C11** (12%), **22C13** (12%), and **24C15** (7%). No significant product formation was observed in the control experiments containing the fatty acid with H_2O_2 but without CYP175A1, or containing the fatty acid with the enzyme but without H_2O_2 in the reaction mixtures (see Figure S3 in the Supporting Information for a typical substrate **16C9**). Thus the oxygenation of the above fatty acids is essentially carried out by the enzymatic action of CYP175A1. These results thus indicate that the substrate binding cavity of the enzyme might be such that it can accommodate the monoenoic fatty acid **16C9** more favorably compared to the larger fatty acids.

We also studied the fatty acid oxygenation in the reductive pathway using putidaredoxin (PdX)-putidaredoxin reductase (PdR) as the redox partners for the class-I type of CYP enzyme system and NADH as the electron donor. PdX-PdR redox partners were earlier[13] shown to drive the reductive pathway for hydroxylation of β-carotene by CYP175A1. The reductive pathway also resulted in the oxygenation of the monoenoic fatty acids. However, the reaction efficiency (e.g., 21% for **16C9**) was lower compared to that in the peroxide shunt pathway (64% for **16C9**), indicating poor redox coupling of PdX-PdR system with CYP175A1 (see Figure S4 in the Supporting Information).

The effects of the experimental conditions on the enzymatic efficiency were assessed by varying the time course, temperature, and H_2O_2 concentration in the reaction mixture without changing the initial concentrations of the enzyme and of the substrate. The percentage yield of the product increased with incubation time and reached a plateau at ~30 min (Figure 2a). Variation of the reaction temperature from 30 to 70 °C also showed distinct temperature dependence of the percentage yield of the product formation and the maximal activity temperature for the monoenoic fatty acid oxygenation was found to be 45 °C (Figure 2b).

Figure 3 shows that with the increase in the concentration of H_2O_2 from 1 mM to 20 mM in the reaction mixture (containing **16C9**), the amount of product (after 1 h reaction) increases and reaches a maximum at 20 mM H_2O_2

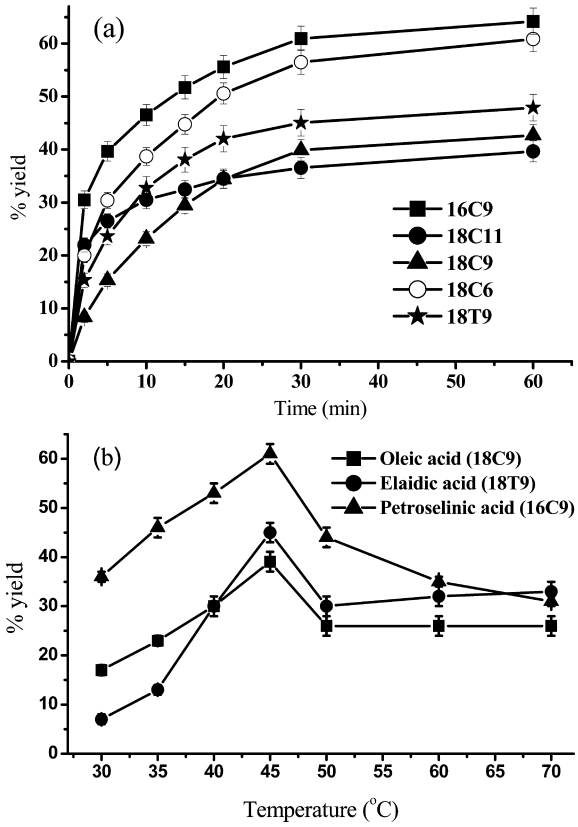

Figure 2. Upper panel (a) shows CYP175A1 catalyzed oxygenation of the monoenoic fatty acids (**16C9**, **18C9**, **18T9**, **18C6**, and **18C11**) at 45 °C as a function of time; lower panel (b) shows CYP175A1 catalyzed oxygenation of three typical monoenoic fatty acids (**16C9**, **18C9**, and **18T9**) in 1 h as a function of temperature. The detailed experimental and analytical conditions are given under Experimental Section.

concentration. When the peroxide concentration was 200 μM and the reaction time was 24 h, the total yield of the oxygenated product for **16C9** was found to be 30% (data not shown). This yield is almost half of that obtained in the presence of 100 times more concentration of H_2O_2 (20 mM H_2O_2) in 1 h reaction time (64% yield for **16C9**). It is however important to note that presence of H_2O_2 causes degradation of the enzyme, which becomes predominant at higher concentrations of the peroxide. Increase of H_2O_2 concentration above 20 mM causes a decrease in the amount of the product formation (Figure 3) possibly due to the H_2O_2-dependent bleaching of the heme chromophore and degradation of the enzyme.[38,40]

We also assessed the heme degradation of CYP175A1 in the presence of the substrate (**16C9**) at different concentrations of H_2O_2 by monitoring the Soret absorption at 417 nm with time (see Figure S5 in the Supporting Information). The results indicated that even with 20 mM H_2O_2, there were ~38% degradation of the enzyme in 1 h, though the overall yield of the enzymatic product at this concentration of peroxide was the highest (64%, Figure 1). Moreover, the degradation of the enzyme by peroxide was not linear with time, and there was only <10% degradation of the enzyme in 1 min in the presence of 20 mM H_2O_2 (see later).

Identification of the Products. *CID-MS/MS Analysis.* Electrospray ionization in the negative ion mode resulted in the production of carboxylate anion of both the substrate (fatty

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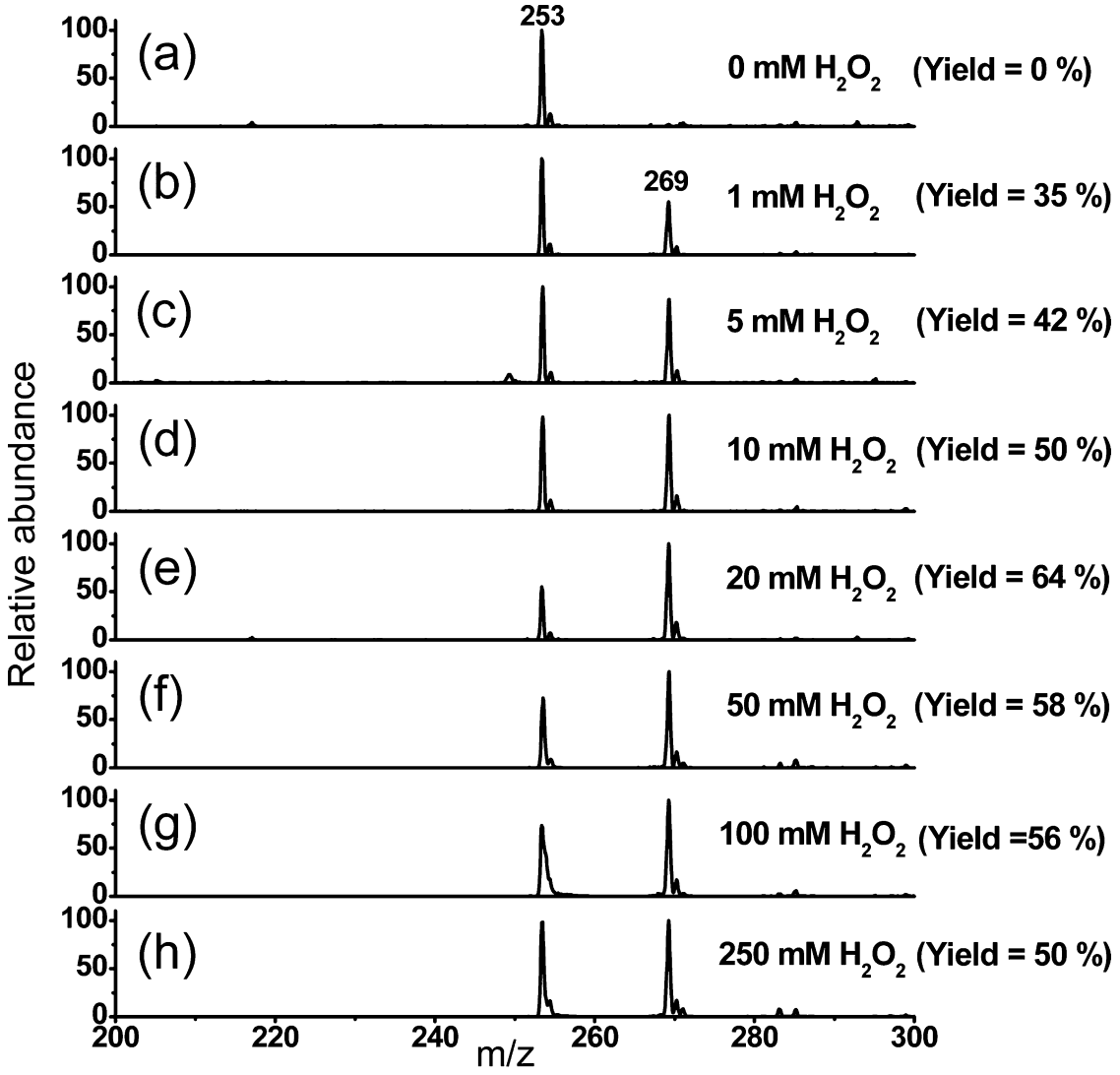

Figure 3. Negative ion ESI-MS study of the enzymatic (CYP175A1) oxygenation of palmitoleic acid (**16C9**) at different H_2O_2 concentrations. The peak at *m/z* 253 and *m/z* 269 corresponds to the unreacted reactant and oxidized product respectively. The detailed experimental and analytical conditions are given under Experimental Section.

acids) and product (oxygenated fatty acids) in the gas phase. In order to identify the site of monooxygenation in the product, the product ion was mass selected and isolated in the ion trap for collision induced dissociation (CID) fragmentation. The CID-MS/MS spectra of two typical monooxygenated fatty acids of **18C9** and of **18T9** are shown in Figures 4 and 5 respectively (see Figure S6 for **16C9**, Figure S7 for **18C6** and Figure S8 for **18C11** in the Supporting Information). Both **18C9** and **18T9** are C18-monounsaturated fatty acid with the double bond at the same position (C9−C10). They only differ in the configuration around the double bond. **18C9** has a cis-double bond and **18T9** has a trans-double bond (see Table 1).

CID-MS/MS spectrum (see Figure 4a) of the enzymatically monooxygenated **18C9** (*m/z* 297, labeled as $[M - H]^-$) shows an intense fragment ion signal at *m/z* 171 (base peak) along with the fragment ion signals at *m/z* 155 as well as at *m/z* 141, *m/z* 157, *m/z* 169, and *m/z* 183. The monooxygenation of the fatty acid may lead to the formation of epoxide (e.g., *cis*-9,10-epoxystearic acid in the case of **18C9**) and/or the allyl-hydroxy derivative with hydroxylation either at C8 or at C11 of **18C9**. In order to identify the monooxygenation product(s) of the fatty acid, we compared the CID-MS/MS spectra of the monooxygenated fatty acid with those of the standard epoxy derivative (Figure 4b) and of the standard hydroxy derivative (Figure 4c) of the fatty acid.

Figure 4b shows that the CID-MS/MS spectrum of *cis*-9,10-epoxystearic acid (epoxy derivative of **18C9**) gives two fragment ions at *m/z* 171 and *m/z* 155 with relative ion counts (relative abundance) 5:1. Figure 4c shows that allylhydroxy oleic acids (hydroxy derivatives of **18C9**) give CID-MS/MS ion fragments at *m/z* 157, *m/z* 183, and *m/z* 169, *m/z* 141, which were assigned to fragments of 8-hydroxyoleic acid and 11-hydroxyoleic acid respectively. Thus the CID-MS/MS spectrum (Figure 4a) of the monooxygenated product of **18C9** consists of fragments from the epoxy derivative (Figure 4b) as well as those from the hydroxy derivative (Figure 4c) of the fatty acid. Analyses of the relative ion counts for each of these derivatives suggested that the enzymatic monooxygenation of **18C9** gives a 7:3 ratio of the epoxy (∼70%) and hydroxy (∼30%) products (see Scheme 1). Moreover, the results indicate that the 8-hydroxy derivative was higher

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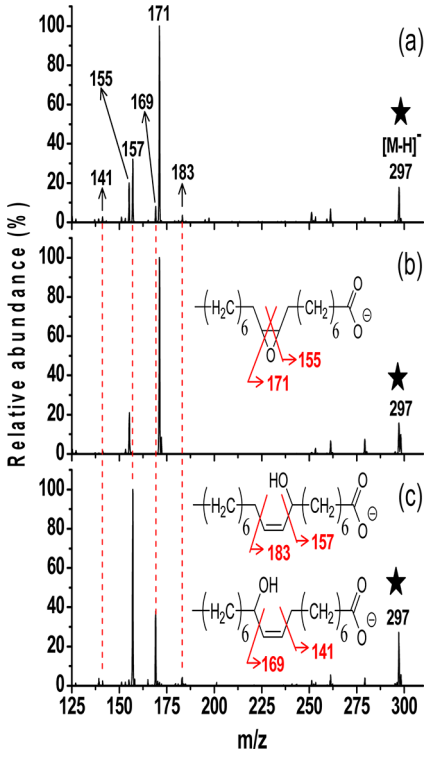

Figure 4. Negative ion CID-MS/MS spectra of the (a) CYP175A1 catalyzed oxygenation product (m/z 297) of oleic acid (**18C9**), (b) standard *cis*-9,10-epoxystearic acid (epoxy-**18C9**, m/z 297), and (c) standard 8-hydroxyoleic acid and 11-hydroxyoleic acid mixture (allyl-hydroxy-**18C9**, m/z 297).

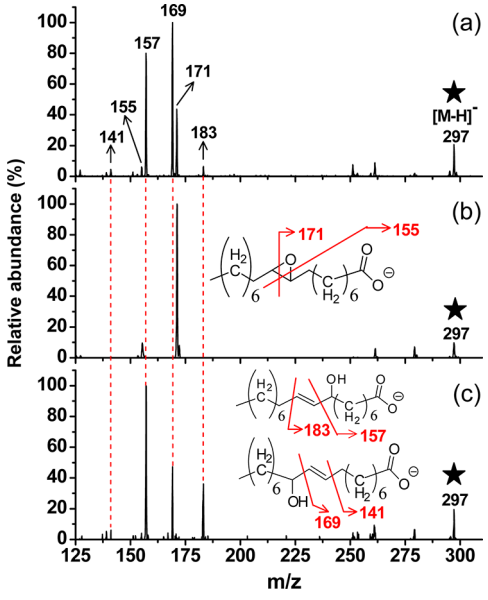

Figure 5. Negative ion CID-MS/MS spectra of the (a) CYP175A1 catalyzed oxygenation product (m/z 297) of elaidic acid (**18T9**), (b) standard *trans*-9,10-epoxystearic acid (epoxy-**18T9**, m/z 297), and (c) standard 8-hydroxyelaidic acid and 11-hydroxyelaidic acid mixture (allyl-hydroxy-**18T9**, m/z 297).

(~24%) compared to the 11-hydroxy derivative (~6%) of the fatty acid.

Analogous to the cis-isomer (**18C9**), the CID-MS/MS spectrum of the enzymatic monooxygenation product of **18T9** shows six ion fragments (Figure 5a) with the m/z values identical to those of the cis-isomer. However, the relative ion counts of the ion fragments from the trans-isomer (**18T9**) were distinctly different from those obtained from the cis-isomer (**18C9**). Analogous to the cis-derivative, the CID-MS/MS spectrum of the standard *trans*-9,10-epoxystearic acid (epoxy derivative of **18T9**) gives two fragment ions at m/z 171 and m/z 155 (Figure 5b) with slightly different relative ion counts (relative abundance) of 10:1. Similarly, Figure 5c shows that chemically synthesized allylhydroxyelaidic acids (hydroxy derivatives of **18T9**) give the same CID-MS/MS ion fragments as those observed in the case of the cis-isomer (Figure 4c), but the relative ion counts of the ion fragments are different than those observed for the allylhydroxyoleic acid (Figure 4c). Analyses of the relative ion counts for each of the fragment ions obtained from the enzymatic monooxygenation of **18T9** (Figure 5a) with the help of the standard epoxy- and allylhydroxy- derivatives suggested that the enzymatic monooxygenation of **18T9** gives a 1:4 ratio of the epoxy (20%) and hydroxy (80%) products (see Scheme 1). The 8-hydroxy derivative was lower (~35%) compared to the 11-hydroxy derivative (~45%) of the fatty acid. The formation of the different fragment ions by collision induced dissociation of the cis- and trans-isomers of the monooxygenated fatty acids is shown in Scheme S1 in the Supporting Information.

These results thus suggest that monooxygenation of **18C9** catalyzed by CYP175A1 yields *cis*-9,10-epoxystearic acid as the major product (70%) and 8-hydroxyoleic acid (24%) and 11-hydroxyoleic acid (6%) as minor products. Analogously, CYP175A1 catalysis gives 8-hydroxyelaidic acid (35%) and 11-hydroxyelaidic acid (45%) as major products and *trans*-9,10-epoxystearic acid (20%) as the minor product on enzymatic monooxygenation of **18T9** indicating high regioselectivity of the enzymatic reaction of CYP175A1.

In a similar manner, by comparing the CID-MS/MS spectra of the CYP175A1 catalyzed oxygenation products of the other fatty acids (**16C9**, **18C6**, and **18C11**) with the standard samples, we observed that **16C9**, **18C6**, and **18C11** (cis-configuration of the double bond) yield epoxy fatty acid as the major product and allylhydroxy fatty acid as the minor product upon enzymatic action of CYP175A1 (Figures S6, S7, S8 in Supporting Information).

The enzymatic reaction products obtained by the fatty acid oxygenation in the reductive pathway using putidaredoxin (PdX)-putidaredoxin reductase (PdR) as the redox partners were also analyzed by ESI-MS and CID-MS/MS to identify the products. The results showed that the products of the enzymatic monooxygenation by the reductive pathway were the same as those obtained by the oxidative (driven by H_2O_2) shunt pathway (data not shown).

GC-MS Analysis. The products of the CYP175A1 catalyzed monooxygenation of the fatty acids were further characterized by GC-MS. Fatty acids require esterification and/or other derivatization to make them sufficiently volatile for detection by GC technique.[46] The carboxylic acid group of the fatty acids and their derivatives were esterified (methyl ester) to detect them in the GC. The unreacted fatty acid as well as the corresponding epoxy-product could be detected by esterification (Figure S9a,b in Supporting Information). The total-ion current (TIC) gas chromatogram of the enzymatic oxygenation (Figure S9a in Supporting Information for a typical fatty acid **16C9**) shows distinct GC peaks for unreacted fatty acid ester (e.g., at retention time 9.9 min for **16C9**) and for the epoxy derivative of the fatty acid ester (e.g., at retention time 11.92

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min for methyl 9,10-epoxypalmitoleate). The TIC of the peaks in the gas chromatogram of the methyl esters of a known mixture of the fatty acid and the corresponding standard epoxy derivative (Figure S9b in Supporting Information) match with that of the chromatogram of the methyl esters of the enzymatic reaction solution, supporting the formation of the epoxy product by the enzyme. It is important to note that unlike in the case of the soft ionization method of ESI-MS, the TIC in the GCMS analyses cannot be used to get the quantitative estimates of the relative concentrations of various products of the fatty acid in the present case. Moreover, simple esterification of the sample could not detect the allyl-hydroxy products of the fatty acids.

The formation of the allyl-hydroxy products could be detected by silylation of the hydroxyl group (Figure S9c,d in Supporting Information). When the esterified monooxygenated product (produced enzymatically from **16C9**) was further derivatized by silylation reaction, two additional peaks at retention time 11.47 min and 11.59 min appeared along with the previous peak of epoxy-**16C9** at the retention time 11.92 min (see Figure S9c in Supporting Information). The esterification followed by silylation of the chemically synthesized standard allyl-hydroxy derivatives of **16C9** also gave two peaks in GC at retention time 11.47 min and 11.59 min (see Figure S9d in Supporting Information). This result indeed confirmed the formation of two allyl-hydroxy products of **16C9** along with epoxy-**16C9** in the CYP1751 catalyzed oxygenation of **16C9**. The enzymatic products were further confirmed by comparing the GC-mass spectral fragmentation data (see Figure S10 in Supporting Information) of each species with a specific retention time in the TIC chromatogram with that of the standard samples having the same retention time (Figure S11 in the Supporting Information). An intense ion signal at m/z 213 (retention time 11.47 min.) corresponding to $[CH_3\text{-}(CH_2)_5\text{-}CH{=}CH{-}CH{-}O\text{-}SiMe_3]^+$ (see Figure S10a and Figure S11a in Supporting Information) was observed in the enzymatic reaction solution as well as in the standard allyl-hydroxy derivative of **16C9** suggesting the presence of the hydroxyl group at C8 position in the monooxygenated **16C9**.[47] Similarly an intense ion signal at m/z 285 (retention time 11.59 min) corresponding to $[Me_3Si{-}O{-}CH{-}CH{=}CH\text{-}(CH_2)_7\text{-}COOMe]^+$ (see Figures S10b and S11b in Supporting Information) was observed both in the enzymatic product and in standard allyl-hydroxy derivative of **16C9** suggesting the hydroxyl group at C11 position in the monooxygenated **16C9**.[47] Analogously, the species with retention time 11.92 min was confirmed as the epoxy derivative of **16C9** (see Figures S10c and S11c in Supporting Information).

Molecular Docking of the Substrates. Molecular docking of the monoenoic fatty acids on the CYP175A1 (PDB: 1N97) was carried out to assess the structural basis of the interaction of the fatty acids with the enzyme. The docking results showed that fatty acids adopt "U"-shaped conformation inside the active site of the protein (see Figures 6 and 7 for two typical fatty acids **18C9** and **18T9** respectively). This "U" shaped conformation of the substrate possibly arises due the restricted rotation of the fatty acid around the double bond inside the active site. The docking results with several fatty acids (**16C9, 18C9, 18T9, 18C6**, and **18C11**) showed that there are two possible types of interactions between the substrate and the enzyme in the present case, *viz.*, (1) the hydrophobic interaction of the alkyl chain with the hydrophobic amino acid residues (e.g., Leu, Ile, Val, and Ala) at the

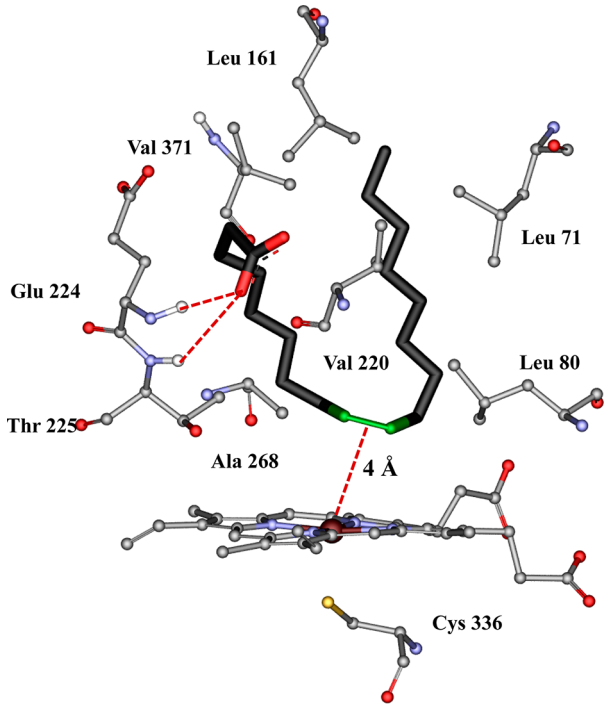

Figure 6. Docking model of the oleic acid (**18C9**) in the active site of CYP175A1 obtained by the GOLD program. The substrate (oleic acid) is shown at the top of the heme ring. The most probable site susceptible to oxygenation is the ethylenic (double) bond (shown in green color; nearer to the heme iron) in the oleic acid.

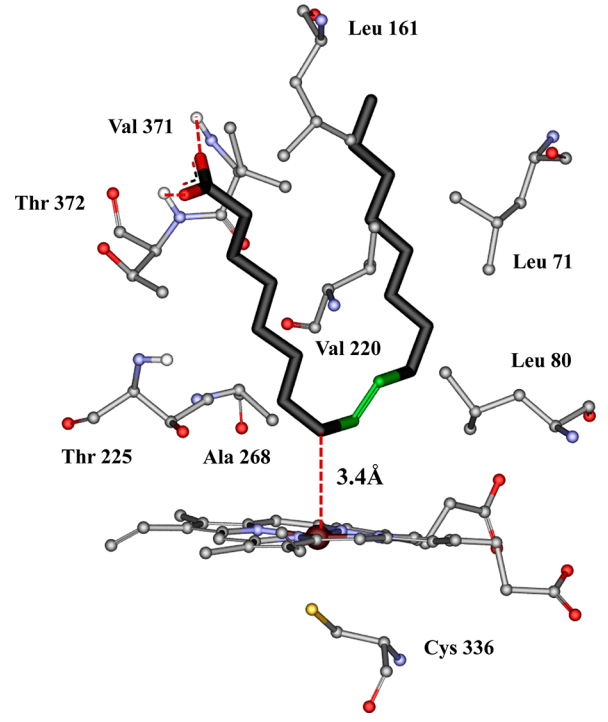

Figure 7. Docking model of the elaidic acid (**18T9**) in the active site of CYP175A1 obtained by the GOLD program. The substrate (elaidic acid) is shown at the top of the heme ring. The most probable site susceptible to oxygenation is the allylic methylene group (nearer to the heme iron; the ethylenic double bond in the elaidic acid is shown in green) in the elaidic acid.

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substrate binding site (see Figures 6 and 7 for two typical fatty acids **18C9** and **18T9**), and (2) the ionic interaction (hydrogen bonding) of the carboxylate group of the fatty acid with the amide hydrogens of nearby amino acids (e.g., Glu 224 and Thr 225 in Figure 6 and Val 371 and Thr 372 in Figure 7) and with water inside the active site.

Since no binding of the saturated fatty acids (e.g., palmitic acid, stearic acid, etc.) to the enzyme was observed, the double bond in the monoenoic fatty acid possibly helps in taking the "U" shape and thus facilitates binding of the substrate at the enzyme pocket. The docking results also indicated that depending on the chain length, double bond position, and stereochemistry of the substrate, different monoenoic fatty acids adopted different spatial orientations in the enzyme pocket (data not shown). Thus the polar residue(s) of the enzyme, which are involved in the interaction with the carboxylic acid group of the substrate, may be different for different fatty acids of varied length and geometry. The docking result of **16C9** also exhibited a possibility of polar interaction involving Thr168 and the carboxylate group of **16C9** (see Figure S12 in the Supporting Information) in the active site of the enzyme pocket, while the same residue is not involved in interaction with other fatty acids. Furthermore, the docking results indicated that the double bond of the *cis*-monoenoic fatty acid (**18C9**, see Figure 6) and allylic-methylene group of the *trans*-monoenoic fatty acid (**18T9**, see Figure 7) reside nearer to the heme iron (at ∼4 Å for **18C9**, and at ∼3.4 Å for **18T9**) making them as the potential site for oxygenation, which agrees with the experimental results (Figures 4 and 5, and Scheme 1). The binding of the fatty acid to the enzyme is however weak (large K_d values, Table 1), and thus the fluctuations in the conformation of the fatty acid inside the enzyme cavity could account for observation of both the epoxy- as well as allyl-hydroxy products on enzymatic monooxygenation by CYP175A1.

It is however important to note that the present *in-silico* analyses of the structures of the enzyme and the fatty acids only provide the possible conformations of the substrates at the active site of the enzyme, and the exact disposition of the fatty acid inside the enzyme cannot be predicted without the crystal structure of the enzyme−substrate complex.

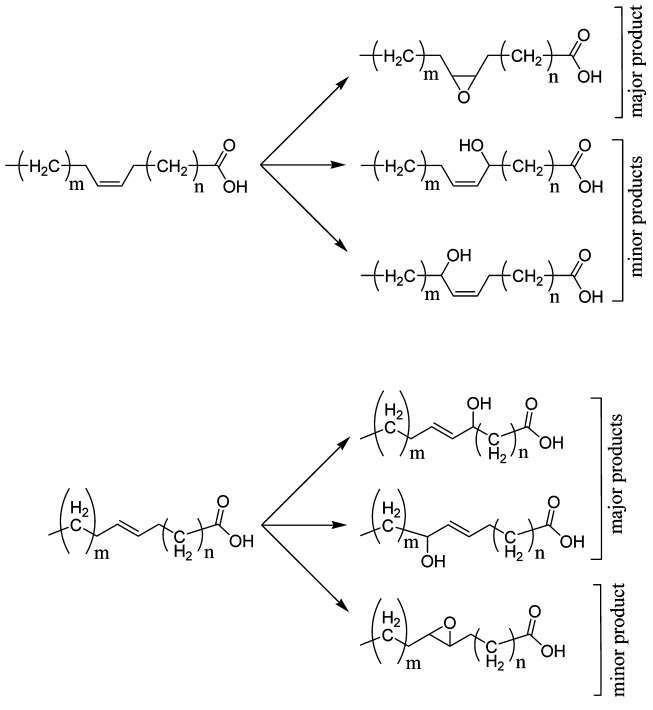
Activity Assay of the Enzyme. The enzymatic activity of CYP175A1 for monooxygenation of the monoenoic fatty acids by H_2O_2 following the oxidative shunt pathway of the enzymatic cycle was determined at 45 °C by Lineweaver−Burk method from the linear fit to the plot of 1/rate (V) vs 1/[substrate] (see Figure S13 in Supporting Information). The values of K_m and k_{cat} for enzymatic reaction of various monoenoic fatty acids with H_2O_2 catalyzed by CYP175A1 are given in Table 2. The results indicate that CYP175A1 show highest enzymatic efficiency (k_{cat}/K_m) for fatty acids **16C9** and **18C9**, while that for the trans- isomer **18T9** is the lowest. The enzymatic activity (k_{cat}) was highest for **16C9** with the turnover number ∼16 min^{-1}. Earlier studies on enzymatic reaction of β-carotene by CYP175A1 following the reductive pathway (i.e., with NADH, PdX, PdR, and oxygen) reported the turnover rate of 0.23 min^{-1}, which is much lower than that observed for reaction of the monoenoic fatty acid by the enzyme following oxidative shunt pathway (i.e., with H_2O_2) in the present study. The reductive pathway of the enzymatic reaction using PdX and PdR was also found to be less efficient in the present case possibly because of weaker redox coupling between PdX and CYP175A1. However, it is important to note that unlike in the reductive pathway the peroxide induced oxidative shunt pathway of the enzymatic reaction was associated with partial degradation of the enzyme (see Figure S5 in Supporting Information). Nevertheless, there was <10% degradation of the enzyme by 20 mM H_2O_2 in the initial 1 min of the reaction (see Figure S5 in Supporting Information), and hence the initial rates (Table 2) determined at this time period may be assumed to have almost no effect of degradation of the enzyme. The apparent rates of the reaction over 1 h (e.g., from the percentage yield data shown in Figure 1) would however be much less than that estimated in a short time period (Table 2) because of partial degradation of the enzyme (see Figure S5 in Supporting Information) as well as because of saturation of the reaction (Figure 2) at longer time scales.

Table 2. Kinetic Parameters of the Peroxide Mediated Fatty Acid Oxygenation at 45 °C by the Enzymatic Action of CYP175A1

substrate	K_m (mM)[a]	k_{cat} (min^{-1})[a]	k_{cat}/K_m (mM^{-1} min^{-1})
palmitoleic acid (**16C9**)	2.89 ± 0.30	16.67 ± 0.50	5.76 ± 0.62
oleic acid (**18C9**)	0.60 ± 0.10	2.94 ± 0.20	4.90 ± 0.88
elaidic acid (**18T9**)	5.18 ± 0.40	5.00 ± 0.30	0.96 ± 0.09
petroselinic cid (**18C6**)	2.82 ± 0.20	7.14 ± 0.40	2.53 ± 0.23
cis-vaccenic acid (**18C11**)	3.43 ± 0.20	10.00 ± 0.50	2.91 ± 0.22

[a]Apparent values of K_m and k_{cat} were calculated from Lineweaver−Burk plots by monitoring the reaction for 1 min using ESI-MS as described in the Experimental Section. Results are the mean ± standard deviation of three independent sets of experiments.

Scheme 1. Product Distribution of the CYP175A1 Catalyzed Oxygenation of *cis*-Monoenoic Fatty Acid (Upper Panel) and Trans-Monoenoic Fatty Acid (Lower Panel) under Investigation



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CONCLUSIONS

The thermostable enzyme CYP175A1 catalyzes monooxygenation reaction of various monoenoic fatty acids by H_2O_2 in the oxidative shunt pathway as well as by oxygen and NADH in the reductive pathway (in the presence of PdX and PdR). This monooxygenation reaction exhibits regioselectivity and the product distribution depends on the configuration of the ethylenic double bond in the substrate (monoenoic fatty acid). Mass spectrometric studies of the enzyme (CYP175A1) catalyzed oxygenation reaction showed that the epoxy fatty acid (double bond oxygenation) is the major product for the *cis*-monoenoic fatty acid substrate, while the allyl-hydroxy fatty acids are the major products for the trans-monoenoic fatty acid substrate. This regioselectivity is consistent with the substrate docking analyses. The specificity of the enzyme also varies with the chain length and position of the double bond (C═C) in the monoenoic fatty acids. Among all the monoenoic fatty acid substrates, palmitoleic acid (**16C9**) was found to be the better substrate than others. The results further support that CYP175A1 may have an important role in lipid metabolism in *Thermus thermophilus* HB27, as it can efficiently metabolize various fatty acids. The present work further suggests that the natural substrate of this orphan enzyme might have a "U" shape structure for efficient binding to the active site of the enzyme.

ASSOCIATED CONTENT

Supporting Information

ESI-MS of CYP175A1, binding titration of various fatty acids with CYP175A1, ESI-mass spectra of the control reactions of **16C9**, enzymatic reaction of **16C9** with CYP175A1 using reductive pathway, H_2O_2 dependent heme degradation, CID-MS/MS of enzymatic and standard products of **16C9**, **18C6** and **18C11**, TIC gas chromatogram of enzymatic and standard products of **16C9**, GC-MS of enzymatic and standard products of **16C9**, docking result of palmitoleic acid (**16C9**), Lineweaver−Burk plot for various fatty acid kinetics, and possible mechanism of fragmentation of epoxy- and different allyl-hydroxy fatty acids during CID/MS/MS study. This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

#These two authors contributed equally to this work.

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Notes

The authors declare no competing financial interest.

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ABBREVIATIONS

heme, iron protoporphyrin IX regardless of oxidation and ligation state; WT, wild type; T_m, melting temperature; PDB, Research Collaboratory for Structural Bioinformatics (RCSB) Protein Data Bank, http://www.rcsb.org/pdb/; PMSF, phenyl methyl sulphonyl fluoride; IPTG, isopropyl-β-D-1-thiogalactopyranoside; TBHP, *tert*-butyl hydroperoxide; *m*-CPBA, *m*-chloroperbenzoic acid; EDTA, ethylenediaminetetraacetic acid dipotassium salt; H_2O_2, hydrogen peroxide; NADH, β-nicotinamide adenine dinucleotide, reduced dipotassium salt; ESI-MS, electrospray ionization mass spectrometry; GC-MS, gas chromatography mass spectrometry; CID, collision induced dissociation; GOLD, genetic optimization for ligand docking

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