His-311 and Arg-559 Are Key Residues Involved in Fatty Acid Oxygenation in Pathogen-inducible Oxygenase*

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Pathogen-inducible oxygenase (PIOX) oxygenates fatty acids into 2R-hydroperoxides. PIOX belongs to the fatty acid α-dioxygenase family, which exhibits homology to cyclooxygenase enzymes (COX-1 and COX-2). Although these enzymes share common catalytic features, including the use of a tyrosine radical during catalysis, little is known about other residues involved in the dioxygenase reaction of PIOX. We generated a model of linoleic acid (LA) bound to PIOX based on computational sequence alignment and secondary structure predictions with COX-1 and experimental observations that governed the placement of carbon-2 of LA below the catalytic Tyr-379. Examination of the model identified His-311, Arg-558, and Arg-559 as potential molecular determinants of the dioxygenase reaction. Substitutions at His-311 and Arg-559 resulted in mutant constructs that retained virtually no oxygenase activity, whereas substitutions of Arg-558 caused only moderate decreases in activity. Arg-559 mutant constructs exhibited increases of greater than 140-fold in $K_m$ whereas no substantial change in $K_m$ was observed for His-311 or Arg-558 mutant constructs. Thermal shift assays used to measure ligand binding affinity show that the binding of LA is significantly reduced in a Y379F/R559A mutant construct compared with that observed for a Y379F/R558A construct. Although Oryza sativa PIOX exhibited oxygenase activity against a variety of 14–20-carbon fatty acids, the enzyme did not oxygenate substrates containing modifications at the carboxylate, carbon-1, or carbon-2. Taken together, these data suggest that Arg-559 is required for high affinity binding of substrates to PIOX, whereas His-311 is involved in optimally aligning carbon-2 below Tyr-379 for catalysis.

Pathogen attack on plants brings about the activation of multiple enzyme systems that results in the production of oxylipins from 18-22 carbon fatty acid precursors. The generation of these bioactive lipid mediators initiates and sustains the defense reaction of the plant against insects, bacteria, fungi, and other pathogens (1, 2). One of the enzymes up-regulated during the host defense response is pathogen-inducible oxygenase (PIOX), which catalyzes a non-lipoxygenase type of fatty acid oxygenation (3). PIOX belongs to a larger family of heme-containing proteins that oxygenate fatty acids (4), which include the mammalian cyclooxygenases (COX-1 and COX-2; (5)), linoleate diol synthase (LDS) from the fungus Gaeumannomyces graminis (6, 7), and a Pseudomonas alcaligenes protein of unknown function encoded by OrfX (8). PIOX has also been identified in many plant species, including Nicotiana attenuata (9), Nicotiana tabacum (3), Arabidopsis thaliana (3, 10), O. sativa (11), Capsicum annuum (12), and Lycopersicon esculentum (13).

PIOX utilizes stereoselective oxygenation to convert linoleic acid (LA) (18:2, n-6) and other fatty acid substrates to their corresponding 2R-hydroperoxides, generating a novel class of oxylipins (3, 11, 14, 15). The resulting 2R-hydroperoxides undergo spontaneous decarboxylation to shorter aldehydes and fatty acids (11, 14). Using stereospecifically deuterated LA, Hamberg and colleagues (16) showed that the dioxygenation catalyzed by PIOX resulted in the stereoselective removal of the 2-pro-R hydrogen from carbon-2 of the fatty acid substrate, followed by the stereospecific addition of molecular oxygen. Interestingly, the reaction catalyzed by PIOX differs from that observed for plant lipoxygenases and the COX enzymes in that a saturated region of the fatty acid substrate is targeted for oxygen addition (10, 14).

Although it was previously thought that plants do not possess a homolog equivalent to the COX enzymes found in mammals, analysis of the sequence of PIOX and other members of the fatty acid α-dioxygenase family indicate catalytic and structural similarities (5, 10). Three residues crucial for activity in the COX enzymes are strictly conserved within the family; the distal and proximal histidines involved in heme binding (His-207 and His-388, respectively) and Tyr-385, which is responsible for initiation of the cyclooxygenase reaction via hydrogen abstraction (17). The complete conservation of these residues within the fatty acid α-dioxygenase family suggested that the dioxygenation carried out by PIOX was initiated by the conserved tyrosine and that all of these proteins bound a heme prosthetic group (5). Subsequent mutagenesis of the equivalent residues in A. thaliana PIOX (His-163, Tyr-386, and His-389) and O.

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§ The abbreviations used are: PIOX, pathogen-inducible oxygenase; COX, cyclooxygenase; LDS, linoleate diol synthase; LA, linoleic acid; C$_{10}$M, decyl maltoside; BisTris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxy-methyl)propane-1,3-diol.
\section*{EXPERIMENTAL PROCEDURES}

\textbf{Materials}—Linoleic acid (9Z,12Z-octadecadienoic acid), \(\alpha\)-linolenic acid (9Z,12Z,15Z-octadecatrienoic acid), dihomo-\(\gamma\)-linolenic acid (8Z,11Z,14Z-eicosatetraenoic acid), arachidonic acid (5Z,8Z,11Z,14Z-eicosatetraenoic acid), \(\gamma\)-linolenic acid (6Z,9Z,12Z-octadecatrienoic acid), eicosapentanoic acid (5Z,8Z,11Z,14Z,17Z-eicosapentaenoic acid), 11Z,14Z-eicosadienoic acid, stearidonic acid (6Z,9Z,12Z,15Z-octadecatetraenoic acid), eicosatrienoic acid (5Z,8Z,11Z-eicosatrienoic acid), oleic acid (9Z-octadecenoic acid), linoleic acid (9Z,12Z-octadecadienoic acid), 9,12-octadecadiynoic acid, palmitic acid (hexadecanoic acid), pinolenic acid (5Z,9Z,12Z-octadecatrienoic acid), and 2-linoleoyl glycerol (9Z,12Z-octadecadienoic acid, 2-glycerol ester) were purchased from Cayman Chemical Company (Ann Arbor, MI). 11Z-Eicosanoic acid, stearic acid (octadecanoic acid), palmitoleic acid (9Z-hexadecanoic acid), myristic acid (tetradecanoic acid), methyl linolenic acid (1Z,9Z,12Z,octadecatrienoic acid), methyl linoleic acid (methyl 9Z,12Z-octadecadienoic acid), methyl oleic acid (methyl 9Z-octadecenoic acid), 2-methyloctadecanoic acid, and SYPRO Orange were purchased from Sigma. Decyl maltoside (C_{10}M) was purchased from Anatrace (Maumee, OH) and TALON metal affinity resin was purchased from Clontech (Palo Alto, CA). Oligos used for site-directed mutagenesis were purchased from Integrated DNA Technologies (Coralville, IA).

\textbf{Site-directed Mutagenesis}—The variants H311A, H311E, H311L, H311Q, H311R, Y379F, R558A, R558K, R558L, R559A, R559E, R559K, R559L, and the double mutants H311R/R559H, R379F/R558A, and Y379F/R559A were created with the QuikChange Mutagenesis Kit (Stratagene, La Jolla, CA) following the manufacturer’s protocols using wild-type \textit{O. sativa} PIOX DNA in a pQE-30 vector as the template (27) and the following primers (note: the site of mutation is marked in bold and underlined; forward primers are listed above reverse primers): H311A, 5'-GCTGTTATTTGCAAGGCTCGAAGCAGAT TTGGAAT-3'; H311E, 5'-CTGCTGTTAATGGCATCAAGGCTCGAAGCAGATTTGGAAT-3'; H311L, 5'-CTGCTGTTAATGGCATCAAGGCTCGAAGCAGATTTGGAAT-3'; H311Q, 5'-GCTGTTATTTGCAAAAGGCTCGAAGCAGATTTGGAAT-3'; R558A, 5'-CATTTTCATTTTAATGGCATCAAGGCTCGAAGCAGATTTGGAAT-3'; R558K, 5'-CATTTTCATTTTAATGGCATCAAGGCTCGAAGCAGATTTGGAAT-3'; R558L, 5'-CATTTTCATTTTAATGGCATCAAGGCTCGAAGCAGATTTGGAAT-3'; R559A, 5'-CATTTTCATTTTAATGGCATCAAGGCTCGAAGCAGATTTGGAAT-3'; R559E, 5'-CATTTTCATTTTAATGGCATCAAGGCTCGAAGCAGATTTGGAAT-3'; R559K, 5'-CATTTTCATTTTAATGGCATCAAGGCTCGAAGCAGATTTGGAAT-3'; R559L, 5'-CATTTTCATTTTAATGGCATCAAGGCTCGAAGCAGATTTGGAAT-3'. Each constructed mutant was verified by DNA sequence analysis.

\textbf{Protein Expression and Purification}—Wild-type and mutant constructs were transformed into \textit{Escherichia coli} (strain M15) and purified as described in Ref. 27. The purified mutant proteins were pooled and dialyzed against 50 mM BisTris, pH 7.0, 150 mM NaCl, 0.1% C_{10}M. Cells containing an empty pQE-30 vector were induced and carried through purification for use as a negative control and to establish a baseline for oxygen consumption measurements.

\section*{RESULTS}

\subsection*{Identification of Mutants with Reduced Peroxidase Activity}

Members of the plant fatty acid \(\alpha\)-dioxygenase family are distinct with respect to the mammalian COX enzymes in that they exhibit a reduced peroxidase activity, which functions to reduce the developing hydroperoxide during oxygenase catalysis. Liu and colleagues (10) have extensively characterized the heme environment of \textit{A. thaliana} PIOX using spectroscopic, mutagenic, and functional assays to investigate its connection with oxygenase catalysis. Although the heme does play a functional role via the initiation of catalysis, they surmise that the reduced peroxidase activity observed for PIOX is likely a result of restricted access to the distal face of the ferric heme due to a large insert that is present in the PIOX sequence, but lacking in COX-1, COX-2, and other myeloperoxidase family members (10).

Previous biophysical studies and functional analyses have utilized sequence comparisons between the COX enzymes and PIOX to both identify the catalytic residues His-157, Tyr-379, and His-382, and confirm the necessity of these residues for optimal oxygenation of fatty acids by PIOX. Although these studies have been proven to establish that PIOX and the COX enzymes share a basic overall mechanism of catalysis, they have been lacking in the identification of additional residues within PIOX that participate in the dioxygenation reaction. Significant characterization of the residues that line the cyclooxygenase channel of COX-1 and COX-2 has been carried out and molecular determinants governing substrate binding, stabilization, specificity, and product formation have been identified (17–26). These studies were aided by the use of both computational and experimentally derived structural models to guide and validate functional characterizations.

We report here studies designed to identify additional residues within the \textit{O. sativa} PIOX active site that play a role in the \(\alpha\)-dioxygenation of fatty acids and to characterize the substrate specificity of the enzyme. Bioinformatics techniques were used to construct a model of \textit{O. sativa} PIOX, and subsequent analysis of the model identified a putative hydrophobic groove below Tyr-379 into which LA was placed and minimized utilizing experimentally derived constraints. Based on the model of LA bound in the groove of PIOX, we identified candidate residues that could potentially interact with the carboxylate of the fatty acid substrate, performed site-directed mutagenesis, and followed with functional analyses to characterize the effect each mutant had on its ability to bind and oxygenate LA. We also determined the substrate specificity of \textit{O. sativa} PIOX toward a variety of 14–20-carbon fatty acids with varying levels of unsaturation, and tested the ability of the enzyme to bind and oxygenate fatty acids with modifications to the carboxylate, carbon-1, or carbon-2 of the substrate.
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Western Blotting—Purified wild-type and mutant proteins were resolved on a 4–20% SDS-PAGE gel and transferred electrophoretically to polyvinylidene difluoride membranes using a Semi-Dry Transfer Apparatus (Bio-Rad). Membranes were blocked in 5% dry milk, 0.1% Tween 20, and Tris-buffered saline and rocked at 4 °C overnight, followed by four 5-min washes with TBST (1% dry milk and 0.1% Tween 20). The polyvinylidene difluoride membranes were then probed for 1 h with a 1:2000 dilution of a mouse monoclonal antibody directed against polyhistidine tags (Sigma). Following incubation, the membranes were washed and probed with a 1:2000 dilution of rabbit anti-mouse IgG horseradish peroxidase conjugate for 30 min. The polyvinylidene difluoride membranes were then washed and immunodetection was carried out using ECL chemiluminescent reagents (Amersham Biosciences UK Ltd.) followed by exposure to film.

Oxygenase Activity Assay—The enzymatic activity of wild-type and mutant *O. sativa* PIOX proteins was tested by measuring the initial rate of O$_2$ uptake at 30 °C using a YSI model 5300 biological oxygen monitor (Yellow Springs Instrument Co., Yellow Springs, OH), equipped with an oxygen electrode. Each standard assay mixture contained 3 ml of 100 mM Tris, pH 8.0, and 500 μM LA as the substrate. Reactions were initiated by the addition of 2–17 μg of protein in a volume of 5–20 μl. $K_m$ and $V_{max}$ values for native and mutant proteins were determined by measuring oxygen uptake using 1–500 μM LA and fitting the data to the Michaelis-Menten equation using GraphPad Prism 5.0 for Windows (GraphPad Software, San Diego, CA). For substrate specificity studies, 100 μM substrate was utilized in the assay and carried out as described above.

Thermal Shift Assay—Thermal shift assays were performed using the Stratagene Mx3005P real-time PCR instrument (Stratagene, La Jolla, CA). Y379F, Y379F/R558A, and Y379F/R559A mutant constructs were added to a final concentration of 1 μM in a 30-μl total reaction volume containing 0.1 mM Tris, pH 8.0, and 0–50 μM fatty acid. SYPRO Orange was added as a fluorescence reporter at a 1000-fold dilution from its stock solution. The change in fluorescence was monitored using the Cy3 filter, with excitation and emission wavelengths of 545 and 568 nm, respectively. Temperature was raised from 25 to 98 °C in 0.5 °C intervals over the course of 45 min, with fluorescence readings taken at each interval. The fluorescence data were plotted, normalized, and the first derivative of the curve calculated to provide the melting temperature ($T_m$) using GraphPad Prism 5.0 as detailed in Ref. 28. The change in melting temperature ($\Delta T_m$) was calculated as the difference between the $T_m$ of the mutant construct in the presence of fatty acid compared with the mutant construct alone.

Secondary Structure Prediction and Homology Modeling—Prediction of the secondary structural elements of *O. sativa* PIOX were carried out using the PSIPRED protein structure prediction server (29, 30). Confidence levels reported for the prediction of helices in the sequence were calculated by adding the numerical “confidence indicator” assigned by the program for each residue within a predicted helix (a value ranging from 0 to 9, with 9 relating to the highest confidence), computing an average, and dividing by the maximum confidence level (29, 30).

For homology modeling, the *O. sativa* PIOX protein sequence was submitted to a structure prediction meta-server (31), in which *O. aries* COX-1 (Protein Data Bank code 1DIY (32)) was found among the best-ranking homologous proteins and a corresponding sequence to structure alignment was retrieved. The alignment covered 74% of the input sequence with the average sequence identity between aligned segments equal to 19.8%. The output alignment was subsequently used by the program MODELLER (33) to construct a homology model of PIOX. A model of the enzyme-substrate complex was built by importing the structure of LA into the PIOX homology model. LA was manually docked such that carbon-2 of the fatty acid substrate was placed ~2.8 Å below the hydroxyl group of Tyr-379. This constraint is based on: 1) the experimental observation by Hamberg and colleagues (16) that the 2-pro-R hydrogen of fatty acid substrate is abstracted by a tyrosyl radical centered on Tyr-379 of PIOX to initiate catalysis; and 2) by the distances observed between the hydroxyl group of Tyr-385 and carbon-13 in the cocrystal structures of COX-1 with fatty acid substrates (20, 26, 32). The conformation of LA within the PIOX model was further energy minimized using the Amber7 molecular modeling package (34), with the distance between the hydroxyl group of Tyr-379 and carbon-2 of LA fixed as defined above. Finally, the entire hydrophobic pocket, consisting of a 5-Å radius around and including LA, was energy minimized, with no additional distance constraints beyond that defined above for the hydroxyl of Tyr-379 and carbon-2 of LA. General Amber force field parameters were used for LA and parm99 force fields for the protein.

RESULTS

Structure Prediction and Identification of Candidate Residues for Mutagenesis—In the absence of an experimentally derived structure for *O. sativa* PIOX (27), we carried out structural predictions to generate a model for PIOX that could serve as the basis for the design of experiments to further study the function of the enzyme. PIOX and the COX enzymes share sequence similarity of ~20% (3, 5, 15). Analysis of the sequence alignment between *O. sativa* PIOX and *O. aries* COX-1 indicates that significant sequence similarities exist between residues 123–609 of PIOX and residues 135–584 of COX-1, which corresponds to the catalytic domain of the COX enzyme (17). Secondary structure prediction utilizing the PSIPRED server identified 19 helical segments, with a confidence level of greater than 70% for each predicted helix, within the complete sequence of *O. sativa* PIOX (Fig. 1). Seventeen of the 19 predicted helices are located within residues 123–609 of *O. sativa* PIOX. Of the 17 helices predicted within this region, 15 align with a structurally observed helix in the catalytic domain of COX-1 (Fig. 1). A structural model for *O. sativa* PIOX was then independently generated with the program MODELLER (33) using the crystal structure of *O. aries* COX-1 (PDB 1DIY (32)) as a template. Subsequent analysis of the in silico generated PIOX model also suggests a predominantly α-helical fold for the overall structure, which is consistent with the results obtained from the PSIPRED server. Indeed, 15 helices in the PIOX model correspond to observed helical regions in the crystal structure of COX-1. No β-sheet secondary struc-
Three residues crucial for activity within the catalytic domain of COX-1 and COX-2 include the distal and proximal histidines involved in heme binding (His-207 and His-388) and the catalytic tyrosine (Tyr-385; Table 1). In the crystal structure of O. aries COX-1, His-207 is located in helix H2, whereas Tyr-385 and His-388 are located in helix H8 (as defined in Ref. 35) (Fig. 2A). The analogous residues in O. sativa PIOX (His-157, Tyr-379, and His-382; Table 1), which are strictly conserved within the known PIOX sequences to date, reside in sequences that are predicted to have α-helical secondary structure by the PSIPRED server, with a confidence level greater than 95% (Fig. 1). Moreover, our PIOX structural model predicts that His-157 and His-382 lie within helices that are the equivalents to H2 and H8 in COX-1, with their side chains poised to bind the heme prosthetic group (Fig. 2B). The strict sequence conservation of His-157, Tyr-379, and His-382, and the above independent structural predictions suggest that the structural architecture of the heme-binding cleft and position of the catalytic tyrosine is conserved within the α-dioxygenase family and analogous to that observed for COX-1 and COX-2 (17).

Tyr-379 has been identified by us (this study) and others (3, 10, 11) as being critical for the catalytic activity exhibited by PIOX enzymes. Moreover, it has been shown using stereospecifically deuterated LA that PIOX enzymes initiate dioxygenation of the fatty acid substrate via the abstraction of the 2-pro-R hydrogen by a radical centered on Tyr-379 (16). In the cocystal structures of COX-1 with arachidonic acid, LA, eicosapentaenoic acid (20:5, n-3), and dihomo-γ-linolenic acid (20:3, n-6), the fatty acid substrate is positioned such that carbon-13 (carbon-11 for LA) lies ~2.8 Å below the hydroxyl group of Tyr-385 such that hydrogen abstraction can occur by the incipient Tyr-385 radical and initiate cyclooxygenase catalysis (17, 20, 26, 32). Inspection of the region around Tyr-379 in our PIOX structural model identified a hydrophobic groove below Tyr-379 that is bordered by predicted helices that are the equivalents of helices H2, H6, and H17 in COX-1 (Fig. 2B). This hydrophobic groove is large enough to accommodate a 16–20-carbon fatty acid substrate. Based on the experimentally derived information described above, we built LA...
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TABLE 1
Sequence alignment of PIOX and other members of the fatty acid α-dioxygenase family

| Protein        | Helix 6     | Helix 7     |
|----------------|-------------|-------------|
| COX-1          | A Q H F T H O F | I V E Y V Q O L S G Y |
| COX-2          | A Q H F T H O F | I V E Y V Q O L S G Y |
| PIOX           | A Q H F T H O F | I V E Y V Q O L S G Y |
| A. thaliana    | I Q F M H D W | I V E T D W T V E L L K T |
| N. tabacum     | I Q F M H D W | I V E T D W T V E L L K T |
| L. esculentum  | I Q F M H D W | I V E T D W T V E L L K T |
| O. sativa      | I Q F M H D W | I V E T D W T V E L L K T |
| C. annuum      | I Q F M H D W | I V E T D W T V E L L K T |
| L. esculentum  | I Q F M H D W | I V E T D W T V E L L K T |
| OrfX           | I Q F M H D W | I V E T D W T V E L L K T |

Utilizing the constructed enzyme-substrate model, we sought to identify polar residues lining the hydrophobic groove near Tyr-379 and the carboxylate end of LA that could potentially interact with the carboxylate group of LA. We identified three candidate residues that were strictly conserved within the PIOX sequences in the α-dioxygenase family: His-311, Arg-558, and Arg-559 (Table 1) (5). In our enzyme-substrate binding model, the side chains of His-311 and Arg-559 are predicted to contact the carboxylate of LA. Both side chains extend into the hydrophobic groove, His-311 from the helix H6 equivalent and Arg-559 from the helix H17 equivalent and both side chains are within bonding distance of the carboxylate of LA (Fig. 2B). The side chain of Arg-558, located in the helix H17 equivalent, is also in the vicinity of the defined hydrophobic groove. However, our model does not predict that the side chain of Arg-558 interacts with the carboxylate of LA, despite the strong sequence conservation within members of PIOX in the α-dioxygenase family (Table 1).

Based on the above analyses, we hypothesize that His-311 and Arg-559 may be important amino acid determinants within the PIOX active site architecture that could interact with the carboxylate of LA and function to stabilize it or potentially play a role in the dioxygenation reaction. As a corollary to this hypothesis, we further predict that O. sativa PIOX would not utilize fatty acids containing modifications at the carboxylate, carbon-1, or carbon-2 as substrates, given the space constraints imposed by the location of helix H2 (Fig. 2B). To test the validity of our hypotheses, we carried out site-directed mutagenesis on His-311, Arg-558, and Arg-559 and functionally characterized the effects of these mutants on substrate binding and catalysis. We further characterized the ability of wild-type O. sativa PIOX to bind and oxygenate 18-carbon fatty acids containing modifications at the carboxylate, carbon-1, or carbon-2 of the substrate.

Site-directed Mutagenesis and Mutant Construct Analysis—Site-directed mutagenesis was carried out on His-311, Arg-558, and Arg-559. Specifically, His-311 was substituted with Ala, Glu, Leu, Gln, and Arg. Arg-558 was substituted with Ala, Lys, and Leu, whereas Arg-559 was substituted with Ala, Glu, Leu, and Lys. In addition, the double mutant H311R/R559H was constructed to characterize the effect of swapping the positions of the His-311 and Arg-559 side chains. Finally, Tyr-379 was mutated to Phe to verify previous studies identifying this residue as the initiator of oxygenase catalysis (10, 11) and to serve as an internal control for our methodology.

Wild-type and mutant PIOX constructs were expressed in E. coli and purified using immobilized metal affinity chromatography and gel filtration. With the exception of the H311E, H311R, H311L, and the H311R/R559H substitutions, all mutant constructs were expressed at levels equivalent to the wild-type enzyme as measured by Western blot analysis utilizing an anti-HIS monoclonal antibody (Fig. 3). In addition, the mutant PIOX constructs were judged to be greater than 80% pure based on visual inspection of the bands on an SDS-PAGE gel (Fig. 3). Previous studies have established that PIOX exhibits a UV-visible absorbance maximum between 410 and 413 nm, which corresponds to the presence of a ferric heme, which is necessary for the initiation of catalysis (10, 11). UV-visible absorbance spectra of the wild-type enzyme and the mutant constructs were indistinguishable from one another (data not shown) and identical to spectra observed for wild-type O. sativa PIOX by Koeduka and colleagues (11), suggesting that heme was properly incorporated into the mutant constructs.

Oxygenase Kinetics of Mutant Constructs—Wild-type and mutant PIOX constructs were functionally characterized by determining oxygen consumption kinetics using an oxygen electrode and LA as the fatty acid substrate. Replacement of His-311 with neutral and charged side chains had strikingly different effects on the rate of oxygenation of LA. Attempts to substitute a larger, charged side chain (Arg or Gln) at position 311 was lethal to the enzyme, with no soluble protein expressed (Table 2, Fig. 3). Substitution of a neutral side chain (Leu or Ala) at this position led to mutant enzymes that were expressed at levels equal to wild-type PIOX, but which retained less than 3% of their oxygenase activity toward LA (Table 2, Fig. 3). Replacement of His with Gln at position 311 produced a mutant enzyme that retained the greatest rate of oxygenation of LA among the His-311 mutant constructs generated (Table 2). Replacement of His-311 with Ala or Gln did not lead to large increases in Km, for these mutant constructs, with only a 1.6-fold increase in Km for the H311A mutant construct and no change in km for the H311Q mutant construct compared with the wild-type enzyme (Table 2).

Surprisingly, all substitutions of Arg-559 led to dramatic decreases in the rate of oxygenation of LA and corresponding large increases in Km, with respect to wild-type enzyme (Table 2). Replacement of Arg-559 with the small, neutral side chains Ala or Leu effectively abolished the ability of the mutant...
enzyme to oxygenate LA. Conservative replacement of Arg-559 with either Lys or Glu, which maintains a charged side chain at this position, also resulted in mutant constructs with minimal oxygenase activity. Most notable is the greater than 140-fold increase in $K_m$ for LA observed for the R559E, R559K, and R559A mutant constructs (Table 2). Binding affinities for these mutants were reduced to such an extent that the velocity of the reaction did not reach saturating levels utilizing the highest substrate concentrations allowed under experimental conditions.

Substitutions of Arg-558 had varying effects on the rate of oxygenation of LA. Replacement of Arg-558 with Ala or Leu resulted in mutant constructs that maintained greater than 45 and 70%, respectively, of their oxygenase activity toward LA (Table 2). Surprisingly, the conservative replacement of Arg-558 with Lys resulted in a mutant construct that had the least oxygenase activity toward LA of the three, with 18% activity compared with the wild-type enzyme (Table 2). Although oxygenase activity toward LA was altered to varying degrees with these three mutant constructs, all exhibited lower $K_m$ values for LA than wild-type enzyme (Table 2).

**Substrate Specificity of O. sativa PIOX**—To gain insight into substrate specificity, we measured the ability of O. sativa PIOX to oxygenate a variety of fatty acid substrates using an oxygen electrode. For this study we chose 18 different fatty acids that were between 14 and 20 carbons in length and that also varied in both the type and number of unsaturated bonds within the fatty acid. The panel of substrates comprised above was generated to also include a subset of fatty acids utilized in an analogous experiment that was performed with A. thaliana PIOX (15), so that comparisons could be made between the two enzymes with respect to their substrate preferences. We also included 5 novel 18-carbon substrates in our panel that have modifications at either the carboxylate group, carbon-1, or carbon-2 (Table 3). Inclusion of these fatty acids were aimed at testing the hypothesis that modifications to the carboxylate end of the molecule would prohibit O. sativa PIOX from utilizing them as substrates.

The ability of O. sativa PIOX to oxygenate the panel of 23 fatty acid substrates is summarized in Table 3. Overall, detectable oxygenase activity could be measured for all of the non-modified fatty acid substrates. Of the 18 non-modified fatty acids tested, the enzyme exhibited the highest oxygenase activity when the 18-carbon fatty acids LA, linoelaidic acid (18:2,
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n-6; trans double bonds), and the 14-carbon fatty acid myristate (14:0) were utilized in the reaction. However, 7 additional fatty acids, with chain lengths between 16 and 20 carbons and varying degrees of unsaturation, were oxygenated at levels greater than 65% that observed for LA. These include palmitic acid (16:0), palmitoleic acid (16:1, n-9), oleic acid (18:1, n-9), α-linolenic acid (18:3, n-3), 11-eicosenoic acid (20:1, n-9), 11,14-eicosadienoic acid (20:2, n-6), and dihomo-γ-linolenic acid. The remaining 8 fatty acids were oxygenated at levels between 11 and 34% of that observed for LA. None of the 5 modified fatty acid substrates were oxygenated by O. sativa PIOX.

Substrate Binding Analysis—Kinetic analyses described above for the His-311, Arg-558, and Arg-559 mutant constructs suggests that Arg-559 is involved in the binding of LA within the hydrophobic groove of O. sativa PIOX. To further investigate the role that Arg-559 plays in substrate binding, we performed a simple thermal shift assay using SPYRO Orange dye and a real-time PCR machine. The assay measures the change in fluorescence during the thermal denaturation of a protein, with the midpoint of the unfolding transition taken as an approximation of the melting temperature ($T_m$) (36–38). Ligand binding affinity is then estimated from the change in melting temperature ($\Delta T_m$) obtained in the presence of ligand relative to that obtained in the absence of ligand, with the extent of the shift proportional to the affinity of the ligand for the protein (39). For known specific ligands with affinities in the micromolar range, shifts in $T_m$ greater than 2 °C are considered significant (38).

For these studies, two additional double mutant constructs were prepared, Y379F/R558A and Y379F/R559A. The coupling of the Y379F mutation with Arg-558 and Arg-559 mutations allows for fatty acid substrate to potentially bind, but not be converted to product during the assay. Both double mutant constructs were expressed and purified at levels equivalent to their single mutant counterparts as judged by Western blot, SDS-PAGE, and UV-visible absorbance spectral scans (data not shown). We screened the Y379F, Y379F/R558A, and Y379F/R559A constructs for their ability to bind LA at concentrations varying from 0 to 50 μM using the thermal shift assay. Both the Y379F and Y379F/R558A constructs exhibited a concentration-dependent increase in thermal stability as the concentration of

### Table 2

**Kinetic properties for oxygenation of LA by wild-type, His-311, Tyr-379, Arg-558, and Arg-559 mutants of PIOX**

Oxygenase activity was measured using an oxygen electrode and LA as the substrate as described under “Experimental Procedures.” $V_{max}$ and $K_m$ values represent the mean values from a minimum of five and three separate determinations, respectively. A value of 100% is assigned for the oxygenase activity of wild-type PIOX, with mutant activity normalized to wild-type after background correction.

| Enzyme          | Relative $V_{max}$ | $K_m$ (μM) | $V_{max}/K_m$ |
|-----------------|--------------------|------------|---------------|
| Wild-type       | 100                | 7.1 ± 1.0  | 14.1          |
| Y379F           | 0.0                | ND         | ND            |
| H311Q           | 11.5               | 7.0 ± 0.6  | 1.6           |
| H311A           | 2.6                | 12.7 ± 1.5 | 0.2           |
| H311L           | 0.0                | ND         | ND            |
| H311E           | Insoluble          | ND         | ND            |
| H311R           | Insoluble          | ND         | ND            |
| R558L           | 74.2               | 2.4 ± 0.4  | 30.9          |
| R558A           | 46.7               | 2.8 ± 0.5  | 16.7          |
| R558K           | 18.4               | 1.3 ± 0.3  | 14.2          |
| H311R/R559H     | Insoluble          | ND         | ND            |
| R559E           | 1.4                | >1000*     | 0.0           |
| R559K           | 0.9                | >1000      | 0.0           |
| R559A           | 0.4                | >1000      | 0.0           |
| R559L           | 0.0                | ND         | ND            |

* ND, not determined.

### Table 3

**Substrate specificity of O. sativa PIOX**

The ability of O. sativa PIOX to oxygenate fatty acid substrates with varying carbon lengths, unsaturated bonds, and modifications to the carboxylate end was measured using an oxygen electrode as described under “Experimental Procedures.” Each substrate was assayed in triplicate at a concentration of 100 μM. A value of 100% is assigned for the oxygenase activity of PIOX towards LA, with the oxygenase activity of the other substrates normalized to this value. The numbers depicted for the location of the unsaturated bond represent the first carbon from the carboxylate end of the substrate that is involved in the bond. The nomenclature for the type of unsaturated bond present in the substrate is as follows: A, acetylene; E, trans; Z, cis. The relative activities for A. thaliana PIOX are taken from Ref. 15 and listed here after re-normalizing the values to the oxygenase activity reported for LA in their study.

| Fatty acid substrate | Number of carbons: unsaturated bonds | Location and type of unsaturated bond | Relative activity |
|----------------------|--------------------------------------|--------------------------------------|------------------|
| Linoleic acid        | 18:2                                 | 9E, 12E                              | 1.11             |
| Myristic acid        | 14:0                                 |                                      | 1.03             |
| Linoleic acid        | 18:2                                 | 9Z, 12Z                              | 1.00             |
| Dihomo-γ-linolenic acid | 20:3                           | 8Z, 11Z, 14Z                         | 0.85             |
| α-Linolenic acid     | 18:3                                 | 9Z, 12Z, 15Z                         | 0.82             |
| Palmitic acid        | 16:0                                 | 9Z                                   | 0.81             |
| Oleic acid           | 18:1                                 | 9Z                                   | 0.79             |
| 11-Eicosenoic acid   | 20:1                                 | 11Z                                  | 0.78             |
| 11,14-Eicosadienoic acid | 20:2                       | 11Z, 14Z                             | 0.75             |
| γ-Linolenic acid     | 18:3                                 | 6Z, 9Z, 12Z                          | 0.67             |
| 9,12-Octadecadienoic acid | 18:2 | 9A, 12A                            | 0.34             |
| Stearic acid         | 18:0                                 |                                      | 0.23             |
| Stearidonic acid     | 18:4                                 | 6Z, 9Z, 12Z, 15Z                     | 0.22             |
| Eicospentaenoic acid | 20:5                                 | 5Z, 8Z, 11Z, 14Z, 17Z                | 0.22             |
| Arachidonic acid     | 20:4                                 | 5Z, 8Z, 11Z, 14Z                     | 0.14             |
| Pinolenic acid       | 18:3                                 | 5Z, 9Z, 12Z                          | 0.13             |
| Eicosatrienoic acid  | 20:3                                 | 5Z, 8Z, 11Z                          | 0.13             |
| 2-Linoleoyl glycerol | 18:2                                 | 9Z, 12Z                              | 0.11             |
| 2-Methyl octadecenoic acid | 18:0                      | 9Z, 12Z                              | 0.00             |
| Methyl linoleic acid | 18:2                                 | 9Z, 12Z                              | 0.00             |
| Methyl α-linolenic acid | 18:3                          | 9Z, 12Z, 15Z                         | 0.00             |
| Methyl oleic acid    | 18:1                                 | 9Z                                   | ND               |

* ND, not determined in Ref. 15.
LA was increased, whereas no concentration dependence was observed for the Y379F/R559A (data not shown). Specifically, values for $\Delta T_m$ of 3.8 ± 0.9°C and 6.5 ± 1.1°C were observed for the Y379F and Y379F/R558A mutant constructs, respectively, at a LA concentration of 50 μM, when compared with the values calculated for these mutant constructs in the absence of LA (Fig. 4A). In contrast, the Y379F/R559A mutant construct exhibited a $\Delta T_m$ of 1.1 ± 0.7°C under the same experimental conditions.

We also utilized the thermal shift assay to assess the ability of the Y379F mutant construct to bind two of the modified fatty acids tested as substrates for O. sativa PIOX above. Neither, methyl linoleic acid nor 2-linoleoyl glycerol showed a significant change in $\Delta T_m$ (values of $-3.3 \pm 1.1$ and $-0.2 \pm 1.1$ °C, respectively) when their $T_m$ values were compared at fatty acid concentrations of 0 and 50 μM (Fig. 4B). Moreover, the negative $\Delta T_m$ values observed for the modified fatty acids is an indication that these compounds actually decrease the stability of the protein.

**DISCUSSION**

COX-1 and COX-2 are the closest homologs to members of the fatty acid α-dioxygenase family that have had their structures experimentally determined. As such, they have served as starting models to further understand the functional and mechanistic nuances of PIOX catalysis (3, 10, 11). Based on the shared catalytic features identified between these enzymes, we sought to further investigate the potential structural similarities between PIOX and the COX enzymes and identify additional molecular determinants within the O. sativa PIOX sequence that are involved in the dioxygenation reaction.

Comparison of the secondary structure prediction for O. sativa PIOX with the secondary structure observed in the crystal structure of O. aries COX-1 and that observed in the independently generated structural model suggests a conservation of α-helical secondary structure elements. Moreover, the identification of COX helix equivalents in O. sativa PIOX containing the required catalytic residues and the maintenance of the spatial relationship of these residues in an analogous manner to that observed in the COX enzymes suggests that the structural architecture of the active site machinery required for oxygenation of fatty acid substrates is conserved between the two enzymes. As such, we further analyzed this area of our structural model to investigate how fatty acid substrates may bind to O. sativa PIOX.

A hydrophobic groove was identified in our structural model below the catalytic tyrosine and LA was subsequently modeled into this groove, with carbon-2 constrained 2.8 Å below the hydroxyl group of Tyr-379. In this orientation, the carboxylate of LA is buried deep within the hydrophobic groove, with the carboxylate group abutting side chain residues contained within helix H2 (Fig. 2B). This is contrary to what is observed in the COX-1 fatty acid cocrystal structures, where the ω end of the substrate is buried deep within the cyclooxygenase channel, whereas the carboxylate is stabilized by polar interactions with Arg-120 and Tyr-355 at the channel opening (Fig. 2A) (20, 26, 32). As such, PIOX is predicted to have evolved to bind fatty acid substrates in the opposite orientation to facilitate oxygenation at carbon-2 of fatty acid substrates. Further data supporting the conformation of LA built within the hydrophobic groove comes from our studies using fatty acids modified at the carboxylate, carbon-1, or carbon-2. Inspection of the hydrophobic groove in the area surrounding the carboxylate of LA suggests that there is limited space to accommodate modifications. Indeed, O. sativa PIOX did not oxygenate any of the 5 modified fatty acids tested. Similar results were observed for A. thaliana PIOX, which did not oxygenate methyl linoleic acid and methyl α-linolenic acid (15). The lack of oxygenase activity against these modified substrates suggests two possibilities: 1) the substrates are too bulky to access and bind within the hydrophobic groove of the PIOX active site, or 2) the substrates bind within the hydrophobic groove, but do not properly orient carbon-2 for subsequent hydrogen abstraction by the Tyr-379 radical. The latter case would be analogous to the binding of eicosapentaeonoic acid in the cyclooxygenase channel of COX-1, where it acts as an inhibitor rather than a substrate (20). When methyl linoleic acid and 2-linoleoyl glycerol were tested for their ability to bind to the Y379F construct in thermal shift assays, neither exhibited an increase in $\Delta T_m$, suggesting that these substrates do not bind to O. sativa PIOX.

In our structural model, the side chains of His-311 and Arg-559 extend into the hydrophobic groove from helices, which are the equivalents of helix H6 and H17 in the COX enzymes. Both helix H6 and H17 contain critical residues, including Val-349, Val-523, and Ser-530, which protrude into the cyclooxygenase channel of COX-1 and COX-2 and act to affect the conversion of arachidonic acid to prostaglandin G2 (24, 25, 40). Mutations of both His-311 and Arg-559 led to constructs that were significantly reduced in their ability to oxygenate LA, suggesting a role for each during oxygenation of the fatty acid substrate. The significant reduction in the oxygenation kinetics observed for substitutions of arginine at position 559 coupled with the lack of a significant shift in $\Delta T_m$ for the Y379F/R559A construct in thermal shift assays strongly suggest that Arg-559 is required for high affinity binding of fatty acid substrates to PIOX. Our model suggests that Arg-559 forms an ionic bond with the carboxylate group of fatty acids to provide the necessary stabilization of the carboxylate in an otherwise hydrophobic environment, which is analogous to the role that Arg-120 plays in the binding of fatty acids to COX-1 (41). Substitutions of histidine at position 311 also significantly reduced the rate of oxygenation of LA. However, there was no appreciable decrease in $K_m$ for either the H311A or H311Q constructs indicating that His-311 is not required for high affinity binding of fatty acid substrates. As such, the reductions in $V_{max}/K_m$ observed for these constructs could be attributed to an altered binding mode for LA, resulting in the perturbation of the alignment of carbon-2 of LA below Tyr-379 for optimal hydrogen abstraction.

His-311 and Arg-559 are also conserved in a protein of unknown function encoded by the open reading frame OrfX from *P. alcaligenes* (Table 1) (5, 8). The conservation of these residues along with the proximal and distal histidines and catalytic tyrosine in OrfX suggests that this protein oxygenates fatty acid substrates at or near the carboxylate end of the substrate in a manner similar to that utilized by PIOX. LDS also exhibits homology to PIOX and the other members of the fatty acid substrate binding family encoded by the open reading frame OrfX.
acid \( \alpha \)-dioxygenase family and catalyzes the dioxygenation of LA into 8R-hydroperoxylinoleic acid (5, 6). LA is oxygenated by LDS in a manner similar to that observed for COX enzymes, with the generation of ferryl intermediates and a protein radical (42). Although there has not been a detailed analysis of the residues involved in substrate binding within the active site of LDS, the lack of conservation of His-311 and Arg-559 in the LDS sequence likely rules out these residues as molecular determinants that govern the binding and orientation of LA in a manner similar to that of PIOX.

\( O. \) sativa PIOX utilized the 13 common fatty acids tested in both our study and that of Liu and colleagues (15) as substrates to varying degrees, which is in agreement with that observed for the \( A. \) thaliana enzyme. However, \( A. \) thaliana PIOX showed an equally high preference for both LA and \( \alpha \)-linolenic acid as substrates, whereas there was a clear distinction in substrate preference between LA and \( \alpha \)-linolenic acid for \( O. \) sativa PIOX (Table 3). In addition, \( O. \) sativa PIOX was able to oxygenate myristic acid at a level equal to LA, whereas \( A. \) thaliana PIOX showed a 4-fold decrease in relative activity with respect to LA. Interestingly, \( A. \) thaliana PIOX also exhibited 4.4- and 1.8-fold decreases in relative activity for palmitic acid and stearic acid, respectively, suggesting that the \( O. \) sativa enzyme could better accommodate the inherent flexibility associated with these saturated fatty acid substrates during catalysis. Finally, \( O. \) sativa PIOX oxygenated 20-carbon fatty acids more efficiently than the \( A. \) thaliana enzyme, regardless of how many unsaturated bonds were present in the substrate (Table 3). The most striking example is the use of dihomo-\( \gamma \)-linolenic acid by \( O. \) sativa PIOX, which oxygenates this substrate at a rate of 85% relative to LA, compared with the 12% relative rate observed for the \( A. \) thaliana enzyme. Overall, these results indicate that both \( O. \) sativa and \( A. \) thaliana PIOX are promiscuous enzymes, with the ability to utilize a variety of fatty acids as substrates. The substrate preferences observed between \( O. \) sativa and \( A. \) thaliana PIOX are likely governed by subtle differences in the amino acid residues lining the substrate binding cleft in each enzyme.

In summary, the bioinformatics and functional analyses presented here provide further insight into the mechanism by which \( O. \) sativa PIOX oxygenates fatty acids. Our data suggests
that substrates are bound deep within a hydrophobic groove such that carbon-2 is placed below Tyr-379 for stereospecific abstraction of the 2-pro-R hydrogen. In this orientation, the carboxylate of the fatty acid binds to and is subsequently stabilized by the side chain of Arg-559 and carbon-2 of the substrate is optimally positioned for hydrogen abstraction by interactions with the side chain of His-311. These studies provide an initial starting point for more detailed structure and functional analyses that will be necessary to fully understand the mechanistic nuances by which fatty acid substrates are oxygenated by O. sativa PIOX and other members of the fatty acid α-dioxygenase family.

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