PROTEIN STRUCTURE REPORT

Crystal structures of α -dioxygenase from *Oryza sativa*: Insights into substrate binding and activation by hydrogen peroxide

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Abstract: α -Dioxygenases (α -DOX) are heme-containing enzymes found predominantly in plants and fungi, where they generate oxylipins in response to pathogen attack. α -DOX oxygenate a variety of 14–20 carbon fatty acids containing up to three unsaturated bonds through stereoselective removal of the *pro-R* hydrogen from the α -carbon by a tyrosyl radical generated via the oxidation of the heme moiety by hydrogen peroxide (H_2O_2). We determined the X-ray crystal structures of wild type α -DOX from *Oryza sativa*, the wild type enzyme in complex with H_2O_2 , and the catalytically inactive Y379F mutant in complex with the fatty acid palmitic acid (PA). PA binds within the active site cleft of α -DOX such that the carboxylate forms ionic interactions with His-311 and Arg-559. Thr-316 aids in the positioning of carbon-2 for hydrogen abstraction. Twenty-five of the twenty eight contacts made between PA and residues lining the active site occur within the carboxylate and first eight carbons, indicating that interactions within this region of the substrate are responsible for governing selectivity. Comparison of the wild type and H_2O_2 structures provides insight into enzyme activation. The binding of H_2O_2 at the distal face of the heme displaces residues His-157, Asp-158, and Trp-159 ~2.5 Å from their positions in the wild type structure. As a result, the O δ 2 atom of Asp-158 interacts with the Ca atom in the calcium binding loop, the side chains of Trp-159 and Trp-213 reorient, and the guanidinium group of Arg-559 is repositioned near Tyr-379, poised to interact with the carboxylate group of the substrate.

Keywords: α -dioxygenase; fatty acids; oxylipins; X-ray crystallography; cyclooxygenase

Abbreviations: Ath, *Arabidopsis thaliana*; C₁₀M, decyl maltoside; IMAC, immobilized metal affinity chromatography; Osa, *Oryza sativa*; PA, palmitic acid; rms, root-mean-square; α -DOX, α -dioxygenase; β OG, β -octylglucoside; Δ 9N, *Oryza sativa* α -dioxygenase with the first 9 amino acids at the N-terminus removed; COX, cyclooxygenase.

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Introduction

α -Dioxygenases (α -DOX) are heme-containing proteins that generate potent lipid mediators, termed oxylipins, via the oxygenation of 14–20 carbon fatty acid substrates. α -DOX is generally found in plants and fungi, where they are upregulated during the host defense response against pathogen attack.^{1–3} These enzymes represent a subfamily within the cyclooxygenase-peroxidase superfamily, which include the mammalian peroxidases and cyclooxygenases (COX).⁴

The structure of *Arabidopsis thaliana* (Ath) α -DOX consists of two predominantly α -helical domains.⁵ The base domain is comprised of eight α -helices and is postulated to govern the interaction of this enzyme with the lipid bilayer in a manner similar to that of the membrane-binding domain of COX.⁶ The catalytic domain is comprised of 22 α -helices and exhibits a significant structural homology to the catalytic domain of COX and myeloperoxidase, despite having a sequence identity of $\sim 15\%$ to these enzymes.⁴ In particular, there is high conservation of four helices that constitute the heme-binding and substrate-binding clefts.^{5,7}

α -DOX converts palmitic acid (PA; 16:0), linoleic acid (LA; 18:2 ω -6), and a variety of other fatty acids into their corresponding 2(*R*)-hydroperoxides.⁸ The *pro-R* hydrogen from carbon 2 of the substrate is stereospecifically removed utilizing a tyrosyl radical centered on Tyr-379 that is generated by the oxidation of the heme moiety by hydrogen peroxide (H_2O_2).^{8,9} The 2(*R*)-hydroperoxides undergo spontaneous decarboxylation to chain shortened aldehydes, with minor amounts of 2(*R*)-hydroxy and C_{n-1} products produced as well.⁸

The reaction catalyzed by α -DOX is unique in that oxygen addition takes place at the α -carbon instead of at an allylic or bis-allylic position as observed for lipoxygenases and COX.¹⁰ As such, α -DOX must bind the fatty acid with the carboxylate group bound deep within the active site cleft near the catalytic tyrosine. This is in contrast to COX, which buries the ω -end of the fatty acid deep within the cyclooxygenase channel and places the carboxylate near the channel opening.^{11,12} Previously, we generated a homology model of *Oryza sativa* (Osa) α -DOX bound with LA to identify potential residues involved in high-affinity binding and catalysis.⁷ Osa α -DOX has $\sim 63\%$ sequence similarity with Ath α -DOX.¹³ Subsequent analysis of the model, coupled with mutational and kinetic analyses, identified His-311 and Arg-559 as molecular determinants of the α -dioxygenase reaction.⁷ We report here three X-ray crystal structures of Osa α -DOX determined to resolutions greater than 2.1 Å using synchrotron radiation. The structure of the catalytically inactive Y379F Osa α -DOX mutant in complex with the fatty acid PA confirms our previous hypothesis of how

substrate binds to the enzyme and details the interaction of PA with residues lining the active site cleft. The structure of the wild type enzyme complexed with H_2O_2 provides further insight into the structural changes that occur in both the heme binding and active site clefts when the enzyme is activated by H_2O_2 . Collectively, the structures identify the structural nuances associated with the mechanism of α -dioxygenation.

Results and Discussion

A codon-optimized version of Osa α -DOX, with the first nine amino acid residues truncated from the N-terminus ($\Delta 9\text{N}$), was cloned and expressed in *E. coli*. Kinetic characterization of the $\Delta 9\text{N}$ construct, utilizing LA as the substrate, yielded values for k_{cat} , K_{M} , and $k_{\text{cat}}/K_{\text{M}}$ of $17.7 \pm 0.2 \text{ s}^{-1}$, $10.8 \pm 0.8 \mu\text{M}$, and 1.6, respectively, which are in line with the values calculated previously for the full length construct of both Osa and Ath α -DOX.^{5,7} We subsequently utilized the $\Delta 9\text{N}$ Osa α -DOX construct to determine crystal structures of: (a) the wild type enzyme; (b) the wild type enzyme in complex with the activator H_2O_2 (WT: H_2O_2); and (c) the catalytically inactive Y379F construct with the substrate PA (Y379F:PA) (Table I). All three structures were solved using the molecular replacement method and the structure of Ath α -DOX as the search model (PDB id 4HHR)⁵.

$\Delta 9\text{N}$ Osa α -DOX is comprised of 609 residues folded into two domains, a base domain and a catalytic domain. While the $\Delta 9\text{N}$ deletion removes the first predicted α -helix of the base domain, the secondary structure and domain makeup of Osa α -DOX is otherwise conserved with that observed for Ath α -DOX.⁵ The wild type structure has calculated root-mean-square (rms) differences between C_α atoms of 0.321 Å and 0.376 Å with the WT: H_2O_2 and Y379F:PA structures, respectively. The rms difference between C_α positions of Y379F:PA and WT: H_2O_2 is 0.176 Å, which is within the mean positional error of the structures. The major differences observed between the WT structure and the WT: H_2O_2 and Y379F:PA structures are triggered by the binding of H_2O_2 to the distal face of the heme moiety and fatty acid substrate binding in the active site cleft. These structural differences are described below as they pertain to the catalytic mechanism of α -dioxygenation.

The walls and ceiling of the active site cleft of Osa α -DOX are comprised of helices H2, H6, H8, and H17 within the catalytic domain, similar to that previously predicted by homology modeling⁷ and observed for Ath α -DOX⁵ (Supporting Information Fig. S1). Helices H2 and H8 constitute the heme-binding cleft, where His-157 and His-382 serve as the distal and proximal ligands in the wild type structure. PA binds in a linear fashion between helices H6 and H17 in the Y379F:PA crystal structure,

Table I. Crystallographic Statistics for *Osa* α -DOX Structures

Crystallographic parameter	Wild type	WT:H ₂ O ₂	Y379F:PA
Space group	<i>I</i> 222	<i>I</i> 222	<i>I</i> 222
No. in asymmetric unit	1	1	1
Unit cell length (Å)			
a	72.46	72.28	72.89
b	128.58	129.65	130.20
c	188.74	187.71	188.17
$\alpha = \beta = \gamma$ (°)	90.00	90.00	90.00
Wavelength (Å)	1.00	1.00	0.98
Resolution (Å)	106–1.98	94–2.12	50–1.96
Highest resolution shell (Å) ^a	2.08–1.98	2.23–2.12	1.99–1.96
R_{merge} ^b	5.40 (51.60)	4.70 (46.10)	6.30 (56.60)
Total observations	410,176	224,053	377,463
Total unique	61,897	50,366	64,653
$I/\sigma(I)$	22.00 (3.80)	17.80 (2.60)	25.20 (2.80)
Completeness (%)	100 (100)	99.60 (100)	99.90 (99.40)
Multiplicity	6.60 (6.80)	4.40 (4.60)	5.80 (4.60)
Wilson <i>B</i> factor (Å ²)	30.00	39.20	29.00
Number of atoms in refinement	5686	5440	5694
R_{work}	16.60	17.0	15.70
R_{free} ^c	19.20	20.80	18.60
Average <i>B</i> factor, protein (Å ²)	33.90	42.00	32.00
Average <i>B</i> factor, solvent (Å ²)	33.90	46.10	32.50
Average <i>B</i> factor, ligands (Å ²)	53.00	61.30	48.60
Average <i>B</i> factor, ions (Å ²)	42.80	51.90	30.60
RMSD bond length (Å)	0.008	0.007	0.012
RMSD bond angle (°)	1.050	1.150	1.223
Mean positional error (Å) ^d	0.133	0.168	0.121
Ramachandran plot (%) ^e			
Preferred	96.60	96.90	96.80
Allowed	3.20	3.10	3.20
Outliers	0.20	0.0	0.0

^a The values in parentheses represent the values in the outermost resolution shell.

^b R_{MERGE} as defined in Ref. 26.

^c 5.0% of the total reflections were used to generate the test set.

^d Coordinate error as calculated by Luzatti plot.

^e Calculated using MOLPROBITY.

such that the carboxylate group of PA forms three ionic interactions: two with the guanidinium group of Arg-559 and one with the Ne2 atom of His-311 (Fig. 1). These interactions confirm our previous functional studies, which suggested that Arg-559 is involved in high affinity substrate binding.^{5,7}

Overall, PA makes a total of 28 contacts with 13 active site residues (Supporting Information Table S1). Interestingly, 25 of the 28 contacts made by active site residues are with the carboxylate group and carbons 1–8 of PA. Phe-552 is responsible for the majority of these contacts (10 of 25), where the side chain interacts with carbons 2–7 of PA. The C γ 2 atom of Thr-316 makes three contacts with carbons 3–5 of PA. We previously suggested that Thr-316 was also a molecular determinant involved in the α -dioxygenation reaction, given that substitutions at this position resulted in mutants with significantly reduced oxygenase activity.⁵ As there is no observed interaction between Thr-316 and the carboxylate group in the Y379F:PA structure, it is likely that this side chain is critical for properly aligning carbon 2 below the side chain of Tyr-379 for the initiation of catalysis.

α -DOX enzymes are promiscuous with respect to their use of fatty acids with varying chain lengths and unsaturation as substrates for the α -dioxygenation reaction. Previous studies indicate that 14–20 carbon fatty acids are efficiently oxygenated by *Osa* and *Ath* α -DOX if they do not contain a double bond within the first seven carbon positions after the carboxylate group.^{7,14} Fatty acids possessing one, two, or, three unsaturated bonds beyond carbon seven were oxygenated by *Osa* and *Ath* α -DOX. There are only three contacts observed between the active site residues and PA beyond carbon 12 in the Y379F:PA crystal structure (Supporting Information Table S1). The lack of contacts with the ω -end of PA, coupled with the functional observations for the location of unsaturated bonds in substrates suitable for oxygenation, lend further support for the carboxylate interaction and interactions between the enzyme and the first seven carbons of the fatty acid substrate as being critical for binding and catalysis.

Osa and *Ath* α -DOX have a reduced peroxidase activity compared to other members of the cyclooxygenase-peroxidase superfamily, due to the

Figure 1. Fatty acid substrate bound to Osa α -DOX. A: Stereo view of hydrogen peroxide bound to the distal face of the heme moiety. $F_O - F_C$ OMIT electron density (blue), contoured at 3σ , is shown for the final refined model of hydrogen peroxide (red) in the WT:H₂O₂ structure. The heme moiety is colored green. B: Stereo view of PA bound in the active site cleft of Osa α -DOX. $F_O - F_C$ OMIT electron density (pink), contoured at 3σ , is shown for the final refined model of PA (cyan). Carbon-2 of PA lies below the phenolic hydroxyl group of Tyr-379, which is shown modeled from the side chain position of phenylalanine in the Y379F:PA crystal structure. Hydrogen peroxide is modeled as the sixth ligand coordinated to Fe based on its position in the WT:H₂O₂ structure. Portions of helix He, H2, H6, H8, and H17 have been removed for the sake of clarity. Residues involved in heme binding, substrate binding, and catalysis are labeled accordingly. The view in (B) is rotated $\sim 90^\circ$ from the view in (A).

presence of two extended inserts that restrict access to the distal face of the heme.^{5,15} Two small channels exist on the surface of the α -DOX enzymes that facilitate access to H₂O₂ for the generation of the radical on Tyr-379 via the oxidation of the heme.^{9,15} Recently,

stopped flow spectrophotometric experiments have shown that Osa α -DOX is activated by H₂O₂ in a manner that is independent of fatty acid oxygenation.⁹

Comparison of the Osa α -DOX wild type and WT:H₂O₂ crystal structures provides insight into the

Figure 2. The structural basis underlying the activation of Osa α -DOX by hydrogen peroxide. In the wild type structure (A), the N ϵ 2 atom of His-157 is the sixth ligand coordinated to the Fe atom in the heme moiety (green) and the side chain of Arg-559 is located between the side chains of Trp-159 and Trp-213, away from the active site cleft and catalytic Tyr-379. Hydrogen peroxide (red) displaces His-157 at the distal face of the heme moiety in the WT:H₂O₂ structure (B), resulting in a ~ 2.5 Å shift of the C α atoms of His-157 and Asp-158 at the end of helix H2. As a result, the O δ 2 atom of Asp-158 displaces a water molecule and participates as a ligand in the bipyramidal coordination of the Ca atom (blue) in the Ca binding site. The side chains of Trp-213 and Arg-559 also reorient, with the side chain of Arg-559 now pointing into the active site cleft near Tyr-379. Upon substrate binding (C), the carboxylate of PA (cyan) forms ionic interactions with Arg-559 and His-311, and the side chain of Phe-552 reorients to stabilize carbons 2–7. The phenolic oxygen of Tyr-379, modeled from the side chain position of phenylalanine in the Y379F:PA crystal structure, lies above carbon-2, poised to abstract the *2proR* hydrogen. Carbon, nitrogen, and oxygen atoms of residues His-157, Asp-158, and Trp-159 within helix H2, as well as the side chains of Trp-213, Phe-552, and Arg-559 are colored pink, blue, and red, respectively to depict their positions in the wild type structure.

structural changes induced by the binding of H₂O₂ to the heme. In the wild type enzyme, the N ϵ 2 atom of His-157 serves as the sixth ligand coordinated to the Fe atom of the heme moiety [Fig. 2(A)]. The binding of H₂O₂ displaces His-157, resulting in the shift of residues His-157, Asp-158, and Trp-159, within helix H2, ~ 2.5 Å from their positions in the wild type enzyme [Fig. 2(B)]. As a consequence of H₂O₂ binding, the O δ 2 atom of Asp-158 displaces a water molecule, where it now serves as one of the seven Ca ligands in the pentagonal bipyramidal coordination geometry observed within the calcium-binding site.^{5,16} The calcium-binding loop in mammalian peroxidases is responsible for the structural positioning of the distal histidine for catalysis.¹⁶ The calcium-binding loop is conserved within other sequences of the α -DOX family members. Our structural studies suggest that the calcium-binding loop also plays a structural role in α -DOX, where it serves to stabilize residues 157–159 of helix H2 in a conformation that allows H₂O₂ to bind to the heme and subsequently activate the enzyme for optimal catalysis.

We also observe significant differences in the conformation of the side chain of Arg-559 within the active site of Osa α -DOX when the wild type and WT:H₂O₂ structures are compared. In the wild type structure, the side chain of Arg-559 points away from the active site cleft, where it is sandwiched between the side chains of Trp-159 and Trp-213 [Fig. 2(A)]. As part of the choreography of H₂O₂ binding, the side chains of Trp-159 and Trp-213 reorient causing a concomitant shift of the side chain of Arg-559 such that the guanidinium group is now

positioned at the apex of the active site cleft [Fig. 2(B)], where it is poised to interact with the carboxylate of the fatty acid substrate [Fig. 2(C)].

In conclusion, the three crystals of Osa α -DOX presented here provide a detailed structural view of the changes associated with the activation of α -DOX by H₂O₂ and the binding of fatty acid substrate within the active site cleft. Collectively, the structural data supports previous biophysical and functional studies carried out on this family of enzymes and provides a rationale for the role that the calcium-binding loop plays in catalysis.

Materials and Methods

Expression and purification

A codon-optimized version of the Osa α -DOX gene (NCBI database entry NP_001066718.1) was synthesized and subcloned into the pQE30 expression vector (Qiagen, Valencia, CA) utilizing the commercial services provided by GeneScript Co. The pQE30 vector contains a non-cleavable N-terminal sequence (MRGSHHHHHHGS-) containing six histidines for immobilized metal affinity chromatography (IMAC). The first nine residues of Osa α -DOX (MGSGLFKPR-) were deleted using the QuikChange Mutagenesis Kit (Stratagene, La Jolla, CA) to create a Δ 9N Osa α -DOX construct utilized for structural studies. The Y379F mutant construct utilized to form the PA complex was generated using the QuikChange kit and the Δ 9N construct as the template. Each construct was verified by DNA sequence analysis at the Roswell Park Cancer Institute DNA Sequencing Laboratory.

The constructs were transformed into *E. coli* M15 cells. For large-scale expression, 1-L shaker flasks containing 2× YT media were inoculated with starter cultures at 37°C and grown to an OD₆₀₀ of 0.6–0.9. The cells were subsequently induced by the addition of IPTG to a final concentration of 0.1 mM and the temperature was lowered to 20°C. Cells were harvested 16 h post-induction via centrifugation and frozen at –80°C until further use.

The cell pellet from a 2 L growth was utilized for the purification of each construct analyzed in this study. Cell disruption, solubilization with decyl maltoside (C₁₀M), IMAC using TALON resin, and size-exclusion chromatography, using a HiPrep 16/60 Supradex-200 column were carried out as described in Ref. 5 with minor modifications. For the Y379F mutant construct, the protein was eluted from the TALON resin using 100 mM EDTA, followed by size-exclusion chromatography using a running buffer of 20 mM Tris, pH 8.0, 150 mM NaCl, and 0.6% (wt/vol) β-octylglucoside (βOG). All other constructs were eluted from the TALON resin using 125 mM imidazole and run on the HiLoad 16/60 Superdex-200 column in 20 mM Tris, pH 8.0, 150 mM NaCl, and 0.1% (wt/vol) C₁₀M. In all cases, peak fractions were pooled and utilized for structure–function studies.

Crystallography

Initial crystallization leads for wild type Osa α-DOX were identified using protein at a concentration of 3 mg/mL and the 1536 microbatch-under-oil tailored membrane protein screen¹⁷ offered by the High-Throughput Crystallization Laboratory at the Hauptman-Woodward Medical Research Institute.¹⁸ For optimization, crystals were grown at 23°C using the sitting-drop vapor diffusion method. To generate the Y379F:PA complex, a 10-fold moles excess of PA was added to the protein prior to setup. Crystals of the complex were grown by combining 2 μL protein solution and 2 μL reservoir solution consisting of 42.5% polyethylene glycol 400 (PEG 400), 200 mM LiCl, and 100 mM sodium citrate, pH 6.1, followed by equilibration of the drop over 500 μL of reservoir solution. Crystals of the wild-type enzyme were grown in essentially the same fashion, except that 2 μL of protein solution was combined with 2 μL of a drop solution consisting of 50% PEG400, 200 mM LiCl, and 100 mM sodium citrate, pH 6.1, followed by equilibration over 500 μL of reservoir solution. Crystals were looped from sitting drops and flash-cooled directly in the nitrogen stream.

To generate the WT:H₂O₂ complex, 0.5 μL of 45% PEG 400, 150 mM LiCl, 75 mM sodium citrate, pH 6.1, and 7.5% (vol/vol) H₂O₂ was added to the drop containing wild-type crystals. After a 3-min incubation, crystals were harvested and flash-cooled for data collection. Diffraction data for the Y379F:PA

complex was collected on beamline A1 at the Cornell High Energy Synchrotron Source using an Area Detector Systems CCD Quantum-210 Detector. The dataset was integrated and scaled using HKL2000.¹⁹ Diffraction data for the wild type and H₂O₂ complex was collected on beamline 17-ID at the Advanced Photon Source (Argonne National Laboratory) using a Pilatus 6M detector. These datasets were processed using autoProc.²⁰

The structures of Osa α-DOX in this study were determined by molecular replacement methods using PHASER²¹ and Ath α-DOX (PDB id 4HHR)⁵ as the search model. The initial phases were used as input to ARP/wARP,²² which successfully built greater than 90% of the residues into the electron density. Iterative cycles of manual model building and refinement using COOT²³ and REFMAC²⁴ were carried out to fit the remaining residues and to add ligands and water. TLS refinement²⁵ was carried out during the final rounds of refinement. Final crystallographic statistics are summarized in Table I. Model validation was conducted using MOLPROBITY,²⁷ OMIT maps were calculated using REFMAC, and figures were created using PYMOL.²⁸ Coordinates and structure factors for wild type Osa α-DOX, Y379F:PA, and WT:H₂O₂ have been deposited in the Protein Data Bank (entries 4KVK, 4KVL, and 4KVJ, respectively).

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