Crystal Structure of Manganese Lipoxygenase of the Rice Blast Fungus *Magnaporthe oryzae**

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Lipoxygenases (LOX) are non-heme metal enzymes, which oxidize polyunsaturated fatty acids to hydroperoxides. All LOX belong to the same gene family, and they are widely distributed. LOX of animals, plants, and prokaryotes contain iron as the catalytic metal, whereas fungi express LOX with iron or with manganese. Little is known about metal selection by LOX and the catalytic metal, whereas fungi express LOX with iron or with manganese. All LOX belong to the same gene family, but plant FeLOX, mammalian FeLOX, and fungal FeLOX and MnLOX form separate subfamilies (5, 8). The prototype MnLOX is secreted by the take-all fungus of wheat, *Gaeumannomyces graminis* (7). The evolution of this enzyme, 13R-MnLOX, and five members of the MnLOX subfamily are illustrated in a phylogenetic tree together with pro- and eukaryotic LOX, including fungal FeLOX (Fig. 1A).

The three-dimensional structures of 11 eukaryotic and two prokaryotic FeLOX are available. These are four structures of soybean LOX (sLOX-1, LOX-3, VLX-B, and VLX-D) (9–13), three human LOX (15-LOX-2, 12S-LOX, and 5-LOX) (2, 13, 14), coral 8R-LOX and 11R-LOX (15, 16), rabbit arachidonate 15-LOX-1 (17), porcine 12S-LOX (18), 15S-LOX of *Pseudomonas aeruginosa* (19), and linoleate 9R-LOX of *Cyanotheca* sp. (20). Plant and mammalian LOX consist of two domains, a relatively small eight-stranded β-barrel domain with homology to the PLAT (polycystin-1, lipoxygenase, α-toxin) domain and a larger catalytic domain of α-helices containing the substrate-binding channel and the catalytic iron (2). The PLAT domain appears to be absent in fungal MnLOX (5, 6, 21–23). The catalytic domain revealed highly conserved metal ligands and likely oxygen channels to the catalytic center in several LOX structures (2, 10, 24). Iron is usually ligated by three His residues, oxygen of an Asn residue, and a carboxyl oxygen of the C-terminal amino acid (2). A water molecule (Fe(H₂O)₆) or a hydroxide (Fe(OH)₆⁻) provides an additional oxygen ligand to the metal and the catalytic base for hydrogen abstraction (2, 10). Sequence alignment with FeLOX and site-directed mutagenesis suggest that MnLOX have essentially conserved metal ligands (23, 25).

The importance of residues in the active site of LOX has been confirmed by site-directed mutagenesis and recently with three-dimensional structures of bound substrates or inhibitors (13, 24). The regio- and stereospecificity of LOX can be a result of different head-to-tail orientations of the substrate, the depth of the active site, residues positioning the hydrogen for abstraction close to the catalytic metal, and oxygen channels (1, 2). The
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MnLOX and FeLOX reaction mechanisms differ in two principal ways as follows: (i) hydrogen abstraction and oxygen insertion occur in a suprafacial manner in at least five MnLOX and antarafacially in all FeLOX (Fig. 1B) (6, 21, 22, 26); (ii) MnLOX are able to oxidize and rearrange bis-allylic hydroperoxides, a reaction that FeLOX only catalyze to a very low rate (27–29). This difference is possibly related to the redox properties of protein-bound iron and manganese and to structural factors. The adjustment of the different redox potentials iron and manganese is an unresolved issue as well as the metal preference of MnLOX that occurs even though the intracellular iron concentration is higher than the manganese concentration (6, 30).

Magnaporthe oryzae (Mo) causes rice blast disease, and it is listed as the most important fungal pathogen in molecular biology (31). This fungus expresses Mo-MnLOX, which oxidizes 18:2n-6 and 18:3n-3 to 9S-, 11-, and 13R-hydroperoxides with intermediate bis-allylic 11-hydroperoxides as the main product. Mo-MnLOX catalyzes β-fragmentation of these 11-hydroperoxides to cis-trans-conjugated hydroperoxides as end products in analogy with 13R-MnLOX (Fig. 1B) (22). In addition, Mo-MnLOX catalyzes prominent sequential lipoxygenation of 18:3n-3 at C-9 and C-16 (22).

The three-dimensional structure of MnLOX may provide important information on the catalytic mechanism, metal selection, and will allow a comparison between MnLOX and FeLOX. Mo-MnLOX has recently been expressed in Pichia pastoris as a stable enzyme in high yields (22). We therefore selected Mo-MnLOX for three-dimensional structure analysis due to its suitable biochemical properties and biological importance in rice blast disease. We now report the crystallization and 2.0 Å resolution structure of Mo-MnLOX.

Experimental Procedures

Materials—Fatty acids and routine chemicals were from Larodan, Merck, and Sigma. pPICZαA, P. pastoris (strain X-33), phleomycin (Zeocin), SYPRO Orange, and yeast nitrogen base were from InVitrogen. 9S-Hydroperoxy-10E,12Z,15Z-octadecatrienoic acid (9S-HPOTrE) was prepared with potato 9S-LOX and purified by HPLC. Equipment and reagents for SDS-PAGE were from Bio-Rad. Pre-stained protein ladder (Page Ruler) and colloidal Coomassie protein staining (PageBlue) for SDS-PAGE were from Fermentas. Crystal screens were from Hampton Research (United Kingdom), and CYMAL-7 (7-cyclohexyl-1-heptyl-β-d-maltoside) was from Molecular Dimensions (United Kingdom).

Expression and Purification—The Mo-MnLOX precursor consists of 619 amino acids, including a predicted secretion signal of 16 amino acids (GenBank™ accession number AE27899). Mo-MnLOX without the secretion signal was cloned in the pPICZαA expression vector in-frame with the yeast α-secretion signal and was expressed in P. pastoris as described (17). Large amounts of enzyme (70 mg/liter) were obtained by expression in a bioreactor for 3–4 days. The secreted enzyme constituted of 603 amino acids with two additional amino acids (Glu and Phe) from the expression vector at the N-terminal end. Mo-MnLOX was purified essentially as described (27). The enzyme (in the expression medium with added 136 g of (NH₄)₂SO₄ per liter and pH adjusted to 6.8 with 10 M KOH) was captured by hydrophobic interaction chromatography (30 ml of butyl-Sepharose CL-4B), washed with 25 mM KHPO₄ (pH 6.8), 1 M (NH₄)₂SO₄, and eluted with 25 mM KHPO₄ (pH 6.8) using ÄKTA FPLC.

Mo-MnLOX contains seven Asn residues available for N-glycosylation as judged from Asn-Xaa-(Ser/Thr) motifs (NetNGlyc 1.0 Server) and eight Ser/Thr residues for O-glycosylation (NetOGlyc 4.0 Server).

The eluted LOX was concentrated by diafiltration, diluted with 0.1 M sodium acetate (pH 5.0), 20 mM ZnCl₂, and deglyco-
sylated with α-mannosidase (Sigma) and endoglycosidase H (Sigma) in a protein ratio of 1:40 (w/w) at 21 °C overnight. The deglycosylated LOX (in 25 mM HEPES (pH 7.0), 0.1 mM NaCl) was purified by gel filtration (Superdex-200 HiLoad 26/600). Fractions with LOX activity were pooled and concentrated to 8–14 mg/ml by diafiltration (Amicon Ultra 10K) and analyzed by SDS-PAGE.

Site-directed Mutagenesis—Site-directed mutagenesis was performed by whole plasmid PCR technology with Pfu polymerase (16 cycles) according to the QuickChange protocol (Stratagene). 10 ng of the expression vector pPICZαA with the open reading frame of Mo-MnLOX served as a template (27). The desired substitutions, R525A and F526L, were introduced with oligonucleotide primers (44 nucleotides). The PCR products were analyzed by agarose gel electrophoresis to confirm amplification of the desired product by digestion of the template DNA with DpnI (37 °C, 2 h). All mutations were confirmed by sequencing before expression (Rudbeck Laboratory, Uppsala University). Transformants were obtained after linearization with SacI, transformation of P. pastoris (strain X-33), and selection on yeast peptone dextrose agar plates with phleomycin (100 µg/ml) at 28 °C (28). Transformed cells were stored as glycerol stocks at −80 °C, and expression was performed in laboratory bench shakers as described (22). The mutated enzymes were captured by hydrophobic interaction chromatography as above, and protein expression was confirmed by SDS-PAGE.

Enzyme Assay—LOX activity was measured on a dual beam spectrophotometer (Shimadzu UV-2101PC). Enzyme was mixed with 50–100 µM 18:2n-6 or 18:3n-3 in 0.1 M NaBO3 (pH 9.0) at 22 °C, and the UV absorbance was followed from 234 and 237 nm, respectively. The cis-trans-conjugated hydro(pero)xy fatty acids were assumed to have an extinction coefficient of 25,000 cm⁻¹ m⁻¹. Biosynthesis of 9,16-dihydroperoxy-10E,12Z,14E-octadecatrienoic acid (9,16-DiHOTrE) and related trienes was assayed at 270 nm. Oxidation of 20:2n-6, 20:3n-3, and 9S-HPOTrE was studied in the same way and compared with 18:2n-6 and 18:3n-3 with the same amount of Mo-MnLOX. Products were extracted on a cartridge of octadecyl silica (SepPak/C18), and hydroperoxides were reduced to alcohols with triphenylphosphine (22). The detergent sylated with α-mannosidase (Sigma) and endoglycosidase H (Sigma) in a protein ratio of 1:40 (w/w) at 21 °C overnight. The deglycosylated LOX (in 25 mM HEPES (pH 7.0), 0.1 mM NaCl) was purified by gel filtration (Superdex-200 HiLoad 26/600). Fractions with LOX activity were pooled and concentrated to 8–14 mg/ml by diafiltration (Amicon Ultra 10K) and analyzed by SDS-PAGE.

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The thermostability of Mo-MnLOX before and after deglycosylation was determined with SYPRO Orange (Invitrogen) and a thermocycler (CFX Connect real time PCR, Bio-Rad). Fluorescence was monitored using the FAM filter (excitation 495 nm; detection 520 nm) as the temperature was gradually increased from 20 to 90 °C (1.5 °C/min). Samples were prepared in triplicate and contained 5 µM Mo-MnLOX and SYPRO Orange (final dilution 1:700 of 5000 concentrates) in 25 mM HEPES (pH 7.0), 100 mM NaCl, in a total volume of 30 µl. Data evaluation and melting temperature determination were performed using the Bio-Rad CFX manager software.

Crystallization—Initial crystallization screens were performed with sitting-drop vapor diffusion in 0.3–µl drops in a 96-well plate with aid of a Mosquito crystallization robot (TTP Labtech, Cambridge, UK). Crystal optimization was carried out by hanging drop vapor diffusion by mixing 1 µl of 8.5 mg/ml protein with a 1-µl reservoir solution (0.1–0.2 mM ammonium citrate dibasic (pH 6.5), 10–16% w/v PEG-3350) in a 15-well plate.

Data Collection and Processing—Data were collected with the focus of achieving a high sulfur and manganese signal at a wavelength of 1.77 Å at 100 K; one dataset was collected at beam line ID29 at the European Synchrotron Radiation Facility, Grenoble, France, and five additional datasets were collected for the same purpose at beam line I02 at the Diamond Light Source, Oxfordshire, UK.

The datasets were processed using XDS (32), and the integrated data were scaled using AIMLESS (33). A set of 5% of the reflections was set aside and used to calculate the quality factor Rfree. None of the datasets provided sufficient anomalous signal to find the manganese and the sulfur sites. To increase the anomalous signal, all the datasets were analyzed for crystal isomorphism using BLEND (34). Four of the XDS integrated datasets were merged and scaled with Rmerge, and Rfree, values of 0.152 and 0.017 and multiplicity of 77.4.

Structure Determination and Refinement—The positions of manganese and sulfur atoms were determined using the HKL2MAP graphical interface with SHELXC, SHELXD, and SHELXE (35, 36). SHELXC showed an anomalous signal extending to 3.5 Å resolution. Two manganese and 15 sulfur sites were identified in SHELXD. The correctness of the solution was confirmed by SHELXE. Single anomalous dispersion phasing was performed by phenix.autosol from the Phenix suite (37), using the sites obtained by HKL2MAP and the merged dataset. The phases obtained from SHELX and the protein sequence were submitted to phenix.autosol, and phenix.autobuild (37) was able to build 871 out of 1210 residues of the two molecules in the asymmetric unit, with Rwork 0.37 and Rfree 0.39. By using a single dataset to 2.0 Å resolution, we could build 1136 out of 1210 residues with Rwork 0.17 and Rfree 0.21. Model evaluation and manual model building were performed in Coot (38). Refinement was performed with phenix.refine (39). Model quality was evaluated with MOLPROBITY (40). 97% of the residues were in favored regions of the Ramachandran plot. Statistics of data collection, processing and model building are presented in Table 1.

Miscellaneous Methods—SDS-PAGE was performed as described (27). Sequences of FeLOX and MnLOX were aligned with the ClustalW program, and a phylogenetic tree was constructed by MEGA6 with bootstrap tests of the resulting nodes (41). All figures were generated with PyMOL (The PyMOL Molecular Graphics System, Version 1.7.4 Schrödinger, LLC).
**Results**

**Deglycosylation**—Mo-MnLOX retained more than 50% of the enzyme activity after deglycosylation, and the three-dimensional structure discussed below showed that N-acetylgalactosamine residues remained at Asn-72, Asn-150, and Asn-535. The deglycosylation process decreases the melting temperature of Mo-MnLOX from 60 to 56 °C. The protein unfolding in response to temperature (assayed with SYPRO Orange (22)) indicated that the enzyme solution was not fully homogeneous (supplemental Fig. S1, A and B).

**Crystallization of Mo-MnLOX**—Needle-like crystals of Mo-MnLOX were formed after 1 week in polyethylene/ion screen HT (Hampton Research) by 1:1 mixing of 14 mg/ml protein and reservoir solution (4% v/v tacsimate (pH 7.0), 12% (w/v) PEG-3350, or 0.2 M ammonium citrate dibasic (pH 5.1), 12% w/v PEG-3350). Single large crystals grew after 1 month at 8 °C in 0.2 M ammonium citrate dibasic (pH 4.8), 12% (w/v) PEG-3350. Parallel optimization was performed with additive screen MemAdvantage (Molecular Dimensions) in 96-well sitting drop plates at 21 °C and in 15-well hanging drop plates at 8 °C. Crystals were formed after 3 days at 21 °C by mixing 0.1 μl of protein (8.5 mg/ml), 0.1 μl of reservoir solution (0.2 M ammonium citrate dibasic (pH 4.8), 10–14% (w/v) PEG-3350), and 0.02 μl of 1.9 M CYMAL-7. Crystals also appeared after 30 days at 8 °C by mixing 1 μl of the same reservoir solution with 0.1 μl of protein and 0.1 μl of 1.9 M CYMAL-7 in hanging drop 15-well plates (supplemental Fig. S1 C). Crystals were cryo-protected in reservoir solution with 15% (v/v) glycerol and vitrified in liquid N2 prior to data collection.

**X-ray Diffraction Analysis**—The crystals of Mo-MnLOX were relatively insensitive to radiation, and 4800 images were collected with 0.15° oscillation. The best dataset had a completeness of 99.6% and an average multiplicity of 25.3. The crystals of Mo-MnLOX diffracted to 2.0 Å. Mo-MnLOX contains one manganese atom in the active site and 15 sulfur atoms from 3 Cys and 12 Met residues. This made it possible to solve the structure by single anomalous dispersion phasing using both manganese and sulfur atoms as anomalous scatterers. We therefore collected data at 1.77 Å to mitigate the absorption effect at longer wavelengths while still being able to collect a useful sulfur signal. Because the Kα absorption edge of a manganese is at λ = 1.88 Å, the anomalous signal for manganese (f = 3.45 electrons) at λ = 1.77 Å becomes an additional source of anomalous signal, which facilitated the phase determination.

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**TABLE 1**

Data collection, processing, phasing and structure refinement statistics

|                        | S-SAD 1 crystal | S-SAD (4 crystals merged) |
|------------------------|-----------------|--------------------------|
| **Data collection and processing** |                 |                          |
| Beamline               | Diamond I02     | ESRF ID-23, Diamond I02  |
| Detector               | Pilatus 6 m     | Pilatus 6 m              |
| Wavelength (Å)         | 1.77            | 1.77                     |
| Oscillation range      | 0.15            | 0.15                     |
| No. of images          | 3600            | 4800/2400/3600/3600      |
| Space group            | P2,2,2          | P2,2,2                   |
| Cell parameters a, b, and c (Å) | 70.72, 111.37, 171.22 | 70.60, 111.32, 171.25 |
| Resolution range (Å)  | 29.70–2.04 (2.11–2.04) | 48.93–2.53 (2.62–2.53) |
| No. of observed reflections | 1,531,004 (49,813) | 3,535,869 (338,794) |
| No. of unique reflections | 81,415 (3,846) | 45,670 (4,371)          |
| Multiplicity           | 18.8 (13.1)     | 77.4 (77.5)              |
| Completeness (%)       | 98.1 (86.0)     | 99.9 (99.9)              |
| Rmerge                 | 0.12 (0.63)     | 0.15 (0.34)              |
| Rfree(I/I0)            | 17.9 (3.9)      | 44.1 (19.2)              |
| CC1/2 (%)              | 0.997 (0.919)   | 1.0 (0.998)              |

| **Manganese and sulfur phasing** |                 |                          |
| Resolution cutoff       | 3.5             |                          |
| No. of sites            | 2 manganese, 15 sulphur |
| CC anomalous            | 30              |                          |
| Map correlation         | 0.66            |                          |
| Connectivity            | 0.75            |                          |
| Contrast                | 0.54            |                          |

| **Refinement statistics** |                 |                          |
| Resolution used in refinement | 2.04         |                          |
| Reflections in working/test set | 84190/4403 |                          |
| R/Rfree factor (%)         | 16.8/21.3      |                          |
| Molecules in asymmetric unit | 2            |                          |
| No. of atoms              |                |                          |
| Protein atoms             | 9741           |                          |
| Mn                        | 2              |                          |
| N-Glycosylation (NAG) atoms | 112         |                          |
| Water molecules           | 683            |                          |
| Protein residues          | 1136           |                          |
| Wilson B-factor           | 35.9           |                          |
| Average atomic B-factors (Å²) |             |                          |
| Overall                  | 43.5           |                          |
| Protein                  | 42.94          |                          |
| Water                    | 47.27          |                          |
| Mn                       | 33.06          |                          |
| r.m.s. deviation          |                |                          |
| Bond lengths from ideal (Å) | 0.007      |                          |
| Bond angles from ideal (%) | 0.93         |                          |
| Ramachandran outliers (%) | 0             |                          |

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The anomalous signal is weak for sulfur ($f' = 0.7$ electrons) at $\lambda = 1.77$ Å. It was therefore crucial to have high redundancy data to enhance the signal to noise ratio. Four datasets were merged that resulted in a multiplicity of 77.4 and an anomalous signal that enabled us to determine the position of the manganese and sulfur atoms.

The crystals belong to space group $P2_12_12_1$ with unit cell dimensions as follows: $a = 70.7$ Å, $b = 111.4$ Å, and $c = 171.2$ Å. The solvent content was 47% with two molecules in the asymmetric unit with the average $C_r$ root-mean-square (r.m.s.) deviation of 0.147 Å. The final model was refined to $R_{work}$ of 0.17 and $R_{free}$ of 0.21. We were able to build all the residues except for the 37 N-terminal residues of the expressed protein (EFV...PEL), possibly due to its flexibility, as well as $N$-acetylglycosamine groups at Asn-72, Asn-150, and Asn-535.

**Crystal Structure of Mo-MnLOX—**

Mo-MnLOX lacks the PLAT domain found in many FeLOX and phospholipases (2). An illustration of the overall Mo-MnLOX structure is presented in Fig. 2A. The structure is composed of 21 $\alpha$-helices and 7 small $\beta$-sheets. The helices $\alpha 9$ and $\alpha 10$ combined are designated the broken arched helix, and it is sheltering the active site (light green, Fig. 2A). The most striking difference to FeLOX is the orientation of helix $\alpha 2$ with 11 turns between residues 79 and 117 (blue helix, Fig. 2B). This long helix is slightly arch-shaped and runs over the whole length of the protein. Its orientation in animal and plant LOX varies, and in plants it has been reported to be mobile to allow access to the active site, as illustrated by a comparison of Mo-MnLOX with human 5-LOX, sLOX-1, and 15S-LOX of P. aeruginosa (Fig. 2B) (2). This helix is found in all known LOX structures and harbors several invariant hydrophobic residues (2). A structure-based sequence alignment between 8R-LOX (4QWT); the 8R-LOX domain of the allene oxide synthase-LOX fusion protein) and Mo-MnLOX shows conservation of most $\alpha$-helices (supplemental Fig. S2).

**Metal Coordination—**

His-294, His-289, His-469, Asn-473, Val-605, and a water molecule coordinate the catalytic metal $\text{Mn}^{2+}$ (Fig. 3A). The coordinating sphere is similar to coral 8R-LOX, but the metal ligands do not superimpose as neatly as those of coral 8R-LOX, sLOX-1, and 15S-LOX of P. aeruginosa (Fig. 3B) (2, 16, 19, 20). The distances between the coordinating residues, manganese, and water are indicated in Table 2 along with a comparison with three FeLOX.

A small loop of 5 residues connects helices $\alpha 17$ and $\alpha 18$ and harbors the manganese ligand, Asn-473 (Fig. 4, A and B). This loop likely brings Asn-473 to a flexible position near the catalytic metal. The side chain oxygen of Gln-281 forms hydrogen bonds to the amino group of Asn-473 with a distance of 2.91 Å (Fig. 4C). This Gln residue is conserved in all FeLOX and MnLOX. There is also a hydrogen bond between the C-terminal carboxyl (Val-605) and the catalytic water (Fig. 4C).

**Substrate Channel—**

There is a solvent-accessible channel leading into the catalytic center of Mo-MnLOX (Fig. 5A). Arg-525 at the entrance is positioned at helix $\alpha 19$. This Arg is conserved in five out of the six confirmed MnLOX in Fig. 1A (except in MnLOX of Fusarium oxysporum). Arg-525 is positioned close to Arg-182 of 8R-LOX when the two structures are superimposed. Arg-525 likely forms a salt bridge with the carboxyl end of the substrate fatty acid (Fig. 5B) in analogy with Arg-182 (2). The entrance is also defined by residues from helix $\alpha 2$ (Trp-93, Val-98, Ser-101, and Phe-105), helix $\alpha 9$ (Leu-326), and helix $\alpha 11$ (Val-350).

The assumed substrate channel in Mo-MnLOX is composed of several hydrophobic residues (Table 3). The closest residues surrounding the coordinated catalytic water are Phe-526 (4.2 Å), Val-323 (4.2 Å), Phe-332 (5.6 Å), Leu-331 (6.6 Å) and Glu-281 (5.9 Å) as discussed above. These residues likely constitute the direct environment of the pentadiene of the substrate and define the hydrophobic channel.

Leu-331 is situated at the bottom of the arched helix where it shelters the active site, and the corresponding residue of
TABLE 2

Distances (Å) between the catalytic metal, the coordinating ligands, and the catalytic base

|                | Mo-MnLOX | 8R-LOX | sLOX-1 | Pa-LOX |
|----------------|----------|--------|--------|--------|
| His7284(NE2)   | 2.6      | 2.4    | 2.2    | 2.3    |
| His7289(NE2)   | 2.2      | 2.3    | 2.3    | 2.2    |
| His7469(NE2)   | 2.1      | 2.3    | 2.2    | 2.2    |
| Asn7473(OD1)   | 2.9      | 3.1    | 3.1    | 2.2    |
| Val7605(OXT)   | 2.1      | 2.3    | 2.4    | 2.2    |
| H2O            | 2.3      | 2.4    | 2.6    | 2.2    |
| Va7605(O)-H2O  | 2.4      | 2.4c   | 2.5c   | 2.5f   |

*a* The 8R-LOX domain of the allene oxide synthase-LOX fusion protein (UniProtKB/Swiss-Prot: O16025.1) is shown.

*b* Pa-LOX is 155-LOX of *P. aeruginosa*.

*c* The distances between the catalytic water and the C-terminal Ile residue are shown.

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**FIGURE 4. Factors influencing the metal coordination of Mo-MnLOX.** A, unbiased 2Fo — Fc electron density map is shown at contour level of one σ. The metal-coordinating Asn473 residue is situated on a loop and might provide the increased flexibility necessary for the use of manganese as catalytic metal. Gin474 and Ser604 are in close positions. B, comparison of the structure of the loop with Asn473 (light blue) with the corresponding part of 8R-LOX (gray). C, hydrogen bond network close to the active site of MnLOX and Asn473, which forms three hydrogen bonds as follows: with the conserved Gin281, the main chain oxygen of the metal coordinating His469 and the main chain of Glu476. Gin474 is forming a hydrogen bond network with Ser604 proximate to the C-terminal Val605; it also forms a weak interaction with a coordinated water molecule that also interacts with the main chain of Arg528 and the side chain of Asn527.

8R-LOX, Leu-431, has been shown to clamp the substrate in the active site (2, 15, 24). It appears to play the same role in Mo-MnLOX (Fig. 6). The side chain of the next residue, Phe-332, points into the hydrophobic channel and might shield one side of the pentadiene from oxygenation. This Phe residue is conserved in all MnLOX, but not in FeLOX, which have either Ile or Val at this position (Table 3).

Phe-526 is also conserved in MnLOX, whereas the corresponding residue in FeLOX is a conserved Leu residue (Table 3). The distance between the side chains of Leu-331 and Phe-526 is only 3.9 Å. The substrate could be clamped by these residues and bent to allow oxygen to access the 11S position after the hydrogen abstraction. The distance between the two corresponding Leu residues in 8R-LOX is similar, but with the substrate in the active site Leu-627 of 8R-LOX is bent backwards, and the distance is increased to 5.2 Å (24).

**Oxygen Access to the Catalytic Center—** MnLOX utilize suprafacial hydrogen abstraction and oxygenation in contrast to the antarafacial oxidation mechanism of FeLOX (6, 21, 22, 26). This implies that O2 can access the pentadienyl radical from the same side as the catalytic complex, Mn3+OH− (cf. Fig. 1B). A possible oxygen channel has been identified in several three-dimensional structures of FeLOX (2, 10, 24). The Coffa-Brash determinant, Gly-427 of coral 8R-LOX, appears to be in a critical position in its oxygen channel (2). No equivalent channel could be found in Mo-MnLOX, and the corresponding Gly-327 residue may have little influence on the position of oxygenation (42). There are two pockets in the Mo-MnLOX substrate channel that could harbor oxygen if it enters via the substrate channel (Fig. 6). It is tempting to speculate that these pockets could explain the stereospecific oxygenation of all three positions of the pentadiene radical.

**Site-directed Mutagenesis—** The structure discussed above suggested that Arg-525 and Phe-526 might be of structural importance for tethering of the carboxyl group and for oxygenation, respectively. We examined the following two mutations: R525A and F526L. Protein expression was confirmed by SDS-PAGE after protein isolation by hydrophobic interaction chromatography.

The mutant R525A transformed 16, 36, and 100 μM 18:3n-3 to small amounts of 11-HPOTrE without apparent substrate inhibition (24). 11-HPOTrE was detected by RP-HPLC-MS/MS analysis (supplemental Fig. S3). The marked reduced catalytic activities could be in agreement with the proposed
function of Arg-525 in tethering the carboxyl group of 18:2\textsuperscript{\textit{n}-6} and 18:3\textsuperscript{\textit{n}-3}.

We also examined 9\textsuperscript{\textit{S}}-HPOTrE as a substrate of R525A. A substantial fraction of 9\textsuperscript{\textit{S}}-HPOTrE was transformed to 9,16-DiHPOTrE as shown in Fig. 7. RP-HPLC analysis showed that it consisted mainly of the expected 9\textsuperscript{\textit{S}},16\textsuperscript{\textit{S}}-diastereoisomer (22).

We conclude that Arg-525 is not essential for the lipoxygenation of 9\textsuperscript{\textit{S}}-HPOTrE.

To assess the importance of the chain length for the interaction with the Arg-525 residue, we compared the oxidation of 20:2\textsuperscript{\textit{n}-6}, 20:3\textsuperscript{\textit{n}-3}, and 22:5\textsuperscript{\textit{n}-6} with 18:2\textsuperscript{\textit{n}-6} and 18:3\textsuperscript{\textit{n}-3}. The two C20 fatty acids were both oxidized at C-13. 20:2\textsuperscript{\textit{n}-6} was also oxidized at C-11 and C-15 in a ratio of \(\sim 10:1\) (supplemental Fig. S4A). 20:3\textsuperscript{\textit{n}-3} was oxidized at both C-11 and C-15. The latter also formed 11,18-dihydroperoxy-12\textsuperscript{\textit{E}},14\textsuperscript{\textit{Z}},16\textsuperscript{\textit{E}}-eicosatrienoic acid (supplemental Fig. S4B). UV analysis (235 nm) and LC-MS analysis to estimate the relative amounts of bis-allylic hydroperoxides suggested that 20:2\textsuperscript{\textit{n}-6} as oxidized at a rate of 70% of 18:2\textsuperscript{\textit{n}-6}, whereas UV analysis (270 nm) indicated that 20:3\textsuperscript{\textit{n}-3} was oxidized to trienes twice as rapidly as 18:3\textsuperscript{\textit{n}-3}. 22:5\textsuperscript{\textit{n}-6} was oxidized at C-13 and C-17 (supplemental Fig. S4C).

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**TABLE 3**

Comparison of amino acid residues surrounding the active site in MnLOX and FeLOX

| Comment | Consensus MnLOX, Mo-MnLOX | FeLOX |
|---------|---------------------------|-------|
| Channel entrance FeLOX | | Tyr-178 |
| Channel entrance FeLOX | | Arg-182 |
| Interaction with Asn-473 | Gln | 281 |
| Metal coordination | His | 284 |
| Oxygen channel | Val-285 |
| Metal coordination | His | 289 |
| Stereocontrol (Coffa-Brash) | Gly/Ala | Gly-327 |
| Clamp substrate | Leu-331 |
| Supra/antarafacial | Phe/Ile | Phe-332 |
| Pocket depth (Sloane) | Phe-338 |
| Pocket depth | Thr-489 |
| Pocket depth | Gln-519 |
| Channel entrance MnLOX | Leu-522 |
| Steric shielding | Phe/Leu | Arg-525\textsuperscript{\textit{b}} |
| C terminus | Val/Ile | Ile-605 |

* The 8\textsuperscript{\textit{R}}-LOX domain of the allene oxide synthase-LOX fusion protein is shown.

* Arg-525 is positioned close to Arg-182 of 8\textsuperscript{\textit{R}}-LOX (Fig. 5B).

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**FIGURE 6.** Possible oxygen access routes in the U-shaped substrate channel of Mo-MnLOX. Leu-331 from the arched helix is defining the upper wall of the channel at the bottom of the U-shaped substrate channel in analogy with Leu-431 of 8\textsuperscript{\textit{R}}-LOX. Phe-332 may shield the pentadiene for oxygen insertion in an antarafacial way so that oxygen may reach the pentadiene radical from the other side as indicated in by the arrows in the two side pockets. Phe-526 is likely to bend the substrate to allow oxygen access from the same side as the catalytic metal. Arachidonic acid, bound to coral 8\textsuperscript{\textit{R}}-LOX (PDB code 4QWT, chain C), is included for clarity; the natural substrates of Mo-MnLOX are linoleic and \(\alpha\)-linolenic acids, but 20:2\textsuperscript{\textit{n}-6}, 20:3\textsuperscript{\textit{n}-3}, and 22:5\textsuperscript{\textit{n}-6} are also oxidized by the enzyme (supplemental Fig. S4).

**FIGURE 7.** RP-HPLC-MS/MS analysis of the biosynthesis of 9\textsuperscript{\textit{S}},16\textsuperscript{\textit{S}}-DiHPOTrE from 9\textsuperscript{\textit{S}}-HPOTrE by the R525A mutant and an overview of the sequential biosynthesis of 9,16-DiHPOTrE by Mo-MnLOX and the R525A mutant. NL, normalized to 100%. TIC, total ion current.
at about 25% of the rate of 18:2\textsuperscript{n-6}. We conclude that the substrate chain length is not critical for catalysis.

The F526L mutant did not oxidize 18:3\textsuperscript{n-3}, but it transformed 9\textsuperscript{S}-HPOTrE to 9\textsuperscript{S,16\textsuperscript{S}}-DiHOTrE (supplemental Fig. S5), which suggests that the catalytic center was intact.

Discussion

We report as our main finding the first three-dimensional structure of MnLOX. This structure relates to three fundamental differences between MnLOX and FeLOX as follows: (i) the coordinating spheres of Mn\textsuperscript{2+} and Fe\textsuperscript{2+} and the metal preferences; (ii) adjustment of the redox potentials of protein-bound Mn\textsuperscript{2+}/Mn\textsuperscript{3+} and Fe\textsuperscript{2+}/Fe\textsuperscript{3+} and the catalytic base by hydrogen bonds, and (iii) the active sites and the supra- and antarafacial oxygenation mechanisms of MnLOX and FeLOX, respectively. An overview of the active site is shown in Fig. 8, and a comparison of important residues with FeLOX is presented in Table 3.

Adjustment of Redox Potentials—FeLOX and MnLOX catalyze the same enzymatic reactions, and their redox properties are therefore likely similar, about 0.6 V (44). As far as is known, manganese-substituted FeLOX are catalytically inactive (9, 38).

Two differences between MnLOX and FeLOX are the capacity of MnLOX to catalyze the same enzymatic reactions, and their redox properties which are enriched in Mn\textsuperscript{2+} (30). Whether the three-dimensional differences between the coordinating spheres of MnLOX and FeLOX also can affect metal selection will await future studies.

Active Site and the Oxygenation Mechanism—The deduced substrate channel of Mo-MnLOX appears to be similar to the U-shaped channel of coral 8R-LOX and related FeLOX (2). The substrate channel of Mo-MnLOX is solvent-exposed (Fig. 5A), and its interior has spacious pockets close to the presumed position of the pentadiene for hydrogen abstraction and oxygenation (Fig. 6). Arg-525 likely tethers the carboxylate of the C-terminal Val and Ile residue of Mo-MnLOX and coral 8R-LOX, respectively, but there were no additional hydrogen bonds to the catalytic water. We therefore examined the network of hydrogen bonds to the manganese ligands and to the second coordinating sphere, respectively (Figs. 4C and 8). A hydrogen bond likely occurs between the metal coordinating Asn-473 and Gln-281 (2.8 Å) of Mo-MnLOX. A hydrogen bond was also noted between Ser-604 and Gln-474 (2.8 Å), but site-directed mutagenesis of the corresponding Gln residue of 13R-MnLOX did not abolish the catalytic activity (25). The tuning of the redox potential of protein-bound Mn\textsuperscript{2+}/Mn\textsuperscript{3+} will need further investigation. This will include further analysis of the hydrogen bond network.

Crystal Structure of Manganese Lipoxygenase

Crystal Structure of Manganese Lipoxygenase

FIGURE 8. Overview of the active site of Mo-MnLOX. Arachidonic acid, bound in the substrate channel of coral 8R-LOX (PDB code 4QWT, chain C), is included in the U-shaped active site of Mo-MnLOX for clarity. The carboxyl group of arachidonic acid is likely tethered by Arg-525 and the \( \omega \) end by Phe-342. Leu-332 clamps the substrate in position, and Phe-332 and Phe-526 may position pentadiene for suprafacial hydrogen abstraction and oxygenation. Three His residues, Asn-473, Val-605, and the catalytic water are coordinating manganese (pink). Hydrogen bonds are likely formed between Gln-281 and Asn-473 and between Val-605 and the catalytic water (red).
analogy with FeLOX, the depth of the substrate channel is likely controlled by Phe-342 at the position of the Sloane determinant (Fig. 8; Table 3) and by Phe-353 (not shown in Fig. 8).

Two residues, Phe-332 and Phe-526, may directly influence the stereospecific oxygenation of fatty acids. Phe-332 is positioned in the active site above the catalytic metal and likely holds the substrate in place and might shield the opposite side for oxygen insertion (Fig. 8). Mutagenesis of the corresponding Phe-337 residue in 13-MnLOX to Ile, which is found at this position of sLOX-1 and other FeLOX (Table 3), switched the oxygen insertion in relation to hydrogen abstraction from suprafacial to mainly antarafacial (46). Phe-526 is also positioned near the catalytic metal and might position the substrate for oxygenation. Site-directed mutagenesis of Phe-526 to Leu resulted in loss of oxidation of 18:3n-3 but retention of the oxidation at C-16 of 9S-HPOTrE. The altered LOX activity suggested that this residue could be essential for catalysis, but further steric analysis of this mutant could not be performed as 18:3n-3 was not oxidized. The three-dimensional structure of Mc-MnLOX with a substrate or a substrate mimic will be needed to exactly define the structural importance of the Phe-526 residue.

Conclusion

We report the three-dimensional crystal structure of MnLOX of the rice blast fungus M. oryzae. The results confirm that the metal ligands of MnLOX and FeLOX are essentially conserved but with geometric differences between the coordinating spheres. Arg-525 likely tethers the carboxyl group of the substrate, and a pair of conserved Phe residues near the catalytic center of MnLOX might be key contributors to the unique suprafacial reaction mechanism.

Author Contributions—A. W. purified and crystallized the protein, determined the x-ray structure, prepared the figures, and wrote the paper. E. H. O. initiated the study, wrote the paper, and prepared the figures. S. K. provided assistance in crystallization, data collection, and interpretation. Y. C. determined the x-ray structure together with A. W., prepared the figures, and wrote the paper. All authors reviewed the results and approved the final version of the manuscript.

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