The structural basis for the catalytic specificity of manganese lipoxygenases

3D structure analysis of the lipoxygenase of Magnaporthe oryzae

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Abstract

Lipoxygenases (LOX) catalyze regio- and stereospecific oxygenation of polyunsaturated fatty acids to hydroperoxides. These hydroperoxides are further metabolized to leukotrienes and lipoxins in mammals, and are involved in asthma and inflammation. LOX of animals and plants contain iron as catalytic metal (FeLOX). Filamentous fungi use both FeLOX, and manganese containing LOX (MnLOX). The role of LOX in fungi is still not known. This thesis focuses on expression of novel MnLOX, analyses of their reaction mechanism and products by HPLC-MS/MS, protein crystallization and analysis of the first MnLOX structure.

MnLOX from G. graminis, M. salvinii, M. oryzae, F. oxysporum and C. gloeosporioides were expressed in Pichia pastoris, purified and characterized by HPLC-MS/MS. All MnLOX catalyzes suprafacial hydrogen abstraction and oxygen insertion. Replacement of one Ile to Phe in the active site of MnLOX of G. graminis could switch the mechanism from suprafacial to mainly antarafacial. MnLOX of F. oxysporum was interesting since it catalyzes oxygenation of linoleic acid to 11R- instead of the more common 11S-hydroperoxides. This feature could be attributed to a single Ser/Phe exchange in the active site.

We found that Gg-MnLOX utilizes hydrogen tunneling in the reaction mechanism, but was slightly more temperature dependent than soybean FeLOX. It is an intriguing question why some fungal LOX use manganese and not iron as catalytic metal and whether the large redox potential of Mn^{2+}/Mn^{3+} (1.5 V) can be tuned close to that of Fe^{2+}/Fe^{3+} (0.77 V) for redox cycling and catalysis.

We present crystallization conditions for two MnLOX, and the 2.07 Å crystal structure of MnLOX from M. oryzae, solved using sulfur and manganese single anomalous dispersion (SAD). The structure reveals a similar metal coordinating sphere as FeLOX but the metal ligand Asn^{173} was positioned on a short loop instead of a helix and formed interactions with a conserved Gln. This feature could be essential for the use of manganese as catalytic metal in LOX. We found three Phe residues that likely facilitate the suprafacial hydrogen abstraction and oxygen insertion for MnLOX.

These findings provide new insight into the unique reaction mechanism of MnLOX.

Keywords: oxylipin, lipoxygenase, crystal structure, crystallography, HPLC, mass spectrometry, yeast, site-directed mutagenesis, fungi

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List of Papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals. Reprints were made with permission from the respective publishers.


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Abbreviations

COX  Cyclooxygenase
Coxibs  Cyclooxygenase-2 inhibitors
CP-HPLC  Chiral phase HPLC
CYP  Cytochrome P450
DHA  Docosahexaenoic acid
EAS  Epoxy alcohol synthase
EPA  Eicosapentaenoic acid
GPCR  G-protein coupled receptor
H(P)ETE  Hydro(per)xyeicosatetraenoic acid
H(P)ODE  Hydro(per)xyoctadecadienoic acid
H(P)OTrE  Hydro(per)xyoctadecatrienoic acid
HIC  Hydrophobic interaction chromatography
HPLC  High performance liquid chromatography
ICP-AES  Inductively coupled plasma-atomic emission-spectroscopy
KOTrE  Keto octadecatrienoic acid
LOX  Lipoxygenase(s)
MS  Mass spectrometry
NSAID  Non-steroidal anti-inflammatory drugs
PG  Prostaglandin
PLAT  Polycystin-1, Lipoxygenase, Alpha-Toxin
PUFA  Polyunsaturated fatty acid
RP-HPLC  Reverse phase HPLC
SAD  Single anomalous dispersion
SP-HPLC  Straight phase HPLC
SRS-A  Slow-reacting substances of anaphylaxis
TX  Thromboxane
Introduction

Fatty acids

Fatty acids are hydrocarbon chains with a carboxyl group. Polyunsaturated fatty acids (PUFAs) contain more than one double bond; some examples are arachidonic acid (20:4n-6), linoleic acid (18:2n-6), α-linolenic acid (18:3n-3) and eicosapentaenoic acid (20:5n-3). If the first double bond is positioned on the third (n-3) or the sixth carbon (n-6) from the methyl-end, the PUFA are commonly called omega (ω)-3 or ω-6. 18:2n-6 and 18:3n-3 serve as precursors for other fatty acids and are essential, meaning that we have to obtain them from the diet. Fish is considered to be a rich source of long chain ω-3 PUFA such as 20:5n-3 and 22:6n-3, and vegetable oils are rich in ω-6 PUFA [1]. In general, metabolites derived from ω-6 PUFAs have pro-inflammatory and immunoactive functions, whereas metabolites derived from ω-3 PUFAs have anti-inflammatory properties [2].

PUFAs are normally found esterified in phospholipids and are released by phospholipases upon specific signals. Except for their important role in membrane viscosity their oxygenated metabolites, called oxylipins, play a key role as signaling molecules. The double bond in PUFAs are cis-configured and separated by a CH₂ group resulting in a 1,4-cis, cis-pentadiene structure which easily becomes aut oxidized resulting in a variety of hydroperoxy-, hydroxy- and epoxy-oxylipins. Enzymes such as lipoygenases (LOX), cyclooxygenases (COX) and cytochrome P450 (CYP) can control the production of oxylipins [3]. There are over a thousand known oxylipins and there are still molecules to discover.

Mammalian oxylipins

Mammal oxylipins are derived mainly from fatty acids with 20 carbons, e.g. arachidonic acid and eicosapentaenoic acid, and are collectively named eicosanoids from the Greek eicosa (twenty). Important eicosanoids are prostaglandins, prostacyclins, thromboxanes, leukotrienes and lipoxins, which are all oxygenated metabolites of arachidonic acid. In man, eicosanoids are involved in several physiological processes such as immune response, maintenance of normal hemostasis, blood pressure, renal function and reproduction. Excess of these molecules, for example under pathological conditions, can cause pain, fever, and inflammation [4].
COXs are the key enzymes in the biosynthesis of prostaglandin (PG), thromboxane (TX), and prostacyclin (PGI2). COX-1 is constitutively expressed in most cells and COX-2 is induced by inflammatory stimuli [1]. There are also reports about a COX-3, a splice variant of COX-1, found in canine brain tissue [5], but it has been questioned if this splice variant is present or has any COX-activity in humans. COX catalyzes the oxygen insertion into two of the unsaturated double bonds of arachidonic acid, generating the unstable endoperoxides PGG2 and PGH2, which are tissue specifically transformed to PGE2, PGI2, PGD2, PGF2α or TXA2. The formation of eicosanoids in different tissue is controlled by specific isomerases or synthases [6].

In addition to the COX pathway, arachidonic acid can be oxygenated by the LOX pathway and form leukotrienes, lipoxins, hepopxilins, and oxins. Leukotrienes are mainly formed in inflammation cells such as leukocytes, macrophages and mast cells. Leukotrienes are involved in inflammation and asthma and are formed by 5-LOX, which is the most thoroughly investigated human LOX. The different LOX and their biological effect are described in more detail in the section Lipoxygenases.

In addition, metabolism of arachidonic acid and other PUFAs are also performed by the ubiquitous CYP, with formation of hydroxy- and epoxy-metabolites. CYPs are abundant in the liver and kidney and are important for xenobiotic metabolism but their eicosanoid metabolites also have important vascular, renal and cardiac effects [7, 8].

Pharmacological overview

One of the first reports of the medical use of salicylic acid came from Hippocrates (460–370 BC). He recommended willow-tree bark to patients suffering from fever and pain, and since then several observations about the analgesic (pain-killing), antipyretic (fever-reducing) and anti-inflammatory effects of salicylic acid have been reported. Acetylated salicylic acid (aspirin) was synthesized 1897 by the Bayer chemist Felix Hoffmann. The acetylated form of salicylic acid is less irritating for the gut mucosa and has less bitter taste, and has since then been used for relief of pain, fever and inflammation [9].

In 1971, Sir John Vane discovered the inhibition of prostaglandin biosynthesis by acetylsalicylic acid [10]. Acetylsalicylic acid binds and acetylates Ser-530 irreversible in the COX active site and blocks arachidonic acid from binding. Later, other non-steroidal anti-inflammatory drugs (NSAID) were developed, e.g. ibuprofen, indomethacin and naproxen that block the binding of arachidonic acid in COX by competitive inhibition. Low dose aspirin can also be used for prevention of thrombosis by inhibition of biosynthesis of TXA2, which is important for platelet aggregation [11].

When COX-2 was discovered, selective COX-2 inhibitors (coxibs) were rapidly developed, taking advantage of the larger active site pocket in COX-2 [12, 13]. The objective was to reduce the adverse side effects associated
with COX-1 inhibition (e.g. gastrointestinal toxicity, increased bleeding). However, the coxibs had unexpected cardiovascular side effects probably due to decreased synthesis of the cardioprotective PGI2. Consequently, some of the coxibs were withdrawn from the market [14].

Prostaglandins have a short half-life in vivo, but prostaglandin analogues are used clinically. PGE1 analogues (e.g. misoprostol) are used for induction of labor, termination of pregnancy, and gastrointestinal protection [15, 16]. In newborn babies a PGE1 analogue can be used to maintain the patency of the ductus arteriosus if surgical correction is needed [17]. A PGI2-analogue increases vasodilatation and inhibits platelet aggregation and can be used in pulmonary hypertension patient and in kidney dialysis [18]. Eye drops with a PGF2α analogue (e.g. latanoprost) can be used in open-angle glaucoma [19].

There are few drugs developed to target the LOX pathway, one group is the cysteinyl leukotriene receptor antagonists (e.g. montelukast, zafirlukast), which often are used as add-on therapy to prevent bronchoconstriction, inflammation and mucus secretion in chronic obstructive pulmonary disease and asthma [20, 21]. Another asthma drug, zileuton, is a 5-LOX inhibitor and prevents the first step in leukotriene biosynthesis, it has therefore broader actions than the cysteinyl receptor antagonists in that it inhibits the formation of the cysteinyl leukotrienes, hydroxyeicosatetraenoic acid (HETE), and also leukotriene B4 [22]. Zileuton is not available on the Swedish market.

Plant oxylipins

Plant oxylipins can be formed by three major groups of enzymes, α-dioxygenases (α-DOX), LOX, and CYP, or by autooxidation. LOX products were discovered in soybean seed already in the 1930s. Soybean LOX (sLOX-1) was crystallized as early as 1947 and has since then been the prototype LOX in research [23].

In plants, C18 fatty acids are the most abundant. Most enzyme-derived plant oxylipins are synthesized from 18:3n-3, 18:2n-6, or 16:3n-3. There are a variety of plant oxylipins e.g. hydroperoxy, hydroxy-, epoxy-, keto- and oxo- fatty acids, epoxy alcohols, divinyl ethers, volatile alcohols or aldehydes, and jasmonic acid and its corresponding derivatives [24]. Plant oxylipin biosynthesis is often initiated by 13- or 9-LOX or α-DOX, forming highly reactive hydroperoxide metabolites which can act as substrates for other enzymes (e.g. epoxy-alcohol synthases, allene oxide synthases or CYP) [25, 26]. α-DOX shows sequence homology with COX and oxidize fatty acids to unstable 2R-hydroperoxy derivatives and may be involved in plant defense against pathogens [27].

Jasmonic acid is the oxylipin in plants that has received the most attention. Jasmonic acid biosynthesis occurs sequentially with catalysis by several enzymes and is initiated by oxidation of 18:3n-3 by 13S-LOX [28]. Jasmonic
Acid has been shown to mediate hormone-like effects in plants and is an important signaling compound in plant development and stress response [28]. Methyl jasmonate is used in the perfume industry as a fragrance component of jasmine oil.

Fatty acids such as 18:3n-3 can also be autoxidized through reactive oxygen species and form phytoprostanes, suggested to be involved in defense against pathogens and oxidative stress [29].

Fungal oxylipins

Even though the occurrence of oxylipins in fungi has been known for more than 30 years, the knowledge of their biological function is limited. In the last years several fungal genomes have been sequenced, this facilitates the search and identification of potentially new oxylipin-producing enzymes. Enzymes in the fungal oxylipin biosynthesis pathway have been identified as LOX, DOX, and CYP. filamentous fungi contain C18 fatty acids, but only low levels of C20 fatty acids.

Fungi often combine DOX and CYP in DOX-CYP fusion proteins, these enzymes can oxidize fatty acids to diols, epoxy alcohols and allene oxides without the need of an external electron transport system [30]. DOX shows sequence homology to COX and α-DOX. TXA synthase (CYP5A) and PGI synthase (CYP8A1) is the human counterpart to the DOX-CYP, but in humans they function as separate enzymes. DOX and DOX-CYP enzymes form a variety of oxylipins such as, 8-HPODE, 9-HPODE, 10-HODE, 5,8- and 7,8-diHODE, 8,11-diHODE [31-33].

The biological function of oxylipins in fungi is mostly studied in the opportunistic pathogens of Aspergillus spp, which can cause both airway infection (aspergillosis) and contaminate food. One group of oxylipins shown to be critical for regulation of sexual (ascospores) and asexual sporulation (conidia), called the psi- (precocious sexual inducer) factors, was first identified in A. nidulans [34, 35]. They constitute of a mixture of hydroxylated 18:2n-6, 18:3n-3 and 18:1n-6 metabolites e.g. 8R-HODE, 5,8-DiHODE, 8R-HOME. They have also been discovered in many other fungi and the responsible enzyme was identified as 5,8-linoleate diol synthase (also designated ppoA), which is a member of the DOX-CYP fusion enzyme family [36, 37]. Two additional genes related by homology can be found in A. nidulans, whereof one was identified as 10R-DOX (ppoC) [38].

Several fungi (e.g. Fusarium oxysporum and Lasiodiplodia theobromae) have been shown to produce the plant hormone, jasmonic acid and jasmonic acid derivatives, but the complete mechanism is not well understood and the responsible enzymes have not yet been identified [39, 40]. The release of jasmonic acid from L. theobromae during infection inhibits the salicylic acid mediated defense system in plants, resulting in facilitated infection by this fungus [41].
Reports of prostaglandin production has for long been restricted to vertebrates and invertebrate animals. Recently, prostaglandins and a PGHS analogue, with 20% sequence homology with COX, was discovered in red algae [42]. In addition, the fungal pathogens Candida albicans and Candida neoformas, as well as other fungi, have been reported to produce prostaglandins and leukotrienes [43, 44]. However, this has been questioned since fungi contain no or very small amount of C₂₀ fatty acids required for prostaglandin and leukotriene synthesis and the enzymes responsible has not been identified [26]. Is has also been proposed that fungi can utilize the host C₂₀ fatty acids as substrate [45]. C. albicans can synthesize the anti-inflammatory oxylipin resolvin E₁ from eicosapentaenoic acid. In low concentration resolvin E₁ could reduce the immune response against C. albicans [46].

Fungi produce similar LOX products as plants, 9-HPODE and 13-HPODE. Fungi contain both iron and manganese containing LOX. The most investigated fungal LOX is the manganese LOX from the filamentous fungi Gaeumannomyces graminis (Gg-MnLOX). This MnLOX was discovered by Su et al. in 1998 [47] and forms 11- and 13-hydroperoxy metabolites from C₁₈ fatty acids. It forms the 13R- and 11S-HPODE enantiomers [48], as opposed to 13S-HPODE formed by plant LOX. The properties of MnLOX are discussed further in the section Fungal Lipoxygenases.

Fungal secondary metabolites of pharmaceutical importance

The diverse fungal kingdom includes many species with novel biochemical pathways that produce a large number of metabolites. Metabolites that are not absolutely required for survival of the organism but are produced in order to gain a survival advantage are called secondary metabolites. Fungi are effective producers of secondary metabolites that have antibacterial, antifungal and anti-tumor activates. Some of these metabolites can be either beneficial (e.g. penicillin, lovastatin, ergotamine) or harmful (e.g. mycotoxins) to humans [49, 50].

Cross-kingdom oxylipin signaling

An interesting question is whether oxylipins can function as signaling molecules between host and pathogen. In fact, it has been shown that plant LOX produced 13- and 9-hydroperoxides elicit effects on sporulation in Aspergillus spp. [51]. Some fungi secrete phospholipases in order to release the sub-
strate arachidonic acid from the host cell membrane to gain growth and virulence advantages [25].

No fungal oxylipin receptor has been identified to date. In animals most eicosanoids and oxylipins bind to receptors that belong to the G-protein coupled receptor (GPCR)-type. LOX derived 9S-HODE, which is produced by both plant and fungi can bind to the mammalian GPCR, G2A, indicating that mammalian cells might also be able to perceive fungal and plant oxylipins [52]. 9S-HPODE, the corresponding hydroperoxide, has been shown to affect fungal sporulation and mycotoxin production. It is possible that GPCRs might also be involved in fungal oxylipin perception and several genes with homology to mammalian GPCR encoding genes have been detected in filamentous fungi [24].

It is possible that fungal pathogens secrete LOX during invasive growth to generate reactive oxygen species and lysis of membranes. Fungal LOX may also regulate biosynthesis of secondary metabolites and toxins, including biosynthesis of jasmonates [25, 40].

Lipoxygenases

Historical background

The first LOX was identified in soybean and was originally termed a lipoxidase, it was isolated and crystallized by Theorell in 1947 [23]. Several years before, in 1930, Harkavy reported that an alcohol soluble substance from sputum of asthmatic patients could induce smooth muscle contraction [53]. In late 1930s the laboratory of Feldberg and Kellaway noted that a substance released from guinea-pig lungs upon anaphylaxis caused the contraction of smooth muscle [54]. The effect was later separated from that of histamine and showed to induce slow but long lasting smooth muscle contraction, and was hence called slow-reacting substance of anaphylaxis, SRS-A [55]. SRS-A was purified and characterized and was shown to be an acidic low molecular weight compound with a characteristic UV-spectrum of three conjugated double bonds [56, 57]. Further studies showed that SRS-A was produced from arachidonic acid and contained cysteine [58]. Samuelsson and coworkers noted the similarities between SRS-A and leukotrienes (named after their location in leukocytes and their triene structure) and discovered that SRS-A in fact was a group of different leukotrienes [59]. SRS-A was later identified as the cysteinyll leukotrienes, LTC₄, LTD₄ and LTE₄, and turned out to be important mediators of allergic inflammation and asthma [60-63].

In the 1970-1980s many breakthroughs were made in LOX research, 12S-LOX was discovered in human and bovine platelets [64, 65] and 15-LOX activity was found in rabbit reticulocytes [66]. The leukotriene pathway was found to be initiated by a 5-LOX [67]. In the late 90s, a 12R-LOX was de-
tected in psoriasis lesions in the skin and was showed to be involved in maintenance of the epidermal water barrier [68, 69]. The most recent discovery of a human LOX came in 2001 [70], but this enzyme, called eLOX-3 lacks the typical dioxygenation capacity of LOX. In 2003, its activity was linked to 12S-LOX and it was discovered that eLOX-3 was a hydroperoxide isomerase and could transform 12R-HPETE to the epoxy alcohol 8R-hydroxy-11R,12R-epoxyeicosa-5Z,9E,14Z-trienoic acid [71].

Since their discovery, LOX has been studied intensively in order to elucidate their biological function, occurrence, structure, and catalytic specificity.

Lipoxygenase reaction mechanism

In the general FeLOX reaction, the catalytic metal redox cycles between Fe$^{2+}$-OH$_2$ (inactive) and Fe$^{3+}$-OH$^-$ (active). Oxidation to Fe$^{3+}$ is facilitated by reduction of hydroperoxides. The catalytic base, Fe$^{3+}$-OH$^-$, is able to abstract the pro-$S$ hydrogen from a cis,cis-1,4-pentadiene in the fatty acid substrate. An unstable carbon-centered radical is formed and is dislocated over the pentadiene. The radical rapidly reacts with oxygen in an antarafacial manner, which means that oxygen insertion occurs at the opposite side of the substrate than the hydrogen abstraction. Molecular oxygen can be inserted at C-1 or C-5 and more rarely at C-3 of the pentadiene, with formation of a peroxyl radical. The catalytic Fe$^{2+}$ delivers one electron to the radical; generating a peroxyl anion, which is protonated to give a cis-trans, conjugated hydroperoxide as end product. The active form of the metal, Fe$^{3+}$-OH$^-$, is reactivated for further catalysis [72, 73].

MnLOX utilizes a similar mechanism as FeLOX, but with some differences. Oxygen is inserted from the same direction as the hydrogen abstraction, a so-called suprafacial oxygen insertion, and it catalyzes oxidation of bis-allylic hydroperoxides two orders of magnitude more rapidly than the Fe LOX [48, 74]. The LOX oxygenation is usually strictly regio- and stereospecific.

Lipoxygenase specificity

LOX enzymes are conventionally named after their regiospecificity and if necessary also after their stereospecificity (e.g. 12S-LOX or 12R-LOX). 13-LOX in plants corresponds to 15-LOX in mammals due to oxidation of substrates with 18 or 20 carbons. The specificity of LOX has been extensively investigated. Important factors for the specificity are the positioning of the fatty acid substrate in the active site, the head-to-tail (carboxyl-end to ω-end) orientation of the substrate, and the access of molecular oxygen to the position of the oxygen insertion [75].

The depth of the active site hydrophobic pocket can determine the positioning of the substrate and which position that is most exposed for hydrogen
abstraction by the catalytic metal. It has been shown that the exchange of Met\textsuperscript{418} and neighboring residues in 15-LOX, with the corresponding smaller residues in 12-LOX, shifted the oxygenation towards C-12 [76]. These residues are localized in the bottom of the active site and are called the “Sloane determinant” of regiospecificity. This amino acid exchange allows the substrate to enter the active site pocket deeper and exposes the C-12 instead of C-15 for oxygenation. Mutagenesis studies shows that Phe\textsuperscript{353} in 15-LOX-1 plays an important role in defining the depth of the hydrophobic pocket harboring the active site [77]. This was confirmed by the 3D structure of rabbit 15-LOX-1, and an additional Ile\textsuperscript{593} was identified to be important for the size and depth of the substrate-binding site [78].

The importance of different head-to-tail orientation was first deduced in plant 13S- and 9S-LOX, which abstract the 11 pro-S and 11 pro-R hydrogen respectively, from linoleic acid [79]. The reverse binding is also evident in the double oxygenation of arachidonic acid by sLOX-1, with formation of 15-HPETE followed by oxygenation at the 5S or 8S position in the same LOX active site [80]. In sLOX-1, it has been shown that low pH promotes formation of the 9S-hydroperoxide. It was suggested that the carboxylic acid form of the substrate can arrange itself in the active site in either head-to-tail orientation, but the carboxylate anion can only be positioned tail-first with formation of the 13S-hydroperoxide [81].

The access of O\textsubscript{2} to the reactive pentadiene is an additional factor controlling the specificity of LOX. An oxygen channel has been shown in 3D structures of coral 8R-LOX and human 15-LOX [82, 83]. It is suggested that a conserved Gly in the active site of R-LOX allows oxygen to access the R-position, and the corresponding Ala in S-LOX directs the oxygen to the S-position. Exchanging this Ala to Gly in mouse 8S-LOX generated a 12R-LOX, and human 15S-LOX was converted to 11R-LOX by the equivalent mutation. The reverse mutation Gly to Ala turned coral 8R-LOX into a 12S-LOX and human 12R-LOX to 8S-LOX [84]. This region is called the “Coffa-Brash determinant” of stereospecificity. However, this rule has some exceptions e.g. zebra fish 12S-LOX with Gly, mouse 12S-LOX with Ser, in this position [84, 85]. The “Coffa-Brash determinant” can serve as a “switch” in that it directs O\textsubscript{2} to the carbon that is either +2 or -2 from the central carbon of the pentadiene. It is possible that the oxygen channel allows access to the more shallow C-2 carbon for R-LOX and in S-LOX the Ala directs the channel to the deeper C+2 carbon [83].

One helix turn away (4 residues) from the “Coffa-Brash determinant” of stereospecificity is a conserved Leu situated on in an ached helix in the 3D structure of coral 8R-LOX. Mutation of this Leu\textsuperscript{432} to smaller or bulkier residues had minor effect on K\textsubscript{m} but a large effect on k\textsubscript{cat}. This Leu is positioning the substrate close to the catalytic metal. [86].
Another residue of importance is an arginine near the entrance of the cavity that has been shown to stabilize the carboxylate head of the fatty acid in coral 8R-LOX [83].

Human lipoxygenases

Humans express six LOX (5-LOX, 12S-LOX, 15-LOX-1 (12/15-LOX), 15-LOX-2, 12R-LOX and epidermal LOX). 5-LOX initiate the leukotriene biosynthesis and is the most studied enzyme in the mammalian LOX pathway. Activation of inflammation cells induces translocation of 5-LOX to the nuclear membrane. 5-LOX activity is dependent on intracellular Ca\(^{2+}\) concentrations and on a small transmembrane protein, the five lipoxygenase-activating protein (FLAP) [87, 88]. 5-LOX abstracts the pro-S hydrogen at C-7 of arachidonic acid with the formation of 5-HPETE, that either can be reduced to 5-HETE or subjected to an additional hydrogen abstraction of the pro-R hydrogen at C-10 of 5-HPETE, the formed radical migrates to C-6 where it together with oxygen at C-5 forms the epoxide LTA\(_4\) [4]. LTA\(_4\) can be hydrolyzed by LTA\(_4\) hydrolase (LTA\(_4\)H), which yields the chemotactic agent LTB\(_4\) [89]. LTA\(_4\) can be conjugated with glutathione to form the potent bronchoconstriction agent LTC\(_4\), the conjugation to glutathione is catalyzed by LTC\(_4\) synthase [90]. The peptide part of the LTC\(_4\) can be subjected to extracellular metabolism, which results in formation of LTD\(_4\) and LTE\(_4\) [61]. An overview of the leukotriene formation is illustrated in Fig. 1.

Expression of the different LOX varies between different cell types. 5-LOX is most common in leukocyte, mast cells and dendritic cells. 12S-LOX is mainly expressed in platelet cells, and its product 12S-HPETE plays a role in platelet aggregation. There are two types of 15-LOX type 1 and type 2. 15-LOX type 1 is also referred to as 12/15-LOX, as it in humans form 15-HPETE and 12-HPETE in a ratio of 9:1 whereas the corresponding ratio in mice is 1:4. In humans, 15-LOX-1 is found in eosinophils and airway respiratory epithelium, and it has been suggested to be involved in various physiological and pathophysiological processes, e.g. asthma, inflammation and cancer [4]. The 15-LOX type 2 has 40% sequence homology with type 1, but catalyzes only the formation of 15S-HPETE from arachidonic acid. 15-LOX type 2 is expressed mainly in hair roots, prostate, lung and cornea, and has been suggested to be involved in prostate cancer [4]. 12R-LOX is expressed in skin and has important epidermal functions; it was the first mammalian LOX to be found to insert oxygen in the R stereoconfiguration [68]. The epidermis type LOX-3 (eLOX-3) is co-localized with 12R-LOX, it is not a classical LOX instead it function as a hydroperoxide isomerase that catalyzes the formation of epoxyalcohols from hydroperoxides [71].

Dual oxygenation of arachidonic acid by 5-LOX followed by 12S-LOX or by 15-LOX-1 results in the formation of lipoxins, which have pro-resolution and anti-inflammatory properties [91] (Fig. 1). Lipoxin formation often oc-
curs via transcellular pathways through neutrophils, leukocytes, endothelial cells and platelets.

Figure 1. Overview of the human LOX pathway.

In the presence of aspirin, COX-2 is acetylated and can not produce prostaglandins, however it is capable of converting arachidonic acid to 15R-HETE, which can be converted by 5-LOX to the lipoxin 15-epi-LXA₄, also called aspirin triggered lipoxin [92].

Resolvins and protectins, as their names implies, are compounds that have pro-resolving and anti-inflammatory properties. Resolvins are formed from the ω-3 PUFAs, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) by the action of aspirin acetylated COX-2 or CYP, and 5-LOX. Protectins are formed by a LOX pathway and are 10,17-dihydroxy containing metabolites from DHA [91].
The formed leukotrienes are exported over the plasma membrane to neighboring cells. The target cells have GPCRs that recognize specific leukotrienes. For example, LTB₄ attracts immune cells to sites of inflammation and binds to BLT₁ and BLT₂ receptors on nearby cells [93], however the function of the latter is not yet elucidated. The cysteinyl-LTs bind to CysLT₁ and CysLT₂ and mediate pro-inflammatory and bronchoconstrictive effects [94], CysLT₁ is targeted by asthma drugs such as montelukast [21].

Overproduction of leukotrienes has been associated with several human chronic inflammation diseases (e.g. atherosclerosis, asthma, allergic rhinitis, osteoporosis, dermatitis and cancer) [4].

Prototype non-mammalian lipoxygenases
One of the most studied invertebrate LOX is the 8R-LOX from the coral *Plexaura homomalla*, it has 40% sequence homology with human 5-LOX. It has similar Ca²⁺ dependence for membrane binding but is more stable than 5-LOX [95], thus it is a good model for the unstable human 5-LOX.

LOX are ubiquitous in plants; eight different LOX encoding genes have been identified in the soybean, *Glycine max*. One of these is the well-characterized sLOX-1, a 13S-LOX, which was purified and crystallized already in 1947 [23]. Soybean LOX-1 catalyzes oxidation of fatty acids by an antarafacial hydrogen abstraction and oxygenation mechanism, and is referred to as the prototype LOX enzyme [72].

In lower organisms LOX genes are less abundant. The first reported LOX in prokaryotes was discovered in *Pseudomonas aeruginosa*. It is a secretable LOX, which converts arachidonic acid to 15-HPETE [96]. Since prokaryotes have very few endogenous PUFAs the biological role of their LOX is unclear. Cyanobacteria have several LOX genes. A mini-FeLOX, from the cyanobacterium *Cyanothece sp.* has been cloned and expressed and could produce 11R-HPODE [97].

Fungal lipoxygenases
The most characterized fungal LOX is the 13R-MnLOX, which is secreted by the *take-all* fungus *G. graminis* [47, 98]. It has attracted special attention since it uses manganese as catalytic metal and catalyzes suprafacial hydrogen abstraction and oxygen insertion of 18:2n-6 to 13R-HPODE and to the uncommon bis-allylic 11S-HPODE. The latter can rapidly be isomerized to 13R-HPODE by the enzyme, a reaction that most FeLOX are unable to perform at a significant rate [99]. 13R-MnLOX has about 27% sequence homology with animal and plant LOX. MnLOX has the conserved metal coordinating residues and many of the conserved hydrophobic residues in the active site identified in FeLOX, but lacks sequence homology with the N-terminal PLAT domain. A 13-MnLOX has also been found in *Aspergillus fumigatus* [100].
Filamentous fungi also contain FeLOX, e.g. a 13S-FeLOX from the plant pathogen *F. oxysporum* has been cloned and expressed in *E. coli* and might be the enzyme responsible for the initial step of jasmonic acid biosynthesis in *F. oxysporum* [101].

### Crystal structures of lipoxygenases

LOX proteins have a single polypeptide chain of about 75-80 kDa in animals, 100 kDa in plants and 65 kDa in fungi and bacteria. Even though the length of the proteins differs, the sequences are highly related and the catalytic mechanisms are essentially the same. Plant and mammalian LOX have an N-terminal β-barrel domain containing eight anti-parallel β-strands and a larger catalytic domain with mainly α-helices. The β-barrel domain contains Ca$^{2+}$ binding sites and has membrane binding and regulatory functions. It resembles the C2-domain of lipases, and belongs to the family of PLAT (Polycystin-1, Lipoxygenase, Alpha-Toxin) domains [102, 103]. The prokaryotic LOX of *P. aeruginosa* lacks this N-terminal β-barrel domain, and instead has a set of extra α-helices in the N-terminal.

The catalytic domains of the mammalian LOX are more compact than in plant LOX, and typically have shorter loop regions. The catalytic domain contains a hydrophobic channel for substrate binding in close proximity to the catalytic metal. The catalytic metal is coordinated by three His, the carboxyl end of the C-terminal residue (Ile or Val), and a distant Asn or His, (Fig. 2). The metal is also coordinated to a water molecule which functions as the catalytic base in the LOX reaction [104, 105].

![Figure 2. A schematic overview of the metal coordinating sphere in FeLOX.](image-url)

An “arched” helix is situated over the catalytic active site in all LOX structures and at the base of the helix there is a conserved Leu that clamps the
substrate close to the catalytic metal [75]. The most striking difference between different LOX structure is the position of helix α2. In 5-LOX it is a broken helix that covers the entrance to the active site [106], but in other structures it is an unbroken helix leaving the entrance open [75]. This helix has been reported to be mobile in plant sLOX-1 [107].

In 1993, Boyington et al. described the first LOX structure, the L1 isoform of soybean LOX [108]. Since then, several structures of LOX have been solved: soybean LOX-3, isoforms B and D, coral 8R-LOX, coral 11R-LOX, rabbit reticulocyte 15-LOX-1, human 15-LOX-2, porcine 12R-LOX, human 5-LOX, human skin 12S-LOX and one prokaryotic LOX, Table 1.

Table 1. Summary of crystallized LOX with their protein data bank (pdb) code.

<table>
<thead>
<tr>
<th>Lipoxygenase</th>
<th>PDB codes</th>
<th>Resolution (Å)</th>
<th>References</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycine max LOX-1</td>
<td>2SBL, 1YGE</td>
<td>2.6, 1.4</td>
<td>[104, 105]</td>
<td>First crystallized LOX</td>
</tr>
<tr>
<td>Glycine max LOX-3</td>
<td>1LNH, 1IK3,</td>
<td>2.6, 2.0, 2.2,</td>
<td>[109-114]</td>
<td>With 13S-HPODE, or inhibitors</td>
</tr>
<tr>
<td></td>
<td>1HU9, 1JNQ,</td>
<td>2.1, 2.0, 2.1,</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1ROV, 1N8Q,</td>
<td>2.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1NO3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glycine max LOXB</td>
<td>2IUJ</td>
<td>2.4</td>
<td>[115]</td>
<td></td>
</tr>
<tr>
<td>Glycine max LOXD</td>
<td>2IUK</td>
<td>2.4</td>
<td>[115]</td>
<td></td>
</tr>
<tr>
<td>P. homomalla 8R-LOX</td>
<td>2FNQ, 3FG1,</td>
<td>3.2, 1.85, 2.0</td>
<td>[95], [86],</td>
<td>With arachidonic acid</td>
</tr>
<tr>
<td></td>
<td>4QWT</td>
<td></td>
<td>[83]</td>
<td></td>
</tr>
<tr>
<td>G. fruticosa 11R-LOX</td>
<td>3VF1</td>
<td>2.47</td>
<td>[116]</td>
<td></td>
</tr>
<tr>
<td>O. cuniculus 15S-LOX-1</td>
<td>1LOX, 2P0M</td>
<td>2.4</td>
<td>[117]</td>
<td></td>
</tr>
<tr>
<td>S. scrofa 12S-LOX</td>
<td>3RDE</td>
<td>1.89</td>
<td>[118]</td>
<td>Truncated N-terminal</td>
</tr>
<tr>
<td>H. sapiens 15S-LOX-2</td>
<td>4NRE</td>
<td>2.63</td>
<td>[82]</td>
<td>With inhibitor</td>
</tr>
<tr>
<td>H. sapiens 5S-LOX</td>
<td>3O8Y</td>
<td>2.4</td>
<td>[106]</td>
<td>Stabilized by mutations</td>
</tr>
<tr>
<td>H. sapiens 12S-LOX</td>
<td>3D3L</td>
<td>2.6</td>
<td>—</td>
<td>Truncated N-terminal</td>
</tr>
<tr>
<td>P. aeruginosa LOX</td>
<td>4G32, 4G33</td>
<td>1.75</td>
<td>[119]</td>
<td>Lacks the N-terminal β-barrel</td>
</tr>
</tbody>
</table>
Mammalian and plant LOX utilize iron as catalytic metal and have been thoroughly investigated, but the knowledge about fungal LOX is scarce. A LOX with catalytic manganese was first discovered in G. graminis and cloned in 1998. The aim of this thesis was to identify additional members of the MnLOX family in fungal pathogens and to find amino acids critical for their regio-and stereospecific reaction mechanism. Intriguing questions are how MnLOX differs from FeLOX in terms of catalytic function and structure, and why fungi have developed to use manganese instead of iron as the catalytic metal.

Specific aims of this study were to:

- Identify residues in control of stereo- and regiospecificity in MnLOX by using site directed mutagenesis.
- Identify and clone novel members of the MnLOX family in order to find common features shared by the MnLOX.
- Compare the catalytic properties of MnLOX and FeLOX with focus on the deuterium kinetic isotope effect and temperature dependence.
- Express and purify large amounts of MnLOX enzyme suitable for crystallization.
- Crystallize and solve the 3D structure of an MnLOX.
Recombinant protein expression in *Pichia pastoris*

The methylotrophic yeast *P. pastoris* is a single cell organism that is easy to manipulate and culture at high densities. Yeasts are capable of post-translational modifications and are faster, easier and less expensive to use for protein expression than insect cells and mammalian cell lines; usually yeast also gives higher expression levels. In comparison to *Saccharomyces cerevisiae*, *P. pastoris* has the advantage that it does not hyperglycosylate secreted proteins, but do perform O- and N-linked glycosylation that often can be necessary for the stability and activity of the eukaryotic proteins. However the addition of long chains of N-glycosylation of the high mannose type to the expressed protein can limit its potential for clinical applications. There are *P. pastoris* strains engineered to produce humanized glycosylation on expressed recombinant proteins [120]. There are also protease deficient strains to minimize undesired proteolytic degradation of the expressed recombinant protein [121].

Methylotrophic yeasts are able to use methanol as carbon and energy source. There are two genes in *P. pastoris* that code for alcohol oxidase enzymes, *AOX1* and *AOX2*. Expression of these genes are suppressed by glucose, glycerol and ethanol and strongly induced by methanol. Recombinant proteins can be expressed either intracellular or extracellular with the addition of a yeast secretion signal sequence [122]. An advantage in using extracellular expression is that *P. pastoris* only secretes low levels of endogenous protein and when grown in culture medium without additional proteins the secreted recombinant proteins can compromise the vast majority of total protein in the solution.

We used the wild type *P. pastoris* strain X-33, transformed with pPICZαA plasmid for expression and secretion of the enzyme. The plasmid was mutated, amplified and purified in *E. coli* and incorporated into the genome by electroporation and homologous recombination of the *AOX1* gene regions of the yeast genome and the plasmid. Expression was performed in 1-5 L bench shaker flasks or in a 10 L bioreactor. The culture was harvested after 3-5 days. In the bioreactor OD<sub>600</sub> could reach as high as 200 but after the third day the enzyme activity usually declined, possibly due to release of yeast proteases.
Protein purification

Ammonium sulfate was added to the enzyme solution in order to stabilize the expressed enzyme, to precipitate other proteins, and to increase the hydrophobic interactions between the protein and the hydrophobic column. The pH, which often was as low as 4 in the end of expression was adjusted to 6.5, and the enzyme solution was centrifuged and filtered to remove precipitated proteins and large particles. The clear protein solution was loaded on a hydrophobic interaction column using ÄKTA FPLC (Pharmacia). Fractions were assayed for LOX activity with UV spectroscopy at 234-237 nm. The fractions with LOX activity were pooled and the volume was reduced by diafiltration (Ultracel 10K, Millipore).

Prior to crystallization, deglycosylation was performed. The harsh conditions necessary for deglycosylation often decreased the enzyme activity. The protein sample was concentrated and further purified by size exclusion chromatography on a gel filtration column using ÄKTA FPLC. A low loading volume (0.5 mL or 2 mL, respectively) and addition of 100-150 mM NaCl were essential for base line separation of the proteins. Expression and purification were analyzed by SDS-PAGE.

Site directed mutagenesis

The QuickChange site-directed mutagenesis protocol (Stratagene) is an easy and robust method to introduce site-specific replacements of nucleotides, in order to change single amino acids in the protein sequence. This technique allows site-specific mutations of nucleotides directly in a double stranded plasmid. The plasmid pPICZαA, was mutated by utilizing Pfu DNA polymerase (Fermentas) and 41-44 bp primers containing the desired mutations. Often one or two nucleotides were mutated to change a codon sequence. In difficult cases and when more than two nucleotides needed to be replaced, the mutations were performed sequentially. When no or low amounts of PCR product were obtained, optimization was performed by varying annealing temperature, template concentration, or DMSO concentration. Template DNA was restricted by DpnI to remove methylated DNA. The PCR products were used to transform chemically competent E. coli. Mutations were confirmed by restrictions analysis and sequencing.

Enzyme activity assay

Expressed enzyme was incubated with fatty acid substrates in 0.1 M NaBO₃ buffer, pH 9.0. LOX activity was assayed by a UV-spectrophotometer at 237 nm and 234 nm, the characteristic wavelength for absorption of the cis-trans
conjugated dienes of HPOTrE and HPODE, respectively. The reaction was stopped by addition of methanol.

Kinetic parameters for the different enzymes and their mutants were determined by triplicate incubations of constant concentration of enzyme and substrate in different concentrations. Fatty acid concentration over 100 µM could not be used due to micelle formation. Reaction rates were followed by UV-spectroscopy and fitted by non-linear regression to the Michaelis-Menten equation. \( K_m \) and \( V_{max} \) values were estimated from the fitted curve.

Hydroperoxide isomerase activity could be measured from the decrease in absorbance at 234 nm and the subsequent increase at 280 nm, which is characteristic for KOTrE (ketooctadecatrienoic acid) products.

In order to study the oxygen path, in the reaction mechanism, the enzyme was incubated with \(^{18}\text{O}_2\) labeled HPODE, which was prepared by incubation of \(13\text{R}-\text{MnLOX}\) with \(18:2\) \(n-6\) in \(^{18}\text{O}_2\) saturated buffer [48].

### Kinetic isotope effect

Hydrogen transfer is important in many enzymatic reactions. For the LOX reaction, the hydrogen abstraction by the metal hydroxide is the rate limiting step [123]. The difference in reaction rate using isotope labeled or unlabeled substrate is useful to deduce the reaction mechanism of enzymes. The most pronounced kinetic isotope effect (KIE) occurs when the difference in relative mass is large, as with hydrogen and deuterium. The mass changes the vibrational frequency, a heavier atom will vibrate slower and more energy is needed to break the bond. Consequently, the reaction will have higher activation energy (\(E_{act}\)).

The classical view of enzyme reactions involves an activation barrier, which separates reactants from products. Reactants require sufficient energy to overcome the barrier. According to this classical view, enzymes function primarily by lowering the energy barrier by transition state stabilization. A more recent revision suggests that enzymes form distinct pathways involving intermediates that introduce multiple smaller barriers.

However, several observations do not fit this classical model of enzyme reactions. It has been shown that hydrogen can move from the reactant to the product, even though there is not sufficient energy to overcome the activation barrier. The rate of reaction involving breaking a C-H bond is around 6-10 times faster than breaking a C-D bond at room temperature, but the hydrogen abstraction in some enzyme reactions can sometimes differ from deuterium abstraction by 100-fold. The laws of classical mechanics cannot explain this effect. The prototype \(sLOX-1\) enzyme has one of the largest detected primary deuterium isotope effect (dKIE) at room temperature (dKIE=81) and is only weakly temperature dependent [123].
This can be explained by quantum mechanical tunneling of the hydrogen atom. Tunneling occurs when a particle crosses through a potential barrier rather than over it.

The temperature dependence of the sLOX-1 and MnLOX reactions and the kinetic isotope effects were determined by comparing $k_{\text{cat}}$ of $18:2n$-6 and [11,11-$^2$H$_2$]18:2n-6 at temperatures between 8 and 50 °C. An Arrhenius plot (Fig. 3) was used to plot ln $k$ against the inverse temperature according to the following equation:

$$\ln k = -\frac{E_{\text{act}}}{RT} + \ln A$$

$E_{\text{act}}$ and the Arrhenius pre-exponential factor ($A$) can be deduced from the slope and the y-axis intercept (at infinite temperature), respectively. $R$ represents the gas constant ($1.99 \times 10^{-3}$ kcal K$^{-1}$ mol$^{-1}$) and $T$ the temperature in Kelvin. The steeper the slope, the higher activation energy, meaning that the reaction is more temperature dependent.

Figure 3. An Arrhenius plot of ln $k$ against 1/$T$ is a straight line for a typical reaction.

HPLC-MS/MS analysis

Fatty acid metabolites were extracted by solid phase extraction using Sep-Pak/C$_{18}$ cartridge and eluted with ethyl acetate. The volume was reduced and the sample was diluted with desired solvent. Hydroperoxides were occasionally reduced to alcohols by treatment with triphenylphosphine before analysis. As routine, the fatty acid metabolites were first separated by reverse phase high performance liquid chromatography (RP-HPLC) on a C$_{18}$-silica column (Phenomenex) eluted isocratic with methanol/water/acetic acid, 80/20/0.01 or 75/25/0.01. The small amount of acetic acid is added to improve the peak shape and to provide a source of protons. The nonpolar sta-
tionary phase and the polar mobile phase result in elution of the most polar metabolites first. The retention time depends on the polarity of the stationary phase, mobile phase and the chemical structure of the fatty acid. In general the retention time is proportional to chain length and the number of double bonds present in the examined fatty acids. Those with short chains elute before the long chains and double bonds increase the retention time. When analyzing fatty acids with more double bonds addition of less water to the mobile phase could decrease the elution time. Geometric isomerization also plays a role in the elution order; cis isomers of the fatty acids are generally eluted before trans isomers.

For separation of 9-HPODE and 13-HPODE, RP-HPLC was rather poor. For better separation, normal phase-HPLC (NP-HPLC) was performed on a silica column with a mobile phase of hexane, isopropyl alcohol and acetic acid, 97/3/0.05. For separation of hydroxy enantiomers chiral phase-HPLC (CP-HPLC) was performed on Reprosil Chiral-AM column with a mobile phase of hexane/methanol/acetic acid, 95/5/0.01 or hexane/methanol/ethanol/acetic acid, 93/5/2/0.01. For better resolution a Chiralcel OB-H (Diachel), with a mobile phase of hexane/isopropyl/acetic acid, 95/5/0.01, was used. Steric analysis of hydroperoxides enantiomers was performed with a Reprosil Chiral NR (Dr. Maisch), with a mobile phase of hexane/methanol/acetic acid, 99/1/0.02.

To obtain proper ionization, the column eluent was combined (2:1 or 1:1) with isopropyl alcohol/water, 3/2 from a second HPLC pump. The samples were introduced by electrospray into a linear quadrupole ion trap mass spectrometer (LTQ, Thermo Scientific). Since the fatty acid metabolites carry a negative charge on the carboxyl group, the instruments was set on negative ion mode. The isolation width was set to 1.5 and was increased to 5 when hydroperoxides and deuterium-labeled analytes were analyzed.

A mass spectrometer (MS) was used to determine the molecular weight of a compound by separating molecules according to their mass-to-charge ratio \((m/z)\). The instrument is essentially built up of an ion source, mass analyzer and a detector. By using HPLC-MS/MS, the combination of polar/chiral separation of the analytes by the HPLC system and the identification of the analytes by their \(m/z\) ratio by mass spectrometry can be obtained. This is especially valuable when analyzing compounds at a very low concentration.

**Crystallization**

The rate-limiting step in protein structure determination is often to obtain diffracting crystals. Crystallization requires a pure and homogenous protein sample at high concentration. Deglycosylation of the protein expressed in *P. pastoris* was therefore necessary to remove the flexible glycan chains. Vapor-diffusion technique was employed to get the proteins into crystalline
form. For crystallization to occur, the protein solution must reach a state of supersaturation. This could be achieved by mixing protein and crystallization solution in a small drop above a well with crystallization solution in a sealed system. The drop is allowed to slowly equilibrate with the crystallization solution, as the water evaporates the concentrations of protein and precipitant are increased, nucleation can then occur where the protein molecules pack in a well-ordered 3D structure. More often, a disordered precipitation appears. Fig. 4 illustrates a typical phase diagram for protein crystallization.

![Figure 4. A typical phase diagram for protein crystallization. The arrow indicates the route for the drop during the crystallization process.](image)

Numerous parameters such as pH, salt, precipitant, temperature, additives etc. are involved in this process. It is difficult to predict the crystallization conditions, but commercial kits contain the most common conditions for crystallization and allow multiple conditions to be screened. Numerous trials are usually required in order to obtain good diffracting crystals. Initial screens are nowadays performed in 96-well plates using a crystallization robot. Promising crystallization hits can be optimized by grid screens with minor variation of some parameters or by adding different additives. Seeding of micro-crystals into a new drop is a useful technique of introducing crystal nucleuses to the crystallization drop, from which new crystals can grow.
X-ray diffraction data collection

In order for an object to diffract light, the wavelength of the light has to be smaller than the object. Bonded atoms in proteins are about 1.5 Å apart; therefore X-ray is used in protein crystallography. A single molecule is a very weak scatterer, analyzing the data from a crystal in which each molecule diffracts identically solves this problem. The crystal is rotated and several images are collected as effective as possible. The crystal deteriorates as the X-ray generates heat and free radicals. Data collection is often a compromise to obtain good completeness, resolution, and accuracy, and is therefore performed at -195°C to decrease the decay caused by the X-ray beam.

Prior to data collection crystals are cryo-protected in crystallization solution supplemented with 15-20% glycerol and cooled in liquid nitrogen. Data in this thesis were collected at synchrotrons in the Max laboratory in Lund, Sweden, in European Synchrotron Radiation Facility in Grenoble, France, and in Diamond light source in Oxfordshire, England. The aim of the data collection was to obtain data with high multiplicity at 1.77 Å in order to be able to obtain an anomalous signal from intrinsic sulfur and manganese.

Structure determination

From the reflection intensities of the diffracted waves the amplitudes can be derived, but the information of the phases is lost. The phases can only be derived from some prior knowledge of the structure. For protein crystallography phases can be obtained either from a similar structure or by finding the coordinates of electron-dense atoms [124].

The fastest method to find the phases is by molecular replacement with an existing model of a protein with a similar tertiary structure, this often implies a sequence identity of 35-40% or greater. In short, molecular replacement employs a rotational function and a translational function.

When there is no similar structure available, other methods have to be used to solve the “phase problem”. One method is isomorphous replacement when the crystal is soaked in heavy-atom solution to create a derivative crystal with heavy atoms. The intensity changes can be used to determine the positions of the heavy atoms; one of the problems with this method is non-isomorphism between the crystals. To overcome this problem intrinsic heavy atoms such as metal cofactors, sulphur or SeMet can be used in multiple or single anomalous dispersion (MAD or SAD). When using sulphur, the changes in amplitudes are generally small and require high multiplicity and completeness [124]. The anomalous signal is used to find the coordinates for the heavy atom substructure, and from these the phases can be calculated.

In our study we used sulphur and manganese as intrinsic anomalous scatterers. Processing of images was carried out with XDS [125], scaling and
data analysis was performed with AIMLESS [126]. Attempts were made to solve the phases by molecular replacement but it was difficult since MnLOX only has 27% sequence homology with FeLOX.

Additional datasets was therefore collected at 1.77 Å, a wavelength chosen to obtain anomalous signal from both sulfur and manganese. Highly redundant data are necessary for the anomalous signal to rise above the background noise; hence the datasets were analyzed and merged using BLEND [127]. The sulfur and manganese substructure was found with SHELX [128]. SAD phasing and automatic model building were formed using the PHENIX program suite [129]. Manual model building and analysis were made in COOT [130] and refinement was performed with phenix.refine [131]. PyMOL (Schrödinger, LLC) was used for visualizing structures and for making figures.
Results

Identification of a determinant of stereospecificity in 13R-MnLOX of *G. graminis* (paper I)

All LOX have a hydrophobic pocket enclosing the active site with the catalytic metal. The Phe\(^{337}\) in 13R-MnLOX aligns with an Ile or a Val located in the active site of FeLOX. Replacement of Phe\(^{337}\) to Ile changed the steric oxygenation at C-13 of 18:2\(n\)-6 and 18:3\(n\)-3, with the formation of 13\(S\)-hydroperoxy-metabolites. The 13R-MnLOX•F337I abstracts the same 11\(S\)-hydrogen as the wild type enzyme but inserts oxygen from the other direction of the substrate, at the 13\(S\) position. This mutation changed the typical suprafacial mechanism of MnLOX to mainly antarafacial. The oxidation at C-9 was slightly increased for both 18:2\(n\)-6 and 18:3\(n\)-3, oxidation of C-9 of the 18:2\(n\)-6 remained mainly suprafacial, whereas 18:3\(n\)-3 products were formed antarafacial.

Another interesting residue conserved in all LOX is a Leu residue situated in an “arched” helix close to the catalytic metal. This Leu is suggested to clamp the substrate in the hydrophobic pocket in the structure of coral 8\(R\)-LOX [83, 86]. To investigate the importance of this residue in MnLOX, we replaced this Leu\(^{336}\) in 13R-MnLOX of *G. graminis* with the smaller residues Val, Ala and Gly. The most prominent effect was detected with 13R-MnLOX•L336A, which switch the regiospecificity from mainly C-13, to C-9, in a ratio of 1:1. The stereocontrol at C-13 was retained but C-9 oxidation generated a racemic mixture.

An overall observation for all the Leu\(^{336}\) mutants was that both the regioand stereocontrol were retained in a higher degree when incubated with 18:3\(n\)-3 than with 18:2\(n\)-6. The kinetic lag time appeared to be increased with some mutants (e.g., 13R-MnLOX•L336A), but \(K_m\) for 18:3\(n\)-3 remained in the low \(\mu\)M range for all the Leu\(^{336}\) mutants.

Replacement of Leu\(^{336}\) with a Phe residue had relatively small effect on the regiospecific oxidation of 18:2\(n\)-6 and 18:3\(n\)-3 but the kinetic lag phase and the formation of KOTrE products were considerably increased. KOTrE products are likely formed by dehydration of HPOTrE. 18:3\(n\)-3 was oxidized at the same rate as 18:2\(n\)-6 and about 10 times more efficiently that 11\(R\)-HPOTrE.
Discovery of a novel MnLOX and an epoxy alcohol synthase in *M. salvinii* (paper II)

Growth medium of the filamentous fungi *M. salvinii* oxidized 18:2n-6 to mainly 9-HPODE, 9-HODE and an epoxy alcohol. This suggested secretion of both a 9-LOX and an epoxy alcohol synthase (EAS). With CP-HPLC-MS/MS we could show that the 9S-HODE stereoisomer was formed exclusively. LOX activity could be followed by the increase in UV absorbance at 234 nm and the EAS activity by the subsequent decrease in absorbance as the EAS transforms the 9S-HPODE to the epoxy alcohol, 10R(11R)-epoxy-9S-hydroxy-12Z-18:1. Gel filtration was used to separate the two enzymes from the growth medium, 9S-LOX activity eluted with the same retention time as albumin (60-65 kDa) and EAS with the same retention time as carbonic anhydrase (29 kDa).

Recombinant 9S-LOX was expressed in *P. pastoris* and displayed 9S-LOX activity with 18:2n-6. It could rapidly transform 11S-HPODE to 9S-HPODE. It could also transform 13R-HPODE to mainly 9S-HPODE but no 11-HPODE could be detected.

The expression and purification was challenging, since the enzyme activity reached a maximum during expression after 2-3 days and then quickly decreased. Addition of the protease inhibitor, pepstatin A at day 2 of expression and EDTA at harvest, decreased the inactivation rate of the protein. The *k*_cat for 18:2n-6 was determined to ~500 min⁻¹; this value is likely underestimated due to the unstable properties of 9S-LOX. The recombinant enzyme could be purified by hydrophobic interaction chromatography and gel filtration. SDS-PAGE revealed a protein in a broad band at 80-90 kDa, probably due to glycosylation of the expected 66 kDa protein. Manganese was determined to be the catalytic metal by inductively coupled plasma-atomic emission spectroscopy (ICP-AES). The previously characterized 13R-MnLOX from *G. graminis* and this novel 9S-MnLOX could be aligned with 77% amino acid identity, and are hereafter referred to as Gg-MnLOX and Ms-MnLOX.

To investigate if Ms-MnLOX is important during rice infection we compared its mRNA levels in *M. salvinii* grown with and without rice leaves added to the growth medium and found that 9S-LOX was slightly increased (~1.6-2.6 fold) in the sample grown in the presence of rice leaves.

Determinant of regiospecificity

An interesting area in LOX was discovered by Sloane et al. [76], and constitute of two residues situated in the bottom of the hydrophobic substrate channel. The two fungal LOX, 13R-Gg-MnLOX and 9S-Ms-MnLOX, have similar amino acid sequences, but differ in the “Sloane determinant”. This
residue was an interesting target to mutate and site-directed mutagenesis gave Gg-MnLOX•F347L and Ms-MnLOX•L350F.

The Gg-MnLOX•F347L changed the oxygenation from C-13 to C-9, resulting in 9-HPODE as the main metabolite from 18:2\textit{n}-6. The effect was less pronounced with 18:3\textit{n}-3. The extra double bond of 18:3\textit{n}-3 could possibly interact with hydrophobic residues in the active site in order to hold the substrate in the correct position, to prevent the substrate to move more freely in the more spacious active site.

The corresponding Ms-MnLOX•L350F was expressed repeatedly with no or very low enzyme activity, due to the transient activity of the wild type enzyme it was difficult to determine if the lack of activity was a result of the mutation or inactivation of the enzyme. After concentration of the purified protein a low but transient activity could be measured, but mainly 9\textit{S}-metabolites could be detected. The concentrated protein solution was analyzed on SDS-PAGE, which confirmed expression of a protein of 80-90 kDa.

Exchange of catalytic metal

To investigate if the Mn and Fe catalytic metals are equivalent and coordinated in the same way, we aimed to exchange Mn with Fe in Gg-MnLOX. Previous attempts to exchange the metal with aid of dialysis have been unsuccessful (unpublished). We intended to facilitate Fe incorporation into Gg-MnLOX by expressing the protein with an extra Thr or Gly in the pentamer motif containing two of the metal coordinating His, generating the hexamer motif found in FeLOX, His-Val-Leu-Phe-Thr-His or His-Val-Leu-Phe-Gly-His (Fig. 5).

Both insertion mutants were inactive with 18:2\textit{n}-6 and 18:3\textit{n}-3 as substrates. Expression was verified by SDS-PAGE. Several attempts were made to measure the manganese and iron content with ICP-AES; unfortunately the
background signal was too high to be able to determine the catalytic metal of the enzyme.

Another attempt to exchange metal was made by protein expression in P. pastoris medium with high concentrations of iron. We could show that yeast cells with a Fe:Mn ratio of 79:1 still expressed LOX with manganese as judged from the enzyme activity and the ability to efficiently transform 11R-HPOTrE to 13R-HPOTrE, a reaction that FeLOX catalyze only at an insignificant rate.

Comparison of kinetic properties of FeLOX and MnLOX (paper III)

The first and limiting step in the LOX reaction is hydrogen abstraction from a methylene group with the formation of a radical intermediate. This hydrogen abstraction is nearly temperature independent in sLOX-1 and associated with an extremely large deuterium kinetic isotope effect (D-KIE) between 30-100 [123].

In order to compare the catalytic properties of MnLOX and FeLOX the reaction rates of sLOX-1 and Gg-MnLOX with 18:2n-6 and [11S-2H]-18:2n-6 at different temperatures were compared. We found that the reaction rate and the D-KIE of sLOX-1 were almost temperature independent between 8-40°C which is in agreement with previous studies. In contrast, the reaction rate of Gg-MnLOX increased 7-fold between 8 and 50°C and the D-KIE decreased linearly from 38 at 8°C, to 20 at 50°C. 13R-MnLOX•F337I, which utilizes a similar antarafacial reaction mechanism as sLOX-1, retained the same temperature dependence and the large D-KIE observed with the wild type enzyme.

The kinetic lag phase of 13R-MnLOX was extended at low temperatures whereas the lag phase of sLOX-1 was to short to be measured under this experimental setup. This difference is likely due to the lower redox potential of Fe²⁺/Fe³⁺ compared to Mn²⁺/Mn³⁺ (0.77 V and 1.54 V, respectively). The active form of the metal, Fe³⁺ is easier to obtain than Mn³⁺, by oxidation of hydroperoxides.

Arrhenius plots were constructed with 18:2n-6 and [11S-2H]-18:2n-6 as substrates. The temperature independence of sLOX1 results in a low $E_{act}$ (1.9 kcal/mol), and the D-KIE was estimated to be 56, this is in the same range as previous reported. The activation energy for Gg-MnLOX and Gg-MnLOX•F337I were slightly higher (7.8 kcal/mol and 9.7 kcal/mol, respectively) and D-KIE values were estimated to 28 and 32, respectively. Low $E_{act}$ values and high D-KIE values are consistent with hydrogen tunneling.

The increase in reaction rate and decrease in D-KIE with increasing temperature suggests that Gg-MnLOX utilizes a combination of hydrogen tun-
neling and a semi-classical transitions state mechanism for hydrogen abstraction. In comparison, sLOX-1 was almost temperature independent suggesting a large contribution of hydrogen tunneling in the reaction mechanism.

**MnLOX of *M. oryzae* (paper IV)**

The genome of *M. oryzae* was sequenced in 2005 [132]. It was proposed to contain a gene with homology to Gg-MnLOX. The NCBI predicted intron borders resulted in a translated amino acid sequence without the characteristic LOX features. The location of the first intron border was unclear, but by reverse transcriptase-PCR and comparison with conserved LOX areas of previously characterized LOX, this intron could be deduced. The second intron was deduced by reverse transcriptase-PCR.

The open reading frame was ordered and expressed in *P. pastoris*, both in baffled flasks and in a bioreactor. The bioreactor culture yielded large amounts of enzyme, as much as 70 mg/L, compared with 12 mg/L obtained in baffled flasks on a bench shaker. The catalytic metal was determined to be manganese, and this enzyme is therefore called Mo-MnLOX. Mo-MnLOX secreted to the medium from the bench culture was highly glycosylated with a molecular weight of 110-130 kDa. After purification and deglycosylation it migrated as a single 70 kDa protein on SDS-PAGE. The amount of glycans added to the secreted protein varied with different expression conditions, it was much lower in enzyme expressed in the bioreactor.

Mo-MnLOX oxidized 18:2*n*-6 to a mixture of 9S-HPODE, 11S-HPODE and 13R-HPODE. In the end of the reaction 11-HPODE was transformed to both 9-HPODE and 13-HPODE resulting in a 13-, 11- and 9-HPODE ratio of 6:1:13 (Fig. 6B). Incubation with 18:3*n*-3 gave a UV-trace with a dip in absorbance, due to the conversion of 9S-HPOTrE to 9S,16S-HPOTrE (Fig. 6A). The main remaining hydroperoxy end products were therefore 13R-HPOTrE and 11R-HPOTrE (Fig. 6C).
Figure 6. Oxidation of 18:2n-6 and 18:3n-3 by Mo-MnLOX. A. UV-analysis of cis-trans conjugated hydroperoxides formed with absorbance at 234-237 nm. Relative quantification by SP- and CP-HPLC-MS-MS of products formed from 18:2n-6 (B) and 18:3n-3 (C).

L-α-lysoglycerophosphatidylcholine was oxidized as rapidly as 18:2n-6 suggesting a “tail” first entrance of the substrate in the active site.

Mo-MnLOX display a D-KIE of ~40 and catalyzes hydrogen abstraction and oxygen insertion with a suprafacial mechanism, in analogy with previously reported MnLOX.

In summary, paper IV describes the partial cloning, expression, purification and enzymatic investigation of a novel MnLOX from the number one of the top ten most devastating fungal pathogens in molecular biology, M. oryzae.

MnLOX of F. oxysporum and C. gloeosporioides (paper V)

Since the genomes of several fungi now are available it was possible to identify MnLOX homologues in F. oxysporum and Colletotrichum gloeosporioides. The sequences was ordered and expressed in P. pastoris. Fo- and Cg-MnLOX oxidized 18:2n-6 to a mix of 11-HPODE, 9-HPODE, and 13-HPODE and 18:3n-3 to mainly 11-HPOTrE. CP-HPLC showed that Cg-MnLOX formed 11S-, 9S-, and 13R-HPODE, in analogy with previously reported Gg-MnLOX and Ms-MnLOX. Fo-MnLOX differed from previous
characterized MnLOX since it formed products with the other stereo configuration, 11R-HPODE, 13S- and 13R-HPODE in a ratio of 9:1, and racemic 9-HPODE.

Cg-MnLOX had a large D-KIE of >30 when incubated with [11S-2H]-18:2n-6, whereas the Fo-MnLOX reaction occurred with retention of the label and a low D-KIE of ~2. We confirmed that Fo-MnLOX abstracts the pro-11R-hydrogen of 18:2n-6, in contrast to all other characterized MnLOX that abstracts the pro-11S-hydrogen (Fig. 7).

Fo-MnLOX gave higher apparent expression levels than Cg-MnLOX and has a different reaction mechanism compared to the other MnLOX. The main focus of this paper is therefore on Fo-MnLOX. Fo-MnLOX differs from all other MnLOX in position 348, where a Ser is found instead of a Phe residue. Fo-MnLOX•Ser348Phe maintained the suprafacial mechanism but changed the products to mainly 13R-, 9S- and 11S-HPODE, and thereby produced a similar product pattern as other MnLOX. This is characteristic for an opposite head-to-tail orientation of the substrate in the active site, however incubation with soybean Lα-lyso-glycero-phosphatidylcholine showed that the substrate still enters the active site tail first. The explanation for the stereospecificity switch could instead be that the substrate is rotated in the hydrophobic pocket of the Fo-MnLOX•Ser348Phe, positioning the 11S-hydrogen instead of the 11R-hydrogen for abstraction and 13R and 9S for oxygenation.

Another position that differs in Fo-MnLOX compared to the other characterized LOX is Arg530. However, the Fo-MnLOX•Leu530Arg had little effect on the product profile.

Figure 7. Overview of the oxidation of 18:2n-6 to hydroperoxides by Fo-MnLOX. 11R- and 13S-HPODE are formed by suprafacial hydrogen abstraction and oxygen insertion.
Structure of MnLOX (paper VI and paper VII)

Crystallization and data processing

The first MnLOX to be crystallized was Gg-MnLOX (Paper VI). It formed crystals in the Morpheus screen (Molecular dimension). Diffracting crystals were obtained after one week in 0.1 M MES/imidazole (pH 6.5), 20% (v/v) glycerol, 10% (w/v) PEG 4000, 0.03 M magnesium chloride, 0.03 M calcium chloride [133]. Crystals were taken directly from the 96-well plate and stored in liquid nitrogen.

The crystal of Gg-MnLOX diffracted to 2.6 Å and belonged to the space group C2. Matthews’s probability calculator suggested two or three molecules in the asymmetric unit. The sequence homology to the best available LOX structures was less than 27%, this made it difficult to find the phases by molecular replacement. The Gg-MnLOX crystals obtained with the Morpheus screen were small and difficult to reproduce, thus we moved the attention to Mo-MnLOX in Paper VII. Gg-MnLOX and Mo-MnLOX are similar with a sequence homology of 57%. Mo-MnLOX could be expressed in larger amounts and is of interest because of the pathogenesis of M. oryzae.

Mo-MnLOX formed needle like crystals in several conditions in the PEG/Ion screen (Hampton Research). These conditions were optimized and the best diffracting crystals were obtained by hanging drop vapor diffusion after 3 weeks at 8 °C by mixing 1 µl enzyme (8 mg/ml), 1 µl reservoir solution (14% PEG 3350, 0.2 M ammonium citrate dibasic) and 0.1 µl CYMAL-7. Setting up plates in 8 °C, compared to 20 °C, and adding the detergent CYMAL-7 to the crystallization drops, improved the crystal quality considerably.

Data were collected at Max laboratory (Lund, Sweden), European synchrotron radiation facility (Grenoble, France) and Diamond light source (Oxfordshire, UK). An initial dataset from Mo-MnLOX crystals was collected at 1 Å, and diffracted to 2.4 Å. Molecular replacement turned out to be unproductive for Mo-MnLOX as well, so additional datasets were collected with high multiplicity at 1.77 Å, in order to solve the phases by manganese and sulphur SAD.

The crystals of Mo-MnLOX were not very sensitive to radiation and it was possible to collect data with high redundancy. The crystals of Mo-MnLOX diffracted at best to 2.07 Å. The protein crystallized in P2₁2₁2₁, with unit cell dimensions of a=70.72, b=111.37, c=171.22, and with two molecules in the asymmetric unit resulting in a solvent content of 51.11%. An anomalous signal used to determine the sulfur and manganese substructure could be abstracted by merging four datasets resulting in multiplicity of 77.4.
Overall structure of Mo-MnLOX

The final 2F\textsubscript{o}-F\textsubscript{c} map shows electron density for all main chain atoms except for the 37 most N-terminal residues. The N-terminal is either too flexible to be seen or has spontaneously been cleaved off. Initial analysis of the structure reveals that Mo-MnLOX lack the β-barrel domain with homology to the PLAT-domain found in many membrane or lipid associated enzymes. An illustration of the overall MnLOX structure is presented in Fig. 8.

The long helix in blue is slightly arched-shaped and runs over the whole length of the protein, this α2-helix is also found in animal and plant LOX but varies in orientation. In plants it has been reported to be mobile to allow access to the active site and in 5-LOX it is broken and covers the active site cavity. A shorter broken arched helix (light green), just above the catalytic Mn, is covering the active site. This helix is found in all known LOX structures and harbors several invariant hydrophobic residues.

![Figure 8](image.png)

Figure 8. The overall structure of Mo-MnLOX illustrated in cartoon rendering and colored in rainbow colors with the N-terminal in blue and the C-terminal in red. The catalytic Mn is illustrated as a grey sphere.

The substrate channel in Mo-MnLOX

The metal is coordinated by His\textsuperscript{284}, His\textsuperscript{289}, His\textsuperscript{469}, Asn\textsuperscript{473}, Val\textsuperscript{605} and a water molecule. The substrate channel is composed of hydrophobic residues, e.g., the invariable Leu\textsuperscript{331}, situated on the arched helix that has been shown to clamp the substrate in the active site, Table 2. Next to the Leu\textsuperscript{331} sits Phe\textsuperscript{332}, this Phe is also conserved between MnLOX but not in FeLOX, which have Ile in the equivalent position. This Phe residue has been shown to be important for the suprafacial mechanism in Gg-MnLOX. In the so-called “Sloane determinant” sits Phe\textsuperscript{342}. Most MnLOX has Phe in this position, but there are some exceptions, e.g., Fo-MnLOX and Ms-MnLOX. On the other
side of the substrate channel is the Phe\textsuperscript{526} residue, 3.9 Å away from the Leu\textsuperscript{331}. This Phe is conserved in MnLOX but not in FeLOX, that has a Leu residue in its place.

The oxygen of Gln\textsuperscript{281} forms tentative hydrogen bonds to the amine of the metal ligand Asn\textsuperscript{473}. This Gln residue is conserved between all Fe- and MnLOX, but the distance to the Asn varies between different LOX. The Asn\textsuperscript{473} is situated on a short loop in Mo-MnLOX, this loop brings it closer to the Mn than it would have been if situated on a helix, and the loop may also provide the flexibility necessary for the Mn coordination.

The entrance to the substrate channel is defined by residues from the α2-helix, the arched helix (α10), and Arg\textsuperscript{525}. This Arg is conserved in most MnLOX, but it is not present in Fo-MnLOX that catalyzes the formation of products with the other stereoconfiguration. The lack of this Arg residue in the entrance might be the reason for the more rotational freedom of the substrate in Fo-MnLOX.

Table 2. Comparison of amino acid residues in the MnLOX and FeLOX substrate channel.

<table>
<thead>
<tr>
<th>Comment</th>
<th>Consensus</th>
<th>MnLOX</th>
<th>FeLOX</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cork (5-LOX)</td>
<td>---</td>
<td>Mo-MnLOX</td>
<td>Gg-MnLOX</td>
</tr>
<tr>
<td>Cork (5-LOX)</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>Interaction with Asn</td>
<td>Gln</td>
<td>281</td>
<td>287</td>
</tr>
<tr>
<td>Metal ligand</td>
<td>His</td>
<td>284</td>
<td>290</td>
</tr>
<tr>
<td>Oxygen channel</td>
<td>---</td>
<td>Val-285</td>
<td>Val-291</td>
</tr>
<tr>
<td>Metal ligand</td>
<td>His</td>
<td>289</td>
<td>294</td>
</tr>
<tr>
<td>---</td>
<td>Val/Ile</td>
<td>Val-323</td>
<td>Ile-328</td>
</tr>
<tr>
<td>Coffa-Brash determ.</td>
<td>Gly-327</td>
<td>Gly-332</td>
<td>Gly-427</td>
</tr>
<tr>
<td>Clamps substrate</td>
<td>Leu</td>
<td>331</td>
<td>336</td>
</tr>
<tr>
<td>Supra-/antarafacial</td>
<td>Phe/Ile</td>
<td>Phe-332</td>
<td>Phe-337</td>
</tr>
<tr>
<td>---</td>
<td>Leu-337</td>
<td>Phe-342</td>
<td>Phe-437</td>
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<tr>
<td>---</td>
<td>Phe-338</td>
<td>Trp-343</td>
<td>Val-438</td>
</tr>
<tr>
<td>Sloane determ.</td>
<td>Phe-342</td>
<td>Phe-347</td>
<td>Leu-442</td>
</tr>
<tr>
<td>Pocket depth</td>
<td>Thr-489</td>
<td>Ala-498</td>
<td>Ala-589</td>
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<tr>
<td>Pocket depth</td>
<td>Gln-519</td>
<td>Gln-532</td>
<td>Ala-620</td>
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<tr>
<td>---</td>
<td>Leu-522</td>
<td>Leu-535</td>
<td>Thr-623</td>
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<td>Cork (MnLOX)</td>
<td>Arg-525</td>
<td>Arg-538</td>
<td>Ile-626</td>
</tr>
<tr>
<td>Phe in MnLOX</td>
<td>Phe/Leu</td>
<td>Phe-526</td>
<td>Phe-539</td>
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<tr>
<td>C-terminal</td>
<td>Val/Ile</td>
<td>Val-605</td>
<td>Val-618</td>
</tr>
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</table>
Discussion

The studies in this thesis provide insight to the MnLOX family and what distinguish them from FeLOX found in plant and animals. Genbank lists several putative MnLOX and to date we have identified, expressed and characterized five MnLOX from *G. graminis*, *M. salvinii*, *M. oryzae*, *F. oxysporum* and *C. gloeosporioides*, Table 3. An additional MnLOX from *A. fumigatus* has been expressed by Heshof et al. [100]. The discovery of new members in the MnLOX family, and the crystal structure of the first MnLOX have opened up for comparisons between Mn- and FeLOX. Some interesting questions to be answered are why some LOX enzymes utilize Mn instead of Fe, and how FeLOX and MnLOX differ in catalytic mechanism and structural features.

Table 3. Summary of characterized MnLOX.

<table>
<thead>
<tr>
<th>MnLOX</th>
<th># aa</th>
<th>Predicted pI</th>
<th>Major HPODE products</th>
<th>Homology to Gg-MnLOX (%)</th>
<th>C-terminal</th>
<th>References</th>
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</thead>
<tbody>
<tr>
<td><em>G. graminis</em></td>
<td>618</td>
<td>8.8</td>
<td>13R, 11S</td>
<td>100</td>
<td>…FFLSV</td>
<td>[47]</td>
</tr>
<tr>
<td><em>M. salvinii</em></td>
<td>617</td>
<td>6.4</td>
<td>9S</td>
<td>77</td>
<td>…FFLSI</td>
<td>Paper II</td>
</tr>
<tr>
<td><em>M. oryzae</em></td>
<td>619</td>
<td>6.7</td>
<td>9S, 13R, 11S</td>
<td>53</td>
<td>…FYLSV</td>
<td>Paper IV</td>
</tr>
<tr>
<td><em>C. gloeosporioides</em></td>
<td>609</td>
<td>5.5</td>
<td>9S, 13R, 11S</td>
<td>53</td>
<td>…FYLSV</td>
<td>Paper V</td>
</tr>
<tr>
<td><em>A. fumigatus</em></td>
<td>608</td>
<td>5.4</td>
<td>13</td>
<td>47</td>
<td>…YYLCV</td>
<td>[100]</td>
</tr>
<tr>
<td><em>F. oxysporum</em></td>
<td>610</td>
<td>6.1</td>
<td>13S, 11R, 9S/R</td>
<td>56</td>
<td>…FYLSV</td>
<td>Paper V</td>
</tr>
</tbody>
</table>

Characteristics of MnLOX

MnLOX utilize a suprafacial hydrogen abstraction and oxygen insertion mechanism, whereas all known FeLOX utilize an antarafacial mechanism. A common feature for several MnLOX is the capacity of oxidizing 11- and 13-hydroperoxides of 18:2n-6 and 18:3n-3 to peroxyl radicals. FeLOX do not catalyze this reaction at significant rates. One reason for this can be the difference in redox potential of Mn$^{2+}$/Mn$^{3+}$ compared to Fe$^{2+}$/Fe$^{3+}$, 1.54 V and 0.77 V, respectively. Even though the redox potential most likely is tuned to a lower value by the enzyme, Mn is likely a stronger oxidant than Fe. Another reason for the stronger oxidation capacity of MnLOX (i.e. the ability to
oxidize hydroperoxides) could be that the metal and the hydroperoxide substrate are positioned in a more favorable orientation in MnLOX than in FeLOX. Cg-MnLOX and Fo-MnLOX are also poor catalysts of oxidation of bis-allylic hydroperoxides, this suggests that manganese in the active site is not enough for effective catalysis of oxidation of hydroperoxides; steric factors might also be an important factor.

If the MnLOX active site is more flexible, this could explain the ability to oxidize hydroperoxides more efficiently. This theory is supported by the fact that the reactions rate of 13R-MnLOX was more temperature dependent than the rate of sLOX-1. Close donor acceptor distances are necessary for efficient tunneling of the hydrogen. Since the MnLOX is more temperature dependent the flexibility in the active site might be larger than in sLOX-1, the vibration generated by higher temperatures could increase the probability for the hydrogen donor and acceptor to be close enough for tunneling.

Attempts to incorporate Fe into MnLOX was unsuccessful, this is interesting since the residues coordinating manganese appears to be essentially identical to the residues coordinating iron in FeLOX [134]. However, one noteworthy difference is that two of the metal coordinating histidines in FeLOX is spaced by four residues whereas three residues separate the corresponding histidines in Gg-MnLOX and Ms-MnLOX. This difference generated the idea to use site directed mutagenesis to add an extra residue between these histidines in order to incorporate iron into MnLOX, however when the other MnLOX were discovered they had the same hexamer motif as FeLOX, and we could therefore conclude that the space between the histidines is not crucial for the selection of catalytic metal.

An interesting question is how and why the enzyme selects Mn instead of Fe for catalytic metal. Our results indicate that the choice of metal in fungal LOX is not dependent on the availability of the metal in the cell. However, it is possible that protein folding occurs at compartments in the cell high in manganese even though the overall concentration of iron is much higher, or that other residues than the direct metal coordinating residues control the metal selection.

The crystal structure provides some possible explanation to the flexibility in the active site and the metal selection. The metal ligands of FeLOX are conserved in MnLOX, but the Ans473 metal ligand is situated in a short loop instead of a α-helix as in FeLOX. This loop might provide more flexibility to the active site and also makes it possible for Mn$^{2+}$ to be oxidized to Mn$^{3+}$, which requires a Jahn-Teller distortion of the octahedral coordination. The amine group of Asn473 has possible hydrogen bond interaction (2.7Å) with the side chain oxygen of Gln281, this residue and also other residues in the second coordinating sphere may contribute to the coordination and redox tuning of the metal.
Residues in control of regio- and stereospecificity

Several mammalian LOX determinants of stereo- and regiospecificity are also applicable to MnLOX (Fig. 9). The “Sloane determinants” of regiospecificity control the depth of the hydrophobic pocket harboring the active site in FeLOX. The catalytic specificity of Gg-MnLOX could be changed from 13R to 9S by mutation of one of the residues in the “Sloane determinants”. This switch of regiospecificity is probably due to a frame shift of the substrate in the active site, positioning the substrate further into the substrate channel, and oxygen can therefore be inserted at C-9 instead of C-13.

The MnLOX of *F. oxysporum*, that produces 11R products, harbors a Ser in the position of the “Sloane determinant”, in comparison to Phe in other MnLOX that produce 11S-hydroperoxides (Fig. 9). This Ser turned out to be in control of stereospecificity rather than regiospecificity in this LOX. Since Fo-MnLOX has a suprafacial mechanism in analogy with other MnLOX, we hypothesize that Ser allows the substrate to rotate in the active site and positions pro-11R-hydrogen for abstraction, and the face of 13S position for oxygen insertion. This rotational freedom might compromise the regio- and stereocontrol, which could explain the mixture of products formed by this enzyme. The Phe residue in the other MnLOX might in addition to controlling the depth of the active site also interact with the substrate and hold it fixed for catalysis.

Another interesting region in FeLOX is the “arched” helix that covers the active site on the other side of the hydrophobic channel from the catalytic metal. [86]. This helix is also present in MnLOX and at the bottom of the
helix sits an invariant Leu residue, which seems to clamp the substrate in place for catalysis. Next to this residue is an Ile or Val in most mammalian LOX, but in MnLOX this residue is a Phe. Mutation of this Phe$^{337}$ to Ile in Gg-MnLOX altered the suprafacial oxygen insertion and hydrogen abstraction, to mainly antarafacial in analogy with FeLOX. It would be interesting to investigate if this switch also is applicable the other way around. Mutation of the corresponding Ile$^{442}$ in coral 8R-LOX to a Trp rendered the enzyme inactive [86]; mutation to a Phe could be a more acceptable change. The crystal structure of Mo-MnLOX confirms that this Phe$^{332}$ is pointing down into the hydrophobic channel and it is likely that this bulky residue shields the face of linoleic acid where the positions for 11$R$, 9$R$ and 13$S$ oxygenation sit.

Replacement of the conserved Leu residue, presumed to clamp the substrate, with less bulky residues (Ala, Gly) in Gg-MnLOX altered the regiospecific oxygenation. This effect was more pronounced with 18:2$n$-6 than 18:3$n$-3 as substrate, indicating that the number of double bonds is important for the alignment of the substrate in the active site. Replacement of the Leu$^{336}$ with a more bulky residue results in increased kinetic lag time, probably due to reduced oxygen access to the active site. Less access to oxygen also contributed to an increase in hydroperoxide isomerase activity, resulting in a rapid formation of keto-compounds.

A hydroperoxide isomerase activity with formation of epoxy-alcohols was also seen when mutating Gg-MnLOX Gly$^{332}$ to Ala one helix turn away from Leu$^{336}$ [135]. This residue is situated at the “Coffa-Brash determinant” (Fig. 9), which is known to control the stereospecificity (actually a switch in regiospecificity results in the switch between $R$ and $S$) in mammalian LOX [84]. However, no switch in specificity was detected in Gg-MnLOX instead this mutation resulted in a switch from dioxygenases activity to hydroperoxide isomerase activity, indicating less oxygen access to the active site. This activity could be compared with human eLOX-3 that mainly has a hydroperoxide isomerase activity. eLOX-3 exhibits an extremely long lag phase for the dioxygenation reaction, which could be shortened by $O_2$-saturated buffer. The reverse mutation of the “Coffa-Brash determinant” in eLOX-3, Ala$^{451}$ to Gly, resulted in lower hydroperoxide isomerase activity, increased activation to the ferric enzyme and a shorter lag phase for dioxygenation when incubated with polyunsaturated fatty acid substrates [136]. This position therefore seems to be important for the $O_2$ access to the active site in both mammalian and fungal LOX.

We conclude that the oxygen insertion can be altered by amino acid replacement in three principle ways. First, insertion of a bulky residue can shield some positions from oxygen insertion, favoring other positions. Second, the substrate alignment in the active site can be changed, making the carbon of the opposite end of the 1$Z$,4$Z$-pentadiene available for oxygen
insertion. Third, the substrate can enter the active site in reverse orientation, with the carboxyl end embedded in the protein.

Insights from the structure of MnLOX

We report the first crystal structure of an MnLOX. MnLOX in analogy with prokaryotic LOX lack the N-terminal β-barrel. MnLOX have low sequence homology (25%) with FeLOX, and the active site in the structures did not align well. The most striking difference from FeLOX was the α2 helix that is longer in MnLOX than in FeLOX. This 11-turn helix is slightly bent and runs the whole length of the protein and constitutes a part of the substrate channel entrance, together with an Arg residue conserved in most MnLOX. This Arg 525 3D align well with the FY cork in 5-LOX [106], but seems to define the entrance and not covering it. It most likely has the same function as Arg 182 in 8R-LOX, stabilizing the negatively charged carboxyl group of the substrate [83] (Fig. 10).

The metal coordinating residues are conserved, but differ in that the Asn 473 is situated on a short loop between two helices. The Mn 3+ may require a Jahn-Teller distortion from the octahedral coordination of Mn 2+ [137], and this distortion might be facilitated by placing Asn 473 on a more flexible loop and not on a α-helix, as in FeLOX. We also noted possible hydrogen bonds between Gln 281 and Asn 473 (Fig. 10). This could be important for the control of the Mn redox potential. It is known that hydrogen bond interactions to coordinated solvent can exert strong redox tuning on the metal ion in superoxide dismutase [138]. It is also possible that this tuning can be accomplished by nearby side chains, such as Gln, in the second coordination sphere.

There are three Phe residues (Phe 332, Phe 342 and Phe 526) in Mo-MnLOX structure with side chains pointing into the hydrophobic channel (Fig. 10). The first Phe has been shown to be important for the suprafacial /antarafacial hydrogen abstraction and oxygenation mechanism by site directed mutagenesis, and this can be supported by the structure. The second Phe has been shown to control the penetration depth and rotation of the substrate in the active site, and the third Phe 526 is located on the same side as the catalytic metal in the hydrophobic channel, and we hypothesize that this Phe residue might bend the substrate to allow hydrogen abstraction and oxygenation from the same face, and thereby facilitating the suprafacial mechanism.
Figure 10. The 2Fo-Fc electron density map of selected residues around the active site (contoured at the 1σ level). Arg525 (dark red) likely stabilizes the carboxyl end in the substrate channel entrance. Phe526 (blue) is hypothesized to bend the substrate to allow oxygen access from underneath the pentadiene. Leu331 (yellow) is defining the upper wall of the channel, Phe332 (cyan) may shield the pentadiene for oxygen insertion at the opposite side of the pentadiene, and Phe342 (green) is at the bottom of the hydrophobic pocket where it can determine the penetration depth of the substrate and hold the hydrophobic tail. The manganese (pink) and the catalytic water (red) are illustrated as spheres.

Biological significance of fungal LOX

Rice and crop diseases are a major problem worldwide since one half of the world’s population relies on rice for their primary calorie intake [139]. *M. oryzae* is a fungus that causes rice blast disease, it destroys typically 10-30% of the total rice harvest, but is some areas it can be the cause for a complete loss of harvest. *M. salvinii* is a plant pathogen that causes rice stem rot. Many pathogenic fungi have a broad host range, e.g. *G. graminis*, also called the take-all fungus that causes severe root disease in various cereals and grasses.

To fight these pathogenic fungi we need to know more about how they function and which metabolites are important for the infection process and their survival. The oxylipin pathways are important in man, and many homologues to mammalian enzymes such as LOX, COX and CYP are found in
fungi. The evolutionary conservation of these enzymes between different species confirms the important role of oxylipin signaling in all kingdoms of life.

The role of these enzymes in man are thoroughly investigated but the biological function of LOX in fungi is still unclear, and especially the function of the unique MnLOX. MnLOX contain secretion signals that are cleaved during translocation over the membrane, so they are probable secreted by the fungus to metabolize fatty acids outside the cell, for example the host cell membrane. MnLOX are more stable than mammalian LOX and protected by glycosylation, which make it easier to use them outside the cell.

It has been shown that a LOX deficient Aspergillus sp. produced lower levels of 13S-HPODE and displayed decreased production of mycotoxin, delayed conidia (asexual spores) formation and increased sclerotic production. Wheat seed infected by this Δlox mutant displayed lower levels of 9S-HPODE and lower mRNA expression of typical defense genes, than seed infected with the wild type strain [140]. To take advantage of the differences between fungal and other LOX might be one strategy for development of anti-fungal agents without negative effect on LOX in plants and animals.

Fungi do not only infect plants, several opportunistic fungi, e.g. Candida, some Aspergillus and Fusarium species, are also human pathogens that can cause invasive infections in immunocompromised patents. A potential role for fungal LOX inhibitor could be in the human skin disease Pityriasis versicolor which is caused by infection by the fungus Pitosporum orbiculare. It causes damages on melanocytes and produces white areas on skin. P. orbiculare can oxidize 18:2n-6 to hydroperoxides, which indicates a LOX activity. It is possible that this LOX might be involved in the depigmentation, as bleaching by LOX is well known [141].
Conclusion

We have characterized novel MnLOX enzymes in filamentous fungi and identified important residues for their catalytic activity.

The main conclusions can be summarized as follows:

- The suprafacial reaction mechanism, which is unique for MnLOX, could be altered in Gg-MnLOX by mutation of a single Phe to Ile.
- *M. salvinii* secretes a 9S-MnLOX and a coactive epoxy-alcohol synthase, the former was cloned and expressed in *P. pastoris*.
- A Phe residue in the bottom of the hydrophobic active site controls the regiospecificity of Gg-MnLOX.
- Gg-MnLOX and sLOX-1 display low $E_{\text{act}}$ and high D-KIE values consistent with hydrogen tunneling.
- Gg-MnLOX showed small temperature dependence, not noted in sLOX-1, suggesting a combination of a hydrogen tunneling and a semi-classical transition state mechanism.
- *M. oryzae*, *F. oxysporum* and *C. gloeosporioides* express MnLOX with high sequence identity to Gg-MnLOX (>40%).
- Fo-MnLOX forms 11R-HPODE, this is attributed to a Ser$_{348}$, and mutation of this position to a Phe residue results in catalytic convergence with the other MnLOX.
- The crystal structure of the first MnLOX reveals interesting differences from FeLOX regarding bulky Phe residues in the active site; these differences may contribute to the suprafacial hydrogen abstraction and oxygenation mechanism.
- The location of the Asn metal ligand and its interactions with a conserved Gln might be important for the selection and use of manganese as catalytic metal in LOX.
Populärvetenskaplig sammanfattning på svenska

Fettsyror förkommer i alla levande organismer och utgör en av byggstenarna i cellmembran, de är även viktiga i energiförbrukning och är substrat för bildande av signalsubstanser som prostaglandiner och leukotriener. Omätta fettsyror innehåller en eller flera dubbelbindningar. En viktig fleromätta fettsyra i människan är arakidonsyra som är substrat för enzymerna cyklooxygenaser (COX) och lipoxygenaser (LOX) som bildar prostaglandiner och leukotriener. Prostaglandiner är inblandade i många patofysiologiska processer som t.ex. smärta, feber, inflammation men har även positiva effekter t.ex. vid reglering av blodtryck, vid förlossning och för slemhinnan i mag- och tarmkanalen. Många vanliga NSAID-läkemedel som används vid smärta, feber och inflammation t.ex. acetysalicylsyra, diklofenak och ibuprofen verkar genom att hämma COX och därmed hæmmas produktionen av prostaglandiner. Leukotriener ger kontraktion av luftroren vid astma och allergi. Hämmare av leukotrienreceptorer används som tilläggsbehandling vid astma.

Liknande enzymer, som katalyserar insättning av syre i fettsyror, finns i alla organismer. Vår grupp upptäckte det första LOX som har mangan som katalytisk metall, alla tidgare kända LOX i djur och människor innehåller järn. Målet med mitt arbete har varit att upptäcka och karakterisera ytterligare manganinnehållande LOX (MnLOX) i filamentösa (mög) svampar. I och med att vi har identifierat fler medlemmar i MnLOX familjen går det att se vad som är karaktäristiskt för MnLOX respektive FeLOX. Vi undersöker vilka aminosyror i enzymet som styr vilka produkter som bildas. Vi skulle också vilja utreda varför bara svampar använder mangan i LOX och vilka fördelar det ger svampen. Rent praktiskt identifieras gensekvenserna i databaser, genen klonas och enzymet uttrycks i jäst. Det rekombinanta enzymet renas upp och blandas med olika fettsyror. Produkterna som bildats analyseras bland annat med HPLC-MS/MS och UV-spektroskopi. Likheten i aminosyrasekvens mellan MnLOX och FeLOX är ca 25 %, men reaktionsmekanismen och produktens stereokemi skiljer sig åt. Ett av arbetena beskriver hur en mutation av en enda aminosyra i MnLOX kan ändra produktina till motsvarande produkter bildade av FeLOX. Vi har också visat att MnLOX delvis använder sig av en väte-tunnling i dess reaktionsmekanism, vilket betyder att väteatomen snarare beter sig som en våg än en partikel.
En intressant fråga är vad svamparna använder MnLOX till. Till skillnad från FeLOX så har den en sekretionssignal och är mycket stabil, detta tyder på att de används för att metabolisera något utanför cellen, kanske värdorganismens cellmembran. Detta kan vara en värdefull egenskap t.ex. vid mögelinfektion av växter.

För att få en tydligare bild av hur MnLOX enzymet ser ut har vi kristallisat proteinet och utsatt den roterande kristallen för röntgenstrålar av en viss våglängd, dessa röntgenstrålar diffrakteras och detekteras på en skärm. Genom att sätta ihop många diffraktionsbilder har vi sammanställt en 3D- struktur av proteinet. 3D strukturen av proteinet har bidragit till en tydligare bild av aktiva ytan och vilka aminosyror som är viktiga för de unika egenskaperna hos MnLOX. Denna information kan utnyttjas för att studera vad som skiljer svampenzymerna från likande enzymer i människan, och även för utveckling av specifika hämmare.

Så varför bedriver vi forskning på svampenzyme? Det är möjligt att upptäcka helt nya enzymer som kan ha intressanta egenskaper. I vårt fall med MnLOX så kan den på grund av dess stabilitet användas som modell för de mer instabila humana LOX proteinerna. MnLOX används också industriellt för produktion av oxylipiner samt blekning av t.ex. mjöl och pappersmassa och vid fläckborttagning.

Det finns en enorm mångfald av metaboliter som kan bildas från växter och svampar, många som vi människor kan dra nytta av. Exempel på några läkemedel som isolerats från mögelsvampar är antibiotika som t.ex. penicillin, immunhämmande cyklosporiner, kolesterol sänkande lovastatin och ergotamin som kan användas för att lindra migrän.
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