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The 1.85 Å structure of an 8R-Lipoxygenase suggests a general model for lipoxygenase product specificity

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Abstract

Lipoxygenases (LOX) play pivotal roles in the biosynthesis of leukotrienes and other biologically active eicosanoids derived from arachidonic acid. A mechanistic understanding of substrate recognition, when lipoxygenases that recognize the same substrate generate different products, can be used to help guide the design of enzyme-specific inhibitors. We report here the 1.85 Å resolution structure of an 8R-lipoxygenase from *Plexaura homomalla*, an enzyme with ~40% sequence identity to human 5-LOX. The structure reveals a U-shaped channel, defined by invariant amino acids, that would allow substrate access to the catalytic iron. We demonstrate that mutations within the channel significantly impact enzyme activity and propose a novel model for substrate binding potentially applicable to other members of this enzyme family.

Keywords

Eicosanoids; lipoxygenase; C2-domains; Calcium-dependent membrane binding; arachidonic acid; X-ray crystallography

Lipoxygenases are non-heme iron dioxygenases that catalyze the stereo- and regio- specific formation of fatty acid hydroperoxides from polyunsaturated fatty acids (1, 2). In animals, lipoxygenases (LOX) play pivotal roles in the biosynthesis of leukotrienes and other biologically active eicosanoids derived from arachidonic acid (AA). Consequently, the enzymes are targets for the development of specific inhibitors to modulate the physiological and pathological effects of the potent signaling compounds they generate (3, 4). Since lipoxygenases are iron enzymes they can be inhibited by iron chelators or redox active compounds. However, these modes of inhibition are inherently non-specific. In order to engineer specificity into any inhibitor, more structural information for this enzyme family is required. Yet no LOX structure provides a comprehensive model for how substrate binds in the active site, a model central to the development of isozyme-specific inhibitors. A mechanistic understanding of substrate recognition, when lipoxygenases that recognize the

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same substrate discriminate among the three chemically equivalent carbons of arachidonic acid to generate different products, can be used to help guide the design of novel inhibitors. Work with the plant enzymes has afforded tremendous insight into the mechanism of hydrogen abstraction by the active site iron, but the basis for the specificity of oxygenation in LOX catalysis is still unclear (5).

We report here the 1.85 Å resolution structure of an 8*R*-lipoxygenase from *Plexaura homomalla*, an enzyme that, like human LOX isoforms, has ~40% sequence identity to human 5-LOX, the enzyme that initiates leukotriene biosynthesis. The structure reveals a U-shaped channel, defined by invariant isoleucines and leucines, that allows substrate access to the catalytic iron. We demonstrate that mutations within this channel significantly impact enzyme activity. Based on our results we propose a model for substrate binding in which an invariant Leu points into the U-shaped channel and restrains the targeted pentadiene for hydrogen abstraction. The register of the pentadiene positioned for attack is set by protein-substrate interactions at either end of the U-shaped channel.

The features of an 8*R*-LOX binding site must be able to account for the regio- and stereo-specificity of the enzyme, yet exhibit the potential flexibility to explain the range of specificities found within the LOX enzyme superfamily. Consequently, the model must be compatible with several well-established aspects of LOX catalysis. (i) The non-heme iron, positioned by amino acids that fill five of the six sites of the coordination sphere, interacts with the substrate *via* its sixth position as Fe³⁺-OH. Sequence, structural and biochemical data suggest that the geometry of the catalytic machinery is conserved among LOX enzymes. (ii) Fe³⁺-OH initiates catalysis by stereoselective removal of one hydrogen from the CH₂ in a *cis*-CH₂-*cis* double bond unit (pentadiene) of the substrate. The free radical pentadiene intermediate generated by H abstraction is oxygenated on its opposite face, at one of two possible positions: the +2 or -2 carbon. Reaction at the +2 or -2 positions yields products of opposite stereochemistry (*R* or *S*) (Fig 1). (iii) A conserved active site Gly in *R*-LOX is associated with *R* oxygenation. Its counterpart in *S*-LOX is Ala. A Gly/Ala switch by mutagenesis redirects oxygenation across the face of the pentadiene, changing the positional specificity along with *R* or *S* stereochemistry. (iv) The fatty acid substrate is held in one head-to-tail orientation or the other. While four distinct hydroperoxide products can arise from each pentadiene in the substrate, each LOX generates only a single specific product. For example, LOX catalyzed peroxidation of the central pentadiene of arachidonic acid requires hydrogen abstraction at C10 and could potentially generate 8*R* or 12*S* with AA bound in one orientation, and 8*S* or 12*R* if AA binds in the inverse orientation (Fig. 1). For the enzyme described herein, only the 8*R*-product is observed. (v) Finally, some lipoxygenases produce the identical hydroperoxide product from free arachidonic or linoleic acids or their large esters (e.g. phospholipid esters). This implies that the carboxyl group does not enter the active site, thus the binding orientation for these enzymes (e.g. 15*S*-LOX) has generally been designated as “tail-first” into the active site. In contrast, the 5*S*-, 8*S* or 12*R*- enzymes cannot oxygenate the phospholipid esters, and their product specificities can be explained by “head first” entry into the active site. There are, however, notable exceptions to this last rule which require a rational explanation as we discuss later.

In effect, active site control of LOX product specificity is determined by three factors: which pentadiene of the substrate is positioned for attack, whether the pro R or pro S hydrogen of the pentadiene is positioned for abstraction, and whether O $_2$ has access to carbon C $_{-2}$ or C $_{+2}$. Our model provides a structural framework to understand the determinants of product diversity of lipoxygenases.

MATERIALS AND METHODS

Protein crystallizations

A deletion mutant of *Plexaura homomalla* 8R-LOX engineered for improved solubility, referred to herein as pseudo wild-type (psWT), was concentrated to ~10 mg/ml and crystallized by sitting-drop vapor-diffusion as previously reported(6). The I433W and I433A mutants of psWT were constructed using whole plasmid polymerase chain reaction. Crystals of I433W:psWT and I433A:psWT were obtained by sitting drop vapor diffusion in 5-8% PEG-8000, 5% glycerol, 0.2 M CaCl $_2$, 0.1 M imidazole acetate, pH 8.0. Crystallizations were performed in a Coy anaerobic chamber. The crystals, which grow overnight, were mounted using a cryo-protecting solution of 10% PEG-8000, 25% glycerol, 0.02 M CaCl $_2$, 0.1 M imidazole acetate, pH 8.0.

X-ray data collection and structure determination

X-ray diffraction data (psWT, I433W:psWT; I433A:psWT) were collected at the Gulf Coast Protein Crystallography Consortium beamline at the Center for Advanced Microstructures and Devices (CAMD, Louisiana State University) using 1.38 Å radiation at a temperature of 100 K. Data for psWT, I433W:psWT and I433A:psWT were processed to a resolution of 1.85 Å, 1.9 Å, and 2.3 Å, respectively (Table 1), using the HKL2000 package(7). The structure of psWT was solved by molecular replacement using the previously published 3.2 Å structure of coral 8R-lipoxygenase (PDB: 2FNQ)(8). The two mutant structures were solved by using only the protein atoms from the refined parent structure as a starting model, refining the protein model and adding ions, small molecules, and waters. Model building was done with *Coot*(9) and refinement with REFMAC5 (10) and *Phenix*(11). Model quality was assessed using MolProbity(12). For the psWT structure, 97% of the residues are in the favored region of the Ramachandran plot, with only a single residue outside the allowed region out of 2800 total residues (there are four molecules in the asymmetric unit). The I433W:psWT structure had 96.9% of the residues in the favored region of the Ramachandran plot, with only 1 outside the allowed region out of 2810 residues. The I433A:psWT structure had 96.7% of the residues in the favored region of the Ramachandran plot, with 4 residues outside the allowed region out of 2758 residues.

Enzyme kinetics

The LOX activity assay of 8R-LOX wild-type and mutants was carried out in a 1 ml sample volume (in 50 mM Tris buffer, pH 7.5, 150 mM NaCl) by following the absorbance increase at 235 nm due to the formation of conjugated diene chromophore using a Lambda 35 UV/Vis spectrophotometer (PerkinElmer). The concentration of wild-type enzyme was 12.5 nm; for the less active mutants enzyme concentrations were increased up to 63 nm. Substrate concentration was varied from 10-200 μM. The rates of reaction were calculated from the

initial linear part of the curve. Values for K_m and turnover were determined by nonlinear regression analysis of a plot of velocity *versus* substrate concentration data to the Michaelis-Menten equation. Activity from the I433A:psWT enzyme was only measurable in the presence of detergent and CaCl_2 (50 mM Tris, pH 7.5, 500 mM NaCl, 0.05% emulphogen, 2 mM CaCl_2).

Product Analysis

8R-lipoxygenase mutagenesis was performed using the Stratagene® Quickchange Mutagenesis kit according to the manufacturer's instructions to replace Leu-432 with Ala, Val, Ile or Phe. Expression and purification of wild-type, psWT, and all mutants for product analysis was performed as reported by Boutard et al. (13). Protein was quantified by running on a 20-4% polyacrylamide gel along with a series of known amounts (1-10 μg) of BSA as previously described (14). Incubations were carried out in 50 mM Tris pH 8.0 containing 500 mM NaCl, 2 mM CaCl_2 , and 0.05% Emulphogen (Sigma) with 50 μM Arachidonic Acid containing 5×10^5 cpm of $[1-^{14}\text{C}]$ arachidonic acid. Samples were incubated for 10 min at room temperature (with shaking) and subsequently extracted with a Waters 1cc Oasis HLB cartridge and eluted with 1 ml of methanol. Treatment with triphenylphosphine in methanol at room temperature for 10 minutes was performed to reduce the hydroperoxyeicosatetraenoic acids (HPETEs) to their hydroxy counterparts (HETEs).

Samples were analyzed initially by RP-HPLC using a Waters Symmetry C18 column (250×4.6 mm) with a solvent system of methanol/water/glacial acetic acid (80:20:0.01, by volume) running at 1 ml/min. The eluant was monitored with an Agilent 1100 diode array detector in series with a Radiomatic Flo-One/Beta A100 inline scintillation counter. This allowed determination of the conversion of substrate to H(P)ETEs (8- and 12-HETE are not well resolved on this system; they elute close together, partly resolved with 12- eluting before 8-HETE). The samples were then methylated using diazomethane and the relative amounts of the individual HETE methyl esters (8- and 12-HETE) were determined on normal phase HPLC using a Beckman Ultrasphere Si column 4.6×250 mm in hexane/isopropanol (100:1, v/v) at 1 ml/min. The chirality of the 8-HETE and 12-HETE methyl ester products were analyzed using a Chiralpak AD column (250×4.6 mm) using hexane/methanol solvent (100:2, v/v) at 1 ml/min (15)

RESULTS

High resolution 8R-LOX structure

8R-LOX crystals diffracting to $1.85 \text{ \AA}$ were obtained following modifications to a putative membrane insertion loop and calcium-binding sites as detailed elsewhere(6). The modified protein crystallized in space group $P2_1$ with four molecules in the asymmetric unit. The structure of the mutant protein (Fig 2.) confirmed the supposition that removal of the putative membrane insertion loop and calcium binding amino acids has no structural consequences outside the immediate vicinity of the deletion. Lipoxygenases contain an N-terminal C2-like domain (~110 amino acids) and a predominately helical domain (~550 amino acids in animal lipoxygenases) in which the catalytic Fe is ligated by amino acid side chains and the main-chain carboxylate of the C-terminal amino acid. 8R-LOX has three

Ca²⁺ binding sites flanked by putative membrane insertion loops in the C2-like domain and the deletion mutant used in these studies lacks one of the loops, as well as chelating amino acids from two of the three Ca²⁺ binding sites (the center site and that most distal to the catalytic domain). The Ca²⁺ site proximal to the catalytic domain, defined primarily by main chain contacts, remains intact and occupied in the mutant structure. Additional density which may be a Ca²⁺ ion is associated with E52, but the coordination sphere of the hepta coordinated ion contains only two protein atoms, and these from different molecules, and is not likely a biologically relevant interaction. Ca²⁺ promotes the binding of 8R-LOX to membranes (8). The deletion mutant displays wild-type activity in a membrane-free assay, however, Ca²⁺ does not promote membrane binding of the mutant and does not stimulate enzyme activity in a membrane-based assay. Thus we refer to this enzyme as pseudo wild-type (psWT) in accordance with its native-like structure and catalytic activity in the absence of membranes.

The structure reveals a channel with conserved features

A striking feature of the electron density map obtained for psWT is a U-shaped channel, open at both ends, which runs under an arched helix and allows access to the catalytic iron from two directions. The channel is a culvert which runs underneath the arch provided by the curvature of $\alpha 9$, roughly orthogonal to it (Fig. 2). At the apex of the arch, sheltering the culvert, is the helical turn that includes residues 428-432. From this turn, the side chain of L432 points down into the U-shaped channel. At the base of this U-shaped channel sits the catalytic iron. This arched, or discontinuous, helix (in *P. homomalla* $\alpha 9$ -10, Fig. S1) is found in all available lipoxygenase structures(16-21). The discontinuity is a consequence of a reverse turn insertion previously discussed by Minor *et. al.* (17). The insertion, which corresponds to the highly conserved glycine (Fig. 3) in animal and plant LOX, has the effect of creating a long, curved helix that arches away from the bundle of helices that constitute the catalytic domain. The C-terminal end of the helix is anchored to the LOX body by a salt link between R429 on $\alpha 9$ and E394 on $\alpha 7$ (8R-LOX numbering, Fig. S1). Both the Arg and Glu that make the salt link are conserved in almost all available lipoxygenase sequences.

In the electron density map the U-shaped channel is deceptively large since the side chains of I433 and L432 are not visible, an indication of either static or dynamic disorder. However, even when the volume of the side chains of these amino acids is taken into account, there is room for AA to slide into the channel and position C10 of the central pentadiene for attack. Positively charged residues, R183 and R429, that could neutralize the carboxylate of AA are located on both sides of the channel. Since R429 participates in the invariant salt link described above, anchoring the arched helix (the roof of the channel) to the helical cluster that forms the catalytic domain, it is more likely that R183 is the neutralizing residue that positions the substrate in 8RLOX for attack at C10. Lipoxygenases that vary with respect to positional specificity would be expected to also vary with respect to the positions of their substrate-tethering amino acids. In contrast, the environment that surrounds the pentadiene for H abstraction is likely to be conserved.

Site-directed mutagenesis of the amino acids lining the channel

L432, which points into the culvert and is positioned above the catalytic iron (Fig. 3), is fully solvent exposed and has no side chain density past the C β . There is, however, sufficient space between a Leu side chain and the catalytic iron for the Leu to secure the pentadiene for hydrogen abstraction by Fe³⁺-OH. L432 is invariant in the lipoxygenase sequences available from plants, animals and microorganisms. A role in positioning the pentadiene for attack is consistent with this pattern of conservation. The alternative interpretation, that the Leu is conserved because it plays an important role in the stabilization of the common structural framework, is difficult to reconcile with the fact that the amino acid is both solvent exposed and highly mobile, properties not generally associated with structurally important amino acids.

To investigate a functional role for L432, mutant enzymes with conservative substitutions were constructed by replacement of L432 with Ala, Val, Ile or Phe. The Ala, Ile and Phe mutations produced enzyme with less than 5% of the activity of the wild type enzyme. While the effect of Val was less dramatic, L432V:8R-LOX still displays less than 20% wild type activity. According to the results of kinetic experiments, this loss of activity cannot be attributed to a loss of affinity for the substrate, as the K_m for AA for the mutant enzymes is not significantly changed from that of psWT enzyme (Table 2). Rather, the mutant enzymes have greatly reduced turnover numbers. These data support a role in catalysis for Leu432. In addition, about one-third of the L432F product is 12S rather than 8R, thus both the specificity and the rate of reaction are compromised with this mutation. These results combine to suggest that L432 plays a role in positioning and restraining the substrate pentadiene. The reduced rates of reaction and altered product profiles can be a consequence of the pentadiene being improperly positioned for H abstraction and/or ineffectively restrained for oxygenation.

Mutants in I433 (I433A and I433W), a second channel-lining amino acid for which no electron density is visible past the C β , were prepared in the background of the psWT. The structures of the mutated enzymes were determined, and the effects of the mutations on catalytic activity were evaluated. Both mutations greatly impair enzyme activity, no activity for the I433W mutant is detected, and the I433A:psWT has measurable activity only in the presence of CaCl₂ and the detergent emolphogen.

The crystal structures of the I433W and I433A mutant enzymes confirm that the only structural differences induced by the mutations are localized to the immediate vicinity of the amino acid substitution (Fig. 4). However, the two substitutions have opposite effects: the side chains of the arched helix in the I433W:psWT structure are more ordered, while substitution with an Ala at the same position results in disordering of the helix main chain. In the I433W mutant there is clear density for the Trp side chain (Fig. 5) and it appears immobilized in the U-shaped site, partially filling an otherwise solvent exposed hydrophobic channel. The bulky Trp occupies space required for the AA tail according to our model. In addition, the side chain of L432 is clearly visible in the I433W electron density map and therefore no longer mobile. The replacement of I433 with a Trp appears to stabilize L432

and plug the tail end of the proposed AA binding site. This mutant has no measurable activity, presumably because the Trp side chain has effectively blocked the AA binding site.

In contrast, in the I433A mutant the helical arch which spans the culvert in the wild-type enzyme is not fully defined in the electron density map: there is no density for side chains or backbone from residues 432 to 436. It appears that the absence of the Ile side chain has destabilized the “roof” of the U-shaped channel. This destabilization negatively impacts the catalytic capacity of the enzyme since L432, a critical side chain according to the results described above, is one of the residues destabilized by the mutation. This mutant produces predominantly the 8*R*-product, but at a significantly impaired rate (Table 2).

Thus mutation of amino acids that line the U-shaped channel significantly impact catalytic activity. More importantly, the crystal structures confirm that amino acid substitutions in this region have no structural impact outside the immediate vicinity of the mutated region. Since both L432 and I433 are mobile and solvent-exposed in the pseudo-wild-type structure, a structural role for these amino acids is unlikely. The observation that substitutions have such a dramatic effect on enzyme activity are consistent with functional roles, such as substrate positioning, for these amino acids.

The AA binding site we propose (Fig. 3) based on the 1.85Å 8*R*-LOX structure is lined with invariant amino acids in the immediate vicinity of the site that accommodates the pentadiene. In support of our experimental results, when Evolutionary Trace(22-24) is employed to visualize patterns of sequence conservation in lipoxygenases in a three dimensional context, five hydrophobic amino acids lining the U-shaped channel are strictly conserved in arachidonatemetabolizing lipoxygenases: Leu-386,391,432,628 and Ile 424. The amino acid(s) that set the register of the AA and dictate which pentadiene is available for H abstraction should flank this area. Since LOX isoforms produce a wide array of products, little conservation of flanking amino acids that bind the carboxylate and tail ends of AA is predicted. Indeed, there is a lack of sequence conservation among lipoxygenases in these regions.

DISCUSSION

The U-shaped channel contrasted with the previously described “boot-shaped” cavity

Prior to our work the only animal LOX structure available was that of rabbit reticulocyte 15-LOX in complex with the inhibitor RS7(18). Since both the U-shaped channel and the boot-shaped cavity of the rabbit 15-LOX structure must provide a path for the pentadiene to approach the catalytic iron, there is overlap between the predicted substrate binding sites. In the two lipoxygenase models, the essential difference is that the boot-shaped cavity protrudes past the Fe and deeper into the enzyme, whereas the U-shaped site veers back up towards the surface. An illustration of the amino acid side chains that define the two sites is rendered in Fig. 6. F353 in 15-LOX is proposed to form the floor of the substrate binding site, with the bulky amino acid precluding further penetration of the AA tail. There is a similarly positioned internal cavity in the 8*R* structure, but it is appreciably smaller in volume. Yet according to the 15-LOX-derived model (25), the cavity should be larger in 8*R*-LOX since the AA must enter 3 carbons deeper to allow the positioning of C10, rather than

C13, at the iron coordination sphere. The positional specificity of the 8R-enzyme is inconsistent with a smaller volume of this cavity. In contrast, the volume and details of the U-shaped channel are consistent with a role in AA binding. The U-shaped channel is defined by the side chains of 5 Leu and 4 Ile residues and displays remarkably high sequence conservation (7 invariant/highly conserved in animal lipoxygenases). While there is site directed mutagenesis data that support the boot-shaped cavity (18, 25, 26), some does not (27). Moreover, in no case is structural data presented to evaluate the impact of the mutations on enzyme structure. While some results may be compatible with that model, an alternative such as the U-shaped channel cannot necessarily be ruled out.

Partial overlap of substrate and inhibitor binding sites, as a consequence of the structural adaptability of the ligand binding pocket, has previously been described for the structures of the ecdysone receptor in complex with a hormone or antagonist(28). The details of the 15-LOX:RS7 structure suggest that as was case for the ecdysone receptor, the inhibitor is sufficiently dissimilar in structure from the natural substrate that it is not a true substrate/ligand mimic. In the case of 15-LOX:RS7, two major stabilizing forces of the protein-ligand interaction (charge-charge, aromatic-aromatic) are not relevant to the natural substrate. First, the carboxylate of the inhibitor is neutralized by the catalytic non-heme Fe, limiting access to the space the pentadiene must occupy for H abstraction to occur. And second, the aromatic ring of the inhibitor, which has no counterpart in the natural substrate, is buried deep in an aromatic cluster. Thus it may be that 15-LOX inhibitor does not fully define the substrate binding cavity.

The recent results of photoaffinity labeling of the active site of 15-LOX are readily explained with our U-shaped cavity extrapolated to that enzyme(29). Photo-affinity substrate analogs with a reactive azido group at the methyl end were crosslinked to two peptides in 15-LOX. Only one peptide contains amino acids shown to be important for AA binding and consistent with the boot-shaped cavity model (I593(25)). In contrast, when our proposed AA binding site is modeled in 15-LOX, the binding site is flanked by the two cross-linked peptides (Fig. 6b). Specifically, the 15-LOX counterparts of amino acids I627 and L628 are provided by one of the peptides, while the other includes amino acids that plug the “back door” of our channel in the 15-LOX structure (W145, K146). Importantly, while the U-shaped channel appears open at both ends in the 8R-LOX structure, this need not be the case for other LOX isoforms. The 15-LOX structure vividly illustrates this point. While a similar channel is apparent in the 15-LOX structure described by Choi *et al.* (21) the channel is closed by the presence of W145 and a salt link (K145, D411) at one end. This closing and consequent shortening of the channel is consistent with the fact that AA need not penetrate as deeply into 15-LOX to position C13, rather than C10, for attack.

Comparison with Soybean Lipoxygenase L-1

As a rule, plant LOX enzymes are about 15-25 kD larger than animal enzymes, they utilize linoleic or linolenic acids (rather than AA) as a substrate, and they share <25% sequence identity with animal LOX. The lipoxygenase core represented by the animal enzyme structures defines the core structure common to all lipoxygenases. Given the similarities in terms of both protein fold and catalytic machinery of animal and plant LOX, a model that

provides a basis for product specificity in animal enzymes should also be relevant to the plant enzymes. Currently there are X-ray crystal structures for soybean L-1 (16, 17, 30), which is a 13-LOX with linoleic acid and a 15-LOX with arachidonic acid, and soybean L-3 (19), which catalyzes non-specific oxygenation of fatty acids and their esters, as well as two vegetative forms from soybean (20). There is no structure for a linoleate 9-LOX, an enzyme predicted to hold the substrate in the opposite head-to-tail orientation (31). The amino acids that envelop the pentadiene for attack in our model (L432, L628, and I424) are conserved in the plant enzymes. While the counterparts of L386 and L391 are substituted with Trp and Ala, respectively, in the plant enzymes. Although Trp narrows the channel, the accompanying substitution of Ala for Leu appears to compensate for this loss in volume. Klinman and co-workers(32, 33) have proposed that Leu 546 and Leu 754, the plant counterparts to Leu 432 and Leu 628, are shielding amino acids in soybean L-1. More recently Meyer et. al mutated I553 and found that conservative mutations dramatically affected enzyme activity (34). I553 corresponds to V439 in 8R-LOX. This amino acid is positioned at the C-terminal end of the arch that shelters the U-shaped channel and in our model is a peripheral channel amino acid that helps define the substrate binding cavity.

While there is not a channel *per se* apparent in the Soybean LOX structure, there is a cavity that can be accessed with a nominal conformational change. Furthermore, a sub-cavity which has been described as the O₂ access channel (illustrated in various plant LOX structures in a recent report (20)) appears to overlap with our U-shaped channel. The cavity does not have access to solvent as the additional polypeptide in the plant enzymes (relative to animal lipoxygenases) forms a β -hairpin that blocks channel access from what would correspond to the methyl end (the “back door”) in our model, and the carboxyl end of the putative channel is blocked by the placement of the counterpart of helix α 2. Minor et al. (17) previously suggested that T259, the counterpart of R183 on α 2 in 8R-LOX, can be moved to allow entry into the active site cavity. Helix α 2 is the only helix in the LOX core that has been described in differing positions in various LOX structures (20, 21) and this conformational variability is consistent with the suggestion that a repositioning of this element of secondary structure could allow entry into the internal cavity. Alternatively, access *via* the “back door” of the channel would require the movement of a β -hairpin common to the plant enzymes. There is ample precedent for such a conformational change, and it would not require the repositioning of additional structural elements. Thus one can envision tail-first entry from one of two possible ends of a substrate binding site that runs under the conserved arched helix in the plant enzymes as well.

An explanation for apparently anomalous substrate specificities

The “boot-shaped” cavity model imposes a major constraint on the physical size of substrates that could bind in the reverse orientation as it requires that the carboxyl group is innermost in the binding cavity. When the fatty acid substrates are provided as the corresponding esters, the internal cavity needs to accommodate a bulkier head group. Indeed, this criterion has been used in support of so-called “tail (methyl end)- first” versus “head (carboxyl end)- first” entry of substrate into the active site. LOX enzymes with a predicted “tail-first” substrate entry will specifically oxygenate not only the free fatty acid substrate, but also very large esters such as 1-palmitoyl-2-linoleoyl-phosphatidylcholine.

Furthermore, 5-LOX and 8S-LOX, predicted “head-first” enzymes, have not been found to specifically oxygenate large esters.

There are, however, notable exceptions that challenge the idea implicit in the “boot-shaped” cavity model that the channel penetrates deep into the protein core. The plant 9-LOX are predicted to bind substrate in the reversed orientation to 13-LOX/15-LOX enzymes. Because the 13-LOX/15-LOX all successfully oxygenate very large esters (phosphatidylcholine (PC), *etc*), in their case it is safe to place the PC moiety “outside” the protein core during catalysis, and thus it should be “inside” for plant 9-LOX and like enzymes. Clearly this would be hard to accommodate, and accordingly one would predict “no reaction”. Yet plant 9-LOX do successfully metabolize some bulky esters and amides, for example anandamide (an ethanolamide moiety) and 1-glycerol-linoleate (35, 36). These facts are more readily reconciled with a U-shaped substrate binding mode in which (it is predicted) the ester/amide moiety of these substrates lies “outside” the protein core at the other end of the channel.

A new light on R versus S stereo-control

One turn of the helix removed from L432 is G428, the amino acid that confers *R*-stereochemistry on the 8*R*-LOX product according to Coffa and Brash(37). Their observation that a Gly to Ala mutation at this position results in an enzyme that produces the 12*S* product can be explained in the context of the U-shaped channel. Recall that in our model AA is positioned with its carboxyl at R183 (Fig. 3) and L432 restrains the pentadiene at the Fe. As a consequence of its role in positioning the pentadiene for attack, L432 is also poised to shield the pentadiene with respect to oxygenation. The wild-type enzyme produces exclusively the 8*R*-product, thus L432 shields C12 from oxygenation. The presence of a C-β at residue 428, however, would serve to push L432 away from C12 and result in the leucine shielding C8 instead. In contrast, in the context of the boot-shaped cavity the Gly/Ala switch presents a paradox. How does the presence of the methyl group of Ala in the *R*-oxygen pocket (which is more superficial in the boot shaped cavity) redirect O₂ to a pocket at the *S*-position of a pentadiene (37, 38), which would lie deeper in the protein, past the catalytic iron? In the U-shaped channel, the Ala/Gly switch is centrally positioned (i.e. there is a single O₂ pocket) where it can potentially influence either side of the reacting pentadiene, and thus direct the oxygen molecule to the *R*- or *S*-position.

Concluding remarks: a “general” model for substrate binding—Taken together, the high resolution structure of 8*R*-lipxygenase from *Plexaura homomalla*, analysis of the impact of mutations on enzyme activity, and data in the literature, suggest a general model for substrate binding to lipxygenases in a U-shaped channel (Fig. 7). U-shaped channels have been proposed to bind the substrate of other fatty acid metabolizing enzymes: the ⁹ stearoyl-acyl carrier protein desaturase (39) and soluble epoxide hydrolase (40).

The essential feature of our model is that a conserved arched helix curves over a U-shaped channel lined with invariant leucines and isoleucines. The substrate can slide into this channel, and wrap around the arched helix, positioning the central carbon of a pentadiene above the catalytic iron. The conserved Leu and Ile side chains serve to guide and/or clamp

the substrate in place and the Gly/Ala switch is positioned such that only a single oxygen pocket is required. The LOX enzymes that utilize arachidonic acid must discriminate among three chemically equivalent carbons. The amino acids that set the register, and thus determine which pentadiene is attacked, are on the periphery of the U-shaped channel and would not be expected to be conserved among lipoxygenases catalyzing different regio-specific reactions.

Although the U-shaped channel in 8R-LOX is open at both ends, this need not be the case in other lipoxygenase. We predict each lipoxygenase isoform is defined by a single site of entry and that the channel is either closed at the opposite end or lacks an appropriately positioned side chain to tether the carboxylate of the substrate. The strict regio- and stereo-specificities of the LOX family members can be explained in this structural context. For either of the two possible orientations, the substrate carboxylate would be solvent exposed, circumventing the dilemma of “burying a charge” inherent in earlier models (41, 42). In summary, the discovery of the U-shaped channel in 8R-LOX has allowed us to present a new concept on substrate binding, one that is open to further evaluation in any of the lipoxygenase enzymes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

AA	arachidonic acid
LOX	Lipoxygenase
8R-HPETE	8R-hydroperoxyeicosatetraenoic acid
RP-HPLC	reversed phase high pressure chromatography

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Figure 1. Regio- and stereo- specificity in lipoxygenases

The relationship of iron-catalyzed hydrogen abstraction and oxygenation on the opposite face of the reacting substrate. Top: with arachidonic acid in this head-to-tail orientation, abstraction of 10-H_S (proS) gives either 8R or 12S oxygenation products. Below: 10-H_R (proR) abstraction leads to 8S or 12R products.

Figure 2. The structure of psWT, a soluble form of 8R-LOX

(a) Cartoon rendering of the enzyme. The arches helix that shelters the catalytic Fe (orange sphere) is shown in blue. An orange arrow indicates the location of the U-shaped channel.

(b) A cross section of the U-shaped channel which passes under the arched helix. Figures generated with PyMOL(43) and CastP(44).

Figure 3. The proposed AA binding site in 8R-LOX

(a) A modeled arachidonic acid in the proposed 8R-LOX active site. The site is lined with Leu and Ile side chains. The side chains of L432 and I433 are not visible in the electron density map and the thinner stick rendering corresponds to the most favored rotamer for these amino acids. Side chains identified by Evolutionary Trace(22) as invariant are in blue, while both invariant and highly conserved residues are numbered in blue text. The only residues unique to the 8R-enzyme are R183 and I427. The backbone of G428 is included in stick rendering (C, yellow, O, red, N, blue), and the conserved salt link in which R429 participates is depicted (C, white; O, red; N; blue). A modeled AA is rendered as a transparent surface (C, white; O, red). (b) A partial sequence alignment of lipoxygenases. I424 and L432, which line the putative recognition site, are invariant, while I433 is highly conserved. R429 is part of the invariant salt link that anchors the arched helix to the catalytic core. The conserved G localized in the arch of the helix is indicated in red. Taxonomic identifiers: 9CNID *Cnidaria*; RAT *Rattus norvegicus*; TETNG *Tetraodon nigroviridis*; HUMAN *Homo sapiens*; PONPY *Pongo abelii*; BOVIN *Bos taurus*; PIG *Sus scrofa*; RABIT *Oryctolagus cuniculus*; MOUSE *Mus musculus*; DANRE *Danio Rerio*; MESAU *Mesocricetus auratus*; XENTRO *Xenopus tropicalis*; AT *Arabidopsis thaliana*; SOYBEAN, *Glycine max*; TOMATO *Lycopersicon esculentum*.

Figure 4. Superposition of psWT, I433W:psWT and I433A:psWT

(a) Ribbon overlay of arched helices. There is no density for amino acids 432-436 in the I433A:psWT structure. (psWT, gold; I433W:psWT, teal; I433A:psWT, pink) (b) Detail of arched helix. (c) The overlay of the active sites reveals no structural differences among the mutants. Fe is shown as a transparent sphere.

Figure 5. A portion of an electron density map

A stereo rendering of the region of the Trp for Ile substitution in the 1.9 Å 2Fo-Fc electron density map of I433W:psWT, contoured at 2.0σ

Figure 6. A comparison of the boot-shaped and U-shaped sites for AA binding

(a) Amino acids that line the proposed 15-LOX (orange) and 8R-LOX (pink) sites. The helical arch (421-441) and helix that includes residues 367-401 are shown in tube rendering. The RS7 inhibitor which defines the rabbit site is in red stick, the iron a dotted sphere. **(b)** This view is rotated $\sim 90^\circ$ about the x-axis. Included in tube rendering (blue) are the 15-LOX peptides that have been reported to crosslink with a photoreactive substrate analog. Note that one of these peptides includes W145 and K145, amino acids which together with D411 (blue stick), plug the methyl end of the channel in 15-LOX. No amino acids from this peptide line the proposed 15-LOX binding site. **(c)** A stereo view of the context of the two cavities. The view is similar to that in (b) and looking from above the enzyme as rendered in Fig. 2a.

Figure 7. Schematic of active site features common to lipxygenases

Invariant Leu and Ile side chains (black stick) position the pentadiene (CPK rendering) for attack (numbered according to 8R-LOX). An invariant Leu (L432, replaced with Ala, Val, Ile and Phe in the psWT) is donated by the arched helix that defines the roof of the active site. The Leu is positioned to clamp the substrate in place above the Fe (red sphere). The direction (and register) of the substrate is set by a neutralizing amino acid (R183 in the 8R-enzyme) at one of the two ends of the channel.

Table 1

Data collection and refinement statistics

	psWT	I433W:psWT	I433A:psWT
Data collection			
Space Group	P2 ₁	P2 ₁	P2 ₁
Cell Dimensions			
<i>A, b, c</i> (Å)	103.98, 170.22, 104.50	103.70, 170.21, 104.90	104.36, 170.38, 104.90
α, β, γ (°)	90, 95.88, 90	90, 95.51, 90	90, 95.51, 90
Resolution (Å)	30 – 1.85	50 – 1.90	30–2.30
Rsym	0.068 (0.677)	0.086 (0.693)	0.124 (0.660)
I/ σ I	21.0 (2.32)	17.1 (2.31)	12.8 (2.27)
Completeness (%)	98.8 (98.0)	98.6 (100)	100 (100)
Redundancy	4.0 (4.0)	4.9(5.1)	4.9(4.7)
Refinement			
Resolution (Å)	30 – 1.85	35 – 1.90	30–2.30
No. reflections	287452	263905	150209
R _{work} / R _{free}	0.1740 / 0.2093	0.1591 / 0.2037	0.1675 / 0.2389
No. atoms			
Protein	22141	22299	21805
Ligand			34
Ion/glycerol/acetate	284	250	224
Water	2667	2485	1582
B-factors			
Protein	26	27.48	27.99
Ligand			43.37
Ion/glycerol/acetate	35.68	38.13	38.8
Water	37.26	38.31	30.84
Rms Deviations			
Bond lengths (Å)	0.015	0.016	0.023
Bond angles (°)	1.409	1.5	1.94

^aValues in parentheses are for the highest resolution shell^b $R_{\text{merge}} = \sum |I_i - \langle I \rangle| / \sum I_i$, where I_i is the intensity of the i th observation and $\langle I \rangle$ is the mean intensity of the reflection^c $R = \sum \|F_O - F_C\| / \sum F_O$, where F_O and F_C are the observed and calculated structure factor amplitudes, respectively. R_{free} was calculated using 5% of the total reflections.

Table 2**Mutagenesis**

<i>Activity</i>		
Enzyme	K _m (uM)	turnover (s ⁻¹)
WT	50± 5	206±8
L432A	33±12	12±1
L432F	-----	trace activity
L432I	18±4	7±1
L432V	28± 6	42±3
psWT		
I433A *	135± 4	19±3
I443W	nd	nd

<i>Regio-specificity</i>		
Enzyme	12 (%)	8 (%)
WT	2	98
L432A	10	90
L432F	34	66
L432I	3	97
L432V	1	99
psWT		
I433A	11	89

<i>Stereochemistry</i>				
	8R (%)	8S (%)	12R (%)	12S (%)
WT	100	0	10	90
L432A	99	1	3	97
L432F	95	5	7	94
L432I	99	1	8	92
L432V	100	0	----	----
psWT				
I433A	95	5	16	84

nd, not detected

* assay conditions include 0.05% emolphogen and 2 mM CaCl₂