Crystal Structure of a Lipoxygenase in Complex with Substrate

THE ARACHIDONIC ACID-BINDING SITE OF 8R-LIPOXYGENASE*

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Background: Lipoxygenases (LOX) catalyze the oxygenation of polyunsaturated fatty acids but generate distinct products from a common substrate.

Results: We report the first structure of a LOX-substrate complex.

Conclusion: The structure provides a context for understanding product specificity in enzymes that metabolize arachidonic acid.

Significance: With roles in the production of potent lipid mediators, LOX are targets for drug design.

Lipoxygenases (LOX)2 catalyze the highly regio- and stereo-specific reaction of polyunsaturated fatty acids with molecular oxygen, typically forming a single, chiral fatty acid hydroperoxide (1–3). The diversity is such that individual LOX enzymes are known that can account for oxygenation on almost all the available positions on the common polyunsaturated fatty acid substrates (3–5). The different members of the LOX superfam-

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‡ The abbreviations used are: LOX, lipoxygenase(s); AA, arachidonic acid; HETE, hydroxyeicosatetraenoic acid.
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FIGURE 1. Schematic of the current model for product specificity in lipoxygenases. Cavity depth and substrate orientation combine to confer regio- and stereo-specificity. The substrate (AA shown here) must align between the catalytic iron (red sphere) and O₂ channel (blue peanut). Differences in cavity depth and head-to-tail orientation determine the hydroperoxyeicosatetraenoic acid generated.

ferrous iron to the active ferric state by traces of peroxide, and (iii) the O₂ co-substrate is ubiquitous under standard conditions of crystallography. Therefore, a combination of these factors has thwarted attempts to halt the one-turnover transformation that will metabolize a substrate molecule in the LOX active site. Crystals of an 8R-LOX from Plexaura homomalla, which generates 8R-hydroperoxyeicosatetraenoic acid from AA, proved to be to sufficiently robust to survive the manipulations required to introduce the hydrophobic substrate at concentrations of AA and carrier that do not compromise diffraction quality, but are adequate for complex formation.

Combined with the previously reported structures of the mammalian lipoxygenases with inhibitors, the model provides a robust framework for the design of isoform-specific inhibitors. Furthermore, mutagenesis and activity assays support the proposed model.

MATERIALS AND METHODS

Mutagenesis—Mutants were constructed in pseudo-wild-type 8R-LOX (1) by whole plasmid polymerase chain reaction using Pfu Ultrall Hot Start polymerase (Agilent Technologies). Where possible, silent mutations were included in the primers for rapid identification of mutant plasmids. Plasmid DNA was purified using the Wizard system (Promega).

Protein Expression and Purification—Pseudo-wild-type 8R-LOX (19) and variants were expressed and purified essentially as described in the literature with the following modifications (11). BL21(DE3) cells were transformed with the appropriate pet3a construct. Single colonies were picked to grow overnight starter cultures in Luria Broth, 50 μg/ml ampicillin. The starter culture was then diluted 100× into 2× Yeast Tryptone and grown at 37 °C to an A₆₀₀ of 0.6, at which point the temperature was changed to 22 °C, and expression was induced with 0.25 mM isopropyl 1-thio-β-D-galactopyranoside. The cells were harvested after 22 h, frozen in liquid nitrogen, and stored at −80 °C. The frozen cells were resuspended in B-PER bacterial protein extraction reagent (Thermo Scientific) supplemented with Dnase, PMSF, leupeptin, and pepstatin, according to the manufacturer’s instructions, and sonicated at 5-s intervals and 50% power for 5 min. The cell lysate was centrifuged at 38,000 × g for 30 min at 4 °C. The supernatant was cleared with a 0.45-μm Millipore PES syringe filter, and applied on a Cohn–nitrilotriacetic acid column using AKTA FPLC. The 8R-LOX was eluted using a gradient of 20 mM to 200 mM imidazole in 20 mM Tris (pH 8.0) and 500 mM NaCl. The fractions containing the prominent peak of the absorbance chromatogram were pooled and dialyzed against 20 mM Tris (pH 8.0) overnight with three buffer exchanges. The dialyzed protein was then purified further using a Mono-Q column and AKTA FPLC and a salt gradient of 0 to 500 mM NaCl. Most of 8R-LOX eluted at ~250 mM NaCl. The purities of fractions were evaluated by SDS-PAGE and Coomassie staining; the fractions containing purest 8R-LOX were collected and concentrated to 5–15 mg/ml using Amicon Ultra centrifugal units.

Kinetic Assays—The kinetic measurements were performed on an Applied Photophysics Stopped Flow instrument SX.18MV, monitoring the increase in absorbance at 237 nm over time in the following buffer: 50 mM Tris (pH 7.4), 150 mM NaCl, 0.5 mM EDTA. The concentration of enzyme was 15 ng (native), 20 (R182A), or 200 nm (A589M and A620H). For each enzyme at least 10 AA (Cayman Chemical) concentrations, varying from 1–50 μM, were monitored. Initial rates were measured by fitting the average of four to five measurements of the initial linear region of absorbance change to a linear equation. Most of the variants were analyzed by using the Michaelis-Menten equation to determine Vₘₐₓ and Kₛ values; however, the R182A mutant was analyzed by using the reciprocal plot and substrate inhibition steady state kinetics using the equation shown below.

\[ v = \frac{V_{\text{max}}}{K_m + \frac{[S]^n}{K_i^n}} \]  

(Eq. 1)

Equation 1 has been used before to study other systems that involve cooperative substrate inhibition, and it was the model that gave the best fit to the experimental data (20–22). An extinction coefficient of 23 absorbance units/cm/mM (23) was used for product.
**Product Analyses**—Incubations were carried out in 1 ml of 50 mM Tris, pH 8.0, containing 500 mM NaCl, 2 mM CaCl₂, using 50 μM arachidonic acid (15 μg) added in 5 μl of ethanol. (10-mI incubations were carried out with the slowly reacting A589M and A620H mutants.) The samples were incubated at room temperature for 2–10 min, with the time depending on the rate of reaction observed by UV-235 nm. The incubations were extracted using a Waters 1-cc Oasis HLB cartridge and eluted with 1 ml of methanol. The extracts were treated with triphenylphosphine in methanol at room temperature for 30 min to reduce hydroperoxyicosatetraenoic acid to the corresponding hydroxynexatrienoic acid (HETE). The samples were analyzed (and the individual HETEs collected) by SP-HPLC using a Beckman Ultrasphere 5 μm silica column (250 × 4.6 mm) with a solvent system of hexane/isopropanol/glacial acetic acid (100:2:0.1, by volume) running at 1 ml/min, and the eluant was monitored with an Agilent 1100 diode array detector. The individual HETEs were then methylated with diazomethane, and the stereochemistry of the products (detected in the active site of chain C. An omit map, calculated after three cycles of coordinate refinement, inserted into the common active site to conceal it from bulk solvent. The 8R-Lipoxygenase (3FG1) described previously was used as the starting model for refinement. Iterative model building and refinement were run in PHENIX (28) and COOT (29). Four monomers are located in the asymmetric unit. Residues for the N-terminal His tag were modeled only where sufficient electron density was warranted (one N-terminal His for chains A, B, and D and three N-terminal His for chain C). In addition, residues 307–315 were not modeled in any of the chains, along with residue 316 in chain A, residues 305 and 306 in chain B, and residue 306 in chain D, due to lack of electron density in those regions. Residue 600 was not modeled in chain B, also due to a lack of electron density. Arachidonate was modeled into electron density present in the active site of chain C. An omit map, calculated after three cycles of coordinate refine-

| Data processing | PDB code | 4QWT |
|-----------------|----------|------|
| Wavelength (Å)  | 0.98     |
| Resolution range (Å) | 50–2.0 (2.11–2.0) |
| Space group     | P2₁      |
| Unit cell       | a, b, c (Å) | 104.0, 170.6, 104.7 |
| B (°)           | 95.4     |
| Total reflections | 914,097 (129,971) |
| Unique reflections | 242,473 (34,734) |
| Multiplicity    | 3.8 (3.7) |
| Completeness (%)| 99.5 (96.1) |
| Mean I/σ(I)     | 12.9 (1.9) |
| Wilson B-factor | 27.01    |
| Rmerge          | 0.102 (0.813) |
| Rfree           | 0.119 (0.951) |
| Rp(l,m)         | 0.061 (0.490) |
| CC1/2 (44)      | 0.996 (0.615) |

**Refinement statistics**

| Rwork (%)   | 16.21    |
| Rfree (%)   | 26.36    |
| No. of non-H atoms | 16,218   |
| Protein     | 21,834   |
| Ligands     | 242      |
| Water       | 1795     |
| Protein residues | 242,473 (34,734) |
| Ramachandran| 97       |
| Outliers (%)| 0.33     |
| Average B-factor | 29.30   |
| Protein     | 28.80    |
| Ligands     | 38.10    |
| Arachidonate| 38.96    |
| Solvent     | 34.40    |

**RESULTS**

**Invariant Amino Acids Position the Pentadiene for Attack**—The crystal structure 8R-LOX was determined to 2.0 Å resolution. Lipoxygenases are composed of an amino-terminal β-barrel domain and a largely α-helical catalytic domain, which is ~80% of the polypeptide (6) and harbors the catalytic iron. Those enzymes that metabolize AA are roughly 650 amino acids in length. Although in general the placement of elements of secondary structure in lipoxygenases is conserved, helix α2, which helps define the active site, has been observed in two strikingly distinct orientations: it can be a long single α-helix that runs the length of the catalytic domain or a broken helix in which some side chains are inserted into the common active site to concealing it from bulk solvent. Helix α2 is one long helix in 8R-LOX and substrate can enter without invoking a major conformational change.

The catalytic iron in 8R-LOX is positioned by three invariant His (384, 389, 570) side chains and the terminal main chain.
carboxylate of an invariant Ile (693). A large U-shaped cavity, in which Fe\(^{2+}\)/H11001 sits at the base of the U, is observed in the structure of 8R-LOX in the absence of substrate (30). An arched helix, kinked by a distinct two-amino acid insertion in one helical turn (first described in detail by Minor \textit{et al.} (31)), covers this site and contributes one of several invariant leucines that line the proposed substrate binding pocket.

In the structure of 8R-LOX with AA bound (Fig. 2, A and B) this “arched” helix (420–442) is displaced ever-so-slightly outwards (Ca–Ca distance $\approx 1.3$ Å for Ile-437) and the side chains of Leu-431 and Ile-437, which frame the insertion, are now ordered (Fig. 2, A and B). These amino acids make van der Waals contacts to secure the AA, Leu-431 with the central carbon of the pentadiene centered at C10. Ile-437 is positioned at the top of the “U” between the carboxyl head (C1) and C17 at the tail. These amino acids side chains fill the U from the side opposite of the catalytic iron, where just across from Leu-431 sits invariant Leu-627. Leu-627 and Leu-431 are equidistant from the substrate, and the two side chains appear to clamp the C10 pentadiene in place. The base of the U is positioned by invariant Leu-385 on one side, and the iron and His-384 and His-389 on the other. Leu-385 and the catalytic iron cradle the base of the U (Fig. 2C).

Pocket depth, which allows the tail to slide in so that C10 is attacked, appears to be conferred by Ala-620, which with Ala-589 makes for a very a deep cavity. Leu-381 and Val-428 define the wall of the cavity at C15, and the polar and charged amino acids Gln-380 and Asp-424 (highly conserved) flank the cluster of histidines that hold the iron in place. In the absence of AA, Tyr-181 and Arg-182 of helix $\alpha2$ participate in an interhelical charge cluster with Glu-430 of the arched helix. In the AA-bound structure, the AA carboxylate expands this charge network with only the slight shift in the arched helix described above and the reorientation of the side chain of Glu-430 to allow Arg-182 to interact with the substrate carboxylate as well as Glu-430.

**FIGURE 2. 8R-LOX with AA.** A, ribbon drawings of 8R-LOX with (blue) and without (gray) AA in the active site. Fe\(^{2+}\) is shown as a red sphere. The helical insertion described in all LOX structures is shown in violet. B, detail of the outward shift of the arched helix observed in the presence of AA. C, an omit map contoured $+2/-2\sigma$ (green/magenta) reveals clear electron density for AA. D, the steady state rate dependence on substrate concentration of 8R-LOX and the mutant R182A. 8R-LOX data were fit to the Michaelis-Menten equation, whereas the R182A data were fit to Equation 1. Equation 1 describes cooperative substrate inhibition for the fit, $n = 2.4 \pm 0.3$. 

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and the single amino acid substitution variants are shown in Table 2. The kinetic properties of the enzyme were dramatically altered by mutations deep in the cavity. Whereas the mutation of R182A had only a modest impact on product specificity, the introduction of a histidine at position 620 as compared with Met, His, Val, or Gln in other LOX structures (Table 3). Consistent with tail first entry, the deepest part of the binding site is formed by hydrophobic amino acids around the active site, which is exceptionally deep when compared with other LOX structures (Table 3). Consistent with tail first entry, the deepest part of the binding site is formed by hydrophobic amino acids around the active site, which is exceptionally deep when compared with other LOX structures (Table 3). Consistent with tail first entry, the deepest part of the binding site is formed by hydrophobic amino acids around the active site, which is exceptionally deep when compared with other LOX structures. The resulting free radical is delocalized along the pentadiene, and oxygenation occurs at the carbon +2 from the site of attack. Hydrogen abstraction and oxygenation occur on opposite faces of the substrate, and the structural basis for this relationship is clearly elucidated in the structure of 8R-LOX with AA. The substrate fills a U-shaped cavity, and at the base of the U, the catalytic iron is positioned by the side chains of conserved amino acids. Of the 16 amino acids that define this cavity and make contacts with the AA six are invariant: two His (384, 389) that also bind the iron, three Leu (385, 431, 627), and an Ile (627). All of these amino acids are positioned to envelope the pentadiene centered at C10 for attack. AA enters 8R-LOX tail first and slides deep into the active site, which is exceptionally deep when compared with that described for other LOX structures due to an Ala at position 620 as compared with Met, His, Val, or Gln in other LOX structures (Table 3). Consistent with tail first entry, the deepest part of the binding site is formed by hydrophobic amino acids (Fig. 3A). Moreover, the mutation of A589M, deep in the cavity, gives 11R-HETE, indicating attack at C13, rather than C10, as expected for a shallower cavity with tail-first entry of AA (Fig. 1 and Table 2). The introduction of a histidine at position 620 not only shortens the deepest part of the U-shaped cavity, but adds a positive charge, making it challenging for the substrate to bind in a tail-first orientation. As a result, the $K_m$ increases, and the turnover rate plummets. Similar to A589M, the major product inhibition kinetics. Prior to fitting the data to the substrate inhibition model, the $K_m$ of R182A was first determined by the double reciprocal plot of $1/V$ versus $1/[S]$ as described (32) and fitting a linear line to the data at intermediate substrate concentrations. The $K_m$ determined from the double reciprocal plot was then used as a constant in fitting the substrate inhibition curve using Equation 1.

**DISCUSSION**

**Cavity Depth as a Determinant of Product Formation**—The LOX reaction proceeds with the abstraction of hydrogen from the sp³ carbon of a pentadiene via a water molecule that fills the metal coordination sphere. The resulting free radical is delocalized along the pentadiene, and oxygenation occurs at the carbon +2 from the site of attack. Hydrogen abstraction and oxygenation occur on opposite faces of the substrate, and the structural basis for this relationship is clearly elucidated in the structure of 8R-LOX with AA. The substrate fills a U-shaped cavity, and at the base of the U, the catalytic iron is positioned by the side chains of conserved amino acids. Of the 16 amino acids that define this cavity and make contacts with the AA six are invariant: two His (384, 389) that also bind the iron, three Leu (385, 431, 627), and an Ile (627). All of these amino acids are positioned to envelope the pentadiene centered at C10 for attack. AA enters 8R-LOX tail first and slides deep into the active site, which is exceptionally deep when compared with that described for other LOX structures due to an Ala at position 620 as compared with Met, His, Val, or Gln in other LOX structures (Table 3). Consistent with tail first entry, the deepest part of the binding site is formed by hydrophobic amino acids (Fig. 3A). Moreover, the mutation of A589M, deep in the cavity, gives 11R-HETE, indicating attack at C13, rather than C10, as expected for a shallower cavity with tail-first entry of AA (Fig. 1 and Table 2). The introduction of a histidine at position 620 not only shortens the deepest part of the U-shaped cavity, but adds a positive charge, making it challenging for the substrate to bind in a tail-first orientation. As a result, the $K_m$ increases, and the turnover rate plummets. Similar to A589M, the major product
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**TABLE 3**

The amino acids that define the AA binding site in 8R-LOX and their counterparts in other LOX structures

| 8R-LOX | 11R-LOX (47%) | 5-LOX (38%) | 15-LOX-2 (33%) | 15-LOX-1 (30%) | 12-LOX (30%) |
|--------|--------------|-------------|----------------|----------------|--------------|
| 4QWT<sup>a</sup> | 3FG1<sup>a</sup> | 3O8Y<sup>a</sup> | 4NRE<sup>a</sup> | 2P0M<sup>a</sup> | 3RDE<sup>a</sup> |
| Tyr-178 | Phe-185 | Phe-177 | Phe-184 | Phe-175 | Phe-175 |
| Arg-182 | Gly-188 | Tyr-181 | Ala-188 | Leu-179 | Leu-179 |
| Gln-380 | Gln-369 | Gln-363 | Glu-369 | Gln-357 | Glu-357 |
| His-384 | 373 | 367 | 373 | 361 | 361 |
| Leu-385 | 374 | 368 | 374 | 362 | 362 |
| His-389 | 378 | 372 | 378 | 366 | 366 |
| Ile-423 | 412 | 406 | 412 | 400 | 400 |
| Gly-427 | Gly-416 | Ala-410 | Ala-416 | Ala-404 | Ala-404 |
| Leu-431 | 420 | 414 | 420 | 408 | 408 |
| Ile-437 | Ala-426 | Leu-420 | Val-426 | Ile-414 | Ile-414 |
| Val-438 | Ala-427 | Phe-421 | Val-427 | Phe-415 | Phe-415 |
| Thr-623 | Thr-623 | Met-606 | His-600 | 590 | 590 |
| Ile-626 | Ala-612 | Ala-606 | Ala-606 | Ile-593 | Ile-593 |
| Leu-627 | 613 | 607 | 610 | 597 | 597 |

<sup>a</sup> Protein Data Bank codes.

**FIGURE 3.** The AA-binding site. A, the substrate adopts a horseshoe shape in the U-shaped channel (stereo). The side chains of highly conserved amino acids line the base of the active site, along with Gly-427. Gln-430, part of an inter-helical charge cluster that includes the substrate carboxylate, is shown in line rendering. The Fe<sup>2+</sup> (transparent dark red sphere) is positioned behind the substrate. B, detail of the superposition of inhibitors (rotated 180° with respect to A) observed in 15-LOX-2 (red) 12-LOX (gold, C, red, O) and 15-LOX-1 (teal, C, red, O). The 15-LOX-2 and 12-LOX inhibitors conform to the AA placement, whereas the 15-LOX-1 inhibitor overlaps partially. The Fe<sup>2+</sup>, solid dark red sphere, is in front of the substrate.

is 11R-HETE along with minor amounts of predominantly 12S- and 15S-HETES, all consistent with a shallower tail-first entry into the active site (Table 2).

**The Importance of Substrate Tethering**—Whereas cavity depth determines how deep the AA slides into the active site, our data suggest that van der Waals contacts alone (the steric complementarity of the AA and the U-shaped active site) are not sufficient to adequately fix the geometry of the pentadiene chain that will line the base of the active site, along with Gly-427. Gln-430, part of an inter-helical charge cluster that includes the substrate carboxylate, is shown in line rendering. The Fe<sup>2+</sup> (transparent dark red sphere) is positioned behind the substrate. A, detail of the superposition of inhibitors (rotated 180° with respect to A) observed in 15-LOX-2 (red) 12-LOX (gold, C, red, O) and 15-LOX-1 (teal, C, red, O). The 15-LOX-2 and 12-LOX inhibitors conform to the AA placement, whereas the 15-LOX-1 inhibitor overlaps partially. The Fe<sup>2+</sup>, solid dark red sphere, is in front of the substrate.

Substrate inhibition has been studied in other enzymes (20, 35–37), and its analysis can be complex (32, 38, 39). We observed that the R182A data are best explained by cooperative substrate inhibition (Equation 1). In this case, the cooperativity observed may be due to an untethered hydrophobic substrate that can assume multiple non-productive conformations in the hydrophobic cavity and be retained there without having to reenter the aqueous milieu before positioning itself into an alternate conformation. Thus, we suggest that Arg-182 securely positions the substrate in a catalytically competent orientation at the active site machinery. The modest loss of product specificity (88% versus 98% 8-HETE), is consistent with this interpretation.

**Can Cavity Depth Be Predicted?**—The obvious question, given the structural similarity observed in the LOX family, is whether this 8R-active site is consistent with the other enzyme structures that have been reported. There are now structures for six AA-metabolizing LOX. In addition to the 8R-LOX, an 11R-, a 5S-, two 15S- (15-LOX-1 and 15-LOX-2), and 12S-lipoxygenases have been described (7, 9, 12, 40, 41). With the
exception of the 15-LOX-1/12-LOX pair, which share 79% sequence identity, pairwise identity for these enzymes is at most 47%. It is the 8R-/11R pair that has 47% sequence identity, and in terms of cavity depth, the most significant difference is presence of a Met in the latter enzyme, instead of Ala-620 (Table 3). The Met protrudes directly in U-shaped cavity and limits its depth so that C13 is positioned for attack in the 11R enzyme. For the 15-LOX-2 enzyme, which also must position C13 at the catalytic machinery, the back end of the cavity is shortened by Val-610 and a Leu at 607.

However, this simple relationship between amino acid side chain volume deepest in the U and cavity depth is not straightforward as overall sequence identity decreases. For instance, both porcine 12-LOX and 8R-LOX (~30% identity) are tail first enzymes that position C10 at the catalytic center; thus, they should have similar cavity depths. Yet 12-LOX has a Gln at the deepest part of the cavity (Ala-620 in 8R-LOX). Unlike the Met side chain in 11R-, the Gln lies perpendicular to the tail end of the cavity, resulting in cavity depth comparable with that in 8R-LOX.

Further complicating comparisons among these structures is the fact that porcine 12-LOX and rabbit 15-LOX-1 share ~80% identity and have only one conservative difference in the amino acids that line the U-shaped site (Table 3, Met/Leu-419). Yet in the rabbit enzyme, the AA can only slide deep enough to position C13, rather than C10 at the catalytic iron. One significant conformational difference in this highly homologous pair is a conspicuous constriction conferred by Phe-415 (Val-438 in 8R-LOX). In the 15-LOX-2 enzyme, which also must position C13 at the catalytic machinery, the back end of the cavity is shortened by Val-610 and a Leu at 607.

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![The Enzyme-Substrate Complex of 8R-Lipoxygenase](image)

**FIGURE 4. The antarafacial relationship between Fe^{2+} and a putative O_{2} access channel.** A, in 8R-LOX the direction of the channel is set by Gly-427 and the orientation of the shielding amino acid Leu-431. B and C, similar channels in 15-LOX-2 and 12-LOX. Note the presence of an Ala pushes the tubular opening deeper into the body of the enzyme. D, detail of the relationship between the Gly/Ala switch and the Leu-431 shielding residues. Note the absence of the side chain in the 8R-enzyme (blue) and positioning of Leu-431 (beige, C-2) of the AA unprotected. Blue, 8R-LOX; green, 15-LOX-2; beige, 12-LOX. In contrast, in the 15-LOX-2 and 12-LOX enzymes, the same carbon would be shielded by the Ala and the invariant Leu no longer shields C12 (C+2) at the opposite end of the pentadiene.
helix α2 (10, 40). Additionally, it was based on an inhibitor that lacks the structural flexibility of AA and so might include conformational changes not required for the natural substrate to enter. Subsequently, a U-shaped model was described (30), which included ~60% of the amino acids in Table 3; this model was flawed as well, as it set the direction of the tail of AA more in the direction of the O₂ channel. Eek et al. (7), tested both these models with the 11R structure, and the data are consistent with our 8R-LOX-AA structure. However, 11R-LOX is corked in the absence of substrate so the complete U shape could not be described in that enzyme either. The structure of 8R-LOX with substrate builds on these models to reveal a robust structural context for mechanistic studies that have provided tremendous insight into how similar enzymes can generate distinct products from a common substrate. It is clear that subtle structural differences among family members are not readily inferred with sequence information alone, and the design of isomorph-specific inhibitors that might lead to the development of novel therapeutics remains a challenge.

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REFERENCES
1. Yamamoto, S. (1992) Mammalian lipoxygenases: molecular structures and functions. Biochim. Biophys. Acta 1128, 117–131
2. Brash, A. R. (1999) Lipoxygenases: occurrence, functions, catalysis, and acquisition of substrate. J. Biol. Chem. 274, 23679–23682
3. Ivanov, I., Heydeck, D., Hofheinz, K., Roffeis, J., O’Donnell, V. B., Kuhn, H., and Walther, M. (2010) Molecular enzymology of lipoxygenases. Arch. Biochem. Biophys. 503, 161–174
4. Schneider, C., Pratt, D. A., Porter, N. A., and Brash, A. R. (2007) Control of oxygenation in lipoxygenase and cyclooxygenase catalysis. Chem. Biol. 14, 473–488
5. Andreou, A., and Feussner, I. (2009) Lipoxygenases: structure and reaction mechanism. Phytochemistry 70, 1504–1510
6. Boyington, J. C., Gaffney, B. J., and Amzel, L. M. (1993) The three-dimensional structure of an arachidonic acid 15-lipoxygenase. Science 260, 1482–1486
7. Eek, P., Järving, R., Järving, I., Gilbert, N. C., Newcomer, M. E., and Samel, N. (2012) Structure of a calcium-dependent 11R-lipoxygenase suggests a mechanism for Ca²⁺ regulation. J. Biol. Chem. 287, 22377–22386
8. Garreta, A., Val-Moraeus, S. P., García-Fernández, Q., Busquets, M., Juan, C., Oliver, A., Ortiz, A., Gaffney, B. J., Fita, I., Manresa, A., and Carpena, X. (2013) Structure and interaction with phospholipids of a prokaryotic lipoxygenase from Pseudomonas aeruginosa. FEBS J. 27, 4811–4821
9. Gilbert, N. C., Bartlett, S. G., Waight, M. T., Neau, D. B., Boeglin, W. E., Brash, A. R., and Newcomer, M. E. (2011) The structure of human 5-lipoxygenase. Science 331, 217–219
10. Gillmor, S. A., Villaseñor, A., Fletterick, R., Sigal, E., and Browner, M. F. (1997) The structure of mammalian 15-lipoxygenase reveals similarity to the lipases and the determinants of substrate specificity. Nat. Struct. Biol. 4, 1003–1009; Correction (1998) Nat. Struct. Biol. 5, 242
11. Oldham, M. L., Brash, A. R., and Newcomer, M. E. (2005) Insights from the x-ray crystal structure of coral 8R-lipoxygenase: calcium activation via a C2-like domain and a structural basis of product chirality. J. Biol. Chem. 280, 39545–39552
12. Xu, S., Mueser, T. C., Marnett, L. J., and Funk, M. O., Jr. (2012) Crystal structure of 12-lipoxygenase catalytic-domain-inhibitor complex identifies a substrate-binding channel for catalysis. Structure 20, 1490–1497
13. Kliman, J. P. (2007) How do enzymes activate oxygen without inactivating themselves? Acc. Chem. Res. 40, 325–333
14. Hamberg, M., and Samuelsson, B. (1967) On the specificity of the oxygenation of unsaturated fatty acids catalyzed by soybean lipoxygenase. J. Biol. Chem. 242, 5329–5335
15. Brash, A. R., Schneider, C., and Hamberg, M. (2012) Applications of stereospecifically-labeled Fatty acids in oxygenase and desaturase biochemistry. Lipids 47, 101–116
16. Kühn, H., Sprecher, H., and Brash, A. R. (1990) On singular or dual positional specificity of lipoxygenases. The number of chiral products varies with alignment of methylene groups at the active site of the enzyme. J. Biol. Chem. 265, 16300–16305
17. Egmond, M. R., Vliegenthart, J. F., and Boldingh, J. (1972) Stereospecificity of the hydrogen abstraction at carbon atom n-8 in the oxygenation of linoleic acid by lipoxygenases from corn germ and soya beans. Biochem. Biophys. Res. Commun. 48, 1055–1060
18. Coffa, G., and Brash, A. R. (2004) A single active site residue directs oxygenation stereospecificity in lipoxygenases: stereocontrol is linked to the position of oxygenation. Proc. Natl. Acad. Sci. U.S.A. 101, 15579–15584
19. Neau, D. B., Gilbert, N. C., Bartlett, S. G., Dassey, A., and Newcomer, M. E. (2007) Improving protein crystal quality by selective removal of a Ca²⁺ dependent membrane-insertion loop. Acta Crystallogr. Sect. F Struct. Biol. Cryst. Commun. 63, 972–975
20. Dewal, M. B., and Firestone, S. M. (2013) Site-directed mutagenesis of catalytic residues in N(5)-carboxyaminomimidazole ribonucleotide synthetase. Biochemistry 52, 6559–6567
21. LiCata, V. J., and Allewell, N. M. (1997) Is substrate inhibition a consequence of allosteric in aspartate transcarbamylase? Biophys. Chem. 64, 225–234
22. Willemoes, M., and Larsen, S. (2003) Substrate inhibition of Lactococcus lactis cytidine 5’-triphosphate synthase by ammonium chloride is enhanced by salt-dependent tetramer dissociation. Arch. Biochem. Biophys. 413, 17–22
23. Boutaud, O., and Brash, A. R. (1999) Purification and catalytic activities of the two domains of the allene oxide synthase-lipoxygenase fusion protein of the coral Plexaura homomalla. J. Biol. Chem. 274, 33764–33770
24. Schneider, C., Boeglin, W. E., and Brash, A. R. (2000) Enantiomeric separation of hydroxy eicosanoids by chiral column chromatography: effect of the alcohol modifier. Anal. Biochem. 287, 186–189
25. Kabsch, W. (2010) Integration, scaling, space-group assignment and post-refinement. Acta Crystallogr. D Biol. Crystallogr. 66, 133–144
26. Evans, P. (2006) Scaling and assessment of data quality. Acta Crystallogr. D Biol. Crystallogr. 62, 72–82
27. Evans, P., and Murshudov, G. N. (2013) How good are my data and what is the resolution? Acta Crystallogr. D Biol. Crystallogr. 69, 1204–1214
28. Adams, P. D., Afonine, P. V., Bunkóczi, G., Chen, V. B., Davis, I. W., Echols, N., Headd, J. J., Hung, L. W., Kapral, G. J., Grosse-Kunstleve, R. W., Mc- Coy, C. J., Nicholls, D. G., Pannu, N. S., Read, R. J., Sokolov, A. P., Storoni, L. C., Oeffner, R., Read, R. J., Richardson, D. C., Richardson, J. S., Terwilliger, T. C., and Zwart, P. H. (2010) PHENIX: a comprehensive Python-based system for macromolecular structure solution. Acta Crystallogr. D Biol. Crystallogr. 66, 213–221
29. Emseley, P., and Cowtan, K. (2004) Coot: model-building tools for molecular graphics. Acta Crystallogr. D Biol. Crystallogr. 60, 2126–2132
30. Neau, D. B., Gilbert, N. C., Bartlett, S. G., Boeglin, W., Brash, A. R., and Newcomer, M. E. (2009) The 1.85 Å structure of an 8R-lipoxygenase suggests a general model for lipoxygenase product specificity. Biochemistry 48, 7906–7915
31. Minor, W., Steczko, J., Stee, B., Otwinowski, Z., Bolin, J. T., Walter, R., and Axelrod, B. (1996) Crystal structure of soybean lipoxygenase L-1 at 1.4 Å resolution. Biochemistry 35, 10687–10701
32. Lineweaver, H., and Burk, D. (1934) The determination of enzyme dissociation constants. J. Am. Chem. Soc. 56, 658–666
33. Peng, S., and van der Donk, W. A. (2003) An unusual isotope effect on
substrate inhibition in the oxidation of arachidonic acid by lipoxygenase. *J. Am. Chem. Soc.* **125**, 8988–8989

34. Berry, H., Debat, H., and Larreta-Garde, V. (1997) Excess substrate inhibition of soybean lipoxygenase-1 is mainly oxygen-dependent. *FEBS Lett.* **408**, 324–326

35. Chen, C., Joo, J. C., Brown, G., Stolnikova, E., Halavaty, A. S., Savchenko, A., Anderson, W. F., and Yakunin, A. F. (2014) Structure-based mutational studies of substrate inhibition of betaine aldehyde dehydrogenase BetB from *Staphylococcus aureus*. *Appl. Environ. Microbiol.* **80**, 3992–4002

36. Szegletes, T., Mallender, W. D., Thomas, P. J., and Rosenberry, T. L. (1999) Substrate binding to the peripheral site of acetylcholinesterase initiates enzymatic catalysis: substrate inhibition arises as a secondary effect. *Biochemistry* **38**, 122–133

37. Ziegler, J., Brandt, W., Geissler, R., and Facchini, P. J. (2009) Removal of substrate inhibition and increase in maximal velocity in the short chain dehydrogenase/reductase salutaridine reductase involved in morphine biosynthesis. *J. Biol. Chem.* **284**, 26758–26767

38. Kühl, P. W. (1994) Excess-substrate inhibition in enzymology and high-dose inhibition in pharmacology: a reinterpretation [corrected]. *Biochem. J.* **298**, 171–180

39. Marangoni, A. G. (2003). in *Enzyme Kinetics: A Modern Approach*, Wiley-Interscience. pp 184–190

40. Choi, J., Chon, J. K., Kim, S., and Shin, W. (2008) Conformational flexibility in mammalian 15S-lipoxygenase: Reinterpretation of the crystallographic data. *Proteins* **70**, 1023–1032

41. Kobe, M. J., Neau, D. B., Mitchell, C. E., Bartlett, S. G., and Newcomer, M. E. (2014) The structure of human 15-lipoxygenase-2 with a substrate mimic. *J. Biol. Chem.* **289**, 8562–8569

42. Knapp, M. J., Seebeck, F. P., and Klinman, J. P. (2001) Steric control of oxygenation regiochemistry in soybean lipoxygenase-1. *J. Am. Chem. Soc.* **123**, 2931–2932

43. Borngräber, S., Browner, M., Gillmor, S., Gerth, C., Anton, M., Fletterick, R., and Kühn, H. (1999) Shape and specificity in mammalian 15-lipoxygenase active site. The functional interplay of sequence determinants for the reaction specificity. *J. Biol. Chem.* **274**, 37345–37350

44. Karplus, P. A., and Diederichs, K. (2012) Linking crystallographic model and data quality. *Science* **336**, 1030–1033