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Review

A comprehensive model of positional and stereo control in lipoxygenases ☆

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Abstract

The lipoxygenase gene family can synthesize an array of chiral hydroperoxy derivatives from polyunsaturated fatty acids. An individual enzyme, however, reacts molecular oxygen on a single position on the carbon chain and in a single stereo configuration. Regiospecificity is regulated by the orientation and depth of substrate entry into the active site. Stereospecificity is a different issue and only recently has experimental support emerged to explain the conceptual basis of stereo control. A key determinant is a single active site residue conserved as an Ala in *S* lipoxygenases and a Gly in *R* lipoxygenases; this residue controls *R* or *S* stereochemistry by switching the position of oxygenation on the reacting pentadiene of the substrate. In this review, we meld together the factors that control product regio- and stereochemistry into a general model that can account for the specificity of individual lipoxygenase reactions.

Keywords: Lipoxygenase; Stereospecific; Linoleic acid; Arachidonic acid; Structure–function; Substrate orientation

The lipoxygenase (LOX) gene family exhibits a remarkable scope for the production of specific fatty acid hydroperoxides of high chiral purity. Via non-enzymic autoxidation, one of the prototypical polyunsaturated fatty acids, arachidonic acid, is oxygenated to a mixture of 12 distinct mono-hydroperoxide derivatives (six pairs of enantiomers). Individual LOX enzymes, on the other hand, typically form a single distinct hydroperoxide enantiomer. Of the 12 theoretical possibilities from arachidonic acid, enzymes with a distinct oxygenation specificity have been identified that can account for nine of the available possibilities. Namely, LOX enzymes have been characterized with 5*R* specificity, or 5*S*, or 8*R*, or 8*S*, or 9*S*, or 11*R*, or 12*R*, or 12*S*, or 15*S* specificity [1–8].

The challenge of putting together a model that can account for the diversity of LOX enzyme specificities is heightened by the close conservation of structure within the LOX family. This is especially true around the active site. The main catalytic domain of LOX enzymes contains a single non-heme iron held in place by a conserved group of amino acid ligands, predominantly histidines plus the C-terminal carboxyl group of the polypeptide [9]. Based on sequence alignments it is clear that this center of catalysis is of very similar structure throughout the LOX gene family. With the catalytic machinery largely in the same place, therefore, how is it possible to synthesize such a range of specific products including individual hydroperoxides that are the mirror image of each other? It is partly because there are so many possibilities that have to be accounted for, that changes in the modes of substrate binding in the active site have to be included in any modeling of the different LOX reactions. In the following account, the development of a universal model that can rationalize the

☆ *Abbreviation:* LOX, lipoxygenase.

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distinct specificities of LOX catalysis is given partly in a historical context of what ideas came when.

Lipoxygenase X-ray crystal structure

Crystallization of soybean lipoxygenase-1 was first reported in 1947 [10], although it was not until the 1990s that crystals suitable for X-ray analysis were prepared and the three-dimensional structure solved [11,12]. Currently also reported are the crystal structures of soybean LOX-3 [13] and rabbit reticulocyte 15-LOX-1 [14]. The overall LOX fold consists of a N-terminal eight-stranded antiparallel β-barrel domain and the larger catalytic domain formed predominantly by α-helices (Fig. 1A). The β-barrel is not essential for catalysis; it is related in structure to the C2-like domains in lipases, and as such is implicated in membrane interactions and acquisition of substrate [15,16]. Despite a difference in size between animal and plant LOX (75–80 and 94–104 kDa, respectively), their overall structure is very similar, with the exception of some superficial extra helices in the plant enzymes. The similarity is even higher in the catalytic domain in region of the active site, with the iron ligands almost super-imposable (Fig. 1B). The iron ligands are in an octahedral arrangement with one position "open" and with sufficient space to allow for the approach of substrate. Currently, there are no LOX structures available with bound substrate, and, given the inherent flexibility of fatty acid carbon chains, this leaves many uncertainties associated with the positioning of the substrate during catalysis.

Insights from substrate-product transformations

Well before it was possible to understand or manipulate LOX enzyme structure, many fundamental concepts of lipoxygenase catalysis were developed from study of substrate transformations. A fundamental tenet of the LOX mechanism is that the reaction is initiated by stereospecific removal of a hydrogen from the CH_2 group between two *cis* double bonds, and is completed by the reaction with O_2 on the opposite face of the substrate [17]. This antarafacial relationship between hydrogen abstraction and oxygenation is a universal facet of LOX catalysis, holding true in the synthesis of *R* or *S* hydroperoxide products [4,18]. All concepts on the basis for positional and stereo control have to be made compatible with this antarafacial "rule." In different LOX enzymes, no doubt the active sites have modifications to effect specific reactions, but with the basic enzyme structure conserved, it is re-alignment of substrate that permits the range of specific oxygenations found within the LOX gene family. Reversed substrate orientation and frameshift repositioning are key to this flexibility.

Earlier experiments comparing the metabolism of linoleic acid by corn LOX and soybean LOX showed that the two enzymes abstract the opposite C-11 hydrogen in forming their respective 9*S*-hydroperoxide and 13*S*-hydroperoxide products; reversed substrate orientation in the two enzymes provided a neat rationalization, while conserving the fundamental enzymic mechanism (Fig. 2) [19]. The same idea helps simplify the understanding of many other LOX reactions, including the catalysis of multiple stereospecific oxygenations by an individual LOX enzyme [9,20], and the switching of 8*S* and 15*S* reaction specificities by site-directed mutagenesis [21,22].

Some individual LOX enzymes synthesize two chiral hydroperoxide products. Prototypical examples of such dual positional specificity lipoxygenases are the enzyme known as the "leukocyte-type" of 12-LOX and its homologue called 15-LOX-1 [7,23]. Each forms a mixture of 12*S* and 15*S* hydroperoxides from arachidonic acid (the main one in each case giving rise to the

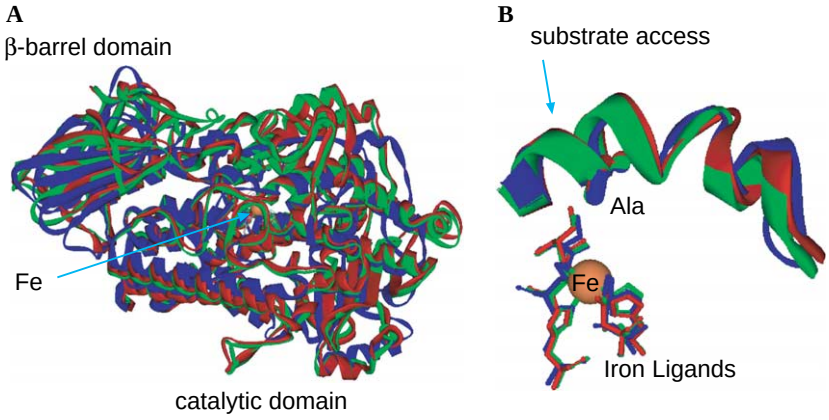

Fig. 1. Structural conservation of lipoxygenases. (A) Superimposed X-ray structures of Soybean LOX-1 (red), soybean LOX-3 (green), and rabbit reticulocyte 15-LOX-1 (blue). (B) The X-ray structures were stripped of most of the amino acids to show the relative position of the Iron (orange), the iron ligands, and the Ala residue (discussed later). The structures used were 1F8N for soybean LOX-1, 1LK3 for soybean LOX-3, and 1-LOX for rabbit reticulocyte 15-LOX-1.

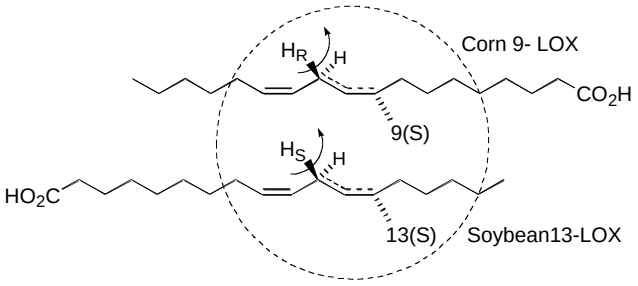

Fig. 2. Reversed orientations of substrate in two LOX active sites conserve the spatial elements in catalysis [19]. Corn 9-LOX (top) removes the 11-*pro-R* hydrogen from linoleic acid and catalyzes 9*S* oxygenation. Soybean 13-LOX (below) removes the other hydrogen, 11-*pro-S*, and catalyzes 13*S* oxygenation.

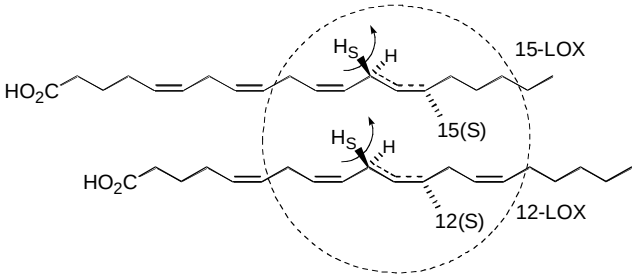

Fig. 3. "Frameshift" re-alignment of substrate in the active sites of 15-LOX and 12-LOX conserves the spatial elements in catalysis [25].

enzyme's name). A frameshift re-positioning of substrate provides a ready explanation (Fig. 3). This concept was strongly substantiated by experiments with synthetic fatty acids having frameshifted double bonds [24], and by mutagenesis experiments founded on conserved differences between 12-LOX and 15-LOX sequences [25]. By changing conserved differences between 12-LOX and 15-LOX, Sloane and colleagues showed that mutation of larger residues in 15-LOX-1 (Ile418 and Met419) to the smaller equivalents in human 12-LOX (Val and Ala) converted the enzyme specificity from 15*S* to 12*S* [25]. It is now confirmed from the X-ray structure of 15-LOX-1 that the key amino acids are located at the interior end of the substrate-binding channel and regulate the in-to-out alignment during catalysis [26].

Compelling arguments to support altered substrate orientations

While tail-first entry of polyunsaturated fatty acids into the LOX active site has always been readily accepted, the concept of carboxylate end-first entry has met with more of a challenge based on the supposed difficulty of accommodating a charged carboxylate group within the active site. The physico-chemical properties of long chain fatty acids are, however, quite compatible with carboxyl end-first binding. The free acids are quite soluble in hydrophobic solvents such as benzene and hexane. In such a hydrophobic environment, ionization of the carboxylate group is suppressed and the protonated COOH moiety is readily dissolved. There is also no problem in transferring the free acids from an aqueous to a hydrophobic environment within the typical range of physiological pH. The pK_a of long chain fatty acids is high, measured as around pH 8 in concentrated solution [27], with an apparent tendency to drop towards pH 7 at high dilution (10 μM) [28]. Either way, there will be a large percentage of uncharged molecules at or near neutral pH. These uncharged molecules can easily travel into the hydrophobic LOX active site where there should be no tendency for re-ionization to occur.

The interaction of fatty acid and esterified substrates with LOX enzymes also provides compelling evidence for the carboxyl end-first orientation as one mode of substrate binding: (i) The ability of a single enzyme, typified by soybean LOX-1, to catalyze specific 15*S*, 5*S*, and 8*S* oxygenations [9,20,29]. (ii) A closely related issue, the improved 5*S* oxygenation of methyl ester substrates compared to the free fatty acids by 15*S*-LOX [30]. (iii) That several LOX enzymes which we would propose to use carboxyl end-first binding show a pH optimum at low pH (∼pH 6), and thus are optimized to function with a high percentage of uncharged substrate molecules [31–33]. (iv) That a pH-dependent change in product profile with soybean LOX-1, switching from a product of tail-first binding (13*S*-hydroperoxy-linoleic acid) to carboxylate end-first binding (9*S*-hydroperoxy), parallels the pH-dependent suppression of ionization [34]. (v) That there is no such pH dependence in metabolism of esters [35]. (vi) That the metabolism of phosphatidylcholine esters of polyunsaturated fatty acids (too large to be accommodated by carboxylate end-first binding) is observed only for enzymes with a proposed tail-first substrate binding (8*R*-LOX, 12*S*-LOX, and 15*S*-LOX) and not for reactions that require the reversed orientation (5*S*-LOX, 8*S*-LOX, and 12*R*-LOX) [36–38].

An explanation for *R* or *S* chirality: switching oxygenation to opposite ends of a pentadiene

All the examples quoted so far involve changes in the regio-specificity of LOX enzymes and its rationalization in terms of substrate binding. In every case, the original *S* chirality of the hydroperoxide products is preserved. How can the different *R* or *S* stereospecificities be explained? To address this question, we searched for conserved differences between *R* and *S* lipoxygenases in residues in and around the active site. This resulted in identification of a candidate residue conserved as an Ala in *S*-LOX and Gly in *R*-LOX [38]. The conserved difference was consistent among dozens of sequences

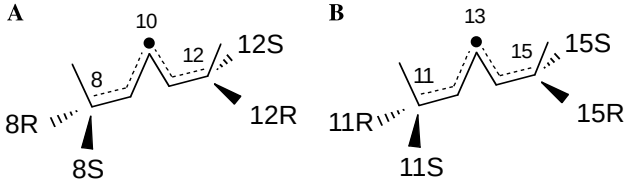

Fig. 4. Relationship between *R* and *S* stereospecificity in lipoxygenases. Reaction is illustrated at the 8,11 (A) and 11,14 (B) double bonds of a polyunsaturated fatty acid substrate such as arachidonic acid. In a lipoxygenase catalyzed reaction, following the initial hydrogen abstraction, oxygen can react on the opposite face of the substrate at one or other end of the pentadiene radical. Thus, there is a relationship between 8*S*/12*R* and 8*R*/12*S* (A), and in the same way between 11*R*/15*S* and 11*S*/15*R* (B). Reproduced from [38].

of *S*-LOX and the four available sequences of *R*-LOX. Our results with mutation of the conserved Ala to Gly in two *S*-LOX (mouse 8*S*-LOX, human 15-LOX-2) and the corresponding Gly-Ala substitution in two *R*-LOX (human 12*R*-LOX, coral 8*R*-LOX) revealed that the basis for *R* or *S* stereocontrol also involves a switch in the position of oxygenation on the substrate. In 8*S*-LOX, for example, the Ala-Gly switch changed the product profile from arachidonic acid substrate from purely 8S-hydroperoxide to a mixture of 8*S*- and 12*R*-hydroperoxides. The 8*S* and 12*R* positions on the fatty acid carbon chain share a spatial relationship of being at opposite ends of the same pentadiene unit involved in the LOX reaction and associated with the same hydrogen abstraction from the 10-carbon, Fig. 4. Equivalent findings pertained in the Ala-Gly mutated 15*S*-LOX (giving 11*R* and 15*S* hydroperoxide products, cf. Fig. 4), as well as in the Gly-Ala mutated 8*R*-LOX (changed largely to 12*S* product), and 12*R*-LOX (8*S* and 12*R* products).

The Ala-Gly residue is located opposite to the nonheme iron with part of the substrate-binding cavity in between (see Fig. 1B). This position is suitable to influence oxygenation on the side of the pentadienyl radical closer to the surface of the enzyme. As it turns out, in putting together a model that can rationalize all known possibilities, this more superficially located end of the reacting pentadiene is always associated with *R* chirality oxygenation, whereas *S* chirality oxygenation always occurs on the other end of the pentadiene, lying deeper in the active site pocket.

Regio- and stereospecificity are linked: toward a general model of LOX catalysis

So, *R* and *S* chiralities can be interchanged by site-directed mutagenesis, but never directly by switching from one enantiomer to the other (not by going from 8*R* to 8*S*, for example). To make a credible model that can account for all possibilities, the relatively new concept of a functional relationship between 8*S*/12*R* or 8*R*/12*S* has to be melded together with changed orientations of substrate in the active site of different LOX enzymes (Fig. 5) [38]. Although this model has a daunting and intricate appearance, some simple generalities can be dissected out of it. As noted, the Ala or Gly residue is situated near the entrance to the active site. In *R*-LOX, it is always the smaller Gly, and oxygenation on that near side of the pentadiene is favored, giving *R* specificity. By contrast, in *S*-LOX the larger Ala takes up space, and oxygenation deeper in the pocket is favored, giving *S* specificity. To change from 8*R* to 8*S*, for example, a reversed orientation of substrate is brought into the picture. This also switches the hydrogen presented to the activated iron for abstraction (and, in agreement with the facts mentioned earlier, the different hydrogen abstraction in 8*R*- and 8*S*-LOX is well established [18]). In sum, this model of LOX

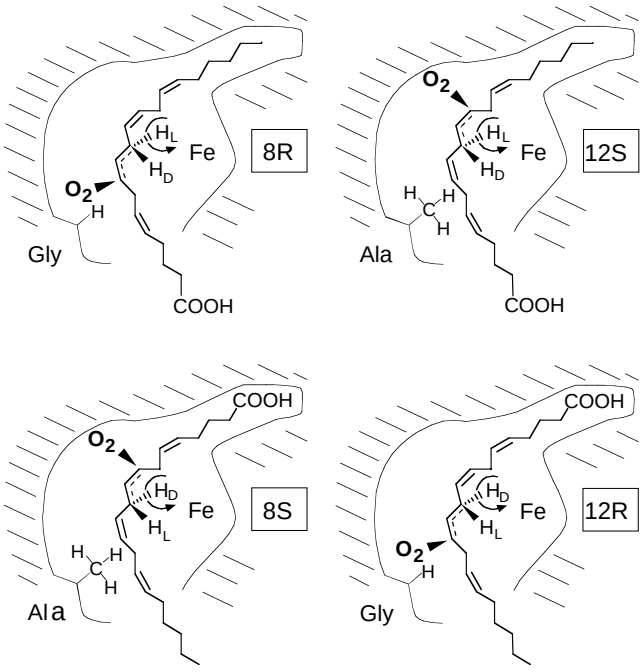

Fig. 5. A basis for *R* or *S* stereospecificity in the lipoxygenase active site. Formation of four different products is represented in lipoxygenase active sites of related structure. In 8*R*-LOX (top left) and 12*S*-LOX (top right), arachidonic acid has the same tail-first orientation in the active site and the reaction is initiated with the same L hydrogen abstraction from one face of the substrate molecule. A Gly residue in the critical position in the proximal area of the active site allows antarafacial oxygenation at the proximal end of the reactive pentadiene in the 8*R* configuration (top left). Substitution with the larger Ala residue prevents oxygen insertion at C-8 and instead the antarafacial oxygenation occurs deep in the binding pocket in the 12*S* configuration (top right). Below, in 8*S*-LOX and 12*R*-LOX, the substrate binds in the reverse orientation with the carboxyl end in the active site, allowing removal of the D hydrogen from C-10. A Gly residue allows oxygenation at the proximal end of the reacting substrate in the 12*R* configuration (lower right), whereas the larger Ala residue prevents this and the antarafacial oxygenation occurs deeper in the active site in the 8*S* configuration (lower left). Reproduced from [38].

reaction specificity proposes that product regio- and stereochemistry is determined by fixed relationships between substrate orientation, hydrogen abstraction, and the Gly or Ala residue we have identified [38].

Control of molecular oxygen and where it reacts

From a global perspective, this model can account for how polyunsaturated fatty acid substrates interact with LOX enzymes and get converted to one of several specific hydroperoxide products. What remains to be explained are molecular details of the enzyme–substrate interactions that direct the stereospecific hydrogen abstraction and the stereospecific oxygenation. We are particularly interested in the mechanism(s) that control the site of the specific reaction with O_2. There is no evidence for binding of O_2 to active site amino acids (none in the vicinity are suitable) nor to the non-heme iron [39,40]. What, therefore, controls the targeting of O_2 to a specific position on an activated pentadiene radical? The possibility of oxygen channeling through the protein is one potential means of achieving the targeting of O_2 onto a particular carbon of the reacting fatty acid radical [11,41–43]. The influence of protein residues in shielding some of the reactive carbons must also figure into the control of product regiochemistry and stereochemistry. The critical Ala or Gly residue is postulated to control oxygenation in the near side of the binding pocket, but what allows or prevents reaction deeper in the pocket remains to be established [38]. New insights from X-ray structures of *R*-LOX and of LOX enzymes with bound substrates or substrate analogues will help probe these unresolved and fascinating mechanistic issues.

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