Minireview

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Lipoxygenases: Occurrence, Functions, Catalysis, and Acquisition of Substrate*

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Lipoxygenase reactions may initiate the synthesis of a signaling molecule or be involved in inducing structural or metabolic changes in the cell. For signaling, synthesis of a single fatty acid hydroperoxide is required. For inducing structural changes, synthesis of a particular product may not be so important as the ability to induce what amounts to an enzyme-catalyzed lipid peroxidation. Reflecting these different functions are lipoxygenases with different characteristics in catalysis. There are enzymes that tightly control the reaction with molecular oxygen and others that form mixed products and permit the release of free radicals. In this review the diversity of lipoxygenase expression will be highlighted and the several facets of lipoxygenase function considered, concluding with a discussion of issues related to the acquisition of substrate in a cellular environment.

The Lipoxygenase Superfamily

Occurrence—Lipoxygenases are found widely in plants, fungi, and animals (1–5). The suitable substrates are polyunsaturated fatty acids containing a series of *cis* double bonds. These are the essential fatty acids in humans. These substrates are not present in most bacteria (cyanobacteria and some marine species excepted (6, 7)), and yeast also lack the necessary desaturases for their synthesis. In accord with this absence of substrate, there is no lipoxygenase in the yeast genome (*Saccharomyces cerevisiae*), and lipoxygenases are also absent from typical prokaryotes. There is no definitive account of a lipoxygenase in insects, although a typical arachidonic acid-derived lipoxygenase product (hydroxyeicosatetraenoic acid (HETE)[1]) is identified in the primitive insect *Thermobia domestica* (8). There is a lipoxygenase in the unicellular *Chlorella* (9) and a partial lipoxygenase cDNA sequence in the data bases from the slime mold *Dictyostelium discoideum*. Higher plants contain multiple lipoxygenases with at least eight identified in soybean, *Glycine max*. In the mouse there are seven genes that express lipoxygenase proteins, and five homologues (and an expressed pseudogene) are characterized in humans (10–12).

Lipoxygenases are expressed in some plant and animal tissues in high levels; they constitute a few percent of the protein in soybeans, and a 15-lipoxygenase (15-LOX) represents one of the main proteins besides hemoglobin in rabbit reticulocytes during anemia (13). Lipoxygenase expression may also be more subtle and low level, as in the cell-specific expression of specific isozymes in soybean leaves (14) or the discrete expression of distinct lipoxygenases in mammalian skin (*e.g.* Ref. 15).

The phylogenetic tree separates the plant and animal enzymes and forms several subgroups within each kingdom (Fig. 1). The formation of a particular lipoxygenase product is not necessarily associated with closely related sequences. For example, the soybean L-1 enzyme, a 15-LOX, has only 25% identity to any mammalian 15-lipoxygenase, and the two human 15-LOX share only 35% identity with each other. By contrast, the close functional homologues across species, forming distinct subgroups, share 70–95% sequence identity.

Nomenclature—In practical usage this is based on the specificity of the enzyme acting on its substrate, and although this can become slightly awkward, it conveys a simple and useful message. 12-LOX oxygenates arachidonic acid at carbon-12, and when necessary, the stereoconfiguration is specified (12*R*-LOX or 12*S*-LOX (Scheme 1)). The differing chain lengths of the most common substrates of plants (linoleate, linolenate, 18-carbon) and animals (arachidonate, 20-carbon) result in a plant 13-LOX corresponding to a mammalian 15-LOX; these particular lipoxygenases "count" the substrate carbons from the tail end of the chain, and both react oxygen at the ω-6 position. Complications can arise, for example, when there is more than one 12-LOX in the same species. To get around this problem, currently the mammalian 12-lipoxygenases are named after the prototypical tissues of their occurrence (hence, the platelet, leukocyte, or epidermal type of 12-LOX (5)). These are distinct enzymes by sequence, catalytic activities, and function. Some lipoxygenases may form a mixture of products, *e.g.* the mammalian reticulocyte type of lipoxygenase catalyzes C-12 and C-15 oxygenation, with the relative proportions varying among species. In rabbits and humans the major product is 15-HPETE, and hence the enzyme is designated a 15-LOX. The most closely related enzyme in the rat, mouse, pig, and cow is the leukocyte type of 12-LOX, an enzyme that catalyzes mainly C-12 oxygenation with some reaction also at C-15 (5).

Role of Lipoxygenases and Their Products

Formation of Biological Mediators/Signaling Molecules

This is usually characterized by synthesis of *a single specific hydroperoxide* from free fatty acid substrate (Fig. 2). Two subcategories are recognized with the lipoxygenase product either an intermediate or end product in the metabolic pathway.

The Lipoxygenase Product as an Intermediate in a Pathway—Some examples are jasmonic acid and aldehyde biosynthesis in plant signaling (1) and leukotriene or lipoxin synthesis in vertebrate animals (16, 17). In human beings, activation of the 5-LOX of leukocytes produces the leukotrienes and these lipid-peptide conjugates and dihydroxyeicosanoids provoke bronchoconstriction and inflammation. Current medications for asthma include 5-LOX inhibitors and leukotriene receptor antagonists (18). Synthesis of these end products represents the best recognized and most firmly established functions of lipoxygenases.

The Lipoxygenase Product (or Its Reduced Hydroxy Derivative) as End Product—An example is 12-HETE synthesis by the platelet 12*S*-LOX. Numerous biological activities are ascribed to individual HETEs and HPETEs, and the weight of evidence now suggests these products act as discrete signaling molecules.

Fast, Potent Actions—Here the actions are mediated at the cell surface on receptors or channels, and the HETE bioactivity is evident in the nanomolar range. In modulating neurotransmission, 12-HETE and its derivatives act fast (19). The 5-HETE oxidation product 5-oxo-eicosatetraenoic acid has instant receptor-mediated actions on calcium fluxes (20), and the potent effects of 12*S*-HETE on cell adhesion, an activity linked to metastasis in prostate carcinoma (21), are considered to act through cell surface signaling and activation of protein kinase C (22). There is evidence of a G-protein-coupled 12-HETE receptor in melanoma cells (23).

Slow Actions—It is striking how the platelet 12-LOX keeps making product for hours (24), whereas the platelet cyclooxygenase generates a short burst of products and is inactivated. An inference is that 12-HETE may modulate longer term events rather than fast responses such as platelet aggregation. Effects on cell differentiation or survival are examples (25, 26).

HETEs as Nuclear Receptor Ligands?—The search for natural

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[1] The abbreviations used are: HETE, hydroxyeicosatetraenoic acid; HPETE, hydroperoxyeicosatetraenoic acid; LOX, lipoxygenase(s); PPAR, peroxisome proliferator-activated receptor; SH3, Src homology 3 domain; FLAP, 5-lipoxygenase-activating protein.

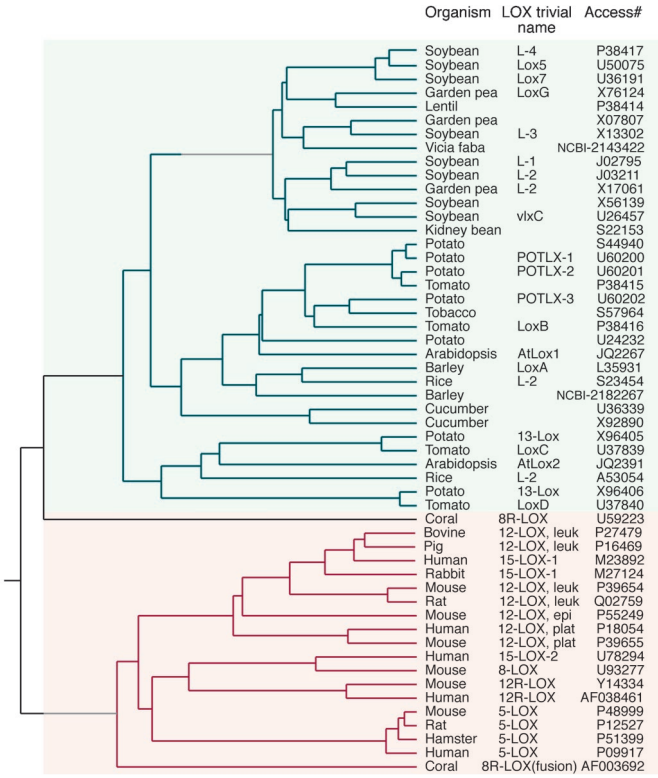
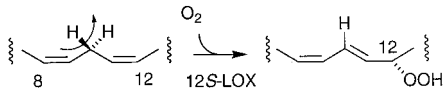


FIG. 1. **Lipoxygenase phylogenetic tree.** Representative plant and animal lipoxygenases were compiled into the phylogenetic tree using MegAlign (DNASTAR, Inc.).



SCHEME 1

ligands for the orphan nuclear receptors has produced some provocative data on activities of the HETEs (25, 27). Yet, only 8*S*-HETE has submicromolar activity in PPAR-reporter assays (25), and 8*S*-HETE is known as a natural product only in mouse skin (28). Linoleate derivatives and other HETEs are significantly weaker PPAR ligands, although it is argued that this has potential physiological significance in relation to oxidation of low density lipoprotein and the initiation of atherosclerosis (29); certainly, high concentrations of hydroxylinoleates can accumulate (30).

Modification of Membrane Structures (Peroxidation Reactions)

Typically this is associated with metabolism of esterified substrate and *often produces a mixture of hydroperoxy products* (Fig. 2).

Lipoxygenase-catalyzed Peroxidation of Membrane Lipids Induces Structural Changes—Here, the objective is to induce physical changes in the cell or change the peroxide tone, and the structure of the hydroperoxy product is not so important as its effects in (*a*) perturbing membrane structure and (*b*) provoking secondary oxygenations (enzyme-catalyzed lipid peroxidation). The concept that a lipoxygenase could peroxidize membrane lipids and thus help induce a series of programmed structural changes in a cell was originally developed around the mammalian reticulocyte 15-LOX and its potential role in red cell maturation (31). Keratinocyte maturation and lens epithelial cell development offer similar possibilities (32). Subsequently, the same 15-LOX enzyme has been implicated in the oxidation of low density lipoprotein, a key event in the initiation of atherosclerosis (33). Similar concepts have evolved independently in the plant literature where a role for lipoxygenases in plant senescence is proposed (*e.g.* Ref. 34).

Gene Knockouts and Lipoxygenase Function—An issue here, and in ascribing functions to other lipoxygenases, is the fact that gene knockout experiments in mice indicate no obvious problems in development or cell differentiation (4). Similarly, transgenic plants

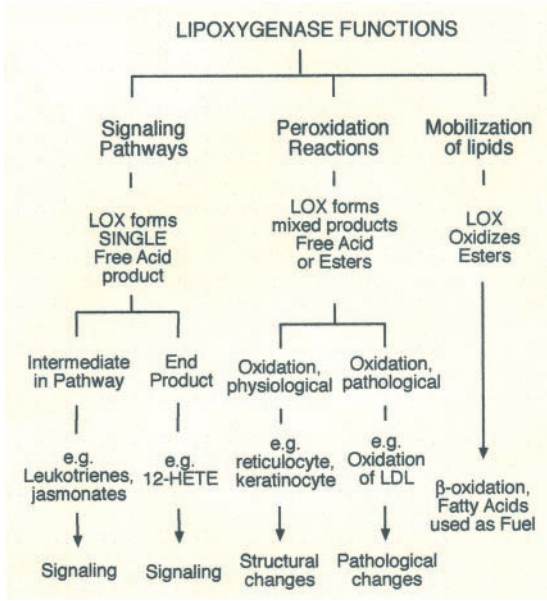


FIG. 2. **Biological roles of lipoxygenases.**

lacking one or several lipoxygenases exhibit subtle changes (35). The lipoxygenases modulate events, and their role may become evident only under physiological or pathological stress (4).

Mixed Products, Short-lived Enzymes—As mentioned earlier under "Nomenclature," the human reticulocyte type of 15-LOX (15-LOX-1) and its animal counterparts form a mixture of 12-HPETE and 15-HPETE. Is this imperfect catalysis by design? It is hard to imagine that the objective is to form mainly one product and a small amount of another. The imperfect fit in the active site and the resulting mobility of the substrate during catalysis might be designed to promote release of free radical intermediates. This accords with the view that the reticulocyte type of 15-LOX functions as a catalyst of lipid peroxidation. It is also notable that this class of lipoxygenase functions for only a minute or two before a turnover-related inactivation kills the enzyme (36). By contrast, lipoxygenases that cleanly catalyze formation of a single product (*e.g.* the platelet type of 12-LOX, the mouse 8-LOX, and the human 15-LOX-2) can keep running near a linear rate for an hour or more (24, 28, 36).

Mobilization of Lipids for Metabolism—Lipoxygenase-catalyzed oxygenation of unsaturated fatty acids esterified in triglycerides is implicated in the germination process in oil-seed plants (37). After conversion specifically to the 13-hydroperoxy esters, the fatty acids become available for β-oxidation and utilization as a fuel source for the developing embryo. An equivalent function has not been ascribed in animal biology, yet this peroxidation of lipid stores serves as a potential model to rationalize, for example, the high lipoxygenase content of certain animal oocytes, which also carry stores for embryo development (*e.g.* Ref. 38).

Lipoxygenase Catalysis

Enzyme Structure—Lipoxygenase proteins have a single polypeptide chain with a molecular mass of ~75–80 kDa in animals and ~94–104 kDa in plants. The proteins have a N-terminal β-barrel domain (Fig. 3, *top panel*, *white*, and discussed later under "Acquisition of Substrate") and a larger catalytic domain containing a single atom of non-heme iron (Fig. 3). The metal is liganded to conserved histidines and to the carboxyl group of a conserved isoleucine at the C terminus of the protein (Fig. 3). As the iron is non-heme, lipoxygenases appear virtually colorless to the eye. The enzymes are usually in the ferrous (inactive) form when isolated. Oxidation to the active ferric enzyme is required for catalysis (Scheme 2).

There are four available crystal structures, of which three are of the arachidonate 15-lipoxygenases, soybean L-1, and rabbit reticulocyte 15-LOX (39–41), and the fourth, soybean L-3, is a catalyst of nonspecific peroxidation (42). There is no consensus on how substrate gains access to the metal center or any definitive information on substrate binding. The reticulocyte 15-LOX structure has a

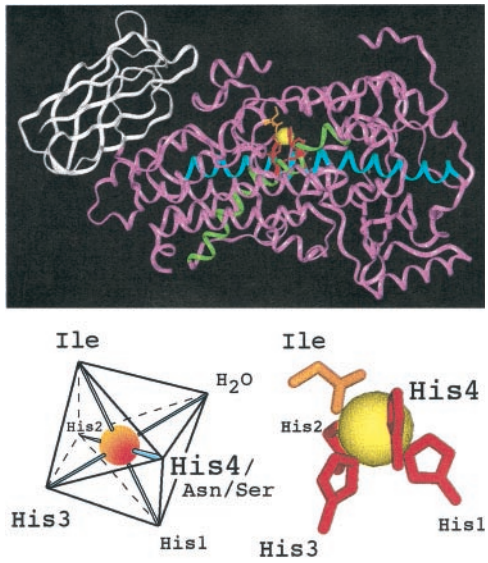
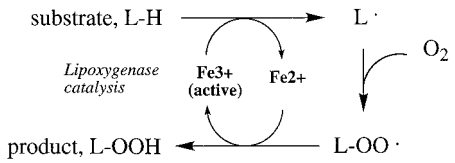


FIG. 3. **Ligands around the lipoxygenase non-heme iron.** In all *panels* the enzyme is in the same view, with the β-barrel on the left in a similar orientation to Ref. 39 (Fig. 2) and Ref. 41 (Fig. 1*b*, Fig. 2). The amino acids close to the iron lie at the corners of a tilted bipyramid, approximately in an octahedral arrangement. This is represented in stylized form in the *lower left panel*, and an example (reticulocyte lipoxygenase) is shown on the *lower right*. For simplicity, the histidine ligands are labeled in order of occurrence in the enzyme primary structure: *His1* is His-361 in the rabbit reticulocyte 15-lipoxygenase (41), which is equivalent to His-499 in soybean L-1 (39); similarly *His2* represents His-366/His-504; *His3* is His-541/His-690; *Ile* is the C-terminal isoleucine; and *His4/Asn/Ser* corresponds either to His-545 of the rabbit reticulocyte 15-lipoxygenase or the equivalent asparagine or serine in the same position in other lipoxygenases; in two crystal structures (39, 42) the Asn-694 of soybean L-1 or L-3 is *not* sufficiently close to ligand with the iron. Two histidine ligands (His-1, His-2) arise from helix 9 (*blue*), which from this view lies on the far side of the iron and slightly below; helix 9 begins on the right where the polypeptide chain turns into this long helix using the conserved peptide sequence WLLAK. His-3 and His-4/Asn/Ser are five amino acids apart in the primary structures and arise from helix 18 (*pale green*), which is situated slightly below and in front of the iron; the polypeptide runs slightly upward, left to right through helix 18. The "H_2O" is seen as a sixth ligand in three of the crystal structures (40–42). The structure shown is the reticulocyte lipoxygenase available under Protein Data Bank accession no. 1LOX; at the top of the structure the apparent two free "ends" are connected by 12 amino acids that did not give clear x-ray diffractions.



SCHEME 2

bound inhibitor lying opposite His-3 as it is designated in Fig. 3 (see legend), and its position helps identify a likely substrate binding site (41). The apparent access channel for arachidonic acid in this model opens onto the top surface of the protein as viewed in Fig. 3. This route is obstructed by overlying helices in the corresponding crystal structures of the soybean L-1 and L-3 enzymes. The soybean enzymes both have a large cavity to the right of the iron as portrayed in Fig. 3 and an opening to the protein surface on the right-hand side. There is a second more restricted route opening to the lower surface of the protein. In addition, the L-3 structure shows potentially accessible space extending left and opening out toward the β-barrel domain (42). Significantly, all three structures are of the ferrous (inactive) form of the enzyme. It is quite likely that movements occur during transition into the active Fe^{3+} form that will result in a unifying model of catalysis.

Multiple Lipoxygenase Activities in the Same Active Site—The soybean L-1 isozyme is a "pure" 15-lipoxygenase, which, at low enzyme concentrations, forms only 15*S*-HPETE from arachidonic acid. It carries out this first oxygenation very cleanly compared with other soybean isozymes or the mammalian reticulocyte type of 15-LOX. Soybean L-1 is, nonetheless, able to catalyze specific 5*S* and 8*S* oxygenations. The initial product, 15-HPETE, is further oxygenated at a much slower rate (overcome by use of higher

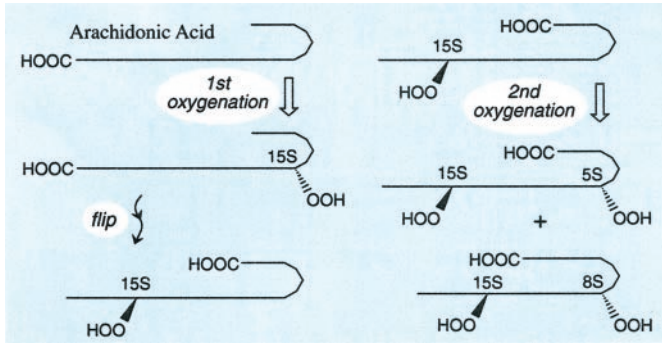


FIG. 4. **Lipoxygenase activities of soybean L-1.** For clarity, arachidonic acid and its derivatives are shown without individual carbons and double bonds. In this model to rationalize the 15*S*, 5*S*, and 8*S* activities of the L-1 enzyme, an initial oxygenation to form 15*S*-HPETE is followed by its reverse orientation ("flip") and 5*S* or 8*S* oxygenation in the same lipoxygenase active site. The concepts illustrated here were developed originally in 1972 based on the finding that the synthesis of 9*S*- or 13*S*-hydroperoxides of linoleic acid involves stereospecific removal of the pro-*R* and pro-*S* hydrogen, respectively, from C-11 (58).

enzyme concentration) to the specific double oxygenation products, 5*S*,15*S*-di-HPETE and 8*S*,15*S*-di-HPETE (43). The important conclusion is that the same enzyme, and presumably the same active site, is able to catalyze stereospecific 15*S*, 5*S*, and 8*S* lipoxygenase reactions. Substrate alignment in the active site is one of the keys to control of the oxygenation reaction (Fig. 4).

R- and S-Lipoxygenases—The best known plant and animal lipoxygenases form products with *S* stereochemistry. Nonetheless, we recognize now that lipoxygenases forming the mirror image *R* configuration products are also widespread, being found among aquatic invertebrates (3), plants (2), and recently in humans (11). *R*-Lipoxygenases contain the same conserved iron ligands and other sequence motifs common to plant or animal *S*-lipoxygenases (44). There are no known sequences or motifs that account for the opposite stereospecificity, and a relatively subtle change in reaction specificity must be capable of giving the mirror image product.

Acquisition of Substrate

Substrate in Membranes—Arachidonic and linoleic acids have a pK_a between pH 7 and 8 (45) and therefore will be partly unionized and quite insoluble in water at the typical physiological pH of 7.4. Under these conditions, the free fatty acids "released" by the action of a lipase will have little tendency to leave the membrane unless by association with a carrier. The free acid might be expected to lie with its long tail in the hydrophobic bilayer and its more polar carboxyl (charged or uncharged) in the aqueous environment or associated with the head groups of phospholipids. These substrates are not necessarily available for lipoxygenase metabolism. In a careful study of the oxygenation of membrane-associated free linoleic and linolenic acids it was found that the soybean L-1 and L-2 isozymes would not metabolize free acid substrates mixed with biological membranes. In the same experiments, the L-2 enzyme (but not L-1) metabolized the membrane esters (46). By contrast, activity of the reticulocyte type of mammalian 15-LOX on free acid substrates is stimulated by association with biomembranes (47), and this enzyme will also metabolize esterified substrates without requiring detergent for solubilization (31).

Lipoxygenase Protein Structure—A potential clue to mechanisms involved in substrate acquisition came from recognition that the conserved β-barrel domain of lipoxygenases shares significant homology to a similar domain located at the C terminus of the mammalian lipases (41). Like most lipoxygenases, the lipases are cytosolic yet need access to substrate in a hydrophobic membrane environment. The lipoprotein lipase β-barrel functions in the acquisition of substrate through protein-protein receptor interactions and by hydrophobic interactions with lipoprotein particles (41). As yet there is no direct evidence of a role of the lipoxygenase β-barrel in binding substrate. Notably, for all lipoxygenases, the understanding of substrate access from the membrane is further clouded by uncertainties regarding the point of substrate entry into the catalytic domain of the protein.

5-LOX and 5-Lipoxygenase-activating Protein (FLAP)—For one

mammalian lipoxygenase, the leukocyte 5-LOX, there is evidence of complex protein-protein interactions in its nuclear membrane translocation, activation, and acquisition of substrate in intact cells. 5-LOX is mainly cytosolic in resting neutrophils yet nuclear in macrophages (48). Cell stimulation results in translocation of 5-LOX to the nuclear membrane and association with FLAP, an 18-kDa integral membrane protein essential for leukotriene biosynthesis in intact cells. FLAP acts as an arachidonic acid transfer protein that "presents" the substrate to the 5-LOX on the leukocyte nuclear membrane (49). It has homology to leukotriene C4 synthase (50) and other microsomal glutathione transferases (51) but no enzymatic activity itself. The inhibitor MK886 binds to FLAP and blocks leukotriene production from either exogenous or endogenous arachidonic acid in intact cells; the inhibitor is inactive in broken cells or with purified 5-LOX.

The involvement with FLAP does not explain the cellular localization of 5-LOX in different cell types or the mechanism of its translocation. Recent evidence implicates at least three 5-LOX sequences in these events: an actin binding sequence, a SH3 binding domain, and a bipartite nuclear localizing sequence (52). An independent study concluded an N-terminal sequence in the β-barrel was necessary although not sufficient for nuclear targeting (53). Three additional 5-LOX-interacting proteins that may be involved in regulation and/or nuclear localization were identified recently using the yeast two-hybrid screen (54).

FLAP appears to be unique. No protein with the equivalent function in substrate handling, or a requirement for one, has been found for any other fatty acid oxygenase. Nonetheless, other mammalian lipoxygenases have the consensus SH3 binding domain, and there is evidence of membrane translocation on cell activation.

Future Insights from Novel Enzymes

The discovery of a manganese lipoxygenase and its unique ability to oxygenate on the fatty acid carbon between the two *cis* double bonds (C-11 of linoleic acid) provides many new questions and issues related to catalysis and reaction mechanisms (55). It remains to be seen if this represents a new class of enzyme or is related to the lipoxygenase superfamily. Similarly the discovery of a plant oxygenase related in sequence to mammalian cyclooxygenase will provide interesting contrasts in the metabolism of the typical lipoxygenase substrates, linoleic and linolenic acids (56). The lipoxygenase-peroxidase fusion protein in a prostaglandin-containing coral raised novel issues of protein-protein interaction and the effects of a peroxide-metabolizing enzyme on peroxide activation of a lipoxygenase (57). Finally, the recent discovery of *R*-lipoxygenase in mammalian systems may spur more interest in these enzymes and their potential utility in unraveling the basis of lipoxygenase stereospecificity (10, 11, 59).

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