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LIPOXYGENASES: Structural Principles and Spectroscopy

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ABSTRACT

Lipoxygenases catalyze the formation of fatty acid hydroperoxides, products used in further biochemical reactions leading to normal and pathological cell functions. X-ray structure analysis and spectroscopy have been applied to elucidate the mechanism of lipoxygenases. Two X-ray structures of soybean lipoxygenase-1 reveal the side chains of three histidines and the COO^- of the carboxy terminus as ligands to the catalytically important iron atom. The enzyme contains a novel three-turn π-helix near the iron center. Spectroscopic studies, including electron magnetic resonance, X-ray absorption spectroscopy, infrared circular dichroism, and magnetic circular dichroism, have been applied to compare lipoxygenases from varied sources and with different substrate positional specificity.

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INTRODUCTION: THE LIPOXYGENASE FAMILY OF ENZYMES

The first X-ray structure of a lipoxygenase (5) and the structure of the catalytic site (45) of the same lipoxygenase were reported in 1993. Solutions of other lipoxygenase structures are in progress with the use of X-ray analysis (76, 79, 81) and homology modeling (62). The major domain of lipoxygenase consists largely of α-helices surrounding a central, long (43 amino acids) helix. Cavities lead to the long helix from two sides and pass close to the catalytic, non-heme iron atom. The fold of the major domain of lipoxygenase is not similar to that of known protein structures and has novel features, including three turns of an apparent π-helix and the carboxy terminus as a ligand to the iron atom (5). Delineation of the protein ligands to iron culminates a long search by spectroscopic techniques to determine the nature of this metal center. Ongoing spectroscopic studies are being used to define the iron center in more detail in terms of exchangeable ligands and catalytic intermediates. These results provide the motivation for reviewing the implications of the lipoxygenase structure. Before the principles exhibited by the lipoxygenase structure are discussed, the history and rationale for considering known lipoxygenases as a family of closely related enzymes are presented.

Crystalline lipoxygenase, in the form of colorless plates, was reported as early as 1947, when formation of crystals from ammonium sulfate solutions was used to improve purification of the enzyme from soybeans (83). In that study, Theorell et al found the molecular weight by sedimentation to be 102 kDa. Although we now know that all lipoxygenases contain one iron atom per molecule, these investigators found only 0.28. The enzyme isolated from soybeans was thought to be similar to the ones responsible for similar activity in other plant preparations. The activity, as it was understood at that time (83), involved oxidation of unsaturated fats, accompanied by bleaching of carotenoids. It was not until 1973–1975 that the presence of one iron per 100 kDa was clearly demonstrated (9, 15, 58, 59) and that changes in iron oxidation state, associated with catalysis, were shown by electron magnetic resonance [(EMR), designated EPR in earlier references for electron paramagnetic resonance] spectroscopy (15, 59). The iron center showed no optical spectrum characteristic of heme, and chemical studies gave no evidence

of an iron–sulfur center (58). The long-standing goal (26) of chemical studies has been to determine the nature of the iron center in lipoxygenases and the involvement of oxidation and reduction of the metal in the catalytic mechanism.

Today, interest in lipoxygenases is fueled by the discoveries, during 1967–1981, of animal lipoxygenases and, more recently, of the involvement of products derived from plant and animal lipoxygenase action in normal and pathologic cell functions (67, 72). Research on formation of prostaglandins from arachidonic acid led to discoveries of animal lipoxygenases from, among others, rabbit polymorphonuclear leukocytes (46), human neutrophils (29a), human platelets (32, 51), and rabbit reticulocytes (84). Many other lipoxygenases have been found since 1981. The first sequence of a lipoxygenase was reported in 1987 (71). Now, more than 30 sequences are known for these enzymes (summarized in 62 and 80), and several genomic sequences have also been reported (13 and references cited therein).

Evidence That Lipoxygenases Are a Family of Enzymes

The evidence is summarized as follows (see also 77):

1. Antarafacial removal of hydrogen and addition of oxygen is exhibited by the enzymes in conversion of (1(Z),4(Z))-double bonds in unsaturated fatty acids to a (1,3 (E,Z))-dienyl-5-hydroperoxy system (39).
2. Pairwise sequence identity is 21–27% between plant and animal lipoxygenases, 43–86% among plant sequences, and 39–93% among animal sequences (62).
3. Residues that provide ligands to the catalytic iron are conserved (13, 67, 80).
4. Genomic structures of several animal lipoxygenases are similar (13 and references therein).

In spite of the similarities indicated above, members of the lipoxygenase family of enzymes differ in the regiospecificity of the reaction they catalyze. In forming lipoxygenase products with arachidonic acid as substrate, there are six possible placements of the hydroperoxy group in the product (carbons 5-, 8-, 9-, 11-, 12-, and 15-). Two stereochemical courses could be taken at each position, thereby making a total of 12 hydroperoxide products possible from arachidonic acid. In humans, enzymes that specifically carry out oxidation at one of three positions are well characterized. The products of these enzymes are 5-D_{S-}, 12-L_{S-}, and 15-L_{S-} hydroperoxides. The stereochemical nomenclature for substituents on fatty acids (39) is such that the 5*S*-product actually

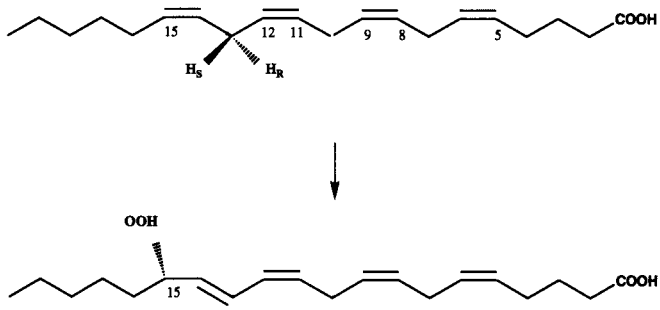


Figure 1 The stereochemical course of the reaction of SBL-1 or reticulocyte 15-LO with arachidonic acid.

has the opposite absolute stereochemistry of that for the 12*S*- and 15*S*-products, so a combination of the D/L Fischer convention with the *R*/*S* nomenclature is used to avoid these complications. The initial removal of hydrogen is antarafacial to the addition of oxygen. Throughout this review, animal lipoxygenases are referred to as 5-LO, 12-LO, and 15-LO. In plants, the substrates are more often 18-carbon chains, so the numbering is different. For simplicity, the major soybean enzyme is referred to as SBL-1, to distinguish it from the other isoforms in soybeans that are designated SBL-2 and SBL-3, for example. (1). Figure 1 shows the stereochemical course of 15-lipoxygenase reactions.

Lipoxygenases also differ in molecular weight. The plant enzymes (90–100 kDa) (71) have an *N*-terminal sequence that is missing in animal lipoxygenases (65–75 kDa) (see references in 13, 62, 80). This region of the plant enzymes is omitted in analysis of sequence identity (62).

BRIEF OVERVIEW OF THE BIOLOGICAL ROLES OF LIPOXYGENASES

Both plants and animals have 5- and 15-lipoxygenases. In each case, there is a cascade of products that results from further enzymatic conversions of the hydroperoxides. In animals, lipoxygenases carry out the first step in the so-called arachidonic acid cascade (67). The branch of this cascade, beginning with 15-LO, leads to lipoxins, whereas the 5-LO branch leads to 5,6-epoxy-leukotrienes. 5-LO activity has been the most studied from the standpoint of pharmacology, because the leukotriene products are involved in a variety of inflammatory responses, including neutrophil chemotaxis, vascular permeability, and smooth muscle contraction, in humans (67). Two reports of mice in

which the gene for 5-LO has been inactivated have appeared recently, and the responses of the mice to challenged states were found to be somewhat abnormal (20, 31). In contrast, Nassar et al (47) suggested that animal 15-LO products act as anti-inflammatory agents. This suggests that the contrasting effects of the 5- and 15-LO pathways may be to regulate the extent and magnitude of inflammatory reactions in humans. It is particularly important, therefore, to develop drugs that are specific for one class of the enzyme and not another (44). Ford-Hutchinson et al (19) recently reviewed the pharmacology and cell biochemistry of 5-LO. Novel inhibitors discussed in that article include those that bind not to 5-LO but to the 5-LO activating protein (FLAP). In addition, articles on advances in studies of arachidonic acid metabolism have appeared in recent monographs (29, 67).

The level of current interest in plant lipoxygenases is also high, because the enzymes start some of the pathways for plant self-defense and signaling mechanisms; these subjects are the basis of papers from a 1994 colloquium (66). Seed industries have an interest in inhibiting lipoxygenase products that contribute to rancidification of oils, but some lipoxygenase activity is thought beneficial in bread flour. One strain of commercial soybean has been genetically altered so that the binding site for the catalytic iron is destroyed (87). Although the mutant protein can be expressed in *Escherichia coli*, the inactive protein does not appear at all in the mature soybeans. It will be of interest in the future to learn whether the defense mechanisms are altered in plants or seeds that contain this defective lipoxygenase gene (82).

LIPOXYGENASE STRUCTURAL PRINCIPLES

Overall Structure

Among single-chain protein structures, the lipoxygenase structure is quite large [839 amino acids (71, 80)]. The overall fold has two domains: an N-terminal β-barrel associated with one helix and a major domain composed of 22 helices and 8 β-strands (5). One problem that was solved by obtaining a lipoxygenase structure concerned the difference in molecular weights between the plant (~90 kDa) and the animal enzymes (~70 kDa). The first 146 amino-terminal amino acids of soybean L-1 form an eight-stranded β-barrel. Most of this region is missing from animal lipoxygenase sequences (62, 71, 80). This domain in L-1 may extend farther than the first approximately 200 amino acids, because one α-helix and a large unstructured region is more closely associated with the β-barrel than with the rest of the structure.

Because of the complexity of the lipoxygenase structure, the buildup of the structure, starting from the N-terminus and proceeding to the nearly completed structure, is shown in four sequential steps in Figure 2 (6). The first panel shows the β-barrel at the left, together with structure in the large domain through helix 7. For reference, the iron atom is shown as a sphere in the center. [Most of the structure shown in the first panel (through the fourth helix) can be removed from the intact protein by proteolysis to leave a catalytically active fragment (63).] The second and third panels extend the structure. Panel four extends the structure as far as the second longest helix, helix 18. Helix 18 crosses helix 9 at the iron-binding site.

The complete structure, including the remaining region of helices -19 to -23, together with a diagram giving the numbering of the helices is shown in Figure 3 (23). This figure includes space-filling representations to highlight significant regions of the structure. A funnel-shaped cavity (cavity I) (5), which extends from the surface at the bottom of the molecule toward the buried iron atom, is shown with light gray side chains. A second cavity (cavity II) (5), which extends from the surface on the right, midpart of Figure 3, past the iron site, and up toward helices -11 and -21, is depicted in medium gray. Labeled residues L480 and M341 (light gray) appear to block the entrance to this cavity. The sites of the four sulfhydryl side chains, some of which have been chemically modified, are also given (dark gray). Sulfhydryl derivatives used in the X-ray structure analysis included C492 labeled with mercury dicyanide, C127 labeled with the same reagent or with mersalyl, and C357 labeled with one of the above-mentioned two reagents or with potassium cyanoaurate (5). C679 in crystalline SBL-1 was not reactive with these heavy atom reagents. Reaction of C492 with methylmercury hydroxide was used in the structure determination by Minor et al (45). Chemical studies have shown that two of the sulfhydryls of SBL-1 can be modified without loss of activity (32b).

The Non-Heme Iron Site

The amino acid side chains that form ligands to iron have been identified from the X-ray structure analyses as three histidines, with $N\epsilon$-

Figure 2 Regions of the lipoxygenase structure are shown with arrows as β-sheets and tubes as α-helices. The sequence of figures shows how the structure progresses from N-terminus through helix 18. (*Upper left*) N-terminus through residue 468; (*upper right*) structure is extended to residue 519 and includes helix 9; (*lower left*) structure is extended to residue 576; (*lower right*) structure is extended to residue 703, thereby giving the structure through helix 18. See Figure 4 for helix numbering. Redrawn from Reference 6.

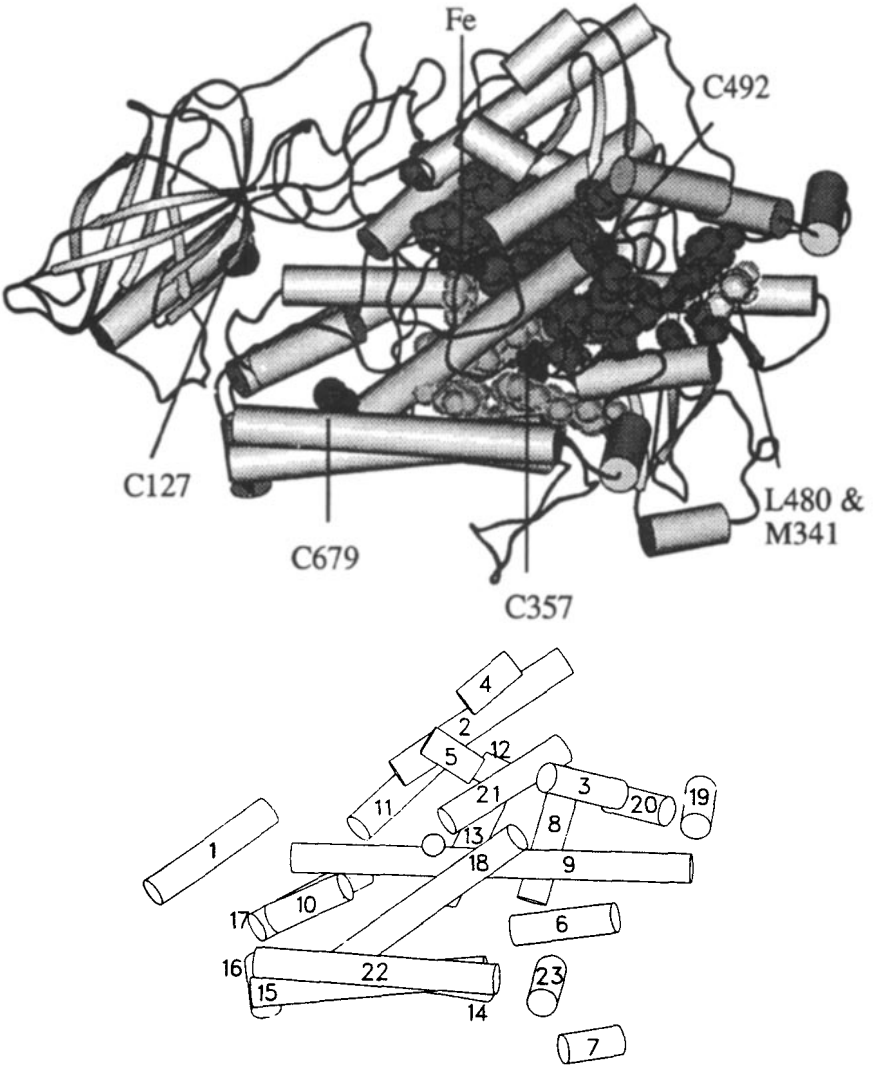


Figure 3 The complete lipoxygenase structure is shown in the upper figure with space-filling representations of side chains that line the cavities that lead to the non-heme iron atom. Light gray = cavity I; medium gray = cavity II. The lower figure gives the numbering of the α-helices. From Reference 23.

ligation, and one oxygen of a carboxyl (5, 45). This carboxyl group is not a side chain but is the COO^- of the carboxy terminus. The terminal COO^- has not been identified previously as a ligand to metal in proteins. The carboxy terminal amino acid is isoleucine in all sequenced lipoxygenases. Of the mutations at the corresponding residue in murine 12-LO, only I663V gave near native activity; I663S and I663N gave 15 and 8% of native activity (13). The group of iron ligands in SBL-1 is chemically the same as the ones in iron superoxide dismutase (4, 81a), although the carboxyl is not the c-terminus in superoxide dismutase and the details of the ligand symmetry appear somewhat different. There are also differences between the two structures of SBL-1 (5, 45). A fifth side chain, asparagine 694, is close enough to be considered an iron ligand in the structure from crystals prepared in polyethylene glycol (pH 5) (45), although it is at least 3 Å from the metal in the structure from crystals in a mixture of salts at high concentration (pH 7) (5). The residue corresponding to this position in some 12- and 15-LOs is histidine. Spectra of these 15-LOs show some differences from those of SBL-1 (see section on *Spectroscopic Studies of Lipoxygenase Structure*). In homology modeling studies based on the SBL-1 structure, two possible conformations, which differ by approximately 90° in χ-1, for this histidine side chain in the modeled human 15-LO (62) were evaluated. Both conformations can be accommodated in the modeled structure, but the δ-nitrogen in the model is predicted to be the iron ligand in the structure with histidine oriented toward iron (62).

Figure 4 gives a view of the iron atom and the side chains of ligands to it from the same perspective as that for Figures 2 and 3. Histidines 499 and 504 lie behind the iron atom on helix 9, whereas the COO^-

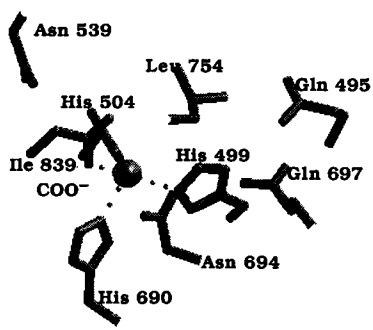


Figure 4 A view of the side chains near the non-heme iron atom (sphere) in SBL-1. The bonds from the iron atom to the terminal COO^- and the three histidine ligands are also shown. The orientation is approximately the same as in Figure 3. Drawing constructed using the program SETOR (18a), with permission.

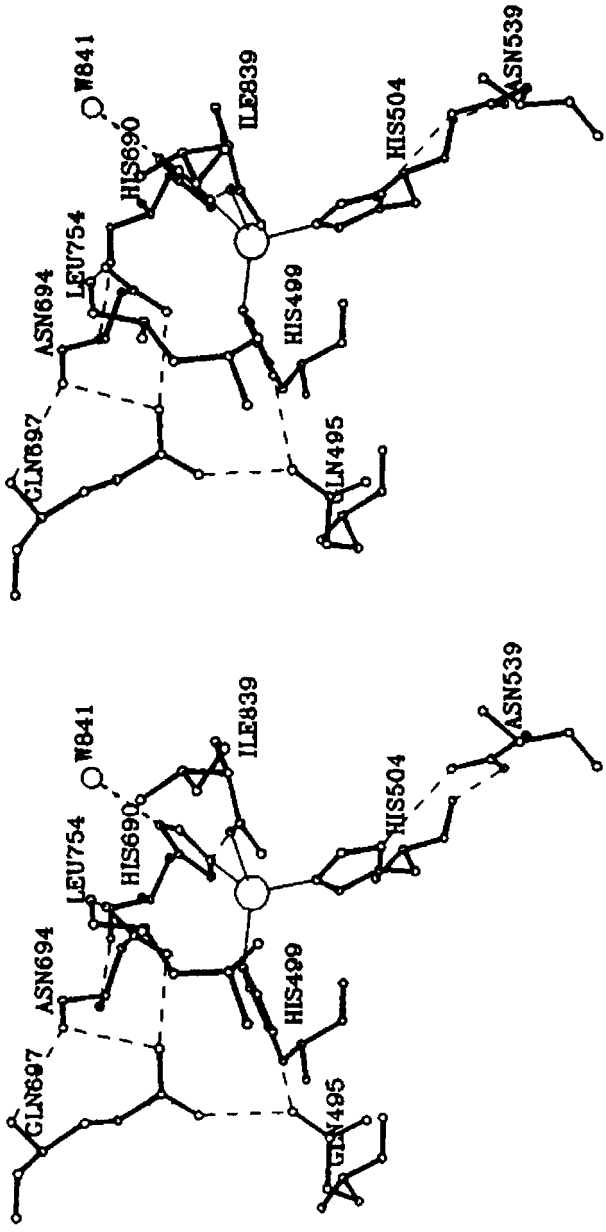


Figure 5 A stereo view of the vicinity near iron from a different orientation than that in Figure 3. Hydrogen bonding near iron (*dashed lines*) is shown. This view also shows the tilt of the His499 ring and the distance between the Asn694 side chain and iron. From Reference 5.

terminus of isoleucine 839 and histidine 690 are in front of the iron atom. Other side chains pictured are involved in hydrogen bonds to the metal ligands. Another view of this region is presented in Figure 5 (5), which displays the hydrogen bonding pattern and has an orientation that shows the space between iron and asparagine 694.

Although the carboxy terminus of lipoxygenase as a metal ligand appears to be a novel structural feature, there are other examples, for instance in hydrogenase (30, 65) and photosystem II (3), in which post-translational modification near the carboxy terminus is essential for assembly and stability of metal binding sites. It is not known whether iron binding to lipoxygenases is a reversible equilibrium; however, the metal in SBL-1 is very tightly bound (58). 5-Lipoxygenase prepared in an expression system incorporates varied levels of metal, and there is evidence that oxidative inactivation of this protein is accompanied by loss of iron (55). These experiments may indicate that iron is bound more weakly in 5-LO than in SBL-1. Loss of iron from reticulocyte 15-LO has also been reported.

A recent example of the *N*-terminus of a protein as a metal ligand was found in cytochrome *f* (42). This ligation imposes a requirement for cleavage by signal peptidase before folding can be complete. Further, this requirement may be a biochemical strategy for maintaining an unfolded state during biosynthesis (42). *C*-terminal ligation to metal can also be a strategy for folding the protein after biosynthesis is complete. Because the carboxy-terminus probably has the only charge associated with the metal ligands in resting lipoxygenase, charge compensation may be an important aspect of assembly of this metal site. It is not yet known whether the carboxy terminus is a metal ligand in those proteins mentioned above in which *C*-terminal processing is important for assembly of the metal site (30, 65).

A Three-Turn π-Helix

Five conserved histidines are found in a short sequence in all but one of the lipoxygenases sequenced (13, 80).When Shibata et al (71) reported the first lipoxygenase sequence, they suggested that some of these histidines might be iron ligands. Results of mutagenesis show that only two of these residues, H499 and H504, are required for enzyme activity in the SBL-1 sequence (80). (Comparisons with other sequences are also given in this reference.) The i and i + 5 spacing of these residues is inconsistent with an α-helical arrangement if both histidines are iron ligands. In fact, these residues are located on the long helix 9, and this helix expands at the metal binding site, as shown

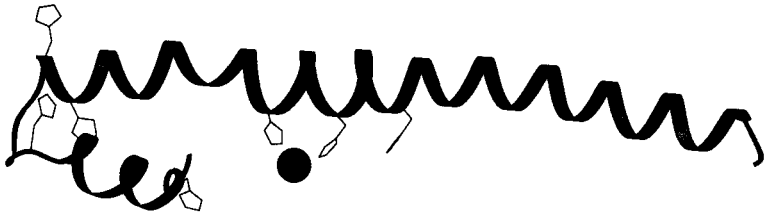


Figure 6 An expanded view of long helix 9, turn, and short helix 10 in SBL-1. Note the expanded, π-helical region. The side chains of the His residues in this region are shown, and the iron atom is represented as a sphere. The left-right orientation is the same as in Figures 2 and 3 (N-terminal end of helix 9 on the right). However, the figure has been rotated about a horizontal axis, relative to the orientation in Figures 2 and 3, to give a better view of the His side chains. Redrawn from Reference 6.

in Figure 6 (5). Most of the carbonyls of the peptide backbone in this region have the same orientation as in an α-helix, and there are eight hydrogen bonds with i to i + 5, instead of i to i + 4 spacing. This is the arrangement of hydrogen bonding in a π-helix. Lipoxygenase has a 14 amino acid stretch, between residues 493 and 506, of π-helix in this region and a shorter, one-turn stretch at H690, where a third histidine-iron bond is located (5). In the 1950s, Low & Baybutt (40) predicted the possible existence of π-helices, and several investigators debated this issue (16, 52, 53). An example of a π-helix with more than one turn has not been reported before in a protein, but a left-handed π-helix with multiple turns has been reported in the synthetic polymer poly(β-phenethyl) L-aspartate (68).

From 1950 to 1952, there was active discussion of possible stable helical arrangements of polypeptides that did not have an integral number of residues per turn. This discussion had a background of 15 years of papers on the principles of protein structure by Huggins, Bragg, Kendrew, Perutz, Pauling, and others. During this period, Pauling and coworkers proposed the α- and γ-helices and evaluated many others (16, 52, 53). Low & Baybutt (40) noted that another helix also fit the criteria for helices laid out by Pauling et al (53) and named it the π-helix. This postulated helix has 4.4 residues per turn and a pitch of 5 Å. Low & Baybutt (40) cited a personal communication from Pauling in which he criticized the π-helix on the basis that it would have a hole down the center. The calculated N-N van der Waals contacts across the middle of the helices are 3.0 Å for a π-helix and 3.2 Å for the γ-helix, compared with 2.9 Å for an α-helix (16). Donohue (16) suggested that water molecules could fill the void in γ-helices but not in π-helices.

Donohue (16) calculated the energetics of π-helices and other helices

Table 1 Helical polypeptide characteristics[a]

Helix name	Atoms in H-bonded ring	H-bond spacing	Residues per turn	Axial translation per residue (Å)	Calculated instability per mole per residue (kcal)
α_{II} ribbon	7		2.2	2.75	0.5
—	8	—	not possible	—	>15.0
3_{10}	10	i → i + 3	3.0	2.00	1.0
—	11	—	not possible	—	>5.0
α	13	i → i + 4	3.6	1.50	0.0
δ	14	i → i − 4	4.3	1.20	2.4
π	16	i → i + 5	4.4	1.15	0.5
γ	17	i → i + 6	5.1	0.98	2.0

[a] From Reference 16.

(Table 1). The calculated stability of the π-helix is only 0.5 kcal/mol less than the α-helix, whereas the 3_{10} helix and the γ-helix are 1.0 and 2.0 kcal/mol less stable. Factors included in the calculation were nonplanarity of the peptide groups, deformation of other single bonds, and nonlinearity of hydrogen bonds. These estimates do not include van der Waals forces or the energies of hydrogen bonds, but ranges of these were discussed by Donohue.

Some irregularities in the lipoxygenase π-helix may be adjustments to maintain a normal protein packing density. Figure 7 shows a quadrant of the Ramachandran plot for the π-helical region compared with that for all of SBL-1. Predicted angles for a π-helix are ϕ=-57, ψ=-70; those for an α-helix are ϕ=-57, ψ=-47; and those for a 3_{10} helix are ϕ=-49, ψ=-26. The rise per residue is 1.15, 1.50 and 2.0 Å for π-, α- and 3_{10}-helices, respectively. Thus, 14 residues of π-helix are shorter along the helix axis than 11 residues of α-helix. The three most abnormal sets of ϕ and ψ in helix 9 of SBL-1 are labeled on the figure. Two of the residues with abnormal angles, M497 and T503, are involved in bifurcated H-bonds from carbonyl oxygens. In addition, residues S498 and N502 have the backbone carbonyl bent out and not participating in a normal H-bond along the helix (S498: ϕ=-63, ψ=-37; N502: ϕ=-65, ψ=-19). This raises the question of whether residues 493–506 in the SBL-1 structure should be regarded as a π-helix or instead form an a-helix with two insertions (32a, 35a).

In the context of this discussion of the π-helical region of the lipoxygenase structure, a mechanistic proposal (62a) based on mutational studies of the first histidine ligand to iron is interesting. Histidine 368

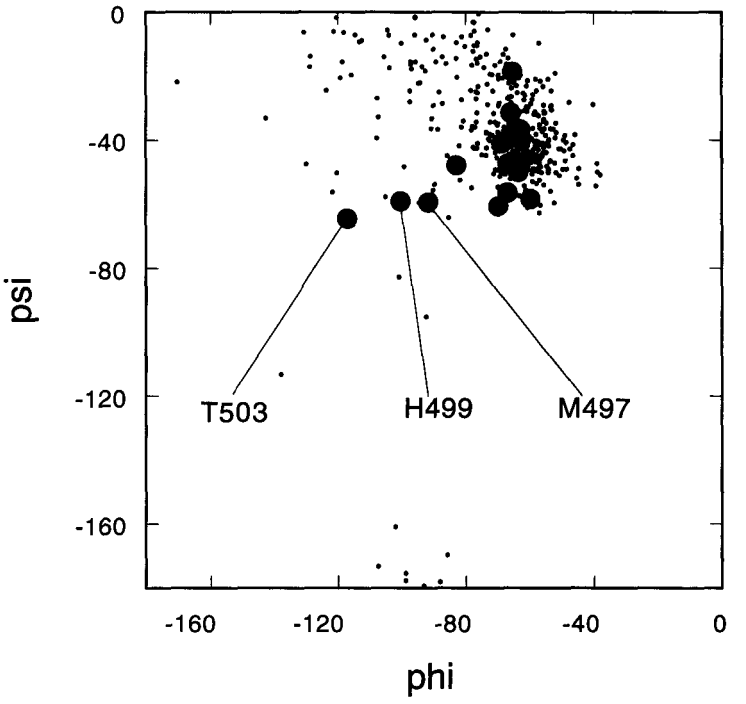


Figure 7 A quadrant of the Ramachandran plot for ϕ and ψ angles of the π-helix (*large dots*) is compared with these angles for all of SBL-1 (*dots*).

in human 5-LO corresponds to H499 in SBL-1. When the mutations H368N, H368Q, or H368S were made in 5-LO, the proteins still could incorporate iron, although the mutated proteins were inactive in catalysis. These results were interpreted to mean that H368 is a replaceable ligand that can be substituted by something else in catalytic intermediates (62a). H499 is uniquely positioned in the SBL-1 structure between helices -9 and -18 and is part of a hydrogen bonding network that involves both helices. It is also tilted from the normal arrangement in which the Fe-N bond is in the plane of the histidine ring. Were the H499 side chain to move substantially, the two cavities in the structure could become connected.

Cavities and Positional Specificity

Before structures were obtained for lipoxygenase, an analysis of sequence differences between 12- and 15-LOs led to a proposal that the 12-/15-positional specificity was governed by residues among the four

Table 2 Residues lining two cavities in soybean lipoxygenase-1[a]

Cavity I:
C357, V358, I359, R360, D408, Y409, I412, Y493, M497, S498, **H499,** L501, N502, T503, V570, N573, W574, V575, D578, Q579, L581, D584, K587, **R588,** Y610, W684, L689, **H690** and V693
Cavity II:
T259, **W340, F346,** E349, M350, G353, V354, **N355,** V358, L407, Y409, L480, **K483,** I487, **D490,** S491, H494, Q495, L496, **H499,** W500, **H504, I538,** L541, A542, L546, I547, I553, T556, F557, **Q697,** G701, I704, M705, **N706,** R707, P708, T709, Y734, S747, L748, V750, I751, I753, **L754** and **I839.**

[a] Residues conserved in known lipoxygenase sequences, regardless of positional specificity, are given in boldface in the preceding lists.

conserved differences between 12- and 15-LOs (77). Mutagenesis was used to test the proposal, and the triple mutant Q416K/I417A/M418V of human 15-LO was found to give 12-LO and 15-LO products in the ratio of 15:1, compared with the wild-type protein, which gave a 1:9 ratio. These residues correspond to T555, T556, and F558 in SBL-1 (21, 80), and the latter two have been identified as among the residues that line one of the cavities in the structure of SBL-1 (5) (see Table 2). The reverse mutation made in human platelet 12-LO (12) (K416Q/A417I/V418M) converted the 12-LO, with negligible 15-LO activity, to an enzyme 15-LO: 12-LO products in the ratio 1:9 to 1:4. To achieve a ratio of 2:1, all residues between 398 and 429 of platelet 12-LO were changed to those of human 15-LO (12). Other investigators have obtained variable results in mutations in the same region of other 12- and 15-LOs (82). Shen et al (70a) suggested that 12-/15-LO activity may not be encoded in the protein sequence but may be a function of protein folding or posttranslational modification. They examined the specificity of the enzyme resulting from expression of a 15-lipoxygenase gene in macrophages of transgenic rabbits and found that the ratio of 12- to 15-LO products varied from 0.3 to 0.4 in different isolates of these macrophages.

The two cavities in the major domain of SBL-1 have been designated cavity I and cavity II, as noted in Figure 3 (23). The residues lining cavity I are given in Table 2. From this list, we find several other candidates that can modulate the positional specificity of lipoxygenases through mutagenesis. For instance, cavity residues T556 and F557 in SBL-1, the sites of significant mutations in other 15- and 12-LOs (12, 77), are located at the end of helix 12, but helix 21 (residues 741–755) is also close to this region (62), as can be seen in Figure 3. Although mutations that succeed in interconverting 15-LO and 5-LO specificity

have not been reported yet (39, 56), the platelet 12-LO sequence has been replaced with 5-LO sequences in the region between residues 399 and 418 (corresponding to residues 538–557 in SBL-1) (39). The mutated proteins had good immunoreactivity but minimal 12-LO activity and no 5-LO activity. These studies were designed to address whether the mechanisms of these two enzymes are similar with respect to substrate binding in cavity II. The opposite stereochemistry (5-D and 15-L) of the products of 5- and 15-LO suggests that the fatty acids may bind in the same manner in both enzymes, except with the carboxyl and methyl ends of the fatty acids in reverse positions in the binding site (39).

An interesting feature of cavity II in SBL-1 is a restriction caused by a salt bridge before the cavity reaches from the surface to the iron center. The salt bridge is formed by D490 and R707 (5), but R707 is not a conserved residue in lipoxygenase sequences. Prigge et al (62) have inspected computer models of other lipoxygenases and have suggested that arginine side chains from neighboring structural elements can occupy the same region as R707 in SBL-1, which implies that the restriction in cavity II is conserved by other arginine to aspartate-490 salt bridges.

SPECTROSCOPIC STUDIES OF LIPOXYGENASE STRUCTURE

Three reviews of non-heme iron proteins that provide background to this section have been published (25, 33, 78).

Characterization

Beginning with demonstrations that the native enzyme gives no significant visible absorption and no EMR signal (15, 59), applications of spectroscopy have been used to examine the SBL-1 structure and function. Addition of one equivalent of 13-L_S-hydroperoxy linoleic acid (HPOD, 13-L_S-hydroperoxy-[*E,Z*]-9,11-octadecadienoic acid) converted the enzyme to a yellow form that gives an EMR signal characteristic of high-spin iron. Magnetic susceptibility studies have since shown that both the ferric and ferrous forms of soybean lipoxygenases are high spin (ferrous form, $S = 4/2$; ferric form, $S = 5/2$) (11, 57). In addition, the level of fluorescence intensity of enzyme preparations is lower for the ferric form than for the ferrous form (18). The ferric enzyme spectroscopic signatures revert to those of the ferrous enzyme with the addition of reducing agents or the anaerobic addition of substrate. A recent addition to spectroscopy of the lipoxygenases is charac-

terization of 5-LO (10). 5-Lipoxygenase exhibited the same spectroscopic properties as SBL-1, with one significant exception: 13-HPOD could oxidize the iron (10) but did not react further to form the purple intermediate (49, 59, 75) that is observed with SBL-1. The purple intermediate is discussed further below. The paper on 5-LO spectroscopy (10) contains references to many of the papers on spectroscopy of lipoxygenase that have been published since the 1970s.

The caveat in most of the spectroscopic studies of lipoxygenases is that frozen samples that have a high concentration of protein are required. There are numerous examples of metal centers in proteins that yield different low-temperature spectra, depending on the solutes present. Two examples in non-heme systems are the manganese cluster in photosystem II (3) and the ferric center in phenylalanine hydroxylase (2, 27, 86). Figure 8 gives an example similar effects in lipoxygenase and illustrates the range of changes possible in the EMR spectra of ferric SBL-1 (24, 25, 73). The well-studied case of the addition of ethanol to SBL-1 samples gives a line shape similar to, but slightly sharper than, the spectrum shown in Figure 8 for imidazole addition (24, 73). Because the SBL-1 spectra are also influenced by the concentration of buffer components, ionic interactions at the surface of the

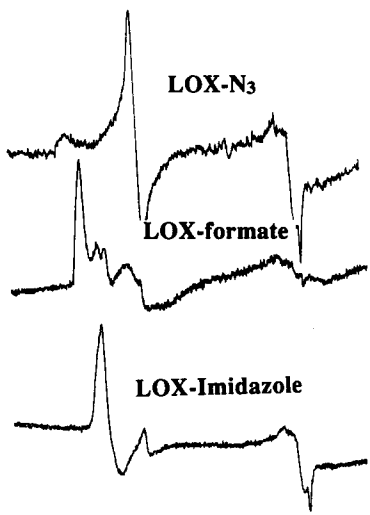
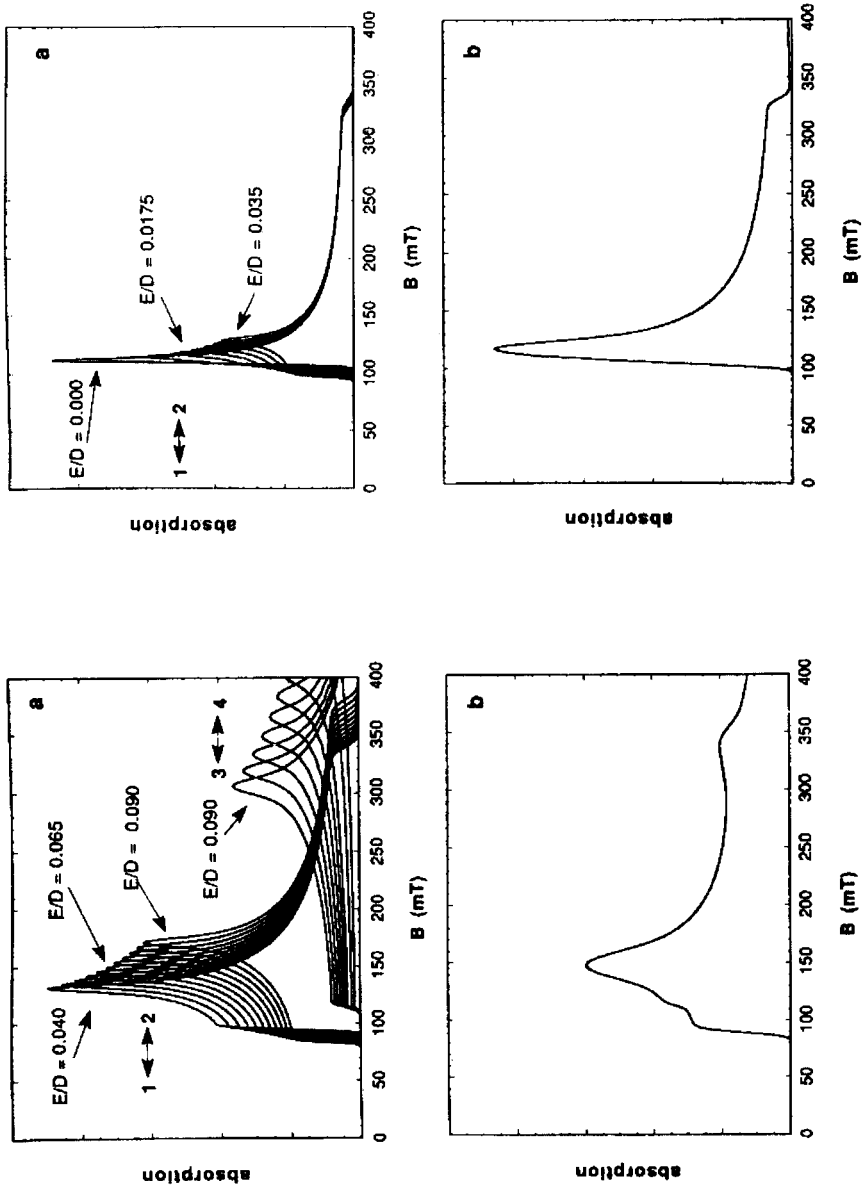


Figure 8 EMR spectra at 4 K of SBL-1 in (*a*) 0.2 M phosphate, 0.5 M sodium azide, pH 7.0; (*b*) 0.2 M sodium formate, pH 7.4; and (*c*) 0.5 M imidazole added to the buffer for *b*. Background signals were not subtracted and contribute to the $g = 2$ region on the right of the spectra. Lipoxygenase was 60 μM. The horizontal axis extends from 0 to 400 mT. From Reference 24.



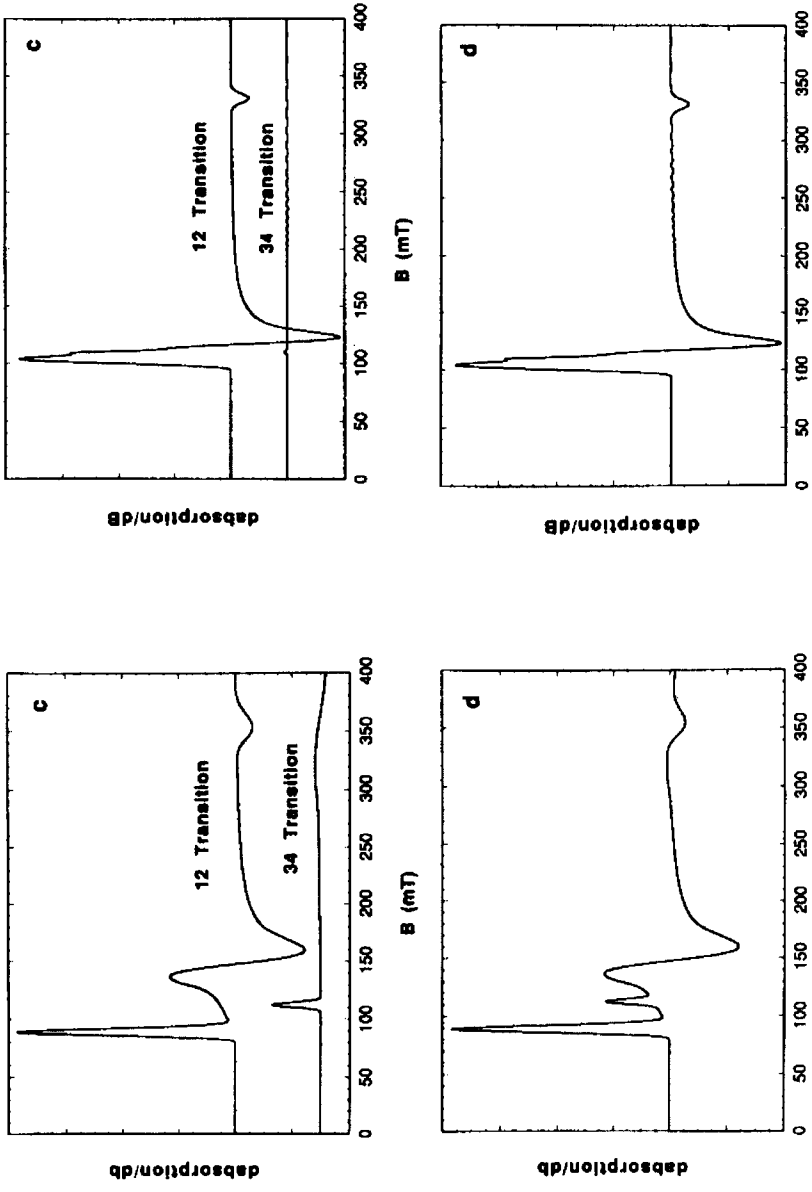


Figure 9 An outline of the calculations of the two components (*left* and *right panels*, respectively) of SBL-1 EMR spectra. (*a*) The distribution of absorption spectra used in the calculation, including absorption between energy levels 1 and 2 and between levels 3 and 4, is shown. (*b*) The signal amplitudes in *a* are multiplied by a Gaussian amplitude factor and added to give the simulated absorption spectrum shown. (*c*) The derivative of the absorption for transitions between each of the pairs of levels is shown separately. (*d*) The sum of the spectra in *c* is shown. From Reference 24.

protein, when it is frozen, may result in altered structural details in the vicinity of the metal center [see Yang & Brill (89) for a discussion of the effects of freezing on hemeproteins]. These effects in SBL-1 studies are not limited to frozen solutions, however, because the effects of glassing agents on solution circular dichroism (CD) and Raman spectra of lipoxygenases have also been reported (61).

Electron magnetic resonance spectra of both SBL-1 (24, 74) and 5-LO (10) have been analyzed quantitatively (90). One study of SBL-1 (24) included the determination of conditions under which the ferric enzyme gave one or the other of two signals but not a mixture of both, e.g. the spectra in Figure 8 for SBL-1 with formate or imidazole (24). Spectra that had been a minor component in other reports were obtained by oxidation of dilute solutions of resting SBL-1 in phosphate or formate buffer (pH 6–8) and rapid removal of fatty acid by-products of the oxidation. Figure 9 (24) shows steps in simulation of the two types of EMR spectra, whereas Figure 10 (24) shows the overlay of the calculated and experimental spectra for the component favored by phosphate or formate buffers. Calculations of the EMR spectra of ferric 5-LO (10) were used to determine the relative proportions of signals at $g_{eff} = 4.3$ (similar to the SBL-1 spectra with azide in Figure 8) and at g_{eff} values greater than 6. In this context, g_{eff} is an effective g-value that satisfies $h\nu = g_{eff}\beta B$, where h is the Planck constant; ν is the spectrometer frequency; β is the Bohr magneton, and B is the magnetic field at which EMR absorption occurs. Both zero-field and Zeeman terms contribute to g_{eff} (25). The conclusion (10) was that the $g_{eff} = 4.3$ signal, although of significant intensity, represented less than 3%

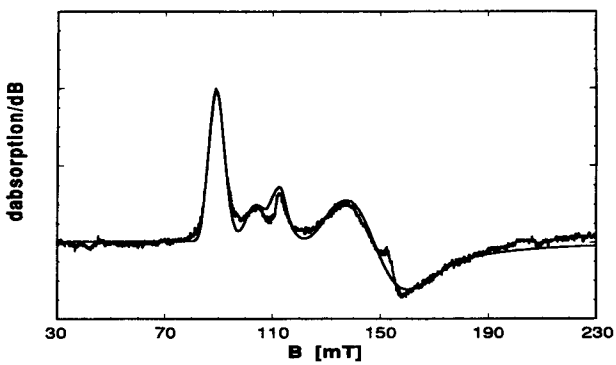


Figure 10 With the use of calculations in the left (93%) and right (7%) columns of Figure 9, a simulated spectrum is overlaid on the low-field portion of the experimental spectrum of SBL-1 in 0.2 M sodium phosphate, pH 7.0. From Reference 23.

of the iron in the sample. The signals at higher g_{eff} values had a shape very similar to the signal from SBL-1. This study, and the ones of His-substituted lipoxygenases (6, 8, 43, 54) that are discussed below, indicate that lipoxygenase EMR signals are related more to the immediate iron ligand environment but do not distinguish between lipoxygenases of different positional specificity (see 38, however, for an exception).

Changes in Iron Ligation

Differences between oxygen and nitrogen ligation to iron are difficult to determine by spectroscopic techniques and usually uncertainties are ± 1 N or O. Nevertheless, the combined techniques of X-ray absorption spectroscopy (XAS) (85), magnetic circular dichroism (MCD) (88, 91), Mössbauer (17, 22), and resonance Raman (of a catecholate complex with SBL-1) (14) together with mutagenesis (mentioned above) were sufficient to identify the nature and location of the potential protein ligands to iron before the lipoxygenase structure was completed. Most spectroscopic studies also suggest the presence of one or two additional oxygen ligands to iron, at least one of which was not apparent in the X-ray structures at 2.6 Å resolution. The earlier results of measurements by XAS, MCD, and resonance Raman have been summarized in several recent papers (54, 69). The discussion here is limited to those experiments that address changes in the coordination environment: an Asn to His substitution in some 12- and 15-LOs and the differences between native ferrous and activated ferric SBL-1.

Changes in Iron Ligation Caused by Amino Acid Substitutions

Two residues in Figures 4 and 5 are not conserved throughout lipoxygenase sequences. In the SBL-1 numbering, these residues are Q495 and N694; in other lipoxygenases, however, these positions may be occupied by E and H, respectively. (In SBL-1, N694 was found at different distances from iron in the two X-ray structures, as noted earlier.) Human and rabbit 15-LOs and some 12-LOs have the histidine for asparagine substitution at the second of these positions (see references in 12, 62, 80) and have been compared with SBL-1 by several forms of spectroscopy (6, 8, 54). Related mutagenesis experiments have shown that when asparagine in this position in 5-LO is changed to glutamine or aspartate (67) or, in another study of SBL-3 (37), to histidine, alanine, or serine, the mutant proteins incorporate iron, but only the histidine mutant has activity similar to the native.

Studies (54) by near-infrared CD (NIR CD) and MCD spectroscopy

are the most detailed to date to address differences in iron center coordination for lipoxygenases in which asparagine, at the position corresponding to SBL-1 694, is replaced by histidine. The comparison was made with samples in the resting, ferrous state. Sucrose was used as a glassing agent for the study, because addition of it to buffered SBL-1 or 15-LOs did not alter the solution (3°C) NIR CD spectra. The spectroscopic results were consistent with six-coordination for the 15-LOs and a mixture of five- and six-coordination for SBL-1. The values of the zero-field splitting parameter, D, are of different sign and magnitude in comparing the His-substituted (D negative) and Asn-substituted (D positive) enzymes, in either five- or six-coordinate species. This finding is consistent with substitution of the stronger His-ligand for an O-ligand or a vacant ligand position. In the same work, the results of XAS were average bond lengths, assuming a hexacoordinate species, of 2.16 ± 0.03 Å for both the soybean and the reticulocyte native enzymes. Results of another XAS study of native SBL-1 (69) in a similar buffer environment led to the same conclusion. For comparison, the average of the iron-ligand bond distances deduced from the 2.6 Å X-ray analysis is 2.2 Å (5). Calculations from XAS data based on fewer than six ligands led to shorter average bond lengths (e.g. 2.12 ± 0.01 Å for five coordinate and 2.0 Å for four-coordinate) (54).

Electron magnetic resonance spectroscopy of lipoxygenases in the ferric form has also been used to examine whether there are spectroscopic differences between proteins with Asn or His in SBL-1 position 694. The EMR spectra of rabbit reticulocyte 15-LO (His) have been reported by two groups (6, 8, 43). This lipoxygenase had little EMR intensity in the resting form and EMR spectra characteristic of high-spin ferric iron after oxidative activation with one equivalent of 13-HPOD, similar to the results obtained with SBL-1 (24, 59, 74) and 5-LO (10) (see above). Electron magnetic resonance spectra of reticulocyte 15-LO were somewhat varied, however, and suggested a broader distribution of spectral parameters characterizing the spectra of the reticulocyte enzyme compared with those for SBL-1. In contrast, EMR spectra of a SBL-3 Asn to His mutant, in the ferric form, resembled those of wild-type ferric SBL-3 more than they did the reticulocyte proteins (37).

Changes Between Ferrous and Ferric States

The second question addressed by spectroscopic studies is whether there are changes in the iron ligation of activated ferric lipoxygenases compared with the resting ferrous ones. Although an X-ray structure of a ferric lipoxygenase has not been reported yet, XAS spectroscopy

has been applied to the question (69, 85), and SBL-1 samples have been used in the studies. One study interpreted differences in data from the two forms of lipoxygenase in terms of replacement of one histidine N-ligand with an O-ligand (85). The results of the more recent study (69) were interpreted in terms of six-coordinate ferric iron with one short bond (1.88 Å), which was not present in the ferrous enzyme sample, and five others with an average bond length of 2.11 Å. The observation of a short bond for ferric SBL-1, but not for the ferrous form, is consistent with a water ligand in ferrous SBL-1 and ionization of this ligand in the ferric enzyme, thereby giving a hydroxyl ligand with the short bond length. The existence of water coordination to iron in ferric SBL-1 was suggested earlier by broadening of EMR signals by ^{17}O-water (48).

RECENT HIGHLIGHTS OF MECHANISTIC STUDIES

A future goal is to relate lipoxygenase structure to mechanism. Some of the insights and unresolved questions regarding the mechanism of lipoxygenases are summarized briefly here.

The current working model of the mechanism of lipoxygenase has the features given in Figure 11. The outer cycle, in which a substrate radical dissociates, has important consequences. Lipoxygenases are inhibited by reducing agents that consume the hydroperoxide, P, and thus trap the enzyme in the ferrous form, E (10, 19, 36). Recently, unusually large kinetic isotope effects have been observed in the SBL-1 reaction (28, 28a, 34), and Figure 11 would have to be modified to be consistent with these effects. The kinetic constants have been found to be a function of temperature, pH, viscosity, substitution of deuterium for hydrogen in the substrate, and substitution of solvent water by deuterium oxide (28a).

Other tests of the mechanism summarized in Figure 11 have focused on two questions: Does lipoxygenation proceed by a free radical mechanism? And, is there involvement of the Δ^9-double bond as well as involvement of the Δ^{12}-bond?

Many investigators have addressed the question of whether lipoxygenation proceeds by a free radical mechanism. Because free radicals are so easily formed by auto-oxidation of polyunsaturated fatty acids (60), it has been challenging to demonstrate that they are true catalytic intermediates of lipoxygenase. In reactions with lipoxygenase, low concentrations of oxygen favor observation of radicals derived from linoleic acid or other substrates. With respect to Figure 11, therefore, either

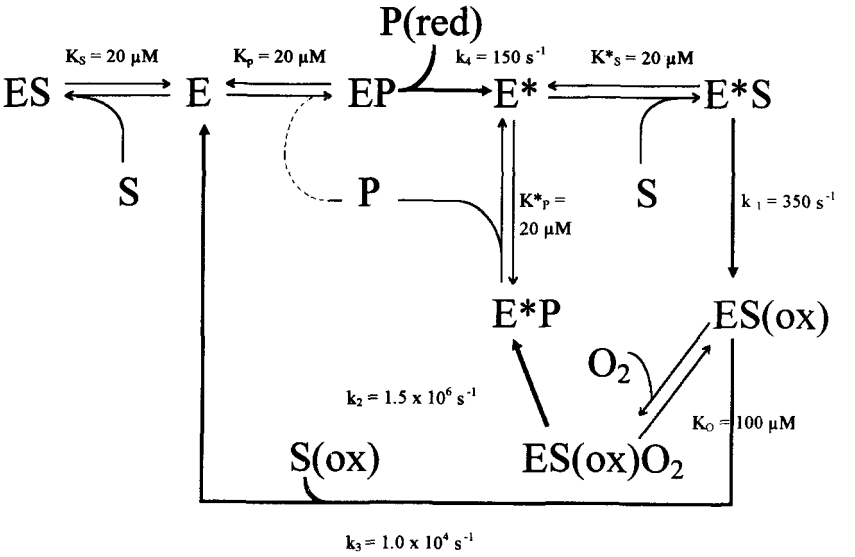


Figure 11 A working model of the SBL-1 mechanism. Ferrous forms of lipoxygenase are designated E and ferric forms are E*. P = product hydroperoxide, and S = substrate. Numerical values shown are for SBL-1 and are taken, largely, from Schilstra et al (70). Kinetic constants are considerably different for rabbit reticulocyte 15-LO (41). Adapted from References 41 and 70.

ES(ox) or S(ox) radicals would build up if conversion to ES(ox)O_2, and then to E*P, is diminished by low oxygen concentrations.

Recent spectroscopic studies of 5-LO provide important new insight into the mechanism regarding formation of a purple intermediate in the lipoxygenase mechanism (10). The oxidation of iron in native SBL-1 is specific for a fatty acid hydroperoxide and cannot be achieved using hydrogen peroxide (59). In a report on 5-LO, Chasteen et al (10) found that this reaction is not specific with respect to the position of the peroxyl group on the fatty acid, as 13-HPOD oxidizes both 15- and 5-LOs. Addition of one equivalent of 13-HPOD to ferric SBL-1, however, gives a purple color (49, 59), whereas the same addition to ferric 5-LO gives no further color over the yellow of the ferric enzyme (10). Assuming that this observation does not simply reflect kinetic differences in the two enzymes, it lends support to the idea that the purple intermediate is a catalytic intermediate in SBL-1 reactions. It also suggests that the structure of the ferric enzyme is different from the ferrous form in ways that affect hydroperoxide binding. Several studies in which free radicals have been detected in purple lipoxygenase are interpreted in terms of conversion of an E*P complex to EP(ox) and subse-

quent free radical reactions (35, 50). This reasoning might add intermediates to the scheme in Figure 11 that include ternary complexes of lipoxygenase, product, and substrate.

The possibility that the lipoxygenase reaction proceeds by a radical pair reaction has been examined by studies of the D/H kinetic isotope effect for reactions in magnetic fields to 0.2 Tesla (34). No effect was found, thereby ruling out a radical pair lifetime in the range of approximately 10^{-10} to 10^{-6} s. Hwang & Grissom (34) note that relaxation by nearby iron might put the radical pair lifetime outside of this range in lipoxygenase. Typical relaxation times at room temperature for ferric and ferrous iron range from 10^{-12} to 10^{-10} s (1a).

Several indications of involvement of two double bonds in the lipoxygenase mechanism have been noted. Although oxygen is added by SBL-1 to C-13 of linoleic acid, a linoleic acid isomer in which the Δ^9-double bond originally had the Z-geometry [(9Z, 12E)-9,12-octadecadienoic acid] was converted to the thermodynamically less favored E-geometry after enzymatic reaction (21). Also, there is some evidence of 9-peroxyl radicals in purple lipoxygenase (50). Secondary steps in converting 15-hydroperoxy linoleic acid to 14,15-leukotriene A_4 also involve hydrogen abstraction three carbons removed from the site involved in the first step in lipoxygenase catalysis (7). Radicals that contain a 12,13-epoxy linoleate, which is a structure related to leukotriene A_4 (LTA_4), have been identified in SBL-1 reactions by combined EMR, gas chromatography (GC), and mass spectroscopic analysis (35).

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