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EPR Spectroscopic Studies of Lipoxygenases

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Abstract

Polyunsaturated fatty acids are sources of diverse natural, and chemically-designed products. The enzyme lipoxygenase selectively oxidizes fatty acid acyl chains using controlled free radical chemistry; the products are regio- and stereo-chemically unique hydroperoxides. A conserved structural fold of ~600 amino acids harbors a long and narrow substrate channel and a well-shielded catalytic iron. Oxygen, a co-substrate, is blocked from the active site until a hydrogen atom is abstracted from substrate *bis*-allylic carbon, in a non-heme iron redox cycle. EPR spectroscopy of ferric intermediates in lipoxygenase catalysis reveals changes in the metal coordination and leads to a proposal on the nature of the reactive intermediate. Remarkably, free radicals are so well controlled in lipoxygenase chemistry that spin label technology can be applied as well. The current level of understanding of steps in lipoxygenase catalysis, from the EPR perspective, will be reviewed.

Graphical Abstract

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Conflict of Interest

The author declares no conflict of interest.

Keywords

Metalloenzymes; Lipids; Enzymes; EPR spectroscopy; Oxygenation

1. Overview of the Active Site in Lipoxigenase and EPR Studies

A lipoxigenase is abundant in soybean seeds (SBL1) and it has been studied from early days of protein chemistry.^[1] It is almost colorless as isolated. Beginning with the earliest structures of lipoxigenases, a cavity with dimensions suitable for binding a fatty acid, winding through a bundle composed largely of helices, has been apparent.^{[2] [3] [4]} The cavities bend and have side branches.^[5] The catalytic metal (usually iron but sometimes manganese^[6]) lies at the internal bend in the cavity. A structure with arachidonic acid bound to anaerobic lipoxigenase,^[7] and several other structures with substrate mimics,^{[4] [8] [9]} reveal the essential features of substrate poised for reaction. The pentadiene reactive moiety lies in the cavity bend on the side of iron facing the water ligand to metal. A branch of the cavity allows oxygen access to an acyl radical intermediate but not to iron.^[5]

Spectroscopy, including EPR, was a major source of information on lipoxigenase structure/function in experiments designed in the period before crystal structures and genomic discoveries of the enzyme in most organisms. Lipoxigenases are large proteins and the ability of EPR to focus closely on a few atoms involved in a mechanism provided impetus for its early application. This review highlights insights obtained in post-structure EPR studies and a very small selection of associated biochemical experiments. Understanding how a catalytic metal and oxygen enter into the chemical mechanism of lipoxigenases is

essential to explaining regio- and stereo-chemical specificity in peroxidation of polyunsaturated fatty acids. Likewise, understanding the orientation and motion of substrate acyl chains within the lipoxygenase cavity is essential.

2. Events at the Catalytic Iron Center in Lipoxygenase

2.1 Biochemical Background about Involvement of iron

Lipoxygenases exercise strict control over stereochemistry of reaction intermediates and products. With arachidonic acid as substrate, one of six bis-allylic C-H bonds is broken in the first step and, after double bond migration, formation of one of twelve unique hydroperoxide products is conceptually possible (Fig. 1).^[10] With linoleic acid and oxygen in large excess over enzyme, the enzymatic reaction product with linoleic acid is 13*S*-hydroperoxy-9,11-(*E,Z*)-octadecadienoic acid (13*S*-HPODE), one of the four possible hydroperoxides (Fig. 1). The figure also shows arachidonic acid labeled on some of the carbons that form the more well-known hydroperoxides in humans. In all, twelve unique hydroperoxides might be formed in β -hydrogen abstraction and antarafacial oxygenation of arachidonic acid and examples of most of these enzyme variants have been characterized.^[11]

Involvement of iron in lipoxygenase reactions with substrate (and product) was established by EPR and other spectroscopies. Most early EPR experiments used the major lipoxygenase isoform of soybean (SBL1).^{[12] [13] [14]} Isolated enzyme has no EPR signal (ferrous) while aerobic reaction with a single equivalent of product leads an EPR spectrum (ferric, high spin activated lipoxygenase). Subsequent (anaerobic) addition of substrate converts the ferric species to an EPR silent one (ferrous). Current understanding is that the lipoxygenase catalytic cycle starts with ferric iron, substrate reduces iron by proton-coupled electron transfer, and the cycle is completed when oxygen adds to a substrate radical and iron returns to ferric by donating a proton/electron to the peroxy-radical.^[15] The nature of this last step (and previous ones) has been investigated by quantum mechanical/molecular mechanical calculations on another lipoxygenase that has specificity in oxidation of arachidonic acid at C-8.^[16] Rotation of two single bonds at C-8 in the peroxy-radical intermediate is able to give a configuration suitable for the final proton/electron abstraction from water bound to ferrous iron of the enzyme intermediate.

There is a pronounced lag in lipoxygenase kinetics when the reaction is initiated by ferrous enzyme.^[12-13, 15] This lag is removed when the reaction begins with preformed-ferric form of the enzyme (activated enzyme). Under saturating concentrations of oxygen and linoleic acid in solution, SBL1 converts the linoleate substrate to a specific HPODE (*vide supra*). But the reaction is different when a single equivalent of HPODE is used to activate the ferrous enzyme: an equivalent of oxygen is consumed and 9(*R,S*)-hydroperoxy-12,13-*trans*-epoxyoctadec-10*E*-enoic acid is formed.^[17] Lack of stereo-specificity at the 9-peroxy radical indicates that an epoxy-radical intermediate has left the ferric enzyme before further reacting in oxygenated solution, thus explaining how SBL1 is activated in a one-electron reaction at iron. Oxygen, when abundant, scavenges the 9-peroxy radical. Under anaerobic conditions, reaction of SBL1 with [¹⁸O₂]hydroperoxy-linoleic acid gives product with high retention of both isotopic oxygens, identified as 11-[¹⁸O]hydroxy-12,13-*trans*-[¹⁸O]epoxyoctadec-9-enoic acid in this case.^[18] Thus, hydroperoxide has been cleaved to generate an alkoxy-lipid

and the equivalent of hydroxyl radical, stored in the form of $\text{Fe}^{3+}\text{-OH}$. In the absence of oxygen, the hydroxyl radical rebounds to complete a peroxidase cycle by reacting with alkoxy radical. Some lipoxygenases, notably eLOX3 of human epidermis, catalyze an alternate hydroperoxide isomerase cycle, most likely because oxygen is not efficiently channeled to the radical intermediate.^[17]

2.2 Characterization of Ferric Iron Centers in Lipoxygenases

What, then, is the nature of "activated ferric lipoxygenase"? How similar is it to the ferrous site in native enzyme? Ferrous iron centers have been characterized by x-ray crystallography for lipoxygenases from many species.^[11] Known active lipoxygenases bind iron with three histidine side chains, the carboxyl terminus, a water, and one other ligand, all held in place by hydrogen bonding to a second coordination sphere (Fig. 2 left).^[19]^[20] The "other ligand" is most often asparagine, in a few cases is a fourth histidine or a serine, or it can be nothing (5-coordinate, 5C). In the latter case, the sixth potential ligand is too far from iron to be a real ligand.^[21]

EPR spectroscopy provides information about the quantity and homogeneity of paramagnetic species. High spin ferric iron samples give a variety of EPR spectra that reflect ligand-metal symmetry from axial to fully rhombic. A protocol in which a single equivalent of HPOD oxidizes dilute SBL1 (1 mg/ml, 10 μM) and the enzyme is rapidly concentrated for measurement, leads to an EPR signal corresponding to rhombic distortion of axial symmetry.^[10] The signal has well-defined maxima (Fig. 2 right). Likely, presence of the large excess of ambient oxygen over enzyme scavenges any radicals released under this oxidation protocol. As isolated, a recent bacterial addition to the lipoxygenase family, *Pseudomonas aeruginosa* lipoxygenase (PaLOX), has a non-substrate phospholipid bound in the active site and a 6C coordination arrangement of iron ligands in the crystal structure of the ferrous enzyme. The EPR spectrum of oxidized PaLOX is nearly identical to that of SBL1 prepared in the same manner (Fig. 2).^[23] The similarity suggests that the same characteristic geometry of ligands is found in ferric forms of both. The upper spectrum (dashed line) is a simulation of the experimental EPR spectra.

From earliest EPR studies of ferric SBL1,^[12-14] spectra have been published showing not one major component (as in Fig. 2) but two (Fig. 3 left),^[24] reflecting sites with different degrees of rhombic distortion from octahedral symmetry, described simply as more axial or rhombic. A smaller portion of the magnetic field range used in Fig. 2 is shown in Fig 3, left and right. Figure 3 right is an expanded region of the spectrum given in Fig. 2 right. Magnetic circular dichroism (MCD) and circular dichroism (CD) spectra also reveal two spectral components in SBL1 (ferric or ferrous forms).^[25]

The EPR experiment illustrated in Fig. 3 left was designed to examine if mutation of residue Ala542 near the substrate cavity entrance, C_{α} ~ 9 Å from iron, leaves the signal of the iron center as well ordered as in the wild type protein. Somewhat surprisingly, the relative amounts of the two components in the iron EPR spectra changed with the mutations distant from iron. In the portion of the spectrum shown in Fig. 3 left, peaks contributed by each component are labelled "1" and "2".^[24] The effect of an A542G mutation (Fig. 3A) is to vary the proportions of spectra labeled 1 and 2, but mutation of this site to Thr led also to

some broad underlying EPR absorption (Fig. 3D), signifying a higher degree of disorder (inhomogeneity) at the iron centers of this mutant. The activity expressed as k_{cat}/K_m was 7.7 for expressed wild-type enzyme and 3.1 for A542G, while the Thr mutant was enzymatically inactive.^[24] The inactivity of A542T can only partially be attributed to inhomogeneity of the sample because small amounts of the wild-type spectra remain.

For comparison, Fig 3 right presents an expanded portion of the spectra in Fig. 2 right. Thus, two spectra of wild type soybean LOX-1 are compared and evidently they are most different in the ratio of components "1" and "2". The samples differ in buffer and in length of time since preparation. Fig. 3B left sample was dialyzed overnight into a Tris buffer (0.2 M, pH 7.5),^[24] while that in Fig. 3 right was prepared within 30 min after iron oxidation by centrifugal buffer exchange into 0.2 M potassium phosphate, pH 6.8.^[23] Even when care is taken to store EPR samples of ferric lipoyxygenase frozen in an EPR tube, time dependent change in the ratio of components "1" and "2" are difficult to prevent (illustrated in a pair of spectra on the frontispiece). Fast (minutes) conversions to EPR spectra dominated by variants of component "2" are observed when glycerol or ethanol are added to either wild type SBL1^[26] or to PaLOX^[23]. Since changes in the iron to 694-residue bond length are well documented in crystal structures^[11], this variation is likely a large contributor to changes observed by spectroscopy, but "hydrogen bonding details and/or the location of waters throughout [the] cavity" are also significant.^[24] Fluctuations in protein backbone, trapped by freezing, also contribute to line widths.^[27] Time dependent changes in EPR of ferric lipoyxygenase spectra may reflect relaxation of a protein environment evolved for ferrous iron to a ferric state. ENDOR is a technique that could contribute understanding to the molecular origin of variations in ferric lipoyxygenase spectra.^[28]

The specificity of redox reactions at lipoyxygenase iron has been examined by EPR. The initial oxidation from ferrous to ferric consumes one equivalent of the enzymatic hydroperoxide product, but other long carbon chain secondary hydroperoxides are reactive too.^[29] Cumene hydroperoxide also forms a ferric SBLI having the same characteristic EPR spectrum (Fig 3 left) as that formed when the enzyme hydroperoxide product is the oxidant.^[30] This is interesting because it suggests that in homolytic cleavage of an organic hydroperoxide by SBL1, it is hydroxyl radical, not the carbon-centered radical, that oxidizes iron. Numerous reducing agents are also active on the ferric iron in lipoyxygenases, and this is the basis of many inhibitors, among which the catechol nordihydroguaiaretic acid (NDGA) is well known.^[31] Substituted ortho-catechols form complexes with iron in SBL1 and a ferric to ferrous reduction is carried out by the stronger of those reductants. Following this process by EPR provided the basis for estimating that the iron redox potential in native SBL1 is 0.57-0.68 V at pH 7.0.^[32] Shifts in His-to-Fe³⁺ charge-transfer optical bands (400-425 nm region) in first-coordination sphere mutants of SBL1 also report on reduction potentials. Reduction potentials correlate well with factors affecting k_{cat} of lipoyxygenase first-coordination sphere variants,^[33] but less well for mutation at more distant sites.^[34]

Some of the human lipoyxygenases have also been examined by EPR to test cycling of the iron oxidation state in mechanistic steps. Human 5-lipoyxygenase (5-LO) oxidizes arachidonic acid at carbon-5 as a first step in leukotriene synthesis.^[35] The amino acid ligands to iron, from the crystal structure, are the same as those in SBL1, but the enzyme is

less stable than SBL1. Residues near the terminal carboxylate ligand to iron are the source of the instability in native 5-LO.^[36] Ferric 5-LO EPR signals are even more axial than "spectrum 2" of SBL1.^[37] An additional 5LO rhombic EPR signal has been attributed to inactivated 5-LO.^[38] On the other hand, EPR spectra of human 15-LO-1, where His replaces the Asn ligand of most other lipoxygenases, suggest a rather inhomogeneous ferric center with both rhombic and more axial species. In this case, conversion of the rhombic species to axial, by further product addition, was demonstrated for the 15-LOs having a fourth His ligand to iron.^[25] Porcine 12-LO also has four His ligated to iron^[39] and EPR spectra reflect "an array of coordination configurations related to one another by the conformational flexibility of the protein".^[40] Several of the human lipoxygenases in which EPR spectra suggest inhomogeneity only yield crystals in the presence of a fatty-acid-mimic inhibitor.^[4, 8] ^[9] Possibly, EPR spectra of these variants of lipoxygenase formed under various preparations might reveal conditions giving higher homogeneity at the iron center.

Effects of changes in the iron first- and second-coordination spheres of lipoxygenases have been evaluated by mutational analysis and spectroscopy of SBL1, and these approaches avoid the variation in preparations and overall protein stability inherent in cross-species comparisons. Evidence that the iron-coordinated water exchanges protons with solvent water was provided by ENDOR/isotope studies of the +2 metal state (Mn²⁺ substituted enzyme),^[28] and by ¹⁷O exchange and broadening of the EPR spectrum of the +3 ferric state.^[41] Mutations of the first sphere ligand Asn694 that retain reasonable enzymatic activity are N694H and N694G, while N694C has very reduced activity.^[20] In the crystal structure of ferrous N694G, a second water, or hydroxyl, replaces the native Asn as a ligand to iron, and other non-bonding waters replace the Asn side chain volume. The result is a first-coordination sphere with two aquo ligands. The EPR spectrum of ferric N694G resembles component "1" in Fig. 3 left more than component "2".^[26] Another example of a ferrous lipoxygenase with two coordinated waters is in the crystal structure of human 15-LO-2 in complex with a fatty-acid mimetic inhibitor.^[9] Since the ferric intermediate in a lipoxygenase catalytic cycle must have a hydroxyl ligand to abstract hydrogen from substrate, which water substituent in the N694G mutant of SBL1 is hydroxyl is an interesting point of discussion regarding the reduced activity of the mutant.^[33] For native SBL1, it could be advantageous to have water/hydroxide at two ligand positions to facilitate generation of the active site hydroxyl in starting the catalytic cycle. Whether this second aquo ligand remains during turnover is a subject for discussion.

Returning to the question on the nature of "activated ferric lipoxygenase", significant factors include the following.

1. Among many ferrous lipoxygenase crystal structures, one metal ligand (N694 in SBL1) is at variable distances from iron, bonding or nonbonding distances.^[11]
2. There are examples of lipoxygenase structures that have two waters bound to the active site ferrous iron. One is a mutant of SBL1^[33] and the other is a natural lipoxygenase (human 15-lipoxygenase-2) complexed with a fatty acid mimic.^[9] Upon oxidation of iron, one of these waters must ionize.^[33]

3. The same EPR spectra of ferric SBL1 are obtained whether the oxidant is the enzyme product, or cumene hydroperoxide.^[30] This identifies hydroxyl radical, generated *in situ*, as the oxidant.^[17] The same conclusion is reached from analysis of organic products resulting from oxidation by the natural product of lipoxygenation.^[17]

4. Reaction of SBL1 with its [¹⁸O] oxidized linoleic acid product, under low oxygen concentrations, gives an oxygen-rebound reaction with high retention of both isotopic oxygens. This shows that oxidation of iron by peroxide product occurs in a controlled environment that protects SBL1 protein from hydroxyl radical damage.^[18]

5. Although mutants of SBL1 uniformly have lower (and much lower) k_{cat} values than native, the rate of iron activation to the ferric form is elevated by a factor of ~2 in N694G compared to native N694.^[33] An increase in a kinetic constant is strong evidence that an observed change does not result from a fraction of inactive enzyme.

Together, these factors suggest that activation of lipoxygenases by one equivalent of product is a reaction of *in situ*-generated hydroxyl radical with 5-coordinate ferrous enzyme, likely at the vacant iron site near Asn694. This first-formed intermediate rearranges to exchange a proton between the two aquo ligands on iron, so that a hydroxyl is in the correct position to abstract hydrogen from substrate. The balance of locations for the hydroxyl may account for the near equally populated ferric states observed by spectroscopies, and/or shifts in the monovalent carboxylate ligand may contribute to those observations of two populations.

3. Specificity of Hydrogen Abstraction from Polyunsaturated Acyl Chains

As lipoxygenase structures from more and more sources are solved, and found to have similar overall folds, questions about the manner by which each one forms a unique hydroperoxide product (Fig.1) have motivated new experimental approaches. A formative experiment addressing this subject used isotope-substituted linoleic acid bearing deuterium on a single bis-allylic methylene hydrogen.^[42] A large isotope effect in SBL1, >40 decrease in k_{cat} , and more recently in other lipoxygenases, was observed, consistent with a quantum tunneling mechanism in the rate-limiting catalytic step. A long, bent cavity filled with waters is observed in crystal structures of SBL1^[3] and other lipoxygenases.^[43] ^[11] and the bend occurs close to the catalytic iron atom. Extensive quantum-chemical calculations have been directed to understanding^[28, 44] ^[45] the isotope effect in SBL1 and, in particular, whether the required donor-acceptor distance (substrate C₁₁ to O of metal coordinated water) for hydrogen tunneling (~2.7 Å) can be met. The relevant lipoxygenase crystal structures with a substrate, or substrate-like, fatty acid bound suggest donor-acceptor distances significantly longer than this, 3.4^[9] and 3.7^[23] Å, respectively.

ENDOR measurements and quantum calculations addressing proton-coupled electron transfer in SBL1 revealed two donor-acceptor distances in the frozen-solution structure of linoleic acid in complex with a manganese-substituted SBL1.^[28] The linoleic acid carbon from which hydrogen is abstracted in forming 13-HPOD is C11. Both linoleic acid C10 and, separately C11, were substituted with ¹³C for the measurements. Using manganese-substituted SBL1 enabled a novel ENDOR procedure in which very low temperature (2K)

was used to suppress population of all but the lowest energy $m_s = 5/2$ sublevel of Mn^{2+} electron spin. The hyperfine interaction between ^{13}C and metal is dominated by a through-space dipolar interaction in this experiment. The ENDOR offsets from the ^{13}C nuclear frequencies depend on the spin quantum number, m_s , of the metal EPR sublevels, and the inverse cube of the distance between nucleus and metal. The result of the experimental design, and associated simulations of orientation-selected ENDOR spectra, is that measurement of nucleus-manganese distances as long as $\sim 6 \text{ \AA}$ was possible. Two orientations of linoleic acid were discovered, with $^{13}\text{C}_{11}$ -Mn distances of 4.9 and 5.7 $\text{\AA}$, and $^{13}\text{C}_{10}$ -Mn distances both of 4.8 $\text{\AA}$. The three-dimensional geometry was supplemented with a proton-Mn ENDOR distance for the water ligand to metal. Molecular dynamics (MD) calculations including these distances gave a donor-acceptor distance of $\sim 3.1 \text{ \AA}$ for the closer C_{11} to O_{water} distance. Calculation of 50 ns MD trajectories, based on the ENDOR-derived distances, gave 0.2 % of the frames reaching a distance $\sim 2.9 \text{ \AA}$, suggesting that the required distance for hydrogen transfer can be attained by conformational sampling.^[28]

4. Spin Label Studies of Lipoxygenase

Stable nitroxide free radicals, $(\text{R}_3\text{C})_2\text{N-O}\cdot$, or spin labels, are easily reduced to a non-paramagnetic hydroxyl amine, $(\text{R}_3\text{C})_2\text{N-OH}$, so the ferrous iron and reactions allowing substrate radicals to escape the active site are potential concerns for spin label studies of lipoxygenases. However, neither is a significant issue in practice. Nitroxides are too bulky to gain access to the iron and even the step that activates the enzyme to the ferric state does not lead to loss of a nitroxide signal as long as the step is performed in excess of oxygen. Spin labeled lipid analogs that are tools used in studies described below are shown in Fig. 4.

An advantage of using spin labels to study large proteins is that localized motion in the vicinity of the probe can be evaluated. While EPR spectra of high spin iron discussed above have a magnetic field breadth covering more than 400 mT, recorded on frozen samples, the spectra of spin labeled molecules examined at common instrument frequency have breadths of $\sim 3\text{-}7 \text{ mT}$ and can be measured frozen or at ambient temperature. Motional averaging of spin label EPR parameters renders the spectra sensitive to motional rates in the nano- to microsecond range.^[46]

4.1. Lipoxygenase as a Fatty Acid-binding Protein

The physiological substrate of lipoxygenases is usually described as an unsaturated fatty acid released from membrane lipids by action of a phospholipase A. However, it has been shown that lysolipids in which the 1-acyl chain has the appropriate *cis,cis*-pentadiene unit also serve as substrates.^[47] In the one crystal structure of a lipoxygenase with a bound phospholipid, the acyl chain that occupies the lipid binding site is the 1-acyl chain,^[23] as it would be with a lysolipid resulting from a phospholipase A_2 reaction. Monounsaturated acyl chains, for instance oleoyl sulfate,^[48] are known lipoxygenase inhibitors, so a spin labeled version of an oleoyl lysolipid, lysooleoylphosphatidylTEMPOcholine (LOPTC), Fig 4, was examined as an inhibitor. Against linoleic acid, LOPTC is a competitive inhibitor with $K_i \sim 20 \text{ }\mu\text{M}$.^[49] Pulsed EPR experiments also provided an estimate of $K_d \sim 60 \text{ }\mu\text{M}$ for binding of LOPTC in frozen solutions of a SBL1 derivative.^[49]

Figure 5A^[49] shows EPR spectra recorded at room temperature of LOPTC in the presence (upper sets of spectra) and absence (lowest spectrum) of SBL1. Although EPR spectra are usually shown as derivatives of absorption, the first integrals are used in Fig. 5 so that the broader signal (~70 mT wide) under the sharp ones is apparent. In the absence of SBL1, LOPTC exists mostly in micelles at 30 μ M (Fig. 5A) and 60 μ M (Fig. 5B) in buffers. The broad signals are characteristic of micelles in which nitroxides at the polar ends of LOPTC are so close-packed that electron spin exchange dominates and the usual three lines are averaged, except for a small sharp component arising from monomer in solution. Titration of micelles of LOPTC with protein (Fig. 5A) reflects transfer of lysolipid monomer from micelle to SBL1, where the spin labeled head group exhibits tethered motion bound to protein. The transfer is more complete at high pH (Fig. 5B).^[49] A series of nitroxide-substituted fatty acids (doxyl stearates, DSAs) (Fig. 4A) also bind to SBL1 with affinities that EPR spectra show increase as the number of methylenes between the carbon-bearing the probe and the methyl end of a stearate increases. For instance, the spin labeled stearic acid derivative 8-DSA, with nine methylenes on the terminal methyl end, has 31 μ M affinity in solution.^[50]

4.2. Location of the Polar End of Lipoyxygenase Substrates

The crystal structure of SBL1 clearly reveals an internal fatty-acid-length cavity but an entrance to the cavity is not apparent and the cavity has several side branches (*vide supra*). Although SBL1 has an internal arginine that could anchor a substrate carboxylate (R707),^[3] this residue is not conserved across diverse organisms. Since phospho- and lysolipids are substrates for which SBL1 has the same specificity as for the free fatty acid, it seems likely that the bulk of the polar end of phospholipids keeps the head group at the protein surface. Indeed, the narrow line widths of the EPR spectra of LOPTC monomers bound to SBL1 (Fig. 5) indicate fluctuations of the head group that are almost as fast as rotational motion of a small molecule in solution. Free fatty acids can be modeled in both orientations (carboxylate-in and carboxylate-out)^[28] and it now seems likely that both orientations are possible for fatty acids and various experimental situations.^[51] It could be argued that the lipoyxygenase activation step would require the oxidizing fatty acid hydroperoxide to have an orientation reversed from that of the product when it is still on the enzyme.

Pulsed EPR Double Electron-Electron Resonance (DEER) and Double Quantum Coherence (DQC) experiments were designed to locate the substrate cavity entrance in SBL1.^[49] In these EPR experiments, after suitable pulse sequences, the through space interaction between two spins in frozen solutions contributes a dipolar component to the microsec decays. Nitroxides were the spins introduced into a cysteine-free SBL1 by site-directed spin labeling (SDSL) or by addition of a spin labeled lipid. Probability distributions of distances between probes, (~10-80 Å), are calculated from the data. The experimental design represents SBL1 as an object with five vertices, each bearing a spin label in a sequestered site near the surface (Fig.6). Docking calculations suggested potential sites where the spins would have limited mobility. The geometric shape of the spin labeled object was determined by measuring all ten pair-wise, two-dimensional distances in separate doubly-labeled constructs. Lastly, the spin labeled substrate analog, was paired with each singly labeled vertex and those spin-spin distances were derived. The coordinates of the polar end of

LOPTC, bearing the spin label choline group, were determined by distance geometry (supplement, ref^[49]). Uncertainty in the measured distances was revealed in the relatively narrow width of the probability distributions of distances. The derived conformational distribution of the LOPTC spin could be approximated as an ellipse with width of the major radius of ~ 6 Å, at 2σ . The volume determined for the LOPTC spin labeled head group overlaps side chains of residues E236, K260, Q264 before and in helix 2 and residue Q544 in helix 11. This solution is illustrated in the Frontispiece figure. The LOPTC head group was found lodged in the region between the two domains of SBL1, just outside of the interface of α -helices 2 and 11, a site that had previously been considered a possible entrance for substrate into the internal cavity.^{[3] [24]} Since the ellipse in which the LOPTC head group undergoes motion overlaps side chains of two residues in helix 2 and one in helix 11, there must be some conformational readjustment of these residues.^[49]

4.3. Protein Motions at the Entrance to the Substrate Cavity

Helices 2 present the most varied structural features in crystal structures of lipoxygenase catalytic domains. Among those showing arachidonic acid 15-lipoxygenase activity, SBL1 has a 21 amino-acid helix with a bulge, or π -helix, in the middle, and as noted earlier, the helix blocks access to the internal substrate cavity.^{[2] [3]} Crystals of 15-LO from rabbit reticulocytes have monomers with helices 2 in different positions: in a closed form and others in open form and containing inhibitor.^[8] The *Pseudomonas aeruginosa* 15-LO has ~ 100 aa preceding an identifiable helix 3, and these residues correspond to a folded group of three helices (α , $\alpha 2A$, $\alpha 2B$) that resemble a lid closing entry to the active site.^[23] Lipoxygenases with different positional specificity, porcine leukocyte 12-LO and human 5-LO, have unique helix 2 configurations as well.^{[39] [36]}

To examine SBL1 in solution at ambient temperature, a series was prepared of spin labeled derivatives of the cysteine-free enzyme, in which each of eighteen amino acids in helix 2 was replaced with cysteine and then spin labeled (SDSL).^[52] The effects of pH variation from 7 to 9 and of lipid addition on the room temperature EPR spectra were examined. In this case, the lipid was not spin labeled because the protein spin labeled cysteine sites were the focus. Cysteines were labeled with 1-oxy-2,2,5,5-tetramethyl-3-pyrroline-3-methyl (MTSL), and the lipid the effects of which were investigated was lyso-oleoyl-phosphatidylcholine (LOPC). Generally, the spectra of helix 2 labeled sites show the effects of motional rates ranging from overall rotational motion of the whole protein (~ 45 ns) to fluctuations almost as fast as rotation of a small molecule in solution (< 2 ns). The middle of helix 2, the π -helix, exhibited the largest position-dependent variation (Fig. 7). (Worth noting is that k_{cat} for each of the spin labeled residues represented in Fig. 7 is within a factor of two of that of the native enzyme.)

At pH 7, spin labeled residues in this region show spectra reflecting the solvent exposure of the corresponding natural side chain in the crystal structure. In contrast at pH 9, where SBL1 is most active, more focused examination of side chain motions associated with lipid binding revealed importance of bending of helix 2 in forming the active site entrance. It appears that the middle of helix 2 in SBL1 has an abrupt change in rapid backbone fluctuations from hydrogen bonds breaking and reforming as pH is raised, and lipid binding influences these

processes. Spin label EPR at the conventional EPR frequency is able to monitor changes in 1 to 100 nsec rates of motion. The results reinforce the notion of helices 2 as a regulators of substrate entry into lipoxygenase binding sites. Viewed on the slower time scale of hydrogen exchange, the whole helix 2-3 region is dynamic compared to the core of the protein.^[53]

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Biography

Biographical Sketch. Dr. Gaffney has studied lipid properties and protein function by electron paramagnetic resonance (EPR) and other biophysical methods. The EPR studies involve development of spin labeling and simulations of EPR line shapes. Iron proteins included in various biophysical studies by Dr. Gaffney are lipoxygenases (primarily), phenylalanine hydroxylase, and transferrin.

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Figure 1. Substrates and product hydroperoxide from the catalytic reaction of lipoxygenases. Reaction of soybean lipoxygenase with linoleate (upper and middle fatty acids). Stereochemical course of reactions of animal lipoxygenases with arachidonate (lower). From ref. [10], with permission.

Figure 2.

Left: The structure of the iron atom and first coordination sphere ligands in SBL1 (from 1YGE coordinates, ref [3]). Right: comparison of EPR spectra of the "activated forms" of ferric lipoxygenases from soybean (SBL) and from *P. aeruginosa* (*PaLOX*). Features of the major EPR component are labeled as "LKD y and x and z". From ref. [23], with permission.

Figure 3.

Left: EPR spectra of ferric SBL1 (B, C) and Ala542 mutants A542G (A), and A542T (D). Note that the magnetic field range shown is condensed from that in Fig. 2. Adapted from ref. [24], with permission. **Right:** A portion of the spectra in Fig. 2 right is expanded to the same scale as Fig. 3 left.

Figure 4. Representative spin labeled lipids applied in studies of SBL1. (A) The doxyl derivatives of stearic acid, where the carbon bearing the doxyl ring is the (n+2) carbon. (B) Lyso-oleoylphosphatidyl choline with a TEMPO group replacing one choline methyl. From ref. [10], with permission.

Figure 5.

Solution EPR spectra of LOPTC as micelles in the absence of protein (lowest spectra in A and B), and bound to cysteine-free SBL1 (upper spectra). The concentration of SBL1 is varied in A and is constant at 100 μM in B. Spectra are given as first integrals in A and B. The usual derivative presentations are in the inset (grey). Adapted from ref. [49], with permission.

Figure 6. Outline of experimental design for pulsed dipolar EPR spectroscopy with a five-sided shape representing the SBL1 structure. The sites shown on the structure (red, oxygen and blue, nitrogen) are computer-docked locations of site-directed spin labels, ((from 1-oxyl-2,2,5,5-tetramethyl-D3-pyrroline-3-methyl)methanethiosulfonate). The inset (lower left) shows a close-up of a space-filling computer fit of one spin label into the protein environment. Adapted from ref. [10], with permission.

Figure 7.

The structure of the pi-helical region of helix 2 in SBL1 (upper). Solution EPR spectra show position-dependent rotational motion (lower); fast motion for solvent exposed normal side chain of SBL1 (*e.g.* I265) and immobilization for solvent inaccessible (*e.g.* S263) at pH 7.2. Changes with pH and lipid binding also shown. From ref. [10], with permission.