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Review

Structural biology of mammalian lipoxygenases: Enzymatic consequences of targeted alterations of the protein structure ☆

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Abstract

Lipoxygenases form a heterogeneous family of lipid peroxidizing enzymes, which have been implicated in the pathogenesis of diseases with major health political relevance (bronchial asthma, atherosclerosis, cancer, and osteoporosis). The crystal structures of one mammalian lipoxygenase and of two plant isoenzymes have been solved and the structural bases of important enzyme properties (reaction specificity, membrane binding, and suicidal inactivation) have been investigated in the past. This review will briefly summarize our current understanding on the structural biology of the most important mammalian lipoxygenase isoforms and will also address selected mechanistic features of the lipoxygenase reaction.

Keywords: Eicosanoids; Oxygenases; Molecular enzymology; Structural biology; Molecular dynamics; Mutagenesis; Inflammation; Atherosclerosis; Protein structure; Catalytic mechanism

Lipoxygenases (LOXs) form a family of fatty acid dioxygenases, which catalyze the stereospecific insertion of molecular oxygen into polyunsaturated fatty acids [1]. The primary products of the LOX reaction are hydroperoxy fatty acids, which usually are rapidly reduced to the corresponding hydroxy derivatives. LOXs occur in plants [2] and animals [3] but have also been detected in selected bacteria [4]. Up to the mid-1970s LOXs were believed to occur only in plant kingdom and lipid peroxidation in animal tissues was usually attributed to hemoprotein catalysis. In 1974, the formation of (12*S*,5*Z*,8*Z*,10*E*,14*Z*)-12-hydroxyeicosa-5,8,10,14-tetraenoic acid (12-HETE) was described in human platelets suggesting expression of a true LOX in these cells [5]. Several months later, a LOX was also detected in rabbit reticulocytes, which was capable of oxidizing phospholipids and biomembranes [6]. This enzyme was later on purified to homogeneity and was comprehensively characterized with respect to its protein chemical and enzymatic properties [7]. The structural identification of the slow reacting substance of anaphylaxis (SRS-A) as a mixture of the peptido-leukotrienes C_4 and D_4 [8] in 1979 and the finding that the 5-LOX is involved in leukotriene biosynthesis were milestones in early LOX research and prompted many researchers to join the field. Today, LOX research is a vital scientific area (2500 papers on LOXs have been published during the last 5 years) and research is currently focused on expression regulation of the enzymes and on the biological relevance of the different LOX-isoforms. In contrast, the structural biology of these enzymes is underdeveloped and only few groups are active in this field. Here, we will summarize the experimental data available at the moment on the structural biology of LOX-isoforms and will discuss the selected aspects of the catalytic mechanism of the LOX reaction.

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Structure of lipoxygenases

Crystal structure of lipoxygenases

The soybean LOX-1 was already crystallized in 1947 but it took 46 years to solve its crystal structure [9,10]. Crystallization of the rabbit 15-LOX was described in 1990 [11] and 7 years later its 3D-structure was solved [12]. Although the amino acid sequences of plant and mammalian LOXs are rather different, the 3D-structures exhibit a high degree of similarity. All LOX-isoforms consist of a single polypeptide chain, which is folded into two major domains. For the rabbit 15-LOX the small N-terminal domain comprises 110 amino acids and is composed of 8 β-barrels. Its sequence, size, and structure is very similar to those of the C-terminal β-barrel domains of mammalian lipases and has been implicated in membrane binding. The β-barrel domain shares a 1600 $Å^2$ interface with the larger C-terminal domain and both domains are interconnected by a small peptide (amino acids 111–124) without defined structure (random coil). The large catalytic domain consists of 18 helices, which are interrupted once by a small β-sheet sub-domain. The center of the C-terminal domain contains 2 long helices, which contain 4 of the 5 protein iron ligands. The fifth iron ligand is the C-terminal isoleucine and water (or hydroxyl) may occupy the sixth ligand position. The iron ligand sphere has octahedral geometry but there appears to be a certain degree of distortion [13]. The overall shape of the enzyme resembles an elliptic cylinder with a large diameter of the ground square of 6.1 nm, a short diameter of 4.5 nm, and a height of 10 nm (Fig. 1). The substrate-binding cleft is a boot-shaped cavity that is directly accessible from the surface of the protein. Its entrance is lined by R403, G407, and L597, and the bottom is defined by the side chains of F353, I418, and I593. The latter residues have been identified as sequence determinants for the positional specificity of the enzyme whereas R403 might interact with the carboxylic group of fatty acid substrates.

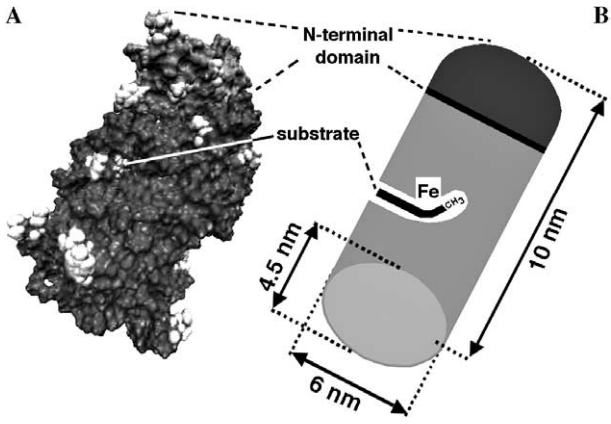

Fig. 1. Structural views of the rabbit 12/15-LOX. The three-dimensional structure of the enzyme was calculated on the basis of the X-ray coordinates [12]. (A) Surface view of the enzyme. The bright residues indicate solvent exposed hydrophobic amino acid and the two-domain structure is indicated. The substrate fatty acid (arachidonic acid) was modeled into the substrate-binding pocket and its carboxy terminus interacting with R403 can be seen. (B) Schematic view of the rabbit LOX structure. The overall shape resembles an elliptic cylinder, the dimensions of which are given in the figure.

Solution structure of lipoxygenases

In crystals, protein molecules are immobilized and this will largely prevent the movement of structural elements. In contrast, in aqueous solutions there may be a higher degree of structural flexibility and thus, the solution structure of an enzyme might differ from its crystal structure. When we investigated the solution structure of the ligand-free rabbit 15-LOX by small angle X-ray scattering [14], we found that at low protein concentrations (<1 mg/ml) and at 10 °C the enzyme is present as hydrated monomer. In fact, the scattering intensities followed closely the Guinier law suggesting a non-aggregated state of the protein solution. Superposition of the high-resolution crystal structure and our low-resolution model of the solution structure calculated from the scattering data revealed that the models are almost perfectly superimposed in the region of the catalytic domain. In contrast, the region of the N-terminal β-barrel domain in the solution structure was stretched out. Such an extension was not observed when a truncated enzyme species lacking the N-terminal β-barrel domain was used. These data might be explained by a high degree of motional freedom of the N-terminal β-barrel domain relative to the catalytic subunit in aqueous solutions. Such interdomain movement may be of functional importance for regulation of catalytic activity and membrane binding. X-ray scattering experiments on the soybean LOX1 did not reveal any evidence for interdomain movement [15]. For this LOX isoform a low-resolution model was calculated, which exactly matched the crystal structure. These results were somewhat surprising since detailed evaluation of the crystal structure suggested the possibility of interdomain movement even for this enzyme [10]. If one compares the crystal structures of the rabbit and soybean LOX, three major differences can be found, which might contribute to prevent interdomain movement for the soybean enzyme: (i) In the soybean structure, residues 295–328 form a small subdomain, which is replaced by a very short loop (three amino acids) in the rabbit enzyme. (ii) Compared with the rabbit 12/15-LOX (1600 $Å^2$) the interdomain contact plane of the soybean enzyme is much larger (2600 $Å^2$). Thus, one may conclude that interdomain contact is more pronounced for the plant enzyme. (iii) The overall structure of the soybean enzyme is more compact than that of the rabbit 15-LOX. For the latter enzyme some structural elements are rather loosely connected to the enzyme core. In fact, the position of the amino acids 177–188 could not even be defined in the crystal structure, what may point towards motional flexibility of this structural element.

Summarizing the recent data on the solution structure of LOXs one may conclude that for the rabbit 15-LOX there

is evidence for an interdomain movement in aqueous solutions. Since these data were not confirmed with the soybean LOX1, additional comparative experiments (NMR studies) are needed to prove interdomain motional flexibility of the rabbit enzyme. Such experiments are currently underway.

Structural basis for the catalytic mechanism

The catalytic cycle of the LOX reaction consists of four elementary reactions (hydrogen abstraction, radical rearrangement, oxygen insertion, and product dissociation) and involves a valence shuttling of the non-heme iron between ferrous and ferric forms. The stereochemistry of all elementary reactions is tightly controlled. Although some structural aspects of the control mechanisms have been investigated in the past, many questions remain unanswered. The LOX reaction is initiated by stereoselective hydrogen abstraction from a bisallylic methylene and this elementary reaction is the rate-limiting step in LOX catalysis [16]. According to the conventional concept of enzyme catalysis formation of the transition state of the LOX reaction requires high activation energy. However, experimentally, rather low values (between 4 and 8 kcal/mol) have been determined. To solve this problem a quantum chemical tunnel mechanism was suggested and kinetic isotope effects provided experimental evidence for the validity of this theory [17]. However, the question about the structural basis for hydrogen tunneling and the potential role of the non-heme iron remain unclear.

Substrate alignment at active site

The crystal structure of a 15-LOX/inhibitor complex was solved for the rabbit 15-LOX but no direct structural data on LOX/substrate complexes are currently available. There is, however, a structural model for the 15-LOX/arachidonic acid complex. This model is based on the X-ray coordinates of the enzyme–inhibitor complex [12] but also considered mutagenesis data [18]. It should be stressed that even in the absence of any structural data on LOXs, there were attempts to describe the alignment of fatty acid substrates at the active site of enzymes [19] and these topological models are close to our current understanding. Experiments with arachidonic acid isomers, in which the double bond system was shifted gradually towards the methyl or carboxyl terminus of the substrate fatty acid, suggested that the oxygenation rate and the reaction specificity of 12/15-LOXs depend on the distance of the bisallylic methylene from the methyl end of the fatty acid molecule [20,21]. It was hypothesized that the active site of LOXs may constitute a hydrophobic pocket and a substrate fatty acid may slide into this pocket with its methyl end ahead. The substrate is then positioned at the active site in such a way that the hydrogen atom, which is abstracted from the bisallylic methylene, is located in close proximity to the non-heme iron acting as electron acceptor [19,21]. According to this theory a dual positional specificity results when two bisallylic methylenes are located in similar distance to the iron (Fig. 2B). To explain the

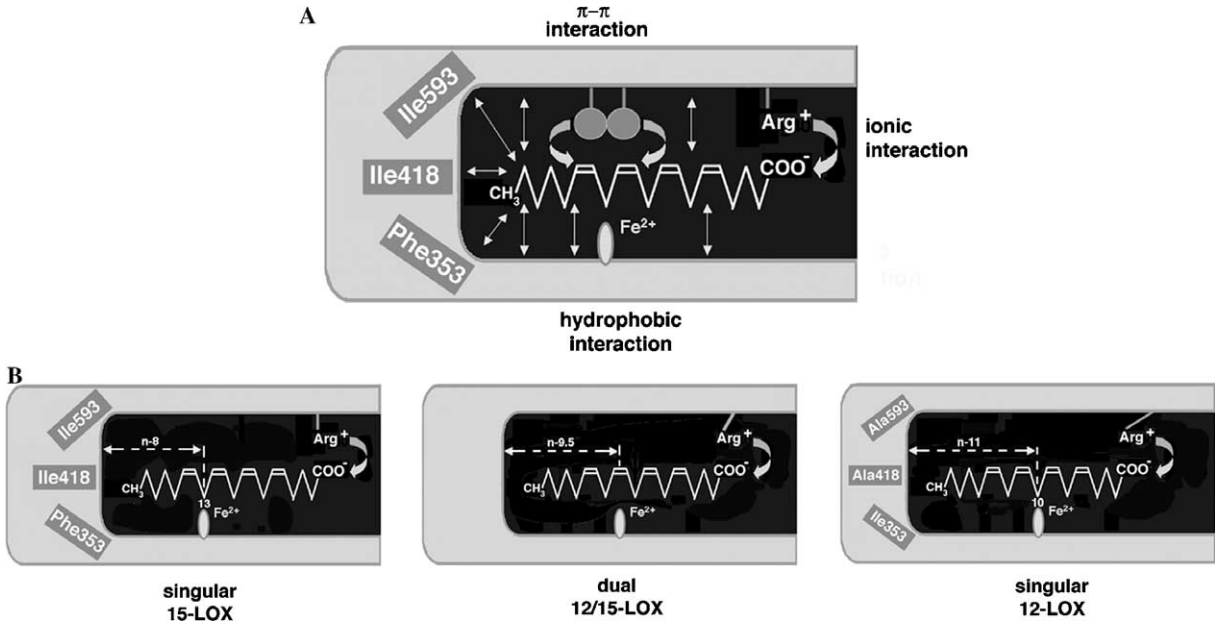

Fig. 2. Three point interaction between the rabbit 15-LOX and its fatty acid substrates. The active site of LOXs is a pocket, which is formed by the side chains of hydrophobic amino acids. The bottom of this pocket is defined by the sequence determinants of the positional specificity (F353, I418, and I593). Polyenoic fatty acid substrates slide into the binding cage with their methyl ends ahead. The bisallylic methylene, from which hydrogen is abstracted, is located in close proximity to the non-heme iron. (A) Fatty acid binding forces. According to our current knowledge three types of binding forces contribute to substrate alignment: (i) hydrophobic interaction between the apolar fatty acid chain with hydrophobic amino acid side chains (represented by the double arrows), (ii) ionic interaction of the fatty acid carboxylate with R403, and (iii) π-electron interaction of the fatty acid double bonds with aromatic amino acids (F415). (B) Substrate alignment at the active site of 12- and 15-lipoxygenating enzymes (singular positional specificity) and for an enzyme species with dual positional specificity (12/15-LOX).

mechanisms that contribute to substrate alignment at the active site, a three point enzyme/substrate interaction has been suggested (Fig. 2A): (i) hydrophobic interaction of the methyl end of the fatty acid substrate with those amino acids forming the bottom of the substrate binding pocket; (ii) ionic interaction of the fatty acid carboxylate with a positively charged site chain; (iii) π-electron interaction of the fatty acid double bonds with aromatic amino acid residues.

To identify amino acids involved in substrate binding, structural modeling of enzyme–substrate complexes was carried out and site directed mutagenesis studies were performed to test the modeling results. As indicated in Fig. 2A a triad of amino acids (F353, I418, and I593) forms the bottom of the substrate binding pocket and the methyl ends of fatty acid substrates appear to interact with these residues. Site-directed mutagenesis of these amino acid to less space filling residues alters the reaction specificity of 12/15-LOX in favor of 12-lipoxygenation [22] and these data can be explained on the basis of the space related hypothesis [23]. Alternatively, alterations in the positional specificity can be explained if an inverse head to tail substrate orientation is assumed [24]. In such cases, substrate fatty acids may enter the substrate-binding pocket with its carboxylate ahead approaching other bisallylic methylenes to the hydrogen acceptor (iron). Structural modeling suggested that R403 interacts with the negatively charged carboxylate of substrate fatty acids and the high degree of motional flexibility of its side chain makes it possible that fatty acids may slide in deeper into the substrate-binding pocket without breaking this ionic interaction. Site-directed mutagenesis of R403 to an uncharged L strongly reduced the catalytic activity of the enzyme [25]. These data appear to suggest the importance of this residue for the catalytic activity. However, it is always dangerous to interpret the data of inactive mutants because of the possibility of unspecific effects. It may well be that mutation might have induced overall structural alterations, which lead to a drop in the catalytic activity. Similar experiments were carried out on F415, the aromatic side chain of which has been suggested to interact with the π-electrons of the fatty acid double bonds [25]. When F415 was mutated to a non-aromatic L (F415L), reduction of the catalytic activity (20% residual activity) and alterations in the product specificity (15-HETE/12-HETE ratio of 1.6:1) were induced. In contrast, when an aromatic W was introduced the resulting mutant was fully functional. Here again, the interpretation of the data obtained with the F415L mutant is rather problematic.

Oxygen movement inside the enzyme

As other dioxygenases LOXs catalyze a bimolecular reaction, and polyunsaturated fatty acids and atmospheric dioxygen constitute the two substrates. Because of their balanced electron configuration both substrates have limited chemical reactivity. LOXs activate the fatty acid substrates by converting them into a radical intermediate but there is no evidence for oxygen activation. Since previous attempts failed to characterize an oxygen-binding site at the enzyme, it was suggested that the enzyme-bound fatty acid radical is the immediate oxygen acceptor. However, it remains unclear how atmospheric oxygen penetrates into the active site. Initially it was believed that oxygen can freely diffuse into the active site and that there is no special path for oxygen movement. On the other hand, random diffusion mechanism appears to be unlikely because of two reasons: (i) The active site of LOXs constitutes a deep hydrophobic cavity, which is not filled with solvent. Thus, oxygen has to travel a long way from the surrounding water to reach the enzyme-bound fatty acid radical, which is buried deeply inside the core of the protein. For oxygenases, which have an active site much closer to its surface, such uncontrolled oxygen diffusion might be more likely. (ii) Assuming random oxygen diffusion it is difficult to explain the high degree of stereoselectivity of most mammalian LOX isoforms. Thus, a more targeted mechanism of oxygen transport is required. For the soybean LOX1 a separate oxygen access channel was recently suggested [26,27]. This channel intersects with the substrate-binding pocket between L546 and Q495, a position that would be occupied by C-13 of an enzyme-bound linoleic acid radical. Moreover, this channel encounters the active site on the face of the substrate-binding cleft opposite to the non-heme iron. Thus, if oxygen employs this channel to travel into the active site it is targeted to the very position where it is needed during catalysis. I553F mutation on the soybean LOX strongly reduced (6.3-fold) the catalytic efficiency of linoleic acid oxygenation (k_{cat}/K_M) but k_{cat} was only reduced 2-fold [26]. According to the authors these data suggested that introduction of a bulky F may impede oxygen access to the fatty acid radical. Such hindrance of oxygen penetration should increase the K_M for oxygen but such constants were not determined in this study.

When we inspected the crystal structure of the rabbit 15-LOX for the presence of such an oxygen access channel, we did not find it to be conserved. Moreover, when we mutated the amino acid that aligns with I553 of the soybean enzyme we did not detect major alterations in fatty acid oxygenation. To trace oxygen movement inside the 15-LOX molecule, we developed an oxygen model suitable for MD simulations and modeled oxygen movement. Simulation of this movement under strongly hyperoxic conditions indicated an oxygen access channel that delivers oxygen to the fatty acid radical exactly at the site where it is needed for 15(*S*)-lipoxygenation of arachidonic acid (Fig. 3). Interestingly, in our simulation experiments oxygen employs the same path for movement regardless of whether it was originally placed in the solvent surrounding the enzyme molecule (inward movement) or in the active site (outward movement). Now we are in the process of testing these findings by site-directed mutagenesis.

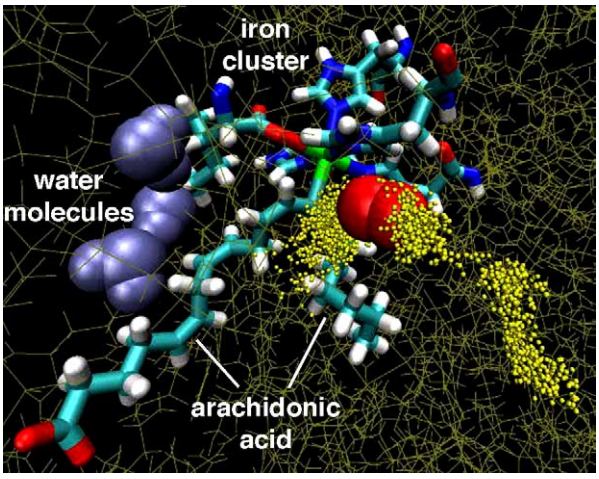

Fig. 3. Oxygen movement within the rabbit 15-LOX. The enzyme substrate complex of the rabbit 15-LOX was modeled in such a way that the hydrogen to be abstracted from the C-13 was arrested in appropriate distance to the non-heme iron (green). An oxygen molecule (red double spheres) was placed next to C-15 of arachidonic acid and its diffusion was studied during MD simulation. The path of oxygen diffusion indicated by the yellow dots extends to the protein surface. When oxygen was initially placed in the solvent surrounding the protein molecule, the same path is taken for oxygen diffusion. Thus, according to this MD simulation, oxygen diffuses through a preformed channel to the very point of the active site (15*S*-position of arachidonic acid) where it is needed for catalysis.

Structural basis for biologically relevant enzymatic properties

Sequence determinants for the positional specificity of various lipoxygenase isoforms

At the early stages of eicosanoid research the positional specificity of LOXs with arachidonic acid as substrate was used as decisive criteria for LOX categorization. 5-LOXs oxygenate arachidonic acid at carbon 5 (C-5) of its hydrocarbon backbone whereas 15-LOXs introduce dioxygen at C-15. Because of the growing diversity of mammalian LOXs, this classification is rather confusing but officially it is still in use.

For the positional specificity of 12/15-LOXs three different amino acids (F353, I418, and I593) are of major importance and these residues are clustered together in the 3D-structure. The bulkiness of their side chains determines the depth of the substrate-binding pocket. When small amino acids are located at these positions, the fatty acid substrate slides in deeper into the substrate-binding cage approaching the bisallylic C-10 of the arachidonic acid molecule to the non-heme iron. This configuration of the enzyme/fatty acid complex favors 12-lipoxygenation of arachidonic acid. In contrast, large amino acids at these positions favor arachidonic acid 15-lipoxygenation. The structural basis for these alterations has been discussed before [22] and will not be addressed in this review.

Despite their different positional specificity the murine epidermis 8*S*-LOX and its human ortholog, the epidermis-type 15*S*-LOX (15-LOX2), share a high degree of amino acid conservation. Site-directed mutagenesis indicated that Y603 and H604 in the 8*S*-LOX appeared to be determinants for the positional specificity [28]. These amino acids align with L589 and G590 of rabbit 15-LOX and thus are close to I593, which has been shown to be important for the specificity of the rabbit enzyme (see above).

Attempts to convert 12/15-LOXs to a 5-lipoxygenating enzyme species were not successful. We recently followed an inverse strategy, trying to convert the human 5-LOX to a 15-lipoxygenating enzyme mutating the previously defined sequence determinants (see above). Initially, these amino acids were mutated separately but only partial alterations in the positional specificity were observed. Next we created double, triple, and even quadruple mutants and observed progressive conversion of the human 5-LOX to an 8- and finally to a 15*S*-lipoxygenating enzyme species. The final, quadruple mutant (F359W + A424I + N425M + A603I) exhibited a somewhat reduced catalytic activity (25% of the wild-type enzyme), but converted arachidonic acid mainly to 15*S*-HpETE (85%), with 8*S*-HpETE (15%) being a minor byproduct [29].

For a long time it was believed that mammalian LOXs exclusively produce *S*-hydroperoxides. However, more detailed search for *R*-lipoxygenating isoforms indicated expression of a 12(*R*)-LOX in murine and human skin [30–32]. Because of their recent discovery little is known on the structural basis for the positional specificity of mammalian *R*-LOXs. To shed light on this topic, we carried out multiple sequence alignments of *S*- and *R*-LOXs, and found that F390, A455, and V631 of the murine 12(*R*)-LOX align with the positional determinants of 12/15-LOXs. Multiple site-directed mutagenesis at F390 and A455 did not induce specific alterations in the reaction specificity but yielded enzyme species with strongly reduced specific activities and unspecific product patterns. In contrast, when V631 (aligns with I593 of the rabbit 15-LOX) was mutated to less space-filling residues (A, G) we obtained enzyme species with augmented catalytic activity and specifically altered reaction characteristics [major formation of chiral 11(*R*)-HETE methyl ester]. These data indicated V631 as major sequence determinant for the specificity of the murine 12(*R*)-LOX [33].

Formally, the positional specificity of a LOX is the resultant of the stereochemistry of two elementary reactions (site of hydrogen abstraction and direction of radical rearrangement). The structural basis for defining the site of hydrogen abstraction (C-13, C-10 or C7 of arachidonic acid) has been explained above. However, it remains unclear how the direction of radical rearrangement is determined [formation of 15-HETE (+2 rearrangement) or 11-HETE (-2 rearrangement) when hydrogen is abstracted from C13)]. The fatty acid radical formed during hydrogen abstraction is generally depicted as a pentadienyl radical, in which the electron density is equally distributed over the entire pentadienyl system generating a higher stability. This configuration would make C-1

(C-11 of arachidonic acid when hydrogen is abstracted from C-13) and C-4 (C-15 of arachidonic acid when hydrogen is abstracted from C-13) of the pentadienyl system equally reactive for oxygen attack and thus similar quantities of 15- and 11-HpETE would be expected. However, if the electron density would be focused at either C-15 or C-11 preferential formation of 15- or 11-HpETE, respectively, becomes plausible. Unfortunately, the way of how the electron density is focused on either carbon is far from clear. It might be possible that electron drawing amino acid side chains delocalize the radical electron. Alternatively, spatial distortion of the planar pentadienyl may favor the formation of an ene-allyl radical (e.g., Δ^9-[11–13] allyl radical), which enables selective 15-lipoxygenation. Similar mechanisms may be discussed as potential reason for the enantioselectivity of these enzymes (see below).

Sequence determinants for the enantioselectivity

As indicated above several amino acid residues influencing the regiospecificity of LOXs have been identified, but the basis of stereocontrol of the LOX reaction is not well understood. For various LOX-isoforms it has been shown that hydrogen abstraction and oxygen insertion proceed from different sides of the plane determined by the double bond system of the fatty acid substrate [34,35]. This antarafacial character of the LOX reaction (Fig. 4) suggests a stereochemical coupling of the two elementary reactions (hydrogen abstraction and oxygen insertion) but the structural basis for this coupling remains unclear. The stereochemistry of hydrogen abstraction is likely to be determined by the special alignment of the fatty acid relative to the hydrogen (electron) acceptor (non-heme iron). If the D-hydrogen[1] of a bisallylic methylene is located in close proximity to the hydrogen acceptor, this hydrogen is likely to be removed. Multiple sequence alignment of *R*- and *S*-LOXs indicated a single conserved amino acid difference between these two enzyme subclasses. At this position an A is present in all *S*-LOXs but a G was found in *R*-LOXs. When this A was mutated to G in the mouse 8*S*-LOX and human 15-LOX-2, the enzymes were converted to 12*R*- and 11*R*-lipoxygenating species, respectively. Furthermore, the coral 8*R*- and the human 12*R*-LOX, which contain a G at the critical position, were transformed to 12*S*- and 8*S*-LOXs when a similar mutagenesis strategy was employed. These data suggested that the basis for *R* or *S* stereocontrol involves a switch in the positional specificity of the enzyme and thus couples positional specificity and enantioselectivity of LOXs [37]. However, when such mutations were performed on the murine 12*R*-LOX (G441A exchange) no major alterations in the positional specificity were observed. The main oxygenation product

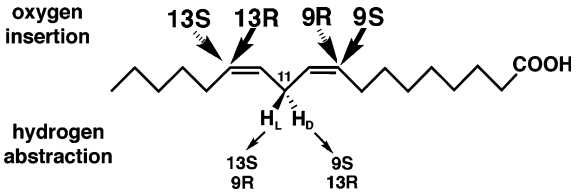

Fig. 4. Antarafacial character of the LOX reaction. When linoleic acid is used as LOX substrate, hydrogen abstraction is only possible from a single bisallylic methylene (C-11). Two hydrogen atoms (pro-D and pro-L) are bound to this carbon but only one is selectively abstracted. Abstraction of the pro-D hydrogen leads to the formation of either 9*S*- or 13*R*-HpODE. In contrast, abstraction of the pro-L hydrogen is involved in 13*S*- and 9*R*-HpODE formation. In both cases, hydrogen abstraction and oxygen insertion proceed from opposite sites of a virtual plane determined by the double bonds.

of arachidonic acid methyl ester by the G441A mutant was still 12-HETE. Interestingly, the 8-HETE formed by the wild-type enzyme was mainly *R* (*S*/*R*-ratio of 2:8) whereas the mutant produced predominantly 8*S*-HETE (*S*/*R*-ratio 70:30). These data confirm the importance of G441 for the enantioselectivity of the murine 12*R*-LOX but they also suggest that for this enzyme there is no strong coupling between positional specificity and enantioselectivity [33].

Structural basis of membrane binding

LOXs are cytosolic enzymes but they have a tendency for membrane association. For the soybean LOX-1 it has been reported that the enzyme binds to biomembranes via calcium dependent mechanisms [38]. Structural modeling suggested the existence of two independent calcium-binding sites, one in the N-terminal β-barrel domain (E21, E106, and E179), the other one in the catalytic domain (E673, D674, and E677). Together with surface exposed hydrophobic amino acids these calcium-binding sites have been implicated in membrane association of the enzyme. Although the role of calcium ions is still not completely understood, they have been suggested to form salt-bridges between acidic surface amino acids and negatively charged constituents of membrane phospholipids.

The human 5-LOX does also have a tendency for membrane binding. In various cells, an increase in the cytosolic calcium concentration triggers translocation of the enzyme from the cytosol to the nuclear envelope [39] and the N-terminal β-barrel domain appears to be essential for membrane association [40]. Structural modeling and site-directed mutagenesis suggested an important role of three surface exposed tryptophanes (W13, W75, and W102) for membrane binding of the 5-LOX [40].

The rabbit 15-LOX (15-LOX-1) is capable of oxygenating esterified polyenoic fatty acids even if they are incorporated in the lipid bilayer of biomembranes and this capability does also require membrane binding of the enzyme. In fact, when the purified enzyme was incubated with sub-mitochondrial membranes in the presence of

[1] For the stereochemical assignment of the two hydrogen atoms at the bisallylic methylenes the Fischer nomenclature should be preferred since it mirrors the steric relations of the enzyme substrate complex in a more suitable way than the Cahn–Prelog convention [36].

0.5 mM calcium the majority of the 15-LOX was recovered from the membrane pellet [41]. In resting cells, the 15-LOX is localized in the cytosol but an increase in cytosolic calcium concentration induces binding to intracellular membranes [41]. To explore the structural basis of calcium-dependent membrane binding, we performed truncation studies and site-directed mutagenesis to define amino acid residues involved in this process [42,43]. Our data indicated that 15-LOX1 associates to biomembranes primarily via hydrophobic interactions between surface-exposed hydrophobic amino acids (Y15, F70, L71, W181, and L195) and membrane lipids. These sequence determinants of membrane binding are located in the N-terminal β-barrel domain (Y15, F70, and L71) as well as in the catalytic subunit (W181, L195). N-terminal truncation of the enzyme (deletion of the β-barrel domain) impaired, but did not abolish, membrane binding. Calcium supports membrane binding probably via the formation of salt-bridges between the negatively charged head groups of membrane phospholipids and acidic surface amino acids, which might contribute to overcome repulsive forces [42,43].

Molecular reasons of suicidal inactivation

LOXs undergo suicidal inactivation during the oxygenation of polyenoic fatty acids [44,45] but the mechanistic aspects of the inactivation process remain elusive [46]. It has been suggested that hydroperoxy fatty acids, the primary products of the LOX reaction, may oxidize catalytically relevant amino acids at the active site, and thus, may contribute to suicidal inactivation. In fact, treatment of the rabbit 15-LOX with 13*S*-HpODE was accompanied by a selective oxidation of a methionine residue [47]. However, site-directed mutagenesis of this methionine to an oxidation resistant alanine did not reduce suicidal inactivation [48]. More recent studies on the inactivation of 12-LOXs by 15*S*-HpETE suggested a covalent linkage of reactive metabolites as mechanistic reason for suicidal inactivation [49]. We also observed covalent modification when incubating the native rabbit 15-LOX with 15*S*-HpETE [50]. Covalent modification was not observed when non-LOX proteins were used and when the enzyme was heat-inactivated prior to incubation. Separation of proteolytic cleavage peptides (Lys-C endoproteinase digestion) by two-dimensional-gel electrophoresis and mass spectral identification revealed covalent modification of several active site peptides (Fig. 5).

It should be stressed at this point that suicidal inactivation of LOX involves several mechanisms depending on the chemical structure of the inactivating agent. Since linoleic acid cannot be converted to epoxy leukotrienes, the above-described mechanism is not possible. However, 13*S*-HpODE, the oxygenation product of linoleic acid, does also inactivate the enzyme, although this reaction is not as efficient as inactivation by 15*S*-HpETE.

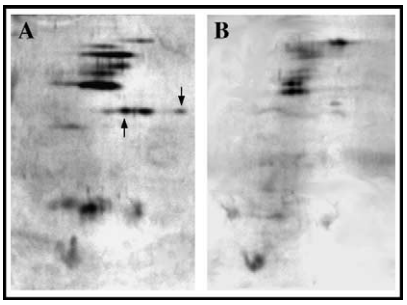

Fig. 5. Inactivation of rabbit 15-LOX by 13S-HpODE. 1.5 mg of rabbit reticulocyte LOX (total volume of 1 ml) was incubated at 25 °C with a 8-fold molar excess of 13-HpODE for 30 min. The protein was precipitated with trichloroacetic acid for 30 min on ice. The precipitate was spun down, washed extensively with acetic acetone, and prepared for Lys-C peptidase cleavage as described in [49]. After Lys-C cleavage, the mixture of cleavage peptides was analyzed by two-dimensional PAGE and representative electropherograms for (A) the native LOX and (B) the inactivated LOX are shown. Arrows indicate the most prominent differences in the cleavage patterns.

Perspectives

The primary structures of more than 20 mammalian LOX-isoforms have been reported so far but only for the rabbit 15-LOX the three-dimensional structure has been solved [12]. To design more detailed studies on the molecular enzymology of the enzyme family and to develop a comprehensive theory on the structural basis of the LOX reaction, additional crystal structures would certainly be helpful. Unfortunately, there is no natural high yield source for any mammalian LOX-isoform (except rabbit reticulocytes) and most recombinant expression systems do not provide sufficient recombinant protein for detailed crystal trials. It would be a big step forward to work out a highly efficient recombinant expression system (e.g., insect cells, *Pichia pastoris*), which yields mg-amounts of enzymatically active LOX protein. Once such a system has been established for one particular enzyme, it may be used to express various isoforms.

Despite our constantly increasing knowledge on the molecular enzymology of LOXs, several aspects of the molecular mechanisms of the oxygenase reaction are not well understood. There are multiple reasons for this lack of knowledge but some open questions will be answered in the near future. Small angle X-ray scattering measurements on two LOX isoforms in aqueous solutions revealed controversial results. For the rabbit enzyme the incongruence of the crystal and the solution structure suggested a significant degree of interdomain movement. Although in the rabbit experiments care was taken to minimize protein aggregation, it could not be completely ruled out. To answer the question on the motional flexibility of the LOX in aqueous solutions, comparative (rabbit vs. soybean LOX) NMR studies should be carried out. Although NMR studies on large proteins (75–100 kDa) are usually difficult to interpret, it should be possible to obtain additional evidence for interdomain movement or to disprove this theory.

The alignment of fatty acid substrates at the active site of LOXs is an essential feature of the LOX reaction and appears to be important for the regio- and stereospecificity of these enzymes. Unfortunately, there are no direct structural data of LOX/substrate complexes. It would be very helpful for the interpretation of past and future mutagenesis data if X-ray coordinates of enzyme/fatty acid complexes would be available. Unfortunately, crystallization studies of enzyme substrate complexes must be carried out under strictly anaerobic conditions to avoid product formation and suicidal inactivation. An alternative approach for obtaining X-ray data on LOX/fatty acid complexes would be crystallization studies with catalytically inactive enzyme mutants. Such mutants must first be X-rayed without substrate to find out whether or not structural alterations were induced by mutagenesis. Besides the fact that such crystal trial must be performed under anaerobic conditions, interpretation of the experimental data might not really be straightforward. The major complication in such experiments is the formation of unproductive enzyme/substrate complexes [51]. Such complexes will only yield limited functional information and quite often it may not even be possible to define productive and unproductive complexes. Moreover, there is a possibility for the existence of several productive [33] and unproductive enzyme/substrate complexes, which makes data interpretation even more complicated.

The driving forces and the path of intra-enzyme oxygen movement during the LOX reaction must be studied in more detail in the future. So far, experimental data on a targeted oxygen transport have only been provided for the soybean LOX1 and thus, it remains unclear whether targeted oxygen movement is a general feature of the LOX reaction. MD simulations on the rabbit LOX/arachidonic acid complex did also provide evidence for targeted oxygen transport but mutagenesis studies, in which the oxygen access channel is narrowed (increase in K_M for oxygen) or opened (decrease in K_M for oxygen), are still pending. Creation of the mutants is not a problem but reliable determination of oxygen K_M. for the various mutants is rather difficult. Nevertheless, such experiments would not only be important for the oxygenases involved in eicosanoid biosynthesis (various LOX- and COX-isoforms) but also for other enzymes reacting with atmospheric dioxygen.

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