Plant lipoxygenase: structure and mechanism

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The presence of a lipid-oxidizing enzyme in plants, then termed lipoxidase, was first described by André and Hou in 1932 [1]. A pigment bleaching property, attributed to a separate enzyme activity described as carotene oxidase, was later found to originate from this enzyme as well [2]. The name lipoxygenase (linoleate:oxygen oxidoreductase, EC 1.13.11.12) is now used for this enzyme.

Lipoxygenase catalyses the dioxygenation of fatty acids containing one or more (1Z,4Z)-pentadiene systems to conjugated hydroperoxydiene derivatives (Scheme 1). In addition to the dioxygenase activity, a hydroperoxide dehydrase activity was found with arachidonic acid (5,8,11,14-eicosatetraenoic acid, ETE) as the original substrate that yields leukotriene A_4 from 5-hydroperoxyeicosatetraenoic acid (5S-HpETE) [3]. The enzyme is ubiquitous in higher plants [4] and has also been found in lower eukaryotes such as yeast [5], algae [6–8] and fungi [9]. Its presence has been reported in several prokaryotes as well [10–12]. Lipoxygenase has been found in mammals [13,14] where it is a key enzyme in the biosyntheses of regulatory molecules such as leukotrienes and lipoxins [15,16]. Mammalian lipoxygenases were recently reviewed by Yamamoto [17].

In plants, usually several lipoxygenase isoenzymes are present that differ in properties such as pH-optimum, substrate preference and product specificity [18]. It has long been thought that lipoxygenase is a unique enzyme, being able to catalyse an oxidation reaction without the assistance of a prosthetic group or a metal cofactor. In the early 1970s, however, the presence of a non-haem iron atom in a molar ratio of 1:1 was reported [19,20], and iron has also been confirmed as being present in mammalian lipoxygenases [15,21].

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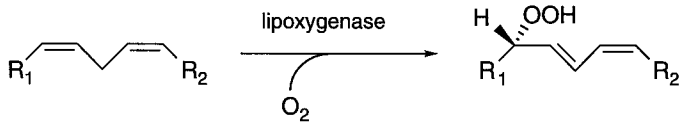
Scheme 1 General reaction catalysed by lipoxygenase

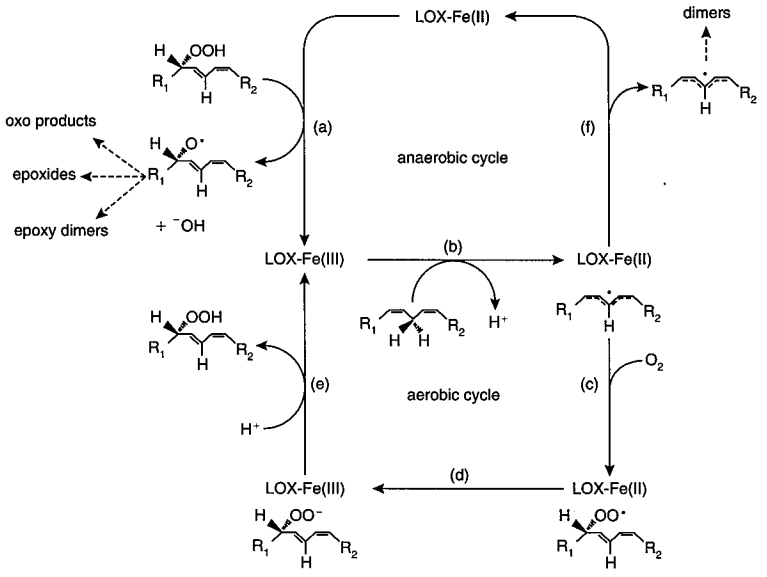


Substrates are unsaturated fatty acids such as linoleic, linolenic and arachidonic acids.

Catalytic cycles

Although soybean lipoxygenase was crystallized as early as 1947 [22], the lipoxygenase isoenzymes from soybean were first purified to homogeneity in the early 1970s [23]. Subsequently, much work has been directed towards lipoxygenase-1 from soybean and the reaction catalysed by this enzyme with linoleic acid as substrate. On the basis of spectroscopic evidence, a model for the catalytic cycles of lipoxygenase was proposed by De Groot et al. [24] (Scheme 2), which accounts for the role of iron, the kinetics observed upon incubation of linoleic acid with lipoxygenase and dioxygen, including the initial lag phase, and for the secondary products formed under anaerobic conditions [25,26].

Scheme 2 Catalytic cycles of the anaerobic and aerobic lipoxygenase reactions



Adapted from [24].

Resting, catalytically inactive, lipoxygenase needs lipid hydroperoxide for activation as was shown with kinetic experiments investigating lipoxygenase [27–31], and are likely to be converted into alkoxy radicals in this process (Scheme 2, step a [24])

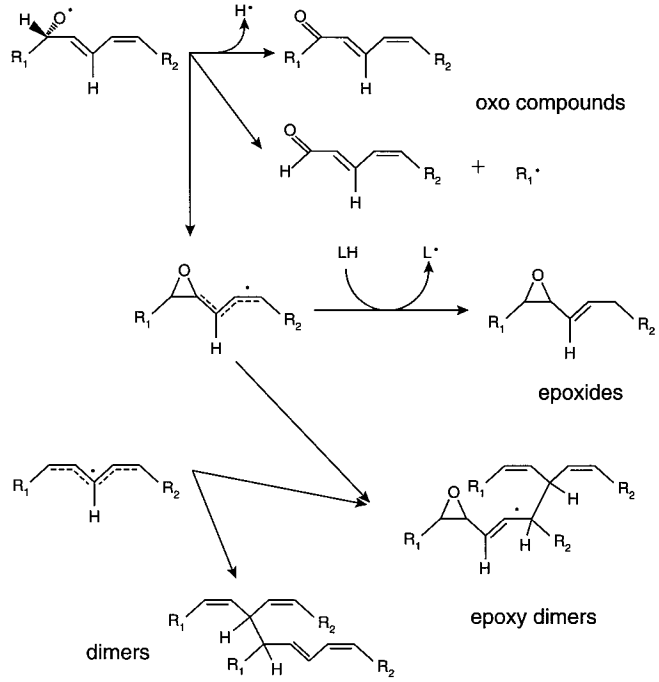
The reaction is either started by traces of hydroperoxides, formed during autoxidation of the substrate, and/or by traces of Fe(III)-lipoxygenase present in the Fe(II)-lipoxygenase preparation [32]. The activation by the product hydroperoxide explains the existence of a kinetic lag-phase in the reaction of resting lipoxygenase with linoleic acid [28,33,34]. The hydroperoxide products formed at the start of the reaction oxidize Fe(II)-lipoxygenase to the active Fe(III)-form; more hydroperoxide is formed by the increasing amount of Fe(III)-lipoxygenase, and eventually all enzyme is activated and no further hydroperoxide is consumed. It has recently been proposed that the hydroperoxide needed to activate the enzyme merely binds to the native enzyme to facilitate activation to the Fe(III)-enzyme [35]. The hydroperoxide was reported by Jones et al. [35] not to be converted during the activation step.

Upon binding of the substrate, a fatty acid radical is formed after the abstraction of a hydrogen atom from the bisallylic methylene group, and concomitantly Fe(III) is reduced to Fe(II) (step b). In the absence of dioxygen, i.e. in the anaerobic cycle, the radical dissociates from the enzyme (step f) and yields a variety of products, part of which are derived from the alkoxy radical formed in step a, via free radical reactions (Scheme 3) [26,36].

In the presence of dioxygen, i.e. in the aerobic cycle, the enzyme-bound fatty acid radical is transformed into a peroxyl radical (Scheme 2, step c). Iron(II) is subsequently oxidized to Fe(III) and a peroxyl anion is formed (step d). The anion reacts with a proton and the resulting hydroperoxide is released from the Fe(III) enzyme, which is then ready for another reaction cycle (step e).

Although this reaction scheme is widely accepted and has been confirmed by recent work [34], some uncertainties exist as to the identity of particular intermediates. In an alternative model, the reduction of Fe(III) to Fe(II) via a single electron transfer from the π-system of the C-12–C-13 double bond in linoleic acid is proposed. This yields a fatty acid radical cation with a lower pK_a for its C-11 hydrogen substituent, thereby permitting its removal as a proton by a base [37]. In another model, it is proposed that the hydrogen abstraction could be provided by the formation of a carbon–iron σ-bond with C-1 or C-5 of the (1*Z*,4*Z*)-pentadiene system of the fatty acid substrate [38]. In contrast to the above-mentioned lipoxygenase mechanisms, this model does not require the reduction of Fe(III) via a single electron transfer. The formation of fatty acid radical cations or organometal intermediates is not consistent with the observed formation of carbon-centred radicals and peroxyl radicals [39–43], and the detection of spin-trapped linoleic acid radicals [44] during the anaerobic lipoxygenase reaction. In addition, the changes in the iron redox state during the lipoxygenase reaction, as proposed in the free radical

Scheme 3 **Product formation in the lipoxygenase-catalysed free radical reaction with unsaturated fatty acids and fatty acid hydroperoxides**

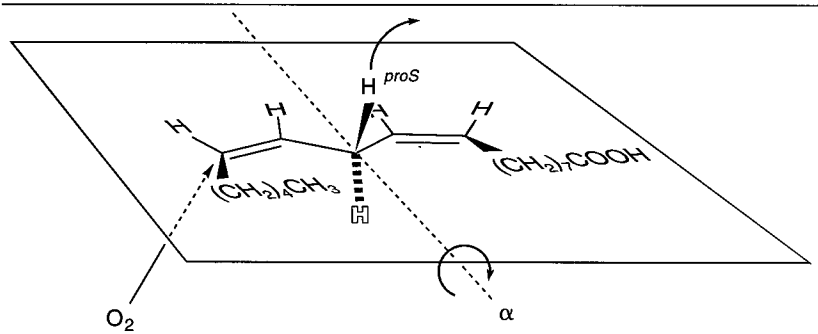


$R_1^{\bullet}$ = alkyl radical, LH = lipid molecule, $L^{\bullet}$ = lipid radical, $H^{\bullet}$ = hydrogen atom. Adapted from [26].

mechanism, have been demonstrated with various spectroscopic techniques [31,45–48]

Most lipoxygenases produce (*S*)-hydroperoxides, and isotopic labelling experiments showed that when a specific hydrogen, the *pro-S* hydrogen, of the bisallylic methylene group in the substrate is replaced by ^3H or ^2H, the lipoxygenase reaction proceeds approximately 8–9 times slower than with the normal substrate. This kinetic isotope effect indicates that the rate-determining step in the lipoxygenase reaction is the stereospecific abstraction of the *pro-S* bisallylic hydrogen (Scheme 2, step b) from the substrate [50–53]. It has furthermore been demonstrated that the hydrogen abstraction precedes the dioxygen binding during the catalytic turnover, and that lipoxygenase is unable to bind dioxygen in the absence of activated substrate [54] The formation of the (*S*)-hydroperoxide can be explained if the abstraction of the *pro-S* hydrogen and the insertion of dioxygen occur in an antarafacial manner with respect to the plane of the pentadiene system (Fig. 1, [49]).

Fig. 1 **The antarafacial character of the lipoxygenase reaction with linoleic acid**



Abstraction of the pro-S hydrogen and subsequent dioxygenation at C-13 yields (9Z,11E)-(13S)-hydroperoxyoctadeca-9,11-dienoic acid. Rotation of 180° around axis α would yield the (10E,12Z)-(9S)-hydroperoxyoctadeca-10,12-dienoic acid. Modified from [49].

Regiospecificity

The different lipoxygenase isoenzymes are named according to their prevalent positional specificity for the dioxygenation of ETE. They are named 15-, 12- or 5-lipoxygenase, depending on whether they attach the hydroperoxide function to carbon atom 15, 12 or 5 of ETE respectively. Irrespective of their positional specificity, the majority of the enzymes generate (*S*)-hydroperoxy fatty acids. Arachidonic acid is converted into (5*Z*,8*Z*,11*Z*,13*E*)-(15*S*)-hydroperoxyeicosa-5,8,11,13-tetraenoic acid (15S-HpETE) by soybean lipoxygenase-1 and rabbit reticulocyte 15-lipoxygenase; into (5*Z*,8*Z*,10*E*,14*Z*)-(12*S*)-hydroperoxyeicosa-5,8,10,14-tetraenoic acid (12S-HpETE) by human platelet 12-lipoxygenase; and into (6*E*,8*Z*,11*Z*,14*Z*)-(5*S*)-hydroperoxyeicosa-6,8,11,14-tetraenoic acid (5S-HpETE) by barley lipoxygenase and human leucocyte 5-lipoxygenase [17,55]. The numbering of the lipoxygenase isoenzymes may be confusing in the case of plant lipoxygenases because these enzymes use linoleic and linolenic acid as substrates and generate products where the hydroperoxide function is attached on to differently numbered carbon atoms with respect to ETE. A more systematic nomenclature has been proposed by Kühn et al. [56]. Soybean lipoxygenase-1 and barley lipoxygenase convert linoleic acid into (9*Z*,11*E*)-(13*S*)-hydroperoxy octadeca-9,11-dienoic acid (13S-HpOD) and (10*E*,12*Z*)-(9*S*)-hydroperoxyoctadeca-10,12-dienoic acid (9S-HpOD) respectively [57].

The regiospecificity of the lipoxygenase product formation depends on the positional specificity of both the initial hydrogen abstraction and the subsequent dioxygen attachment. The formation of the different regioisomeric lipoxygenase products from linoleic acid can be explained by a simple reverse orientation

of the substrate in the substrate binding site (Scheme 1; [49]). This model may be supported by the observation that soybean lipoxygenase-1 produces both 13S- and 9S-HpOD in a pH-dependent manner, suggesting that the protonated fatty acid binds more easily in the reversed orientation compared with the unprotonated fatty acid [58,59]. A similar explanation was given for the difference in regiospecificity of mammalian 5-lipoxygenases on the one hand and the 12- and 15-lipoxygenases on the other. It was assumed that 5-lipoxygenases have a polar substrate binding pocket and 12- and 15-lipoxygenases have a non-polar substrate binding pocket thus allowing reverse orientations of ETE in 5-lipoxygenase, and in 12- and 15-lipoxygenase respectively [60,61].

The regiospecificity of the lipoxygenase reaction may also be explained by assuming that the substrate is bound in one orientation and the free electron of the enzyme-bound fatty acid radical is localized on either C-1 or C-5 of the (1Z,4Z)-pentadienyl moiety. For the linoleyl radical this would be achieved if rotation around the C-11–C-12 or C-10–C-11 blocks delocalization of the free electron over the C-12–C-13 or C-9–C-10 π-orbital respectively [41]. In yet another model the regiospecificity is achieved by the specific approach of dioxygen through steric interference of the enzyme such that a regio- (and stereo-) selective reaction takes place [62].

Soybean lipoxygenase-1 preferentially produces 15S-HpETE from ETE and 13*S*-HpOD from linoleic acid with high stereospecificity. The substrate specificity indicates that substrate recognition proceeds via the methyl-end, as was shown in studies using different polyunsaturated fatty acids. Especially, the presence of *Z*-double bonds at the ω-6 and ω-9 positions is essential for the lipoxygenase reaction [52,63]. Nevertheless, the linoleic acid isomer (13Z,16Z)-octadeca-13,16-dienoic acid was converted into (13*Z*,15*E*)-(17*S*)-hydroperoxyoctadeca-13,15-dienoic acid by soybean lipoxygenase-1 [64], and several substrates wherein the methyl group was replaced by more bulky substituents were also accepted as substrates [64–66]. A decrease in positional specificity was also observed for (8Z,11Z,14Z)-eicosa-8,11,14-trienoic acid, which was converted into the (15*S*)-hydroperoxy derivative by soybean lipoxygenase-1, whereas the regioisomer (9Z,12Z,15Z)-eicosa-9,12,15-trienoic acid was converted into both the (13*S*)- and the (16*S*)-hydroperoxy derivatives [67]. The regioisomer of ETE, (6Z,9Z,12Z,15Z)-eicosa-6,9,12,15-tetraenoic acid, was converted into the 13-and 16-hydroperoxide derivatives by lipoxygenase from rabbit reticulocytes, whereas ETE was converted into the 15- and 12-hydroperoxide derivatives, and it was concluded that improper positioning of the substrate in the active site was the cause of this phenomenon [68].

Novel and more detailed insights into the regiospecificity of the lipoxygenase-catalysed dioxygenation reaction were obtained from site-specific mutagenesis experiments. From comparisons of the amino acid sequences of a number of 15- and 12-lipoxygenases, the amino acid residues Arg-402, Phe-414, Ile-417 and Met-418 of human 15-lipoxygenase were identified as possible determinants for the regiospecificity of the enzyme [69–71]. Human 15-lipoxy-

genase, in which Met-418 was replaced by a valine residue, produced equal amounts of 12- and 15-HpETE. This indicates the existence of a substrate binding pocket in which the substrate binds deeper when less bulky amino acids are at the bottom of the pocket [71]. A nonpolar substrate binding pocket that determines the alignment of the substrate was suggested [60]. A combined mutagenesis and molecular modelling study, in which the three-dimensional structure of soybean lipoxygenase-1 was used as a template for human 15-lipoxygenase, indicated that the interaction of the π-electrons of the double bonds in ETE with the π-electrons of Phe-414 in human 15-lipoxygenase may be important for the regioselectivity of the lipoxygenase reaction. When Phe-414 was replaced by an isoleucine residue, the ratio of 15-HpETE to 12-HpETE changed from 9.4:1 to 1.6:1, whereas replacement of the Phe-414 by a tryptophan residue changed the ratio to 25:1. Especially, the ω-9 double bond of ETE may interact with Phe-414 [70]. The regioselectivity of human 15-lipoxygenase is further determined by Arg-402. The 15-HpETE:12-HpETE product ratios of 9.4:1 changed to 4:1 when Arg-402 was replaced by a leucine residue, and to 11:1 when Arg-402 was replaced by a lysine residue. This indicates that the positive charge of Arg-402 may define the carboxylic acid binding site, and thus may position the substrate accurately in the binding site relative to the catalytic iron [70]. However, it should be stressed that the human and the rat 5-lipoxygenase, for which an inverse orientation of the substrate fatty acid has been suggested [60], does also contain a positively charged amino acid (Lys) at this position which may interact with the carboxylic group. Such an interaction would actually prevent an inverse head-to-tail substrate orientation. Interestingly, Phe-414 and Arg-402 in human 15-lipoxygenase correspond to Trp-500 and Leu-541 in soybean lipoxygenase-1 [70], which also converts ETE into 15-HpETE. Because Trp-500 in soybean lipoxygenase-1 is close to the active site iron (see below, Fig. 4), it is noteworthy that the tryptophan fluorescence of soybean lipoxygenase-1 is quenched when Fe(II) is oxidized to the Fe(III) state by 13-HpOD [34].

Not all lipoxygenases display complete regio- and enantiospecificity in product formation. Soybean lipoxygenase-2 or -3 produce both enantiomers of 13- and 9-HpOD from linoleic acid. The apparent lack of regioselectivity and stereoselectivity exhibited by some lipoxygenases can be explained by assuming that the fatty acid radical dissociates from the enzyme (Scheme 2, step f) and reacts nonenzymically with dioxygen. The lack of regio- and stereoselectivity is usually accompanied by the formation of secondary reaction products such as oxodienes, fatty acid dimers and aldehydes, products most probably derived from free radical reactions (see also Scheme 3, [72–77].) These free radical reactions may also be the active principle of the 'carotene oxidase' activity, and cause the co-oxidation of various organic compounds in the lipoxygenase reaction [2,78–85].

The lipoxygenase-catalysed regio- and stereoselective dioxygenation reaction has long been thought to be limited to fatty acids containing one or more (1*Z*,4*Z*)-pentadiene systems. Interestingly, it has been demonstrated that both the (*Z*,*E*)- and (*E*,*Z*)-isomers of linoleic acid are substrates for soybean lipoxygenase-1

Table 1 Plant lipoxygenase sequences available in major sequence databases

LOX: name for a lipoxygenase – names have been kept as much as possible in line with the names used for the LOXs from a species. For potato tuber LOX, a large number of highly similar sequences are available which have been numbered 1a–1g. Gene: genomic sequences available in the EMBL nucleotide sequence database (release 49.0). DNA: cDNA sequences available in the EMBL nucleotide sequence database (release 49.0) SWISS: protein sequences available in the SWISSPROT protein sequence database (release 34.0). PIR: protein sequences in the PIR protein sequence database (release 50.0). Remarks: between brackets: length in amino acid residues of partial sequences. Includes further names of specific clones, alternative names for a LOX used by the author.

		EMBL/GenBank entries		Protein database entries			
Species	LOX	gene	cDNA	SWISS	PIR	Remarks	References
Soybean	soy 1		J02795	P08170	A28435		[89]
			M16921	P08170	A28435	pal-134 (325)	[112]
			X67304	P08170	S25064		[113]
	soy 2		J03211	P09439	A28161		[90]
			M16876	P09439	A28161	plx-65 (635)	[112]
			D13949			Null-mutant	[114]
	soy 3	X06928	X13302	P09186	S01864		[91]
			U50081				[115]
	soy 4	D13999		P38417			[94]
			U04785			pTK-18	[95]
			S76065			Lox B1 (92)	[116]
	soy 5		U50075			vlxB	[117]
			S76064		S18613	Lox B2 (115)	[116]
	soy 6	X56139	U04526	P24095	S13381	pTK-11	[95,118]
			S76063		S18612	Lox A (599)	[116]
	soy 7		U36191				[119]

Table 1 (Contd.)

		EMBL/GenBankentries		Protein database entries			
Species	LOX	gene	cDNA	SWISS	PIR	Remarks	References
Soybean	soy 8		U36192			(46)	[119]
	soy 9		U36442			(18)	[119]
	soy C		U26457		PQ0291	vlxC	[117,120]
Pea	pea 2	X78580	X17061	P14856	S07065	Lox1:Ps:2	[93,121]
		M80833				Promoter region	[122]
	pea 3	X78581	X07807	P09918	S01142	Lox1:Ps:3	[92,123]
	pea G		X76124		S56655		[124]
	pea P				S18614	Lox P1 (94)	[116]
Lentil	len 1		X71344	P38414			[125]
Bean	bea A	X63525		P27480	S22153	Lox 1	[126]
	bea B		X63521	P27481	S18906		[127]
	bea C		U76687				[128]
Dodder	cus 1		U05041			(385)	[129]
Arabidopsis	ara 1	U01843	L04637	Q06327	JQ2267		[130,131]
	ara 2		L23968	P38418	JQ2391	AtLox2	[132]

(Contd.)

Table 1 (Contd.)

Species	LOX	EMBL/GenBank entries		Protein database entries		Remarks	References
		gene	cDNA	SWISS	PIR		
Cucumber	cuc 1		U25058				[133]
	cuc 2		U36339				[134]
	cuc 3		X92890				[135]
Potato	pot 1a		X79107	P37831	S44940	Lox1:St:1	[88]
	pot 1b		S73865				[136]
	pot 1c		U60201				[137]
	pot 1d		X95511			T4 (447)	[138]
			X95516			T13 (697)	[138]
	pot 1e		X95512			T6 (844)	[138]
			X95515			T12 (130)	[138]
			U60200				[137]
			X95666			(109)	[139]
			X95667			(32)	[139]
	pot 1f		X95113			T8	[138]
	pot 1g		X95114			T10 (454)	[138]
	pot 2		X96405			H1	[138]
	pot 3		X96406			H3	[138]
	pot 4	U24232					[140]
	pot 5		U60202				[137]
Tobacco	tob 1		X84040		S57946		[141]

Table 1 (Contd.)

		EMBL/GenBank entries		Protein database entries			
Species	LOX	gene	cDNA	SWISS	PIR	Remarks	References
Tomato	tom A		U09026	P38415			[96]
		U63117				Promoter region	[96,142]
	tom B		U09025	P38416			[96]
		U63118				Promoter region	[96,142]
			U13681				[143]
	tom C		U37839				[144]
	tom D		U37840				[144]
	tom E		X94945			(246)	[145]
Rice	rice C		D14000	P38419	A53054		[146]
	rice 2		X64369	P29250	S23454		[147,148]
Barley	bar A		L35931				[149,150]
				P29114	S21772	Lox I (21)	[149]
				P29114	S22236	Lox I (14)	[149]
	bar B		L37359			(341)	[150]
	bar C		L37358			(580)	[150]
Wheat	whe 1		U32428				[151]
Red algae	alg 1		U08842				[152]

as well, yielding predominantly 13-hydroperoxy-(9Z,11*E*)-octadeca-9,11-dienoic acid [86]. Moreover, several oxo β-monoenoic fatty acids can serve as lipoxygenase substrates [87], indicating that the lipoxygenase active site can accept a large variety of unsaturated substrates.

Primary structure

The primary structures of a number of plant ([88–96]; Table 1) and mammalian lipoxygenases [97–103] have been elucidated. The plant lipoxygenases all contain 880 ± 40 amino acids and their sequences have 70–90% of the residues in common when two sequences are compared. The mammalian lipoxygenases show less homology (see Chapter 3). They consist of a smaller number of amino acids than the plant lipoxygenases and in particular the first 150–200 N-terminal amino acid residues have been deleted. Comparison of the mammalian and the plant lipoxygenase sequences show, however, several regions of high similarity. All lipoxygenases have a histidine-rich region of about 40 amino acids that contains five conserved histidines, and they all have a C-terminal isoleucine. X-ray-absorption fine structure (XAFS) studies have indicated that Fe(II) in soybean lipoxygenase-1 is bound by 4 ± 1 N (imidazole) atoms and 2 ± 1 O atoms ligands [47,104,105]. The replacement of His-499, His-504 and His-690 in soybean lipoxygenase-1 by glutamine residues resulted in inactive enzymes devoid of iron, indicating that the histidines are iron ligands [106,107]. Similar results were obtained with mammalian lipoxygenases, and in addition the C-terminal isoleucine was identified as an iron ligand [108–111].

In Table 1 the primary structures have been compiled from major sequence databases.

Mammalian lipoxygenase sequences are shorter than their plant counterparts, missing 150–200 N-terminal amino acid residues. Comparison of the sequences (Fig. 2) shows several regions of high similarity. One such region, usually described as the histidine-rich region, contains five conserved histidines. Another conserved region also contains a conserved histidine, and the C-terminus is identical for all lipoxygenases. The presence of conserved histidines, combined with the spectroscopic data (see above), indicating that nitrogen is involved in iron-binding, suggests that these histidines are ligands to iron. The replacement of His-499, His-504 and His-690 in soybean lipoxygenase-1 by glutamine resulted in completely inactive enzymes, devoid of iron [106,107]. Therefore, these three histidines are probably iron ligands. This was confirmed by similar work on mammalian lipoxygenases [110,111,153].

Using the same data as in Fig. 2 and Table 1, Fig. 3 shows a pairwise comparison of plant lipoxygenase sequences known to date, and a separate detailed comparison of the soybean isozymes.

Three-dimensional structure

Although soybean lipoxygenase-1 was already crystallized in 1947 [22] it was not until 1990 that the first lipoxygenase-1 and -2 crystals suitable for X-ray crystallography were obtained [154–156]. The first crystal structure, at 2.6 Å resolution, of soybean lipoxygenase-1 was reported in 1993 [157,158]. Recently, the lipoxygenase-1 structure with a 1.4 Å resolution, and the crystal structure of soybean lipoxygenase-3 with a 2.6 Å resolution have been reported [62,159].

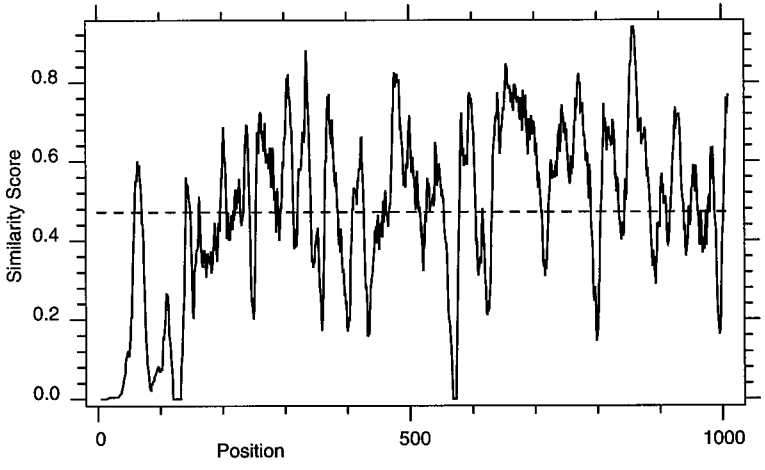
The two soybean lipoxygenase-1 crystal structures that have been reported are similar, but have slightly different iron environments [62,158]. Soybean lipoxygenase-1 is a two domain, 839-residue-long single-chain ellipsoid of dimensions 90 × 65 × 60 Å, with a molecular mass of 95 kDa and a pI of 5.71. The smaller N-domain, which comprises the residues 1–146 (domain I), is an eight-stranded antiparallel β-barrel with a jelly-roll-like topology. Domain I is located at one end of the larger C-domain, which comprises the residues 147–839 (domain II), and is relatively loosely associated to it. Domain II has predominantly a helical secondary structure and includes the catalytic Fe-binding site (Fig. 4).

Domain I

At present, a specific function of domain I is hard to define because mammalian 5-, 12- and 15-lipoxygenases apparently lack this domain. Limited proteolysis of soybean lipoxygenase-1 generated a 30 kDa fragment containing residues 1–274 (domain I plus a portion of domain II), and a 60 kDa fragment containing residues 318–839 (the remainder of domain II). These two fragments remained tightly associated. The cleaved enzyme was still active, but the individual fragments were inactive, indicating that interaction of the two domains is necessary for activity [160]. This is supported by the observation that a recombinant soybean lipoxygenase-1, in which residues 2–140 were replaced by a stretch of five residues, was inactive [161]. It is noteworthy that domain I resembles the C-terminal colipase binding domain of mammalian pancreatic lipases [158]. A recent study on the limited proteolysis of soybean lipoxygenase-3, which has an overall structure almost identical to that of soybean lipoxygenase-1 [159], indicated that domain I is connected across the entire length of domain II to a narrow, tongue-like projection that extends into the vicinity of the entrance to the proposed substrate binding channel [162]. Motions of domain I might be involved in binding, transport and release of substrate and products [62].

Interestingly, a membrane-associated 5-lipoxygenase-activating protein (FLAP) has been discovered that activates 5-lipoxygenase by forming a 5-lipoxygenase–FLAP–membrane complex. The presence of an ETE-binding site on FLAP that facilitates the transfer of this fatty acid to 5-lipoxygenase to allow a more efficient reaction has been demonstrated. More interestingly, the membrane–FLAP–associated 5-lipoxygenase was more efficient in the production of leukotriene A_4 than the free cytosolic enzyme and could, unlike the cytosolic 5-lipoxygenase,

Fig. 2 **Similarity plot for a multiple sequence alignment of all available full-length lipoxygenase sequences**



The quantity plotted for an amino acid residue is the fraction of all the possible amino acid pairs consisting of identical residues, averaged over a window of 10 amino acids. Amino acid residues identical in all sequences are indicated above the graph (upper row), together with the six conserved histidine residues (lower row).

Fig. 3 **Percentage of amino acids found to be identical in pairwise comparisons of plant lipoxygenase sequences**

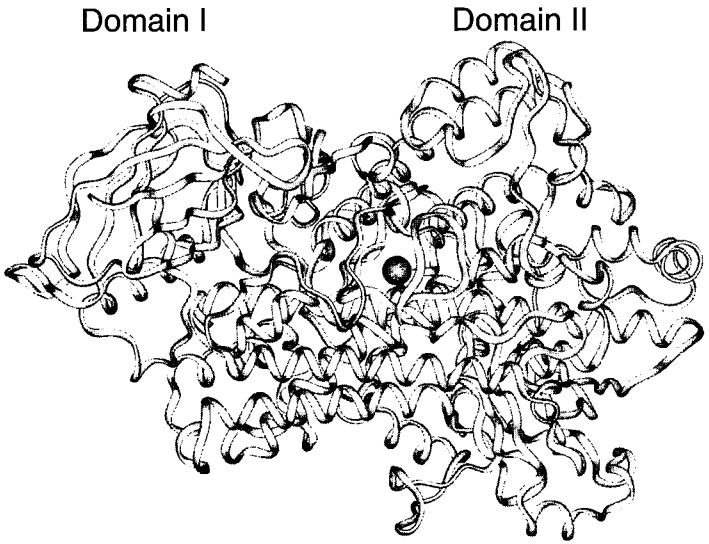
	alg1	barC	barB	barA	ricC	ri	tomC	tomB	tomA	tob1	pot3	pot2	pot1	cuc2	cuc1	araC	ara1	cus1	beaB	beaA	len1	peaG	pea3	pea2
soy 1	23.1	59.0	59.8	55.5	43.9	54.7	61.0	57.8	59.4	60.4	58.2	59.9	58.7	61.0	59.2	44.7	58.6	46.9	66.9	66.1	65.5	64.2	71.4	78.6
soy 2	24.1	59.9	60.6	56.6	43.5	55.3	62.3	59.2	61.4	62.0	59.9	62.7	61.2	61.7	60.4	42.7	60.0	48.3	66.0	72.4	66.1	64.9	75.4	83.7
soy 3	24.2	59.2	62.2	56.6	44.8	55.1	66.1	61.7	63.5	65.0	61.0	64.4	63.5	62.6	60.8	44.3	62.5	45.3	76.5	71.2	71.9	70.7	85.5	74.6
soy 4	24.3	58.2	62.5	54.5	42.8	53.7	66.1	58.1	61.0	60.5	57.8	61.7	61.7	59.8	58.3	42.3	59.7	44.5	89.2	66.4	70.7	71.3	73.0	67.6
soy 5	24.9	58.6	61.0	55.2	44.6	54.0	63.3	57.9	61.2	60.2	57.2	61.9	61.9	59.0	59.5	44.2	58.4	47.9	67.5	71.2	65.5	64.9	71.8	72.7
soy 6	24.5	57.9	59.5	54.6	43.9	53.4	61.6	57.6	61.3	60.3	58.4	61.9	61.8	59.0	58.0	43.4	57.9	48.2	66.9	72.6	65.2	64.8	70.8	71.7
soy 7	24.9	58.9	62.2	56.5	42.2	54.5	66.1	57.8	61.9	60.3	57.6	62.3	61.7	59.9	58.8	42.4	58.5	44.5	66.9	66.0	71.3	72.7	74.2	66.1
pea 2	24.1	58.9	61.9	56.2	42.8	54.2	60.4	59.3	63.1	62.5	59.8	63.2	62.2	63.0	60.6	43.8	59.5	44.2	66.1	71.9	65.9	65.0	74.5	
pea 3	24.7	57.6	59.8	55.6	43.2	54.7	64.1	60.0	61.9	62.9	59.8	63.1	63.0	61.0	59.1	43.0	61.8	46.9	75.7	66.3	71.1	70.8		
pea g	25.5	55.2	59.5	53.5	42.2	53.2	62.9	56.8	58.2	59.5	56.2	58.9	58.7	56.2	54.4	40.7	56.1	42.1	74.1	63.7	82.3			
len 1	25.9	56.4	60.4	55.6	42.2	53.8	65.3	58.6	61.5	62.0	57.1	61.5	62.0	59.6	57.0	42.1	58.2	44.5	73.5	64.9				
bea a	24.3	59.1	61.7	56.7	45.0	55.0	60.8	59.0	61.7	60.7	58.4	62.1	61.5	60.6	58.9	42.4	59.2	47.1	67.3					
bea b	24.7	57.9	61.8	57.3	45.9	56.6	64.4	59.6	63.0	62.6	60.3	63.4	62.7	61.3	59.8	44.2	61.9	43.9						
cus 1	28.0	45.6	45.6	45.3	58.9	43.6	44.1	46.2	49.2	47.1	44.5	49.2	48.4	46.3	48.4	62.3	49.6							
ara 1	24.8	58.9	65.0	57.2	43.5	57.0	67.8	63.6	68.0	69.2	64.3	67.6	66.5	61.9	61.1	45.2								
ara c	26.2	47.1	47.7	42.0	51.6	42.4	47.3	42.5	43.5	44.1	43.6	43.0	43.1	42.9	42.7									
cuc 1	24.7	57.2	59.2	53.8	41.5	54.0	66.1	60.1	63.3	62.0	57.3	64.2	62.8	75.9										
cuc 2	22.5	58.2	59.2	56.1	42.8	55.0	65.3	61.1	63.3	63.0	59.6	64.2	63.5											
pot 1	27.2	60.2	66.2	58.1	44.5	57.5	79.6	72.3	78.3	78.3	66.2	100.0												
pot 2	27.4	60.4	66.8	58.6	44.8	58.3	79.2	72.6	78.1	79.6	67.2													
pot 3	25.4	58.8	63.2	57.6	40.6	53.9	64.8	62.3	66.6	69.2														
tob 1	26.4	61.0	67.4	58.6	43.6	57.7	80.6	79.7	79.3															
tom a	27.7	60.0	66.5	58.6	43.9	57.8	77.6	72.4																
tom b	24.0	57.4	61.2	55.9	42.3	54.9	78.0																	
tom c	28.9	61.6	64.1	62.4	44.1	60.8																		
ric 2	24.7	75.2	70.8	74.9	41.5																			
ric c	27.6	43.9	46.5	41.2																				
bar a	25.4	75.9	76.9																					
bar b	31.9	73.0																						
bar c	31.3																							

	soy7	soy6	soy5	soy4	soy3	soy2
soy 1	67.1	70.3	70.5	67.0	72.2	84.9
soy 2	66.7	72.2	71.6	67.8	75.4	
soy 3	75.0	71.4	71.1	74.3		
soy 4	67.6	68.2	68.3			
soy 5	68.8	86.6				
soy 6	66.4					

65-70% identical
70-80% identical
80-90% identical

Partial sequences shorter than 250 amino acids are not included.

Fig. 4 **Ribbon diagram of the three-dimensional structure of soybean lipoxygenase-1**



The sphere in the middle of domain II represents the active-site iron. Adapted from [158].

metabolize 12S- and 15S-HETE into (6*E*,8*Z*,10*E*,14*Z*)-(5*S*,12*S*)-dihydroxyeicosa-6,8,10,14-tetraenoic acid and (6*E*,8*Z*,11*Z*,13*E*)-(5*S*,15*S*)-dihydroxyeicosa-6,8,11,13-tetraenoic acid respectively. It can be concluded that FLAP is able to influence the substrate specificity of 5-lipoxygenase ([163], and references therein; see also chapter 5 in this volume).

Domain II

Domain II contains 693 residues and consists of 20 helices [62] (23 according to Boyington et al. [158]) and two antiparallel β-sheets. Seventeen of the helices are approximately parallel or antiparallel to each other and surround a 65 Å long central helix (helix 9) that lies in the direction of the longest dimension of the protein. The ends of helix 9 are solvent-exposed, and the middle buried part is not predominantly hydrophobic. A second helix (helix 18) lies parallel to helix 9. Both helix 9 and helix 18 contain highly conserved residues, which serve as iron ligands, in a π helical section in their middle (a π-helix has hydrogen bonding between residues i and $i+5$ instead of i and $i+4$). Helix 9 contributes His-499 and His-504, and helix 18 His-690 as iron ligands.

Iron coordination

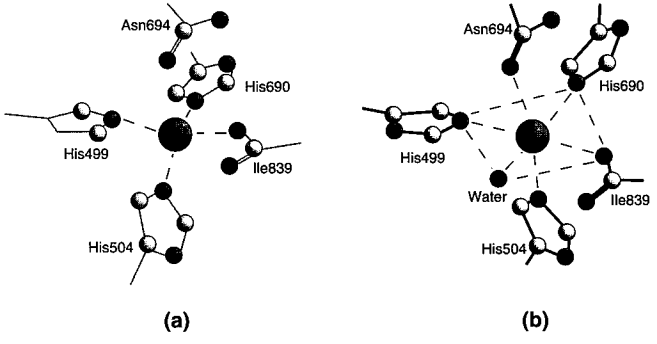
The iron is located between helix 9 and 18 at the centre of domain II. The two currently available crystal structures differ in the iron coordination; [158] describes

a coordination by four ligands, forming a highly distorted octahedron with two adjacent, unoccupied positions. Three of the ligands are formed by the N^{ϵ} of the His-499, His-504 and His-690, and the fourth ligand by an oxygen of the C-terminal Ile-839. The O-δ1 of Asn-694 at 3.16 Å of the iron was not considered as a ligand (Fig. 4). In contrast, [62] describes the iron coordination as an octahedron, with six ligands, that is only slightly distorted. Three of the ligands are formed by the N^{ϵ} atoms of His-499, His-504 and His-690, the fourth ligand is formed by an oxygen atom of the C-terminal Ile-839, and the fifth ligand is formed by the oxygen of a water molecule positioned *trans* to His-690. A sixth potential ligand is the side chain O-δ1 atom of Asn-694 at 3.05 Å distance from the iron (Fig. 3). The water ligand is positioned *trans* to His-690 at the boundary of a large internal cavity that is presumably the fatty acid binding site (Fig. 5).

The latter iron coordination, and especially the presence of a water ligand, is consistent with XAFS and EPR data [164–166]. The coordination observed by Boyington et al. [158] may be the result of the differences in experimental conditions. The XAFS and EPR data, and the X-ray structure of [62] were obtained at liquid nitrogen temperature, whereas [158] performed the X-ray studies at room temperature. Interestingly, Asn-694 is replaced by a histidine in mammalian 15-lipoxygenases [166,167].

The fact that soybean lipoxygenase-1 is relatively easy to purify in large quantities, and moreover is fairly stable, has meant that almost every available spectroscopic technique has been used to study the iron-environment of the enzyme [104]. Iron(II)-lipoxygenase is EPR-silent [24], but ^{1}H NMR spectroscopy on the interaction of alcohols with lipoxygenase and magnetic susceptibility measurements showed that the iron of Fe(II)-lipoxygenase is in a high-spin (S2) state [168]. The Fe(III)-enzyme, obtained by oxidizing Fe(II)-lipoxygenase with

Fig. 5 **Coordination of the iron in the active site of soybean lipoxygenase-1 as reported by (a) Boyington et al. [158], and (b) by Minor et al. [62]**



Redrawn from Minor et al. [62].

one equivalent of HpOD, gives EPR signals around $g = 6$, which indicates the presence of high-spin Fe(III) ($S = 5/2$) in a distorted ligand field, slightly deviating from axial symmetry [169]. Similar EPR data have been obtained with other lipoxygenases from soybean [170]. XAFS-data indicate 4 ± 1 N (imidazole) ligands and 2 ± 1 O-ligands for the Fe(II) enzyme [104], whereupon oxidation to Fe(III) results in one nitrogen being replaced by oxygen [47]. Mössbauer spectroscopy yielded data consistent with six N and/or O ligands for Fe(II) [171]. Magnetic circular dichroism studies indicated an octahedral 6-coordinate system for Fe(II)-lipoxygenase, with a small rhombic distortion [172] and at least two histidines as ligands [174]. Magnetic susceptibility experiments ruled out the binding of molecular oxygen to Fe(II) [174], but the possibility exists that water provides one of the oxygen ligands [164].

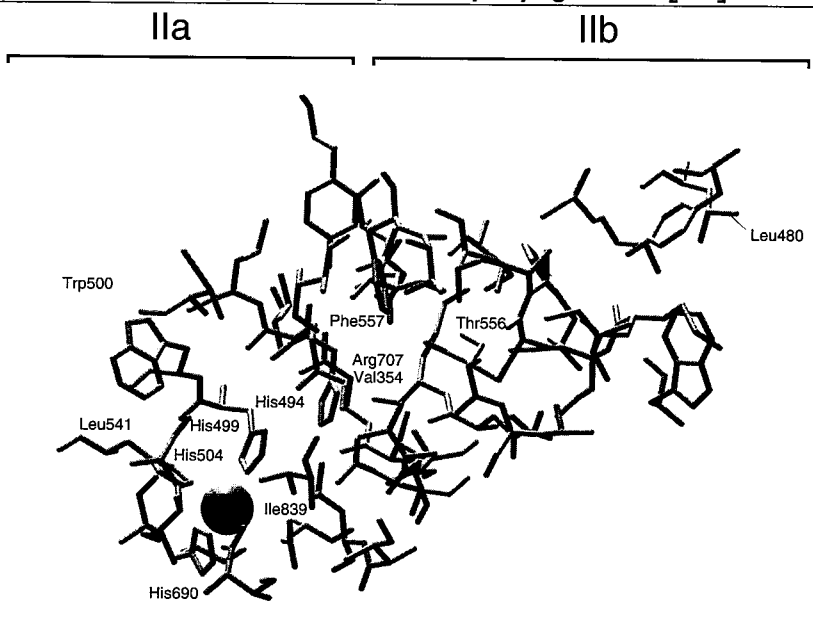
Cavities

Boyington et al. [158] have described two major cavities in domain II of the protein. The first cavity (cavity I) is shaped like a funnel, faces His-499, His-504 and His-690 and forms an 18 Å long hydrophobic tunnel that becomes very narrow (2.5 Å) just before reaching the iron. Cavity I could be the path for the movement of dioxygen. The second cavity (cavity II) faces the carboxyl of the C-terminal Ile-839 and the ligands His-499 and His-690. The 46 amino acids that line cavity II are mainly hydrophobic and highly conserved. The 40 Å long cavity II is narrow (less than 3.5 Å wide in some places) and has two major bends. One bend is adjacent to the iron, very close to the end of the cavity. The second bend is approximately in the middle, where the cavity becomes very narrow. This cavity may function as the substrate-binding cavity (Fig. 6).

Minor et al. [62] disagree on the presence of cavity I because their model does not allow for the transport of dioxygen through this cavity without substantial rearrangement of several residues lining the cavity. Instead they propose that dioxygen may pass through cavity II, or through a 20 Å pathway starting from His-248, and Arg-533 at the surface of the protein, passing between Pro-834 and Ile-839 and ending in the vicinity of the side chain of His-690 (not shown).

Because cavity II is wholly internal, the entry and exit for the fatty acid are difficult to identify. Boyington et al. [158] propose that the substrate enters the cavity after rearrangement of the Met-341 and Leu-480 side chains that block the entry. In addition, rearrangement of the side chains of Arg-707 and Val-354, that make a 3.5 Å contact, would be necessary to allow the substrate to reach the iron. Minor et al. [62] agree on the part of cavity II near the iron (Fig. 6, cavity IIa), but suggest that the part of the cavity II between Met-341/Leu-480 and Arg-707 (cavity IIb) may not be suitable for transport of the substrate. They propose three possible channels (not shown) that reach cavity IIa at the opposite side of that proposed by Boyington et al. [158].

Fig. 6 **Putative fatty acid binding cavity (cavity II) of soybean lipoxygenase-1 as reported by Boyington et al. [158]**



Cavity IIa and cavity IIb were assigned according to Minor et al. [62].

Substrate binding

Although the structure of the enzyme may change upon substrate binding, it is clear that cavity IIa can accommodate C_{18} and C_{20} fatty acids. A molecular modelling study where the three-dimensional structure of soybean lipoxygenase-1 of [158] was used as a template to model human 5-, 12- and 15-lipoxygenases suggests that the positional selectivity of soybean lipoxygenase-1 is largely determined by residues Thr-556 and Phe-557 because bulky amino acids at these positions would not permit the methyl-end of the substrate to penetrate deeply into the binding site [167]. The study indicates that Phe-557 in soybean lipoxygenase-1 corresponds with Met-418 in human 15-lipoxygenase and this corroborates the observation that replacement of Met-418 by a less bulky valine residue yields an enzyme with 12-lipoxygenase activity [71]. If the positional specificity of soybean lipoxygenase-1 is indeed determined by Thr-556 and Phe-557, this would imply that the enzyme-bound substrate is bent in a U-shape in cavity IIa (Fig. 6) with its diene system in the vicinity of the iron opposite His-690 [167]. Minor et al. [62] point out that, given the flexibility of the fatty acid substrates, it is not possible to define a specific mode of binding and that it is possible to bind a substrate in cavity IIa with its methyl-end in the hydrophobic pocket that terminates at Leu-541. Another study indicates that

the substrate may also bind in an orientation where the carboxylic group is located near Lys-260, i.e. close to Leu-541, with the methyl-end of the substrate near Phe-557 [70]. All current models agree, however, on the positioning of the pentadiene system opposite His-690, but co-crystals of lipoxygenase, and preferably Fe(III)-lipoxygenase, with different substrates or substrate analogues are needed to identify the substrate binding site(s). In a recent report an additional sequence determinant of the positional specificity of 12-/15-lipoxygenases has been described for mammalian enzymes [175]. In rabbit 15-lipoxygenase, Phe-353 appears to force the substrate fatty acid into a bent orientation so that the methyl terminal of the substrate comes in contact with Met-418. When Phe-353 was mutated to a less space-filling amino acid, arachidonic acid was oxygenated at C-12. It remains to be investigated whether the corresponding amino acid in the soybean enzyme may also influence the positional specificity.

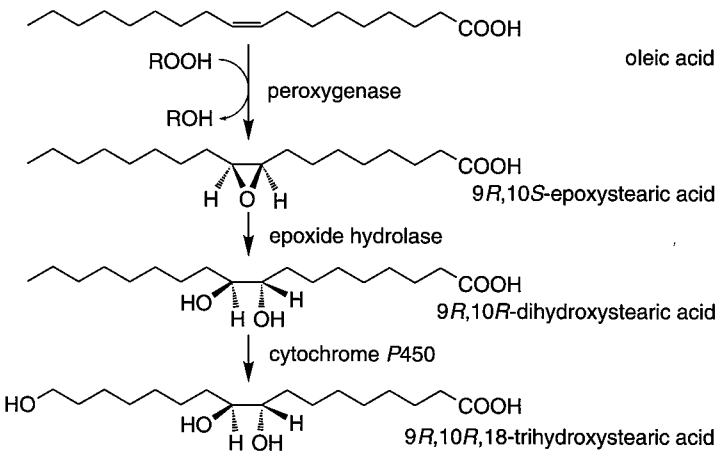
Lipid hydroperoxide metabolism in plants

An obvious way to find out about the physiological function of an enzyme is to study the fate of the products that result from the reaction catalysed by the enzyme. The primary products of the lipoxygenase reaction, fatty-acid hydroperoxides, are potentially dangerous and should be quickly metabolized. Two major routes for metabolizing lipoxygenase products have been identified, collectively known as the lipoxygenase pathway [176]. This was recently extended by the discovery of the peroxygenase cascade [177].

The peroxygenase cascade (Scheme 4) starts with the conversion of unsaturated fatty acids into *cis*-epoxides by a peroxygenase. This peroxygenase requires fatty-acid hydroperoxides as co-substrate [178–180] and the peroxygenase cascade thus depends on the presence of lipoxygenase. The final product of this cascade is (9*R*,10*R*)-18-trihydroxystearate, which is a major component of cutin. Cutin is a polymer found in the cuticle that covers all the aerial parts of the plant and forms the first barrier against plant invasion [181]. Other components of cutin are oleic acid, 18-hydroxyoleic acid, 18-hydroxy-(9*R*,10*S*)-epoxyoleic acid and their 12,13-unsaturated analogues; products that are all present in this cascade [177]. Several intermediate products of the cascade, e.g. linoleic acid epoxides and diols, have antifungal properties [182,183], and may also be involved in the defence system of the plant.

The first route in the lipoxygenase pathway (Scheme 5) involves the cleavage of 13-hydroperoxides into 12-oxo-(9*Z*)-dodecenoic acid and C_6-aldehydes by hydroperoxide lyase, an enzyme that has been found in a large number of plants [176,184,185]. The 12-oxo-(9*Z*)-dodecenoic acid isomerizes readily into the more stable 12-oxo-(10*E*)-dodecenoic acid, which is also known as traumatin [186]. Traumatin has been reported to mimic the physiological effects seen upon wounding of plant tissue and can be converted into traumatic acid, which has

Scheme 4 The peroxygenase cascade

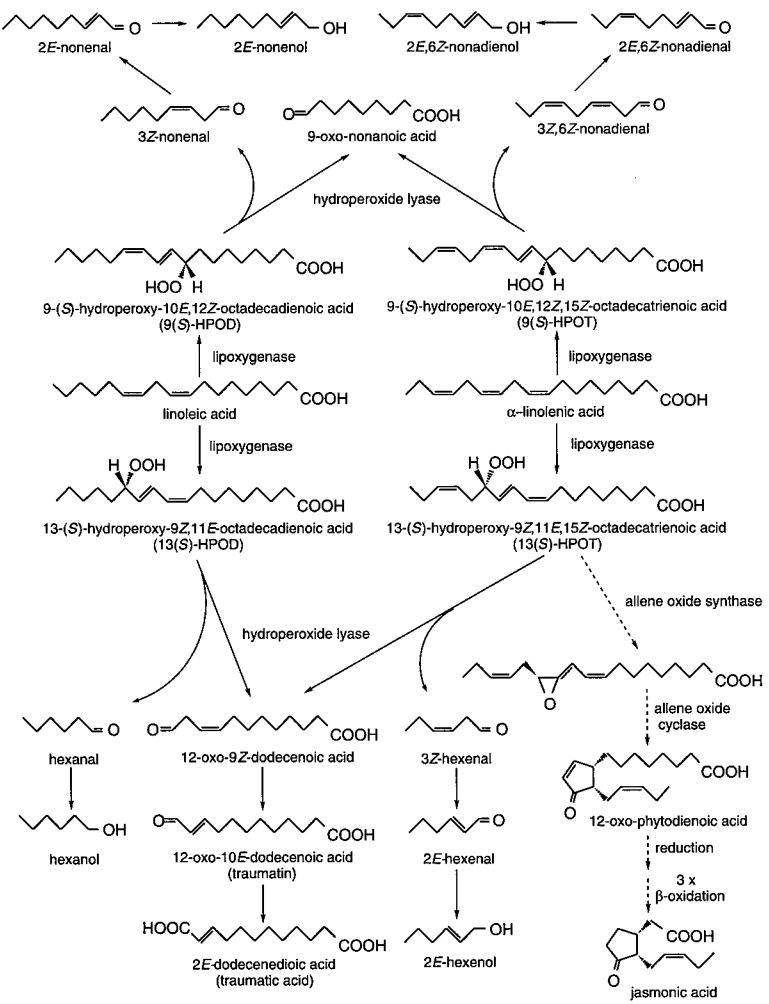


The hydroxylation by a cytochrome-P-450-dependent enzyme may occur at any point in the cascade, thus also 18-hydroxyoleate (if the hydroxylation occurs before the peroxidation) and 18-hydroxy-(9R,10S)-epoxyoleate (if hydroxylation occurs before epoxide hydrolysis) are synthesized. Starting with linoleic acid, the 12,13-unsaturated analogues also will be formed.

similar effects on plants as traumatin [187,188]. The C_6 aldehydes can be converted into their corresponding alcohols. Together these aldehydes and alcohols are responsible for the 'green' odour of damaged green tissues [189]. Hydroperoxide lyase may also convert 9-hydroperoxides, resulting in 9-oxononanoic acid and various C_9 aldehydes and their corresponding alcohols (Scheme 5).

The second route in the lipoxygenase pathway comprises the conversion of 13-hydroperoxyoctadecatrienoic acid into an unstable allene oxide by allene oxide synthase [190–192], which is then converted into 12-oxophytodienoic acid by allene oxide cyclase. 12-Oxophytodienoic acid can be converted into jasmonic acid by reduction of the double bond in the ring and three β-oxidation steps (Scheme 5). Jasmonic acid and its methyl ester have growth inhibitory properties similar to those of abscisic acid. The physiological effects of exogenous addition of jasmonic acid to plants include inhibition of soybean callus growth [193], stimulation of ethylene production in ripening fruits [194] and inhibition of growth in rice seedlings [195]. More recently, jasmonates have been shown to induce the synthesis of proteinase inhibitors [192,196–198] and to trigger the formation of phytoalexins such as flavonoids, alkaloids, terpenoids [199] and their biosynthetic enzymes [199,200], and thionins, proteins with antifungal properties in barley [201]. Furthermore, jasmonic acid stimulates the accumulation of vegetative storage proteins in soybean [202]. Interestingly, one of them has recently been identified as a lipoxygenase [120]. Exogenous addition of jasmonate stimulates lipoxygenase

Scheme 5 Lipoxygenase pathways in plants



The hydroperoxide lyase route may process the products synthesized by 9- and 13-lipoxygenases from both linoleic and α-linolenic acids, resulting in a variety of aldehydes and their corresponding alcohols. The jasmonate route (broken arrows) only processes 13S-HpOT.

activity [116,203] and it seems remarkable that a regulatory molecule should have a stimulatory effect on an enzyme involved in its synthesis.

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