Lipoxygenases from Plant and Animal Origin

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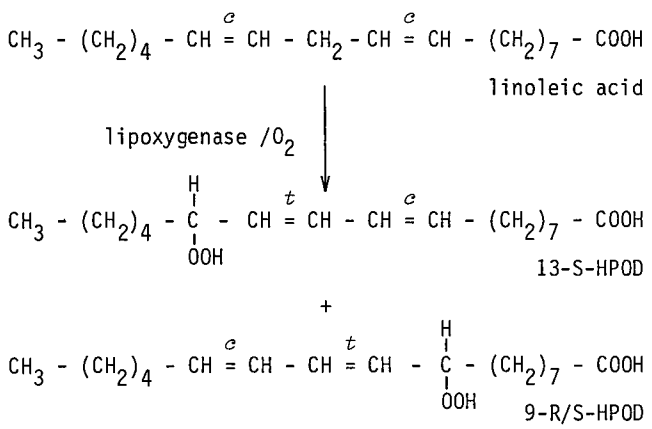
Introduction

Lipoxygenases (linoleate:oxygen oxidoreductase EC 1.13.11.12) catalyze the dioxygenation of polyunsaturated fatty acids which posses a 1,4-*cis*,*cis*-pentadiene system. Under optimum conditions regarding pH, temperature, and concentration of the reactants, the products are optically active *cis*, *trans* conjugated hydroperoxides. For example, the products of the enzymic dioxygenation of linoleic acid are 13-S(L)-hydroperoxy-9-*cis*,11-*trans*- (13-S-HPOD), 9-R(L)-hydroperoxy-10-*trans*,12-*cis*- (9-R-HPOD) or 9-S(D)-hydroperoxy-10-*trans*,12-*cis*-octadecadienoic acid (9-S-HPOD) (Scheme 1).



Scheme 1. The dioxygenation reaction of linoleic acid catalyzed by lipoxygenase

The enzyme is widespread in the plant kingdom, especially in legume seeds and cereals. The existence of lipoxygenase activity has also been demonstrated in a number of animal and human systems, e.g. blood cells. Interestingly, the end product of mammalian enzymes is often a hydroxy-polyene with *cis*,*trans*-conjugated double bonds rather than a hydroperoxy-polyene.

Besides the dioxygenation reaction, several lipoxygenases are capable of metabolizing the primary product, i.e., hydroperoxide. A variety of secondary products, including dimers and oxodienoic acids, can be

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obtained in this way. In animal and human systems the primary products are precursors for compounds which may possess physiological activity, like prostaglandins, thromboxanes and leukotrienes.

In this paper, we present recent developments in the biochemistry and biophysics of plant and mammalian lipoxygenases and related enzymes like cyclooxygenase. An emphasis is given to soybean lipoxygenase, because this enzyme has most extensively been investigated.

Plant Lipoxygenases

Variations in substrate and product specificity, pH-optimum, and stability have been observed for lipoxygenases from different plants. For soybeans, four more or less different isoenzymes have been described. Lipoxygenase-1 from soybeans is the most extensively investigated isoenzyme, due to its high stability and easy accessibility. It was already crystallized as early as 1947. For soybean lipoxygenase-1, several isolation methods have been reported (Vliegenthart and Veldink 1982).

Soybean Lipoxygenase-1

Lipoxygenase-1 consists of a single polypeptide chain and has a molecular weight of approx. 100 000. Theorell et al. (1947) reported an iron content of 0.3 mol per mol enzyme, but considered iron as an impurity. Therefore, for a long time lipoxygenase was regarded as an unique nonmetal dioxygenase. However, in 1973 several research groups (e.g., Roza and Francke 1973; Chan 1973) reported the occurrence of 1 mol iron per mol enzyme. The iron is not part of a heme group, but is directly bound to the polypeptide backbone.

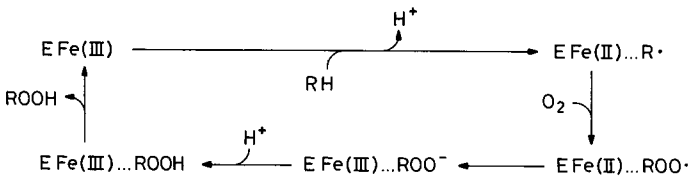
A specific activity of 280 μmol $O_2 \cdot min^{-1} \cdot mg^{-1}$ has been reported as determined by a polarographic method. This activity corresponds to 4.67 μkat$\cdot mg^{-1}$ or to a turnover number of 467 s^{-1}.

The amino acid composition of lipoxygenase-1 has been published by several investigators, but the sequence has not yet been established. Information on the amino acids located in or near the active site of the enzyme has been obtained from various studies. On the basis of fluorescence experiments (Finazzi-Agrò et al. 1975), it has been proposed that soybean lipoxygenase-1 has a large hydrophobic active site, which contains tryptophan residues. A histidine residue has been suggested to have a functional role in the enzymic dioxygenation of unsaturated fatty acids (Yamamoto et al. 1970). Furthermore, it has been reported (Spaapen et al. 1980) that lipoxygenase-1 contains five free sulfhydryl groups and no disulfide bridges. Three sulfhydryl groups react readily with methylmercuric halides leading to significant changes in the catalytic properties of the enzyme.

The optical spectrum of the native enzyme shows only a protein absorption band with a maximum at 280 nm and at high enzyme concentration shoulders are discernable at around 330 nm and 400 nm (Spaapen et al. 1979).

Reactions Catalyzed by Soybean Lipoxygenase-1

1. The Dioxygenation Reaction. The main product of the dioxygenation of linoleic acid catalyzed by soybean lipoxygenase-1 at pH 9.0 is 13-S(L)-hydroperoxy-9-*cis*,11-*trans*-octadecadienoic acid (13-S-HPOD) (Van Os et al. 1979). The kinetics of this reaction have been studied extensively. Using a prochiral ^{3}H-labeling of the methylene group of the pentadiene system, it has been demonstrated that an antarafacial relationship exists between hydrogen abstraction and dioxygen insertion (Hamberg and Samuelsson 1967; Egmond et al. 1972). From studies with substrates ^{2}H-labeled at the n-8 position[2], it could be concluded that abstraction of hydrogen is the rate-limiting step of the reaction (Egmond et al. 1973).



Scheme 2. The aerobic reaction of lipoxygenase-1

Based on qualitative EPR results, a scheme with a key role for iron in the dioxygenation reaction has been proposed (Scheme 2; De Groot et al. 1975). The native enzyme is almost EPR-silent (Fig. 1) and iron is thought to be in the Fe(II) state. A yellow enzyme form is obtained upon addition of one molar equivalent of 13-S-HPOD to native lipoxygenase-1. Yellow lipoxygenase-1 shows an EPR spectrum with resonances around *g* 6 characteristic for high-spin Fe(III) (Fig. 1). This oxidized enzyme form can be reduced by linoleic acid. Anaerobic addition of this substrate leads to an enzyme form which shows no EPR spectrum as reported for the native enzyme. In an aerobic system, the dioxygenation proceeds and the formation of the product hydroperoxide is coupled with a reoxidation of the iron in lipoxygenase and the enzyme becomes available for a new cycle.

2. The Anaerobic Reaction. When dioxygen is depleted during the dioxygenation reaction and both linoleic acid and product (13-S-HPOD) are available, lipoxygenase-1 starts to catalyze an anaerobic reaction (Scheme 3; Garssen et al. 1971, 1972). The yellow Fe(III) enzyme is reduced by linoleic acid and the reduced enzyme form is oxidized by the product 13-S-HPOD. The radicals formed give rise to a variety of products, including dimers and oxodienoic acids. From steady-state kinetics of the anaerobic reaction (Verhagen et al. 1978), it is concluded that lipoxygenase has one active site, which alternatively binds the two substrates of the anaerobic reaction (linoleic acid and 13-S-HPOD).

3. The Conversion of Linoleic Acid Hydroperoxide. In the mechanism of the anaerobic reaction (Scheme 3), the enzymic conversion of 13-S-HPOD in the presence of linoleic acid is shown. However, lipoxygenase also catalyzes the conversion of the product 13-S-HPOD in the absence of

2 Different systems are in use for the indication of the position of atoms in polyunsaturated fatty acids. For plant lipoxygenases, the position is indicated counting from the methyl group. For mammalian lipoxygenases, the positions in the molecule are given by numbers counting from the carboxylic group

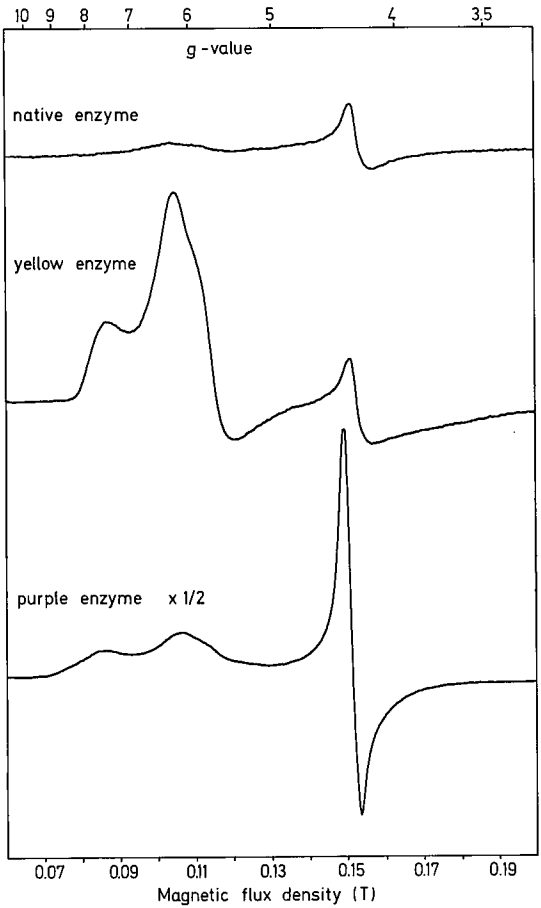
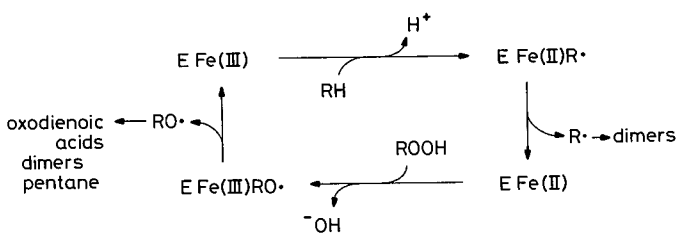


Fig. 1. EPR spectra of various enzyme forms of soybean lipoxygenase-1. *Native enzyme*: 0.54 m*M* in 0.1 *M* sodium borate buffer, pH 9.0. *Yellow enzyme*: 1 molar equivalent of 13-S-HPOD was added to the native enzyme solution. *Purple enzyme*: 3 molar equivalents of 13-S-HPOD were added to the yellow enzyme solution. Microwave frequency, 9.12 GHz; microwave power, 2 *mW*; temperature 15 K. The g_z parts of the spectra are presented in Fig. 3

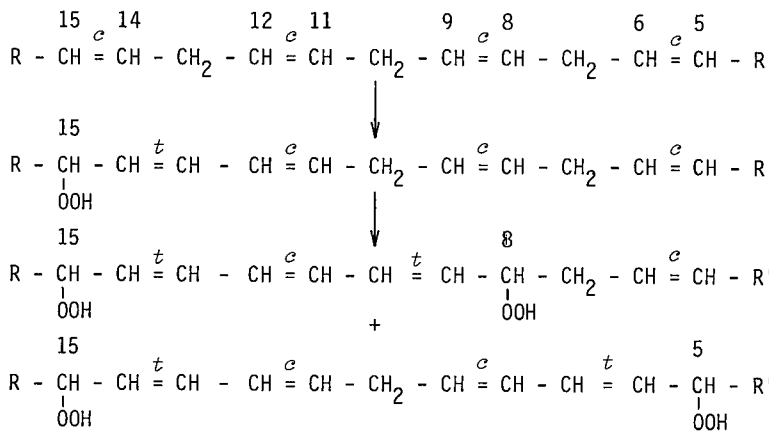


Scheme 3. The anaerobic reaction of lipoxygenase-1

linoleic acid. The conversion of 9-R- and 13-S-HPOD catalyzed by soybean lipoxygenase-1 has been investigated both under aerobic and anaerobic conditions (Verhagen et al. 1977, 1979). These reactions are much slower than the anaerobic reaction in the presence of linoleic acid. Under anaerobic conditions,
13-oxo-9-*cis*,11-*trans*-octadecadienoic acid,
13-oxo-9-*cis*(*trans*),11-*trans*-tridecadienoic acid, and
11-hydroxy-12:13-epoxy-9-*cis*-octadeceoic acid are the main products. In the aerobic conversion of 13-S-HPOD, the reaction rate is at least 4 times slower and no 13-oxo-9-*cis*,11-*trans*-tridecadienoic acid is

formed. The amount of dioxygen consumed is 0.7 mol per mol 13-S-HPOD leading to a large amount of products probably from oxidative polymerization. Two tentative schemes for the action of lipoxygenase on hydroperoxides have been presented, but the detailed mechanism is as yet unknown (Verhagen 1978).

4. Double Dioxygenation Reaction. The reactions described sofar concern the interaction between lipoxygenase and linoleic acid as well as products derived from linoleic acid. In polyunsaturated fatty acids with an 1,4,7-octatriene or an 1,4,7,10-undecatetraene system, like linolenic and arachidonic acid, lipoxygenase has the capacity of incorporating two molecules of dioxygen per substrate molecule (Bild et al. 1977). The first step is the "normal" dioxygen insertion at the n-6 position, followed by a second dioxygenation leading to a dihydroperoxy compound, which has either two conjugated *cis,trans* diene systems or a conjugated *trans,cis,trans* triene system, depending on the substrate and the position of insertion of the second dioxygen molecule (Scheme 4). The second dioxygenation step proceeds with a reaction rate which is several orders of magnitude smaller than that of the first step (Van Os et al. 1981).



Scheme 4. Dioxygenation of arachidonic acid and 15-hydroperoxy-arachidonic acid

Magnetic and Spectroscopic Studies on Soybean Lipoxygenase-1

Lipoxygenase is a metalloprotein which contains one mol nonheme iron per mol protein. In general, the metal environment and the reaction mechanism of metalloenzymes can adequately be studied by magnetic and spectroscopic methods.

1. Description of the EPR Signals Around g 6. Previously, the functional role of iron has been studied by qualitative EPR spectroscopy (De Groot et al. 1975a). The EPR signals around *g* 6 of yellow lipoxygenase result from contributions of high-spin Fe(III) species with more or less axial symmetry (*g* 6.2 - 7.4). The components contributing to the signal are enzyme species differing only in structure of the environment of iron, because the signals could not be attributed to either different enzyme - product complexes or to protonated and nonprotonated enzyme forms (Slappendel et al. 1982a). A shift to an axial type of EPR spectrum is observed after storage of the enzyme in the yellow form at 4°C. Storage of the native enzyme in a sodium-borate buffer solution (pH 9.0) instead of in a sodium-acetate buffer solution (pH 5.0)

brought to 25% saturation with solid ammonium sulfate at 4°C also leads to changes of the metal environment, which become apparent in a similar axial type of EPR spectrum after oxidation.

2. Determination of the Amount of EPR-Visible Iron. For further proof of the main reaction scheme (Scheme 2), it is necessary to quantitate the amount of iron visible in the EPR spectra. Because the signals around *g* 6 (Fig. 1) stem from only one of the three Kramers' doublets, the population of the doublets had to be determined. This requires knowledge of the energy differences between the doublets, which are described by the zero-field splitting constants (*D*) of the different components building up the signal around *g* 6 of lipoxygenase. The *D*-values are determined by two methods: (1) temperature dependence studies of the signal intensity and (2) by establishing *g*-shift upon increasing the microwave frequency (Slappendel et al. 1980). The ranges of *D* for the axial and rhombic species are found to be 1.5 - 3.0 K and 1.8 - 4.4 K, respectively. The absence of a rigid coordination sphere for iron in lipoxygenase like porphyrin in heme proteins might be the reason for the observed width of the ranges of the *D*-values and the variation in the shape of the EPR signal around *g* 6. Heme proteins with iron in the high-spin Fe(III) state also give EPR signals around *g* 6, however, these proteins have much higher *D*-values (approx. 14 K). The intensity of the EPR signals of lipoxygenase has been determined by both double integration and simulation methods. The complex EPR signal around *g* 6 of yellow lipoxygenase could only be simulated by using three components, which differ in degree of axiality (Fig. 2 and Table 1) (Slappendel et al. 1981). The amounts of iron visible in the EPR spectra have been calculated from the spectral intensity using the *D*-values described above and as references a Cu^{2+} solution in $NaClO_4$, pH 2, and a solution of sero-transferrin, an iron-transporting protein, which shows an EPR signal around *g* 4.3 (Table 2). A considerable amount of iron is EPR-visible in the yellow enzyme form which indicates that the qualitative EPR results earlier described dealt with major enzyme forms and this study strongly supports the mechanism of the dioxygenation presented in Scheme 2.

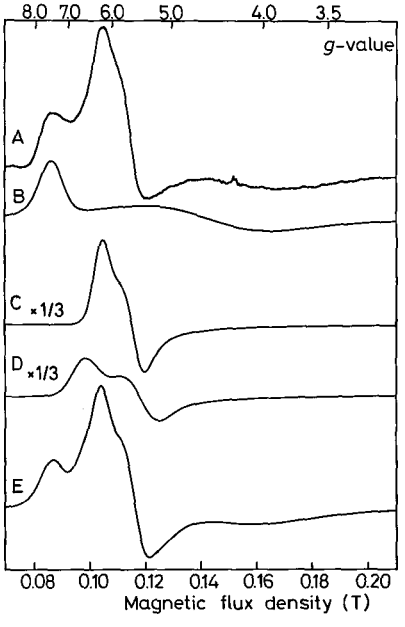


Fig. 2. Experimental and simulated spectra of yellow Fe(III)-lipoxygenase-1. *A* Spectrum of yellow lipoxygenase. The signal at *g* 4.3, which stems for at least 90% from an impurity in the EPR cavity and quartz dewar, is eliminated by subtraction of the spectrum of native enzyme from the spectrum of yellow enzyme. *B*, *C*, and *D* Simulated spectra giving the sum spectrum *E*. Spectrum *C* and *D* are recorded with a gain making their total integrated intensities one-third of the intensity of spectrum *B*. Simulation data is given in Table 1

Table 1. Simulation data of yellow lipoxygenase[a]

Component	g value			Line width (mT)			Relative weight in spectrum E
	x	y	z	x	y	z	
1 (B)	7.35	4.55	1.88	7	35	25	1.0
2 (C)	6.20	5.60	2.00	5.5	6.5	25	0.4
3 (D)	6.55	5.45	1.98	9	10	25	0.5

[a]The letters refer to the spectra presented in Fig.2

Table 2. EPR-visible iron in lipoxygenase-1[a]

Enzyme form	Signal around g 6 Simulation	Signal at g 4.3 Simulation	Integration
Native	<1	0.1	
Yellow	80	0.6	
Purple	76	8	13

[a]The amount of iron visible in the EPR spectra presented in Fig. 1 is given as a percentage of the total iron content. Corrections have been made for a signal at g 4.3 stemming from an impurity in the EPR cavity and quartz dewar (90% of the signal shown for native lipoxygenase)

3. Purple Lipoxygenase. Besides yellow Fe(III)-lipoxygenase, a second colored enzyme form has been described by De Groot et al. (1975b). Upon addition of a molar excess of 13-S-HPOD to native or yellow lipoxygenase, a purple enzyme form is obtained, which shows in the EPR spectrum a more rhombic type of signal around g 6 than yellow enzyme and in addition a signal at g 4.3 (Fig. 1). The line shape of the signal aroung g 6 has strongly changed as compared with yellow lipoxygenase, but the amount of iron visible in this part of the spectrum has only slightly been diminished (Table 2). The EPR signal at g 4.3 is typical for high-spin Fe(III) iron in a ligand field of rhombic symmetry. In the case of lipoxygenase, it has been attributed to an enzyme – 13-S-HPOD complex. Recently, it has been shown that the intensities of the signals around g 6 and at g 4.3 are linearly correlated with the absorbance of yellow lipoxygenase at 370 nm and the absorbance of purple lipoxygenase at 570 nm, respectively (Slappendel et al. 1983). The amount of iron visible in the EPR signal at g 4.3 is only approx. 10% of the total iron content (Table 2). This has led to a reinterpretation of the EPR results concerning the purple enzyme form. Addition of 3 to 4 molar equivalents of 13-S-HPOD to native or yellow lipoxygenase results in an almost complete formation of the enzyme – 13-S-HPOD complex because the affinity of 13-S-HPOD for lipoxygenase is large (K_{aff} = 10 μM; Egmond et al. 1977). This means that only part of the enzyme – 13-S-HPOD complex (approx. 10% cf. Table 2) has a conformation of the environment of iron which leads to an EPR signal at g 4.3 and an absorption at 570 nm (Scheme 5). The molar absorption coefficient ε_{570} is 10^4 $M^{-1} \cdot cm^{-1}$ and the absorption is proposed to originate from charge transfer transitions between iron and amino acids.

4. EPR Spectrum Around g 2. In the EPR spectra of the various forms of soybean lipoxygenase-1, a signal around g 2 can be discerned (Fig. 3). Several different contributions to this signal can be distinguished, including those from contaminants. The latter ones can be analyzed by studying the native enzyme, which is in principle EPR-silent. A

$$\text{E - Fe(III)} + \text{13-L-LOOH} \rightleftharpoons \text{E - Fe(III)}\cdot\cdot\,\text{13-L-LOOH} \longleftrightarrow \text{E - Fe}\cdot\cdot\,\text{13-L-LOOH}$$

YELLOW	YELLOW	PURPLE
EPR AROUND G 6	EPR AROUND G 6 (LESS AXIAL THAN E - Fe(III))	EPR AT G 4.3

Scheme 5. Interaction of lipoxygenase-1 and 13-hydroperoxy-linoleic acid (13-L-LOOH)

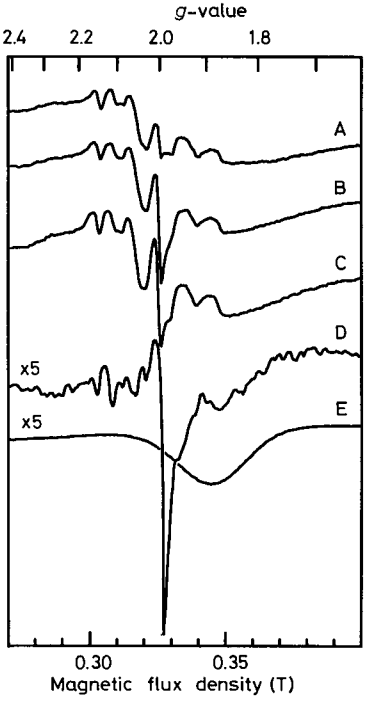


Fig. 3. EPR signals around g 2 of soybean lipoxygenase-1. *A* Native enzyme; *B* yellow enzyme; *C* purple enuyme; *D* spectrum obtained by subtraction of spectrum *A* from *B*. *E* Simulated spectrum of the g_z component of the high-spin Fe(III). Spectra *D* and *E* are presented with a gain 5 times higher than spectra *A* - *C*. For experimental conditions and simulation data see Fig. 1 and Table 1

contamination of manganese (0.07 mol per mol enzyme) cause a signal with a hyperfine splitting in six lines (I = 5/2) (Fig. 3A-C). Contributions from copper (0.006 mol per mol enzyme) and from other impurities were not detectable. Besides the manganese-signal, the signal around g 2 of yellow and purple lipoxygenase consists of two parts:

a. The high-field component (g_z) of the high-spin Fe(III) signal with g_x and g_y components aroung g 6. The g_z component of the high-spin Fe(III) enzyme species having the least axial symmetry is discernable at g 1.9 in the spectrum obtained by subtraction of the spectrum of native enzyme from that of yellow (Fig. 3D) or from that of the purple enzyme. A simulated spectrum of the g_z component of this high-spin Fe(III) enzyme species is given in Fig.3E. The g_z parts of the two more axial species coincide with the signals at g 2.

b. A radical type of signal (Fig. 3B-C). This signal is better recognizable after subtraction of the manganese signal (Fig. 3D). In the different schemes describing lipoxygenase catalysis (Scheme 2 and 3) radicals are present in either an enzyme-bound or in a free-radical form. The dissociation of the enzyme - radical complexes in the aerobic

catalytic cycle is thought to be insignificant, since the high degree of optical purity of the hydroperoxides formed in the lipoxygenase-1-catalyzed dioxygenation indicates that the enzyme controls the way of dioxygen insertion. In the anaerobic reaction of lipoxygenase, the dissociation of the enzyme - radical complex is more pronounced than in the aerobic reaction and part of the radicals are thought to be free radicals which react with each other to form a variety of dimers. Knowledge on the structure of these free radicals in solution could be obtained by a spin-trapping method (De Groot et al. 1973). The water soluble spin-trap 2-methyl-2-nitrosopropanol has been used in the anaerobic reaction. From incubation with linoleic acid, specifically ^{2}H-labeled in the pentadiene system, the trapped radical could be identified as a linoleyl radical. Spin adducts of oxygen-centered radicals are thought to decompose too rapidly at ambient temperature to be observed by EPR spectroscopy. For direct detection of radicals at low temperature (<77 K), rapid-freeze equipment has been used. Exploratory studies with this technique have given experimental evidence for the formation of alkoxy radicals during the incubation of lipoxygenase with 13-S-HPOD.

5. EPR Spectra of the Protein Chain. The EPR spectra of lipoxygenase described sofar contain signals from iron and radicals. However, EPR signals arising from the protein chain of lipoxygenase are observed after storage of a sample of yellow lipoxygenase-1 for 6 months at 77 K (Fig. 4). The signals stem from the protein moiety of the enzyme, because the conversion products of 13-S-HPOD give a different type of EPR signals. According to Schaich and Karel (1976), signals as shown in Fig. 4 could stem from either cysteine or amino acids with nitrogen-containing side chains, but assignment to one particular amino acid can not be made. Either radicals from the conversion products of 13-S-HPOD or iron may be involved in the reaction leading to this new radical signal.

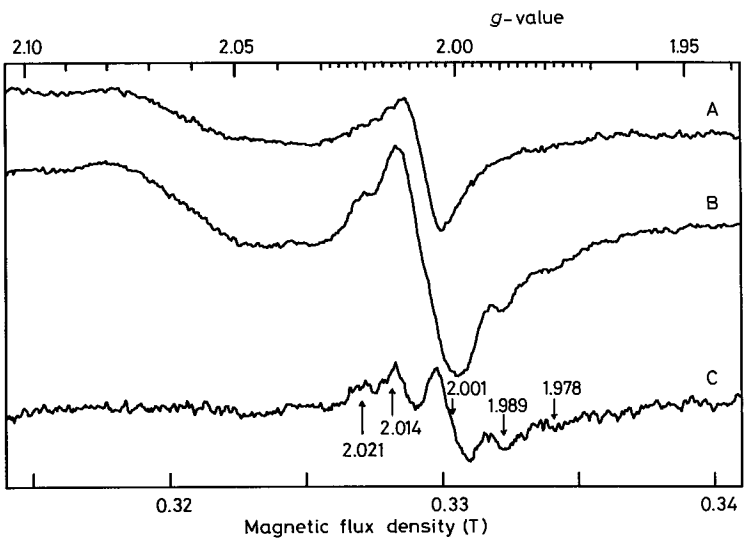


Fig. 4. EPR spectra around g 2 of yellow lipoxygenase. *A* Yellow enzyme; *B* yellow enzyme after storage at 77 K over 6 months; *C* difference spectrum of spectra *A* and *B*. For complete base-line correction, it was necessary to multiply spectrum *A* with a factor 1.5. Microwave frequency 9.251 GHz; microwave power 2 *mW*; temperature 15 K

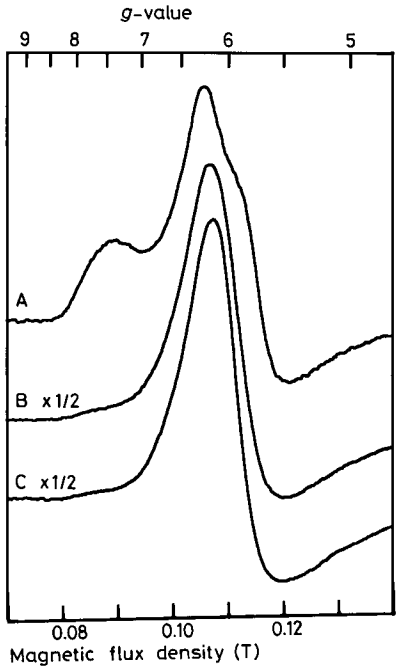


Fig. 5. EPR line-shape changes of yellow lipoxygenase-1. Yellow lipoxygenase (*A*) was incubated with 10 molar equivalents KCN (*B*) and 30 molar equivalents ethanol (*C*). Microwave frequency 9.256 GHz; temperature 15 K

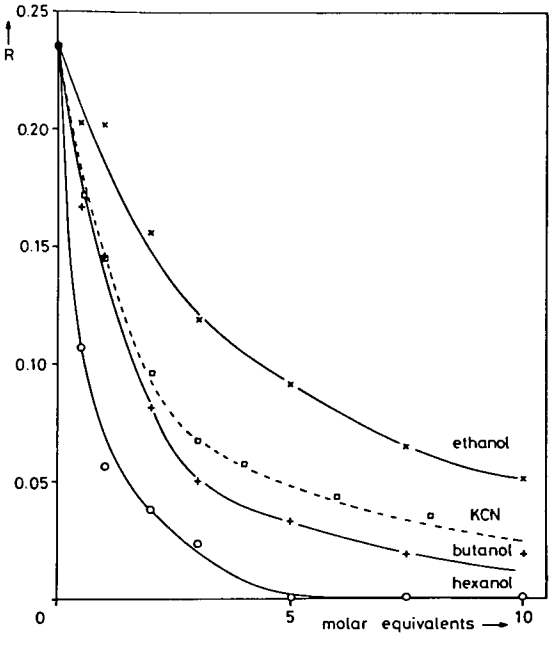


Fig. 6. Titration of yellow lipoxygenase-1 with alcohols and cyanide. On the *ordinate*, the ratio of the rhombic and axial components (*R*) is given. The amplitude of the rhombic part measured at *g* 7.5 is corrected for the contribution of the axial part at this *g*-value

6. The Binding of Alcohols. Interestingly, it has been found that small amounts of ethanol (30 molar equivalents) when added to a yellow Fe(III)-lipoxygenase solution induce a shift to a nearly axial type of EPR spectrum (Fig. 5; Slappendel et al. 1982a). The binding of alcohols to the enzyme has been further studied by ^{1}H-NMR and EPR spectroscopy (Slappendel et al. 1982b,c). Titration curves of yellow lipoxygenase with ethanol, butanol-1, and hexanol-1 are given in Fig. 6, where changes in the EPR spectra are presented as the ratio (*R*) of the amplitudes of the rhombic (at *g* 7.4) and axial (at *g* 6.0 - 6.2) components. ^{1}H-NMR spectra of solutions of butanol-1, to which either native or yellow lipoxygenase has been added, show a line-broadening of the proton resonances, which is due to proton relaxation enhancement from magnetic interaction between iron and protons (Fig. 7). In comparing the same proton resonances, the line-broadening is more pronounced for the yellow than for the native enzyme. This can be explained by one or more of the following reasons: a. the line-broadening is determined by the spin quantum number *S*, which is 2 (see next section) and 5/2 for native and yellow enzyme, respectively; b. a difference in affinity of the alcohol for binding to the enzyme forms; and c. a difference in relaxation times (rotational correlation time, exchange life time, and electron spin relaxation time). For the different proton resonances of the same enzyme sample (native or yellow enzyme added to a butanol-1/D_2O solution), the line-broadening increases going from the methyl protons to the protons bound to carbon

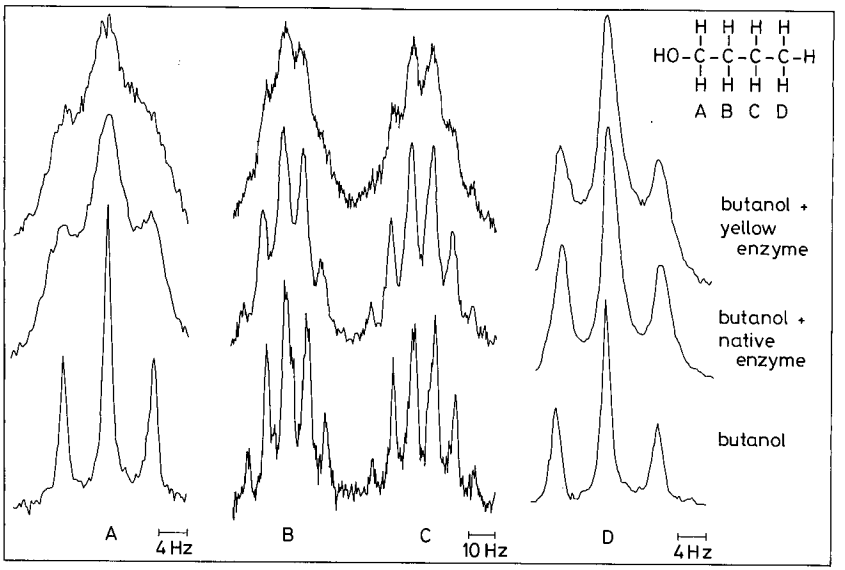


Fig. 7. NMR spectra of butanol-1 showing the effect of paramagnetic iron in native and yellow lipoxygenase on the proton resonances of butanol-1. *A* 8 m*M* butanol-1; *B* and *C* enzyme: 26 µ*M* and butanol-1 22.8 m*M*. Buffer: 0.1 *M* sodium borate/D_2O, pH 9.0; temperature 297 K

Table 3. ^{1}H-NMR study of the binding of various alcohols to yellow lipoxygenase-1[a]

Alcohol	Observed line-broadening (Hz)		K_a (m*M*)	Distance to iron (A)	
	Methyl protons	Protons bound to the carbon atom 1		H_3C-	-CH_2OH
Ethanol	1.5	2.0	260	5.1	4.7
Butanol-1	5.0	11	30	7.2	6.0
Hexanol-1	8	21	3	6.3	5.3

[a]The observed line-broadening of the methyl protons and the protons bound to carbon atom 1 of the alcohol is given after addition of yellow lipoxygenase-1 to an alcohol/D_2O solution; concentrations: alcohol 8 m*M*; enzyme 0.03 m*M*. The affinity constant K_a has been derived from titration curves of yellow enzyme with the alcohols. The distances between iron and methyl protons and protons bound to carbon atom 1, respectively, have been calculated with the Solomon-Bloembergen equation

atom 1. This differential line-broadening implies no difference in mobility of the protons, because from a competition experiment with cyanide, which also gives a shift to an axial type of EPR spectrum (Fig. 5 and 6), it is concluded that the alcohols do not bind via the hydroxyl group. Quantitative results on the binding of various alcohols to lipoxygenase have been obtained from titrations and calculations using the Solomon-Bloembergen equation (Table 3). The affinity of the various unbranched alcohols for binding to yellow lipoxygenase increases with increasing carbon chain length (see also Fig. 6). The branched alcohol t-butanol gives neither a shift to an axial type of EPR spectrum nor a line-broadening in NMR upon addition to a yellow lipoxygenase solution. Remarkably, the calculated distance between the methyl protons of the various alcohols and iron is in

good agreement with the distance between the methyl protons (n-1 position) and the proton at the n-8 position, which is abstracted during the dioxygenation reaction. This could indicate that the binding places of the methyl group of the alcohols and the substrate are similar.

7. Determination of the Spin and Valence State of Iron in Native Lipoxygenase. Magnetic susceptibility measurements have been used to determine the spin and valence state of iron in native lipoxygenase (Slappendel et al. 1982d). The molar susceptibility shows Curie dependence over the temperature range from 40-200 K (Fig. 8). A value for the Bohr magneton equal to 5.2 ± 0.3 has been found, which is typical for high-spin Fe(II) (S=2). The high-spin state of iron in native lipoxygenase is also clear from the line-broadening in the ^{1}H-NMR spectrum (Fig. 6) observed upon addition of native enzyme to a butanol-1/D_2O solution, because Fe(II) in the low-spin state has no unpaired electrons (S = 0) and should not give paramagnetic proton relaxation enhancement.

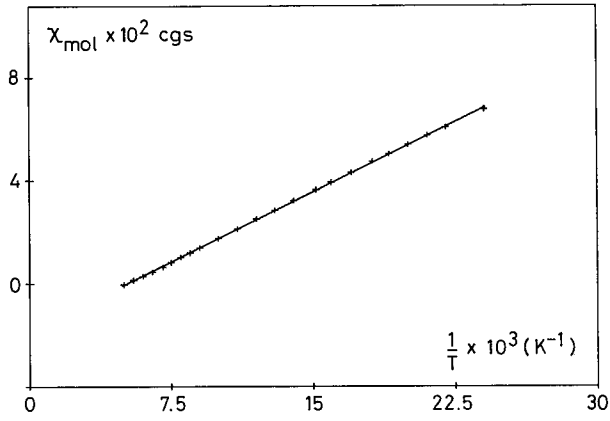


Fig. 8. The temperature dependent contribution of the magnetic susceptibility of native lipoxygenase-1. Enzyme: 1.19 mM in 0.1 M sodium borate buffer, pH 9.0

Physiological Role of Plant Lipoxygenases

Although lipoxygenases are widespread in the plant kingdom and occur in relatively large amounts, especially in legume seeds and cereals, little is known about the physiological role of these enzymes. Hypotheses include a role of the enzymes in various stages of germination and growth, in the process of wound healing via traumatic acid, and in the production of volatile compounds which render legumes and fruits their typical flavors. These functions are possibly also coupled with lipoperoxidase activity of the enzyme.

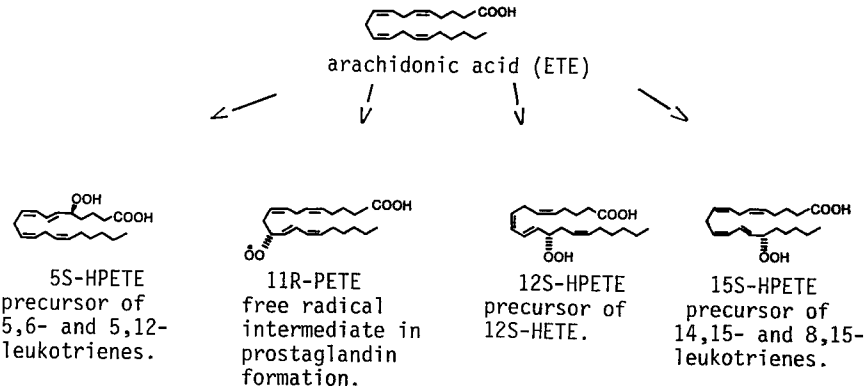
Mammalian Lipoxygenases and Cyclooxygenase

Since 1974, lipoxygenases have also been isolated from mammalian origin, predominantly from various blood cells. As mentioned before, these mammalian enzymes differ in several respects from those of plant origin, i.a., with regard to substrate specificity. However, there are also interesting common features, especially for the lipoxygenase isolated from rabbit reticulocytes and for the related enzyme cyclooxygenase purified from sheep vesicular glands.

In this section, a summary of the metabolic pathways of arachidonic acid catalyzed by cyclooxygenase and mammalian lipoxygenases will be presented. A number of these enzymes will be described. Finally, the physiological role of mammalian lipoxygenases and cyclooxygenase is briefly summarized.

The Metabolism of Arachidonic Acid

The substrate of mammalian lipoxygenases and cyclooxygenase is often arachidonic acid (eicosatetraenoic acid, ETE) rather than linoleic acid, which is the best substrate for soybean lipoxygenase-1. In vivo, arachidonic acid is obtained i.a. from cell membrane phospholipids through the action of phospholipases. Due to the presence of 4 *cis* double bonds in an 1,4,7,10-undecatetraenoic system, lipoxygenase-catalyzed dioxygenation can occur at several positions (Scheme 6). So far, from the 12 possible hydroxy derivatives of arachidonic acid (HETE), the 5-S, 11-S/R, 12-S, and 15-S compounds have been isolated. In reaction with (partly) purified enzyme preparations, the presence of the corresponding precursor hydroperoxy arachidonic acid (HPETE) has been demonstrated (e.g., 12-S-HPETE in platelets). The peroxy radical of 11-R-HPETE is an intermediate in the reaction of cyclooxygenase. 5-S and 15-S-HPETE are the precursors of the leukotrienes. The leukotrienes derived from 5-S-HPETE (Scheme 7) have important physiological functions.

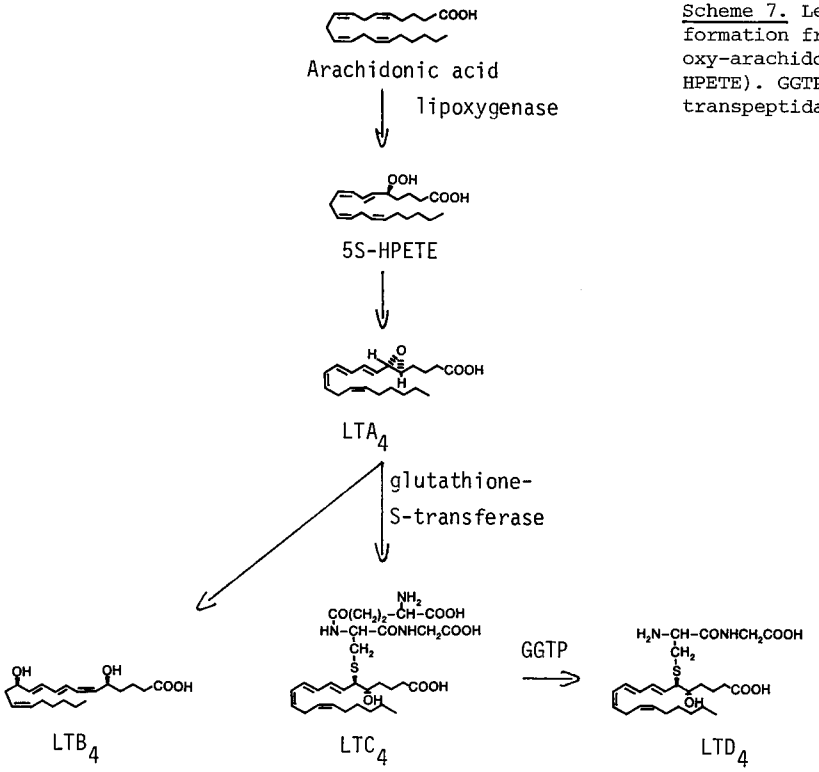


Scheme 6. The hydroperoxydation of arachidonic acid (ETE)

Lipoxygenases from Mammalian Origin

A listing of mammalian lipoxygenases arranged according to the position of dioxygen insertion is presented in Table 4. The lipoxygenases are indicated by the number of the carbon atom (counted from the carboxylic group) to which dioxygen is attached. A number of mammalian enzymes, which have been (partly) purified, will be described with emphasis on the metabolic aspects and compared with soybean lipoxygenase.

15-Lipoxygenase from Reticulocytes. A lipoxygenase has been isolated from rabbit reticulocytes by Rapoport et al. (1979). The enzyme has a molecular weight of 78 000 and an isoelectric point of 5.5. Furthermore,



Scheme 7. Leukotriene (LT) formation from 5-hydroperoxy-arachidonic acid (5S-HPETE). GGTP = γ-glutamyl transpeptidase

Table 4. Sources of mammalian lipoxygenases

5-Lipoxygenase	12-Lipoxygenase	15-Lipoxygenase
Granulocytes	Platelets	Reticulocytes
Lymphocytes	Granulocytes	Lymphocytes
Mast cells	Mast cells	Granulocytes
Mastocytoma cells	Spleen	Monocytes
RBL-1 cells	Aorta	Macrophages
Monocytes	Monocytes	
Macrophages	Macrophages	
Lung tissue	Lung tissue	
Spleen		

it contains 5% neutral sugars and one mol iron per mol protein. The environment of iron is thought to be identical to that of soybean lipoxygenase, because it has similar spectroscopic properties and there is no evidence for either a porphyrin system or an iron-sulfur cluster. Reticulocyte lipoxygenase converts linoleic acid into 13-S-HPOD and arachidonic acid into 15-S-HPETE similar to soybean lipoxygenase-1. However, its substrate specificity is less pronounced compared to the soybean enzyme. All polyunsaturated fatty acids having a 1,4-*cis,cis* pentadiene system and all classes of phospholipids containing polyunsaturated fatty acids are attacked. The enzyme also acts on intact mitochondrial membranes and causes repiratory inhibition of submitochondrial particles of various origin. Besides the dioxygenation reaction reticulocyte lipoxygenase has a number of

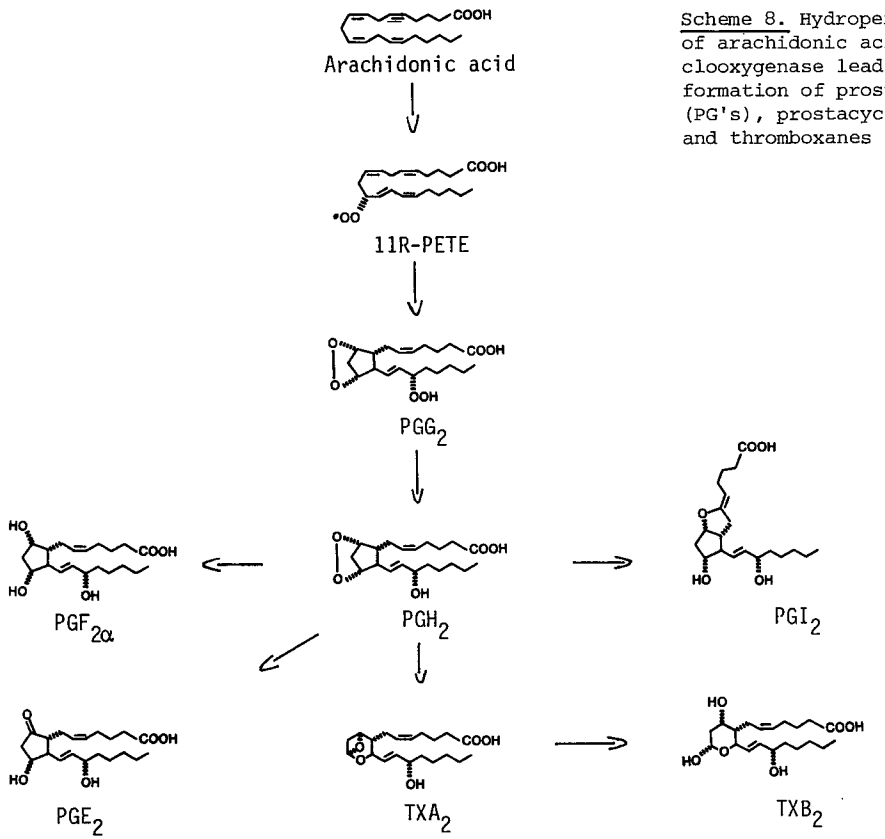
features in common with soybean lipoxygenase (Härtel et al. 1982). For the activation of the enzyme, 13-S-HPOD can be used. Addition of one molar equivalent of 13-S-HPOD leads to the conversion of iron from the Fe(II) into the Fe(III) state, while concomitantly the fluorescence and absorption properties of the enzyme change. The enzyme is also capable of converting 13-S-HPOD in the presence of linoleic acid under anaerobic conditions. The products of this reaction are similar to those obtained in the anaerobic reaction of soybean lipoxygenase (cf. Scheme 3). The catalytic activity of reticulocyte lipoxygenase has a suicidal nature, i.e., an inactivation occurs by the products formed during catalysis at temperatures above 20°C. Both conversion products of 13-S-HPOD as well as the products of the anaerobic reaction are responsible for the inactivation of the enzyme. Self-inactivation has also been reported for cyclooxygenase.

12-Lipoxygenase from Platelets. Hamberg and Samuelsson (1974) were the first to describe the conversion of arachidonic acid into 12-S-HETE catalyzed by a lipoxygenase in human platelet homogenates. Nugteren (1975) purified a platelet lipoxygenase from cows and a novel preparation of human platelet lipoxygenase has recently been described by Wallach and Brown (1981). This 12-lipoxygenase is not inhibited by aspirin and indomethacin like cyclooxygenase, but it is inhibited by tetraynoic acids and a series of phenylhydrazone inhibitors. These compounds also inhibit dioxygenation catalyzed by soybean lipoxygenase and cyclooxygenase. Cyclooxygenase is relatively less sensitive to these inhibitors than human platelet lipoxygenase. Inhibition of platelet lipoxygenase by toluene-3,4-dithiol and other Fe(III) chelators has been reported by Aharony et al. (1981). Fe(II) chelators have no influence on the platelet lipoxygenase catalysis, suggesting that the enzyme activity depends on iron in the Fe(III) state. This is in agreement with the observation that the platelet enzyme is activated by 12-S-HPETE as is seen with soybean lipoxygenase-1 by its product 15-S-HPETE. Hamberg and Hamberg (1980) have investigated the mechanism of the dioxygenation of arachidonic acid by human platelet lipoxygenase. Similar to soybean lipoxygenase-1, an antarafacial relation between the hydrogen abstraction from C-10 and the insertion of dioxygen at C-12 was found. Furthermore, the hydrogen abstraction is probably the initial step in the dioxygenation reaction. The main product of the catalytic reaction with arachidonic acid as substrate is 12-S-HPETE, which is converted by a hydroperoxidase into 12-S-HETE. In addition, minor quantities of epoxy-hydroxy and trihydroxy derivatives of arachidonic acid are formed, possibly via mechanisms similar to those observed with soybean lipoxygenase.

5-Lipoxygenase from Rat Basophilic Leukemia Cells. So far, only a few 5-lipoxygenases have been (partly) purified and little is known about the properties of these enzymes. Rat basophilic leukemia cells (RBL-1 cells) contain a lipoxygenase which catalyzes the conversion of arachidonic acid into 5-S-HPETE. This product is further converted into several products including leukotriene B_4 and D_4 and 5-S-HETE. The 5-lipoxygenase from RBL-1 cells has recently been purified by Parker and Aykent (1982). The enzyme has a molecular weight of 90 000. In the presence of Ca^{2+}, a dimer is formed which possesses the enzymatic activity. So far, most experiments have been done with homogenates and intact cells. As yet, information on the presence and possible involvement of iron in the dioxygenation catalyzed by this enzyme is not available.

Cyclooxygenase

Cyclooxygenase or prostaglandin endoperoxide synthase (EC 1.14.99.1) catalyzes the insertion of dioxygen into polyunsaturated fatty acids. When linoleic acid is the substrate, the main product is 9-R-hydroxy-octadecadienoic acid (9-R-HOD) (Hamberg and Samuelsson 1980), which is also the main product formed by soybean lipoxygenase-2 (Van Os et al. 1979). Arachidonic acid is further converted into 15-hydroperoxy-9α,11α-peroxidoprosta-5,13-dienoic acid (PGG_2) by cyclooxygenase. This hydroperoxy-endoperoxide is then reduced by the same enzyme to a hydroxy-endoperoxide (PGH_2), which is, in turn, the precursor of other prostaglandins, prostacyclin, and thromboxanes (Scheme 8).



Scheme 8. Hydroperoxidation of arachidonic acid by cyclooxygenase leads to the formation of prostaglandins (PG's), prostacyclin (PGI), and thromboxanes (TX's)

Cyclooxygenase has been isolated from sheep vesicular glands and has a molecular weight of approx. 70 000 (Hemler et al. 1976; Van der Ouderaa et al. 1977). Hemler et al. (1976) have reported that the isolated enzyme contains nonheme iron similar to soybean lipoxygenase, but for full activity, hemin has to be bound to the enzyme in at least stoichiometric amounts. However, Van der Ouderaa et al. (1979) have excluded the presence of nonheme iron in the enzyme. Similar to soybean lipoxygenase, the enzyme is activated by hydroperoxide (Hemler et al. 1979). This suggests that iron in the Fe(III) state is a requirement for activity (cf. yellow Fe(III)-lipoxygenase in Scheme 2).

Conversely, Peterson et al. (1980) put forward the hypothesis that Fe(II) is the active form of iron in cyclooxygenase. EPR studies may contribute to settle this controversy. So far, EPR studies on the cyclooxygenase reaction have been confined to only a free radical formed during a reaction of PGG_2 with ram seminal vesicles. For a long time, the radical has been described as an oxygen-centered free radical, which is formed during the reduction of PGG_2 to PGH_2, but recently, Kalyanaraman et al. (1982) have attributed this radical to a hemoprotein free radical, possibly formed by the oxidation of an amino acid, which is a part of the heme environment. Radicals formed during the reduction of PGG_2 to PGH_2 are thought to be responsible for the self-inactivation of the enzyme similar to the suicidal action observed for lipoxygenase from reticulocytes.

Inhibition studies on cyclooxygenase are rather complicated from a mechanistic point of view (Kuehl et al. 1981). For example, reducing agents act both on the peroxides necessary for the activation of the enzyme (inhibition) and on the oxidants released during the reduction of PGG_2 (protection against self-inactivation). The antiinflammatory effect of most nonsteroidal antiinflammatory agents, like aspirin and indomethacin, is based on the inhibition of cyclooxygenase. Differences in the inhibition mechanism of these agents are observed. Aspirin irreversibly modifies the active site by transferring an acetyl group to a serine residue, while indomethacin reversibly blocks the uptake of dioxygen. Furthermore, often different effects of inhibitors on the two steps of the cyclooxygenase-catalyzed reaction are observed.

Physiological Role of Mammalian Lipoxygenases and Cyclooxygenase

Concerning the physiological role of mammalian lipoxygenases and cyclooxygenase, a lot of information has become available when compared to the enzymes from plant origin. Cyclooxygenase plays a key role in the synthesis of prostaglandins, prostacyclin, and thromboxanes. These compounds are involved in many physiological processes (Table 5). This has led to a growing number of medical applications of these compounds and their derivatives.

Table 5. Biological effects of arachidonic acid metabolites

Prostaglandins, prostacyclin and thromboxanes	Leukotrienes LTB_4	LTC_4 and LTD_4
- contraction and relaxation of smooth muscle - stimulation (TXA_2) and prevention (PGI_2) of platelet aggregation - regulation of gastric secretion	- chemotactic for leukocytes - promotes sticking of leucocytes to blood vessel wall (capillary vessels)	- contraction of smooth muscle (slow-reacting substance of anaphylaxis, bronchoconstriction, asthma) - increase of vascular permeability (edema) - induction of TXA_2 formation (guinea pig lung)

An important function of the mammalian lipoxygenases is the production of the precursors to leukotrienes. Known biological effects of the leukotrienes are given in Table 5. The physiologically active compounds probably play a role in asthma and in various allergic reactions. It

is expected that research in the field of lipoxygenase-catalyzed reactions of polyunsaturated fatty acids, especially leukotriene formation, will lead to a better understanding of the biochemical events, physiological processes, and therapies related to different diseases. The fundamental knowledge of the reactions catalyzed by soybean lipoxygenases forms a sound basis for these research activities.

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Discussion

Hamprecht: From one of your tables, I took that in macrophages lipoxygenases act on arachidonic acid at positions 5, 12, and 15. Do you know whether the three different enzymes occur in the same cell or in different subtypes of macrophages or whether they occur under different induction conditions?

Vliegenthart: The production of 5-hydroperoxy arachidonic acid and its derivatives (leukotrienes and 5-HETE) by macrophages from different sources is dependent on the presence of calcium, ionophore, and glutathione. In the absence of calcium, the formation of other hydroxy compounds of arachidonic acid also takes place. Unfortunately, the literature is rather confusing at this point. However, it could well be that some cell types are more outspoken in the production of specific compounds than others.

Stoffel: I have two questions: (1) What is the reason for hydroperoxide formation in different positions of linoleic acid? (2) What is the function of lipoxygenase in soybeans, rich in polyunsaturated fatty acids?

Vliegenthart:
1) The stereospecificity of the product formation from the incubation of linoleic acid is determined i.a. by the source of lipoxygenase and by the reaction conditions (pH, substrate concentration, temperature).
2) The function of lipoxygenase in plants is still obscure. Suggestions given in the literature are dealing with a possible role during the germination of seeds when normal respiration has not yet been started, with an involvement in wound healing through the production of the wound hormone traumatic acid and with the formation of volatile compounds.

Weller: With regard to the lectures this morning, it appears of interest where the iron is bound in the protein. Is there no idea about that, e.g., from Resonance Raman Spectroscopy of the ferric enzyme?

Vliegenthart: The iron coordination to the protein backbone is as yet unknown. Resonance Raman Spectroscopy was not applicable because of strong fluorescence from amino acid residues and instability of the ferric enzyme in the laser beam. Other spectroscopic studies including EXAFS are now in progress.

Walsh: How do you account for the antarafacial stereochemistry on the pentadiene system. Given C-H breakage as $-C^{\bullet} + H^{\bullet}$, where does $H^{\bullet}$ go? To Fe? If so, what coordinates the O_2?

Vliegenthart: For the study of the course of the stereochemical attack of the pentadiene system [$11L_S$-^3H], linoleic acid has been prepared by incubation of [$11L_S$-^3H] stearic acid with the green algae *Chlorella vulgaris*. Incubation of this specifically labelled linoleic acid with soybean lipoxygenase-1 leads to the product $13L_S$-hydroperoxy linoleic acid with a very low tritium content. This experimental evidence has led us to propose an antarafacial relationship between hydrogen abstraction and dioxygen insertion.

The formation of the linoleyl radical in the dioxygenation reaction is coupled with a reduction of iron in lipoxygenase. Concomitantly, the hydrogen atom is converted into a proton. Studies concerning the coordination of dioxygen are in progress. So far, no solid evidence could be obtained for binding O_2 to iron in native lipoxygenase.

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