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LIPOXYGENASES, NONHEME IRON-CONTAINING ENZYMES

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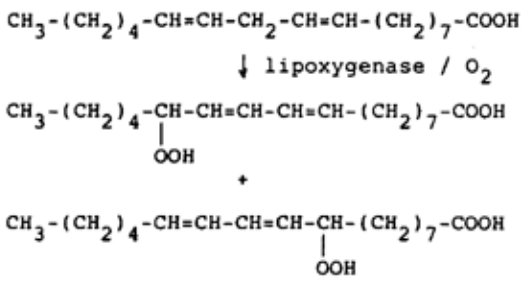
I. INTRODUCTION

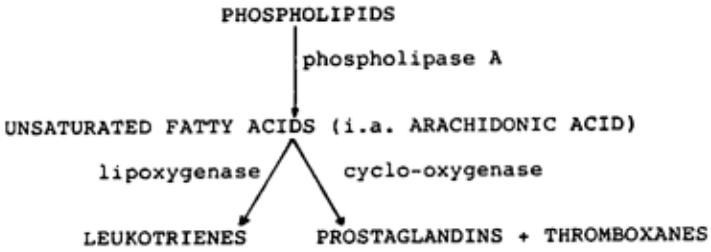
Lipoxygenases (EC 1.13.11.12) constitute a class of metal-containing proteins which catalyze the incorporation of dioxygen into unsaturated fatty acids containing one or more 1Z,4Z-pentadiene systems, e.g., 9Z,12Z-octadecanoic acid (linoleic acid) to yield hydroperoxy fatty acids (Scheme 1). The occurrence of lipoxygenases has been demonstrated in a wide variety of plants, in particular



Scheme 1

in Leguminosae (soybean, pea, horse bean and peanut), Solanaceae (potato, eggplant, tomato), Graminae (wheat, rye, corn, barley), Linaceae (flax), Compositae (artichoke, African daisy) and Cruciferae (cauliflower) (1,2). The purification and properties of a lipoxygenase from soybeans were first described by Theorell et al. (3) and Holman et al. (4,5). It was subsequently established that lipoxygenases and the enzymes involved in the biosynthesis of prostaglandins utilize the same type of substrates, namely unsaturated fatty acids. Although both enzymes also incorporate molecular dioxygen into the fatty acid substrate, the end products differ considerably. Only recently, true lipoxygenases have been discovered in animal tissues which thus exist alongside cyclo-oxygenase (prostaglandin synthetase) and which, in vivo, may have a strong regulatory interrelationship.

The majority of lipoxygenases known to date contain iron as the essential transition metal in a 1:1 molar ratio, although cobalt has also been reported as a required component for a particular lipoxygenase (11). The formation of oxygenated fatty acids constitutes the first step of a number of novel pathways along which certain poly-unsaturated fatty acids can be metabolized (Scheme 2).



Scheme 2

Since the discovery of the biosynthetic pathway leading to the formation of leukotrienes, an appreciable amount of research has been carried out in this particular area, mainly because of the key role leukotrienes are thought to play in the molecular mechanisms underlying vital immunochemical and regulatory processes. In this chapter, attention will be focused primarily on physico-chemical and spectroscopic aspects of lipoxygenase catalysis rather than on the structural characteristics of the various reaction products and their physiological effects and functions.

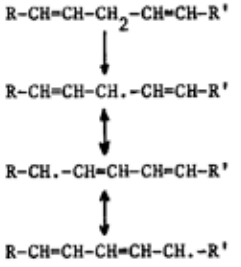
II. METAL CONTENT

Lipoxygenases occupy a unique position in the realm of metal containing proteins. They do not contain a heme prosthetic group nor one of the familiar iron-sulfur clusters nor any other prosthetic group, so the iron in lipoxygenases must be directly bound to functional groups of the protein. The presence of one iron atom per mol of protein had long been overlooked, although in their pioneering work on soybean lipoxygenase Theorell et al. had mentioned the presence of certain metals, including iron, in their preparations (3). Apparently, these results were not recognized at that time as reflecting an intrinsic property of this type of enzyme. As analytical methodology became more sophisticated and an ever-growing number of oxidases and oxygenases were found to contain essential metal cofactors to implement electron transfer processes, the need for a reinvestigation of lipoxygenase seemed obvious. Atomic absorption spectrometry was then applied to a purified preparation of soybean lipoxygenase, and revealed the presence of a significant amount of iron in this protein (12, 13). Subsequent studies have confirmed these results and it is now well established that soybean lipoxygenase-1 contains one mol of iron per mol of protein (40). This enzyme from soybeans has been widely studied because it can be obtained in large amounts relatively easily. Standard methods of protein purification then afford a preparation which is homogeneous by the usual criteria for protein purity. In a typical assay (see Section III), with linoleic acid as the substrate, a k_{cat} of 280 μmol $O_2 . min^{-1} . mg^{-1}$ protein can be found. Several other lipoxygenases, both from plant and animal origin, including prostaglandin synthetase, have been investigated for their metal requirements and from these studies there seems to emerge a consistent pattern of a novel type of iron-mediated electron transfer (14,15).

III. ENZYME-SUBSTRATE INTERACTIONS

Soybean lipoxygenase-1 utilizes both the unsaturated fatty acid and dioxygen as substrates to produce fatty acid hydroperoxides (Scheme 1). Reaction rates can be measured by either monitoring the formation of the conjugated diene chromophore at 236 nm, or the oxygen consumption which is most conveniently done polarographically. Amphiphilic substances like linoleic acid have a strong tendency to form multimolecular aggregates whose structures depend on the concentration of the solute and on the nature of the solvent. If the solvent is water, the polar head groups interact with water while the hydrophobic moieties of the fatty acids associate to form so-called acid soaps and eventually micelles (6). Lipoxygenase-1 has been found to utilize only the monomeric substrate, as is particularly evident from kinetic studies (7). Increasing the substrate concentration beyond the critical micelle concentration of the substrate will no longer cause a corresponding increase of the initial velocity. The critical micelle concentration of fatty acids in water is strongly affected by pH, and thus kinetic studies at lower pH-values are severely hampered. This circumstance also may have led to artifactual values for kinetic parameters like K_m. If the substrate concentration range in kinetic experiments is chosen wide enough so as to include concentrations below 10 μM, and the reaction conditions are otherwise carefully controlled as well, a K_m for linoleic acid of approx. 5 μM is found (8) at pH 9.0.

The mechanism of the dioxygenation of linoleic acid by lipoxygenase has some features in common with the free-radical autoxidation of olefins. The initial and rate-determining step in lipoxygenase catalysis is the fission of the covalent bond between carbon and one of the prochiral hydrogen atoms of the central carbon atom of a 1Z,4Z-pentadiene system, thus producing a fatty acid free radical intermediate (Scheme 3) (9,10).



Scheme 3

The pentadienyl free radical moiety then rearranges and dioxygen is attached to one of the terminal carbon atoms of the pentadienyl fragment. Apparently, this process is under strict stereochemical control of the enzyme as is evident from the high enantiomeric purity (>95%) of the hydroperoxide formed. Lipoxygenase-1 from soybeans abstracts the 11-proS hydrogen atom from linoleic acid and produces the 13S-hydroperoxy-11E,9Z-octadecadienoic acid (13S-HPOD) in a 95% yield (16) and a correspondingly small amount of the 9S-HPOD. On the other hand, lipoxygenase-2 from soybeans, when incubated at pH 9.0, shows a stereoselectivity that is strikingly similar to the cyclo-oxygenase of the prostaglandin synthetase complex (17,18), and it has been claimed that this enzyme from soybeans will indeed catalyze the formation of prostaglandin-like substances (19,20).

It appears that lipoxygenases, including those from mammalian origin, display an antarafacial relationship between abstraction of a specific hydrogen and the insertion of dioxygen; i.e., a proS hydrogen is abstracted and an S-configuration results at the carbon atom bearing the hydroperoxide group (18,21,22).

The mechanism of hydroperoxide formation thus involves an activation of the fatty acid to overcome the spin barrier between ground state triplet oxygen and the fatty acid. In principle, an activated oxygen species like singlet oxygen could also be involved in hydroperoxide formation. However, for lipoxygenases no conclusive evidence has been found for such an activation of oxygen, with the possible exception of a lipoxygenase-like enzyme from Fusarium Oxysporum (23). Magnetic susceptibility measurements have provided evidence that over 80% of the iron in native lipoxygenase-1 from soybeans is in a high-spin Fe(II) state (S = 2), whereas electron paramagnetic resonance spectroscopy (EPR) has shown that the oxidized enzyme (see Section IV) contains high-spin Fe(III) (S = 5/2) (24-26). The latter technique also demonstrated the profound effect of alcohols on the line-shape of the Fe(III)-signals. It was found that the addition of minute amounts of alcohols with alkyl chains of varying length changed the shape of the EPR signal around g6 from rhombic to axial (27,28). The effect of alcohols (ethanol, butanol-1, hexanol-1) increased with their chain length. Mitsuda et al. (29) have reported the inhibitory effect of alcohols on lipoxygenase-catalyzed reactions; this effect was found to be stronger for alcohols with relatively long chain lengths. These interesting observations suggest an affinity of saturated monovalent alcohols for lipoxygenase; this affinity may be caused by the involvement of a hydrophobic binding site for the fatty acid substrate. The effects of hydroxy-octadecadienoic acids (13S-HOD and 9S-HOD) on the EPR lineshape have also been studied. It appears that 13S-HOD induces a change to an axial type of spectrum, whereas 9S-HOD causes a shift to a rhombic

shape. The amounts needed to exert the maximum effect were 5 molar equivalents, the same as with hexanol-1. This equivalence may be related to the common structural feature in these compounds, i.e. a linear saturated chain of five carbon atoms.

Additional paramagnetic resonance spectroscopy (^{1}H NMR) experiments have been carried out to establish whether this binding phenomenon is also reflected in the ^{1}H NMR spectra of the alcohols. It appears that ethanol, butanol-1 and hexanol-1 show a general broadening of the proton resonances upon binding to the enzyme. However, besides an overall broadening effect a differential broadening is observed in going from the methyl protons to protons attached to carbon atom 1, as illustrated in Figure 1 (30). Comparative binding experiments with cyanide and a series of alcohols have indicated that binding of the alcohols to the enzyme most probably involves hydrophobic interactions rather than the direct binding of the hydroxyl group of the alcohol. When the line-broadening effect of the alcohols is quantitatively evaluated with the Solomon-Bloembergen

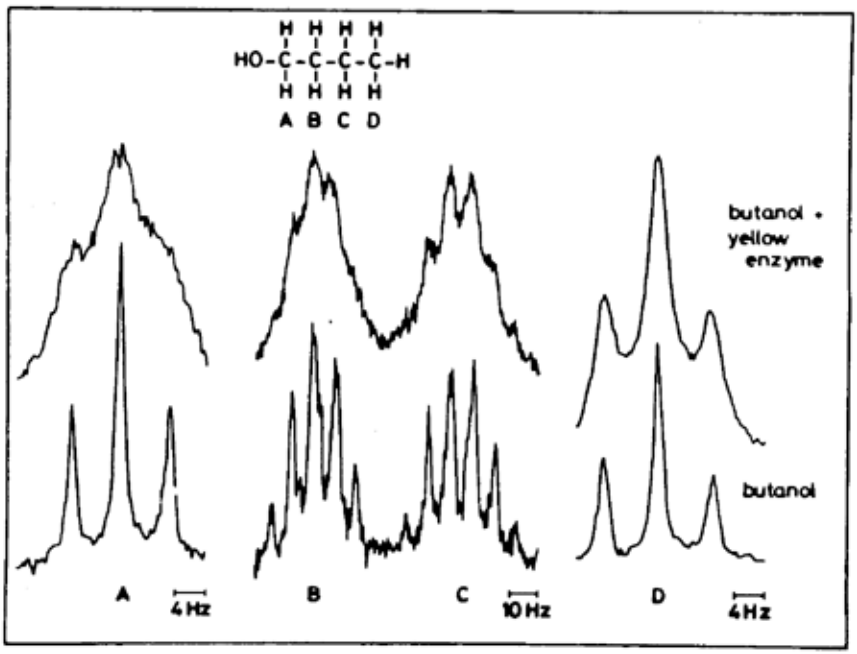


Fig. 1. ^{1}H-NMR spectra of 8 mM butanol-1 (bottom traces) and 26 μM of yellow soybean lipoxygenase-1 plus 22.8 μM butanol-1, in 0.1 M borate buffer/D_2O at pH 9.0 and 297 K.

equation the distances between methyl groups and the paramagnetic Fe(III) can be calculated. For ethanol, butanol-1 and hexanol-1 these values are 51, 72 and 63 nm, respectively. It is interesting to relate these distances to the results from previous fluorescence perturbation measurements (31) on lipoxygenase. From these measurements it was concluded that lipoxygenase contains a large hydrophobic site due to tryptophan residues at a distance of 70 nm from the central iron atom. A model of the linoleic acid molecule clearly shows the relevance of these results in explaining the binding of a substrate molecule, because it then appears that the distance between the methyl group of linoleic acid and the reactive methylene group of the pentadiene system is approximately 80 nm. The affinity of straight-chain alcohols is several orders of magnitude lower than that of linoleic acid. Thus, besides hydrophobic interactions the π-electrons of the double bonds of the fatty acid are also involved in the binding of the substrate to the enzyme. Indeed, ^{2}H-NMR studies with ^{2}H-labeled substrates and inhibitors (32) have shown the affinity of the unsaturated region of the fatty acid chain for the protein molecule.

IV. ENZYME-PRODUCT INTERACTIONS

A specific interaction between the product hydroperoxide and the enzyme lipoxygenase was first noted by Haining and Axelrod (33) in kinetic experiments. At relatively high substrate concentrations the progress curve for the dioxygenation of linoleic acid has a marked sigmoid nature. If, however, product hydroperoxide is added at the beginning of the reaction the initial part of the curve is straight and the enzyme shows normal Michaelis-Menten behavior. The effect of product hydroperoxide is very specific; only the product hydroperoxide actually formed by the enzyme exerts this change in kinetic behavior (34). A similar activating effect of 12-HPETE, the product of a reaction between arachidonic acid (ETE) and a lipoxygenase from human platelets has been reported (22). Soybean lipoxygenase-1 predominantly produces 13S-HPOD, together with only minor amounts of the 9S-HPOD isomer, from linoleic acid. The kinetic lag phase appears to be extremely sensitive to 13-HPOD, the 9S-HPOD being essentially ineffective. Garssen et al. (36,37) have described the formation of a complex mixture of products from a system containing lipoxygenase-1, linoleic acid and 13S-HPOD, while excluding dioxygen from the reaction mixture. The principal reaction products formed under these anaerobic conditions are C36 fatty acid dimers containing both hydroperoxide and fatty acid moieties. In addition C18 and C13 products are formed, both absorbing at 280 nm, and n-pentane (Scheme 4). The latter compounds appeared to be derived from the 13S-HPOD. These observa-

$$CH_3-(CH_2)_3-CH_3$$

+

$$O=CH-CH=CH-CH=CH-(CH_2)_7-COOH$$

+

$$CH_3-(CH_2)_4-\underset{\underset{O}{\|}}{C}-CH=CH-CH=CH-(CH_2)_7-COOH$$

+

Fatty acid dimers linked through $C_{11}-C_{13}$, or $C_{11}-C_9$, or $C_{13}-C_{13}$, or $C_{13}-C_9$,

Scheme 4

tions have substantiated the idea of a direct interaction between the enzyme and the hydroperoxide.

A. Reactive Intermediates

One of the key steps in both the aerobic and the anaerobic reaction is the electron transfer from Fe(II) to the hydroperoxide (Scheme 5).

$$E\text{-}Fe(II) + ROOH \longrightarrow E\text{-}Fe(III) + RO\cdot + OH^-$$

Scheme 5

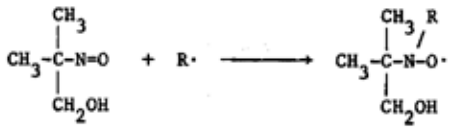
In the other half-cycle of the anaerobic reaction the Fe(III) species is then reduced by the linoleic acid, or other unsaturated fatty acid, (Scheme 6) to yield again the Fe(II)-species and a fatty acid free radical.

$$E\text{-}Fe(III) + RH \longrightarrow E\text{-}Fe(II) + R\cdot + H^+$$

Scheme 6

The occurrence of free radicals in the anaerobic reaction has been demonstrated by De Groot et al. (38) by using a spin-trapping technique. Lipoxygenase

was allowed to react in the complete absence of oxygen with natural linoleic acid or with ^{2}H-labeled linoleic acids in the presence of the water-soluble spin trap 2-methyl-2-nitrosopropanol and fatty acid hydroperoxide as the second substrate (Scheme 7).



Scheme 7

The EPR spectra clearly proved that the trapped radical stemmed from linoleic acid and not from the fatty acid hydroperoxide. The use of the ^{2}H-labeled linoleic acid further demonstrated that the spin trap had reacted at either carbon atom 13 or 9 (cf. Scheme 3). Recently, similar experiments have been conducted for autoxidizing unsaturated fatty acids (39).

The mechanism of the formation of the very first molecules of fatty acid hydroperoxide might involve the small fraction of enzyme molecules that contain Fe(III). Once some HPOD, either present as a contamination or produced enzymically , has started the activation process, it may proceed as depicted in Scheme 5 and on completion of the activation phase the rates become linear.

Fluorescence spectroscopy has provided direct evidence for the interaction of the fatty acid hydroperoxide and the enzyme (40). The native enzyme shows a fluorescence maximum at 328 nm which is partly quenched by the addition of product hydroperoxide. The extent of the fluorescence quenching is directly related to the amount of product hydroperoxide added, while the total concentration of hydroperoxide needed to cause the maximum quenching effect equals the concentration of the enzyme. Interestingly, the same effect can be seen when linoleic acid is used instead of 13S-HPOD which, in turn, exerts its quenching effect. Figure 2 demonstrates the effects of 13S-HPOD on the protein fluorescence spectra. The quenching of the protein fluorescence is parallelled by changes in the optical spectrum of lipoxygenase-1. On mixing equimolar amounts of native protein with 13S-HPOD, a yellow species with a maximum absorbance at 360 nm is formed (41).

The interaction between the enzyme and the product hydroperoxide has further been investigated by EPR to gain more detailed information on the properties of

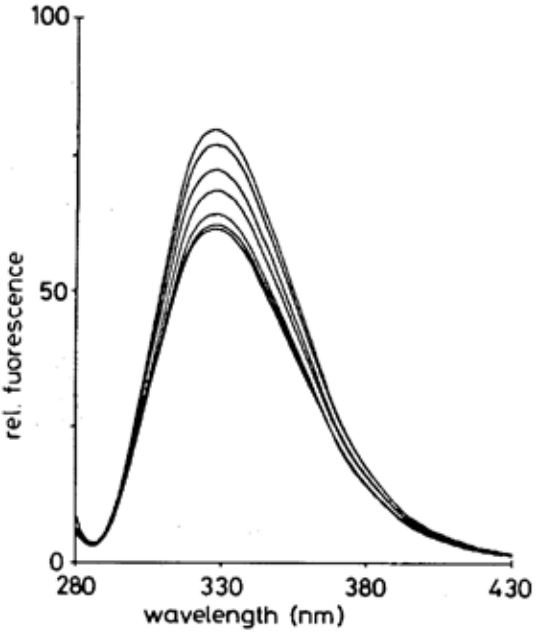
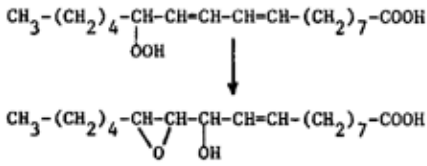


Fig. 2. Quenching effect of 13S-HPOD on the fluorescence of native soybean lipoxygenase-1. Top curve: native enzyme; each successive trace was obtained by adding 0.2 molar equivalent of 13S-HPOD to 0.001 mM of enzyme.

the iron in the protein. The native resting enzyme is EPR-silent and shows only a weak Fe(III)-signal at g4.3 and a number of lines around g2 stemming from traces of Mn and Cu occurring as impurities. In order to get a qualitative insight into the role of iron, low-temperature EPR-experiments were carried out analogous to those described above for other spectroscopic techniques; namely, combining the native enzyme with a stoichiometric amount of 13S-HPOD. This treatment caused the appearance of new signals around g6, reminiscent of those observed with certain heme proteins.

The signal is considerably rhombic in nature, but its shape can be profoundly influenced by small amounts of hydrophobic solutes, e.g. certain alcohols, and by cyanide (28). On adding more than one molar equivalent of 13S-HPOD the color of the solution becomes purple and another EPR signal appears at g4.3, in conjunction with deformation of the g6 signal. This new enzyme species is not stable (42,43). At room temperature it gradually loses its purple color to become yellow again and, simultaneously, the g4.3 signal in the EPR spectrum vanishes. Eventually, the spectrum is indistinguishable from that of the original

yellow protein. In the slow transition from purple to yellow the 13S-HPOD is transformed into an epoxy-hydroxy fatty acid (Scheme 8).



Scheme 8

The half-life of the purple species at room temperature is about 2 min. The epoxy-hydroxy fatty acid has retained both oxygen atoms of the hydroperoxy-group, as has been demonstrated by Garssen et al. (42) using $^{18}O_2$-labeled 13S-HPOD. The retention of both oxygen atoms indicates that this transformation takes place in a solvent cage which effectively prevents interactions with an aqueous environment. The purple species can thus be described as a complex between the yellow enzyme and 13S-HPOD. Figures 3 and 4 give the optical and EPR spectra of the various enzyme species.

EPR spectroscopy was also used as a tool to investigate the quantitative aspects of the iron in soybean lipoxygenase-1, in particular with regard to the amount of Fe(III) that is visible in the EPR spectra of the various species. This procedure required knowledge of the zero-field splitting parameters of the various Fe(III)-species. Protocatechuate dioxygenase, another nonheme iron dioxygenase recently investigated by EPR, was found to have a negative D-value (44,45), so that the investigation of lipoxygenase became even more urgent. The D-values for lipoxygenase were determined from the frequency dependence and the temperature dependence of the Fe(III) signals. The frequency dependence was established by taking spectra at 9 GHz and 35 GHz. The temperature dependence could not be measured directly because the sample and the temperature probe occupy relatively remote positions in the helium flow system. It was therefore necessary to include cytochrome c as a reference in the sample and to utilize its known temperature dependence as a thermometer. By combining both methods certain ranges could be defined for the D-values of the axial and the rhombic parts of the spectrum (Table I).

The uncertainties in the determinations of the D-values caused corresponding uncertainties in the calculated amounts of Fe(III) responsible for the various EPR lines. If the extremes of the D-values are considered, a deviation of about

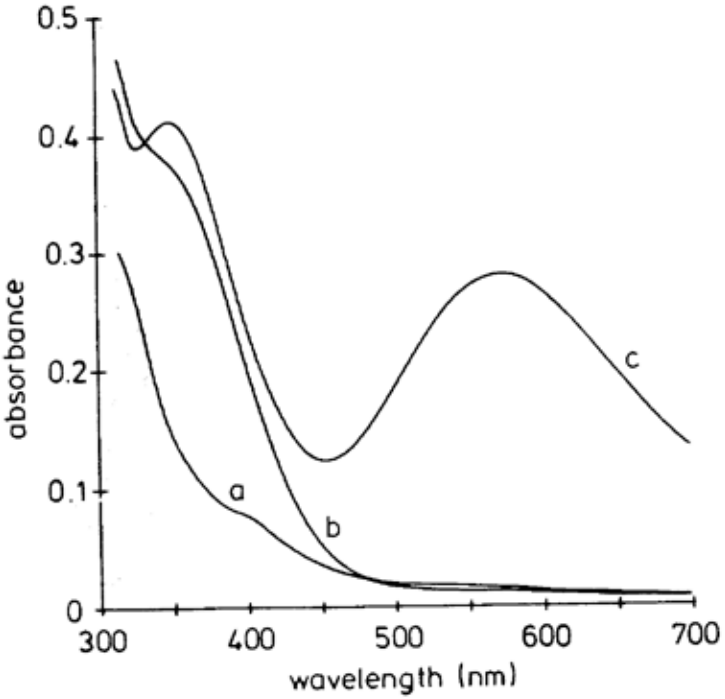


Fig. 3. Optical absorption spectra of lipoxygenase-1 species. a, native enzyme; b, yellow enzyme; c, purple enzyme. (lipoxygenase, 0.21 M, pH 9.0).

10% in the calculated amount of EPR-visible Fe(III) arises. The complexity of the EPR signal of Fe(III)-lipoxygenases poses a special problem when a full simulation of the signal is attempted. When the signal around g6 is predominantly 'rhombic' a satisfactory simulation can only be obtained with at least three

TABLE I

ZERO FIELD SPLITTING CONSTANTS[a] FOR YELLOW SOYBEAN LIPOXYGENASE-1

Species	D-values from T dependence	T dependence combined with g shift method
Rhombic	4.4 > D > 1.5	4.4 > D > 1.8
Axial	3.8 > D > 0.8	3.0 > D > 1.5

[a] D in K.

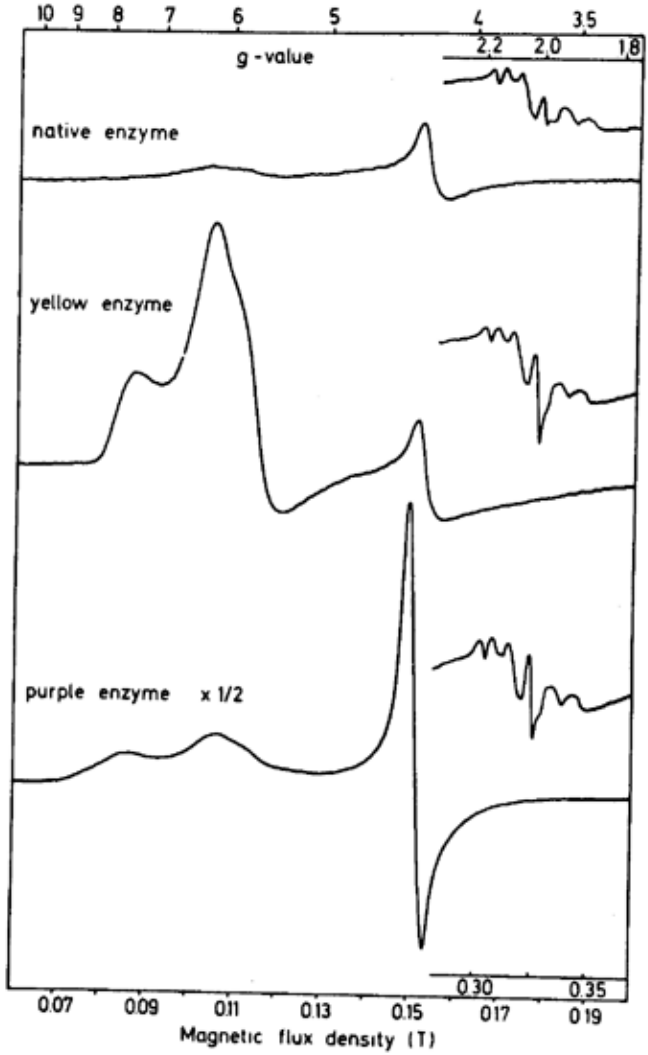


Fig. 4. First derivative EPR spectra of lipoxygenase-1 species at 15 K. Enzyme concentration 0.53 mM, pH 9.0, microwave frequency 9.12 GHz, microwave power 2 mW, modulation amplitude 2 mT.

components, whereas an 'axial' signal requires two components. Table II contains the amounts of EPR-visible Fe(III) obtained by simulating and integrating the EPR signal of the various enzyme species.

TABLE II

EPR-VISIBLE IRON IN SOYBEAN LIPOXYGENASE-1[a]

Species	g6 signal	g4.3 signal
native	<1	0.1
yellow	80	0.6
purple	76	8, 13[b]

[a]Amounts are given as a percentage of the total iron content.
[b]Results of simulation and integration, respectively.

It should be noted that the total amounts of EPR-visible iron are virtually the same for the yellow and purple species. Linear correlations have been observed between the absorbance at 360 nm and the g6 signal, and between the absorbance at 570 nm and the g4.3 EPR signal as a function of the amount of 13S-HPOD added (35). The EPR spectrum of the purple enzyme (Figure 4) shows a very prominent signal at g4.3. However, this signal integrates to about only 10% of the total iron content. Thus, the complex between Fe(III) and 13S-HPOD appears to consist of a yellow fraction with an EPR resonance at g6 and a purple fraction with a pronounced g4.3 EPR signal.

V. LIGHT EMISSION DURING LIPOXYGENASE CATALYSIS

The occurrence of a low-level chemiluminescence during lipoxygenase-catalyzed reactions has been observed by several investigators (46,47). The time course of the light emission has some remarkable features. Depending on the relative amounts of substrate fatty acid and dissolved molecular oxygen, the chemiluminescence shows different patterns. Immediately after mixing the reactants a peak is observed (48). The intensity then drops to a steady level but may eventually rise again to give a second peak. The occurrence of this second peak is observed only if the amount of substrate fatty acid exceeds the amount of available oxygen. As soon as the system is devoid of oxygen, light emission ceases (Figure 5). The occurrence of a luminescence peak just before anaerobiosis is reached is characteristic for lipoxygenase catalysis.

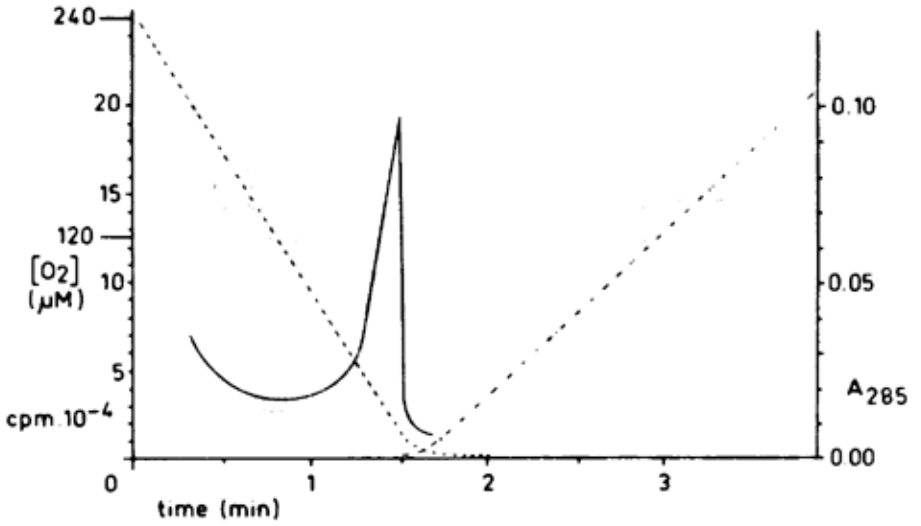


Fig. 5. Chemiluminescence during lipoxygenase catalysis. Oxygen concentration (---), light emission (solid line) and absorbance change at 285 nm vs. time. Starting conditions: Linoleic acid, 1.85 mM; lipoxygenase-1, 5×10^{-9} M; oxygen, 240 μM; borate buffer, pH 9.0.

This phenomenon has been described by Veldink et al. (49) and has since been confirmed by others (48,50). Boveris et al. (48) and Nakano and Sugioka (51) have analyzed the spectral composition of the emitted light and found maxima around 450 and 550 nm and a weak shoulder at 630 nm. However, the exact nature of the light-emitting species is as yet unclear. Veldink et al. (49) have described a quenching effect of superoxide dismutase which may point to involvement of superoxide. Boveris et al. (48) suggested the involvement of small amounts of singlet oxygen on the basis of the effect of DABCO (1,4-diazabicyclo-2,2,2,-octane). The 450 nm peak might stem from excited carbonyl groups formed after decomposition of fatty acid peroxy-radicals.

VI. MULTIPLE DIOXYGENATIONS BY LIPOXYGENASES

Lipoxygenases show considerable affinities for both polyenoic fatty acids like linoleic acid and their primary reaction products, mono-hydroperoxy fatty acids. If the primary substrate contains multiple 1Z,4Z-pentadiene moieties, this type of enzyme is also capable of introducing a second hydroperoxy-group.

Using methyl α-linolenate and an aqueous extract of soybean flour, Roza and Francke (52) have shown that, in addition to monohydroperoxides, hydroxy-endoperoxides are formed. This reaction appeared not to be catalyzed by purified soybean lipoxygenase-1. Bild et al (53), using soybean lipoxygenase-1, reported on a twofold dioxygenation of arachidonic acid. It was subsequently shown by Van Os et al. (54) that this system leads to the formation of a mixture of 8S,15S-DHPETE and 5S,15S-DHPETE. The second dioxygenation step proceeds at a considerably lower rate than the first one, and therefore Bild et al. (53) could clearly show the stepwise fashion of the double dioxygenation of arachidonic acid. This difference in the dioxygenation kinetics is shown in Figure 6.

The multiple dioxygenation capacity is not confined to soybean lipoxygenase-1. Enzymes from mammalian origin can also transform HETE's and/or HPETE's into the corresponding DH(P)ETE's. For example, enzymes from polymorphonuclear leukocytes (PMNL) forming leukotriene B_4 (LTB_4; 5S,12R-DHETE) also produce the double dioxygenation product 5S,12S-DHETE (55). Conversely, when the primary reaction product from ETE is 12S-HPETE (56) it can be shown that, on incubating 5S-HETE with an enzyme preparation from porcine PMNL, a 5S,12S-DHETE results. Although the reaction products have common structural features, important

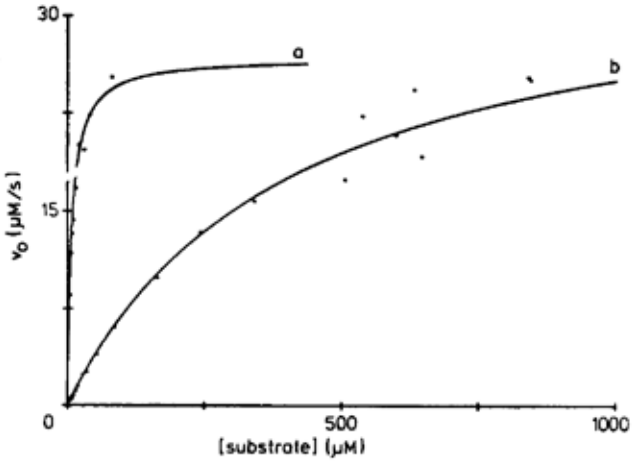
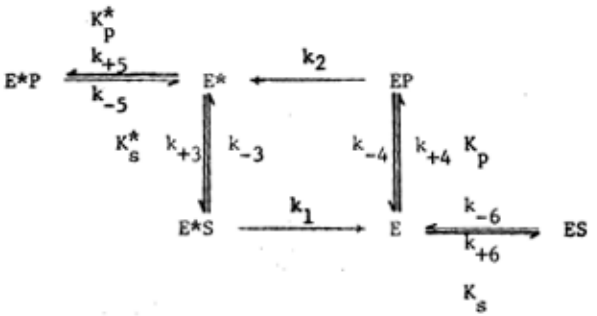


Fig. 6. Double dioxygenation kinetics of arachidonic acid (ETE) catalyzed by soybean lipoxygenase-1. (a) First step: lipoxygenase, 2 nM, substrate ETE; (b) second step: lipoxygenase, 25 nM, substrate 15S-HPETE. All experiments: $[O_2]$, 240 μM, 0.1 M sodium borate buffer (pH 8.7), 25 °C.

stereochemical differences exist that are also reflected in their various biological effects. Interestingly, the 8S,15S-DHETE, one of the double-dioxygenation products of ETE formed by soybean lipoxygenase-1 (53), proved to possess considerable chemotactic activity for human polymorphonuclear leukocytes (57).

VII. KINETICS OF THE ANAEROBIC REACTION OF LIPOXYGENASE

In previous sections of this chapter, evidence has been presented for the interaction of both the fatty acid and the fatty acid hydroperoxide with lipoxygenase. This evidence was based on the results of the static use of spectroscopic methods like electron paramagnetic resonance, fluorescence and absorption spectroscopy. The spectral characteristics of the various reaction products do, however, permit a sensitive and continuous monitoring of the reactions of lipoxygenase, so that they are very convenient for a kinetic study. For the calculation of the initial velocities of hydroperoxide conversion and carbonyl formation molar absorbances of 25000 $M^{-1}.cm^{-1}$ for both types of compounds were used. This two-substrate reaction required a large number of individual assays, the results of which were ultimately fitted to the mathematical model of a substituted enzyme mechanism in which inhibition by both substrates was accounted for. The kinetic scheme for such a model is given in Scheme 9.



E*, E-Fe(III); E, EFe(II); S, linoleic acid; P, 13-L-hydroperoxylinoleic acid.

Scheme 9

Table III contains the calculated kinetic constants. The true dissociation constants K_s^* and K_p have been calculated as 163 µM and 110 µM, respectively. It then appears that OD and 13S-HPOD have similar affinities for the enzyme, although the Fe(II) species shows a slightly higher affinity (58).

Direct evidence for the involvement of the yellow and purple enzyme species was obtained from stopped-flow kinetic experiments (59). Figure 7 contains

TABLE III

KINETIC CONSTANTS OF THE ANAEROBIC REACTION OF SOYBEAN LIPOXYGENASE-1, 13S-HPOD AND LINOLEIC ACID[a] AT pH 10.0

K^*_{ms}	55 ± 6 μM
K_{pm}	73 ± 8 μM
K_s	111 ± 14 μM
K^*_p	153 ± 25 μM
V	143 ± 11 $\mu M.min^{-1}$
K^*_s	163 ± 22 μM
K_p	110 ± 14 μM
k_1	309 ± 10 s^{-1}
k_2	156 ± 16 s^{-1}

[a] cf. Scheme 9.

typical examples in which the course of the reaction was monitored vs. time at the appropriate wavelengths of the colored species.

The anaerobic reaction has also been found with a lipoxygenase from rabbit reticulocytes (60). Furthermore, the optical and fluorescence spectroscopic features of this enzyme show a great similarity with those of soybean lipoxygenase-1.

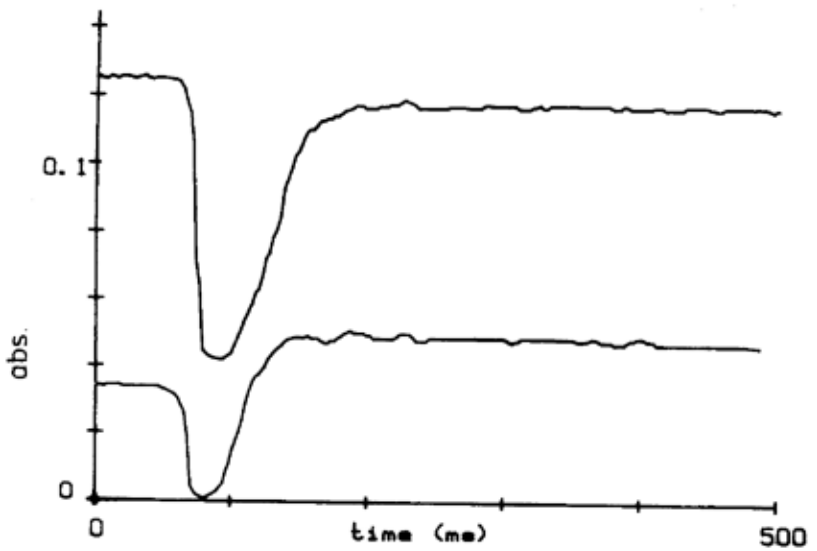


Fig. 7. Formation of yellow (330 nm), top, and purple (580 nm) enzyme species in a stopped-flow experiment. Lipoxygenase-1, 30 μM; $[O_2]$, 240 μM; linoleic acid, 200 μM; pH 9.5, 25 °C.

VIII. STATE OF IRON IN NATIVE SOYBEAN LIPOXYGENASE-1

Numerous examples exist of iron-containing proteins in which the Fe(II)-form reversibly binds dioxygen. As yet, no direct evidence is available which favors the binding of dioxygen to native lipoxygenase. It has been suggested that the role of certain transition metals, including iron, in initiating autoxidation of olefins might involve a metal-dioxygen complex (61). In the case of Fe(II), a free-radical chain reaction may then be initiated as depicted in Scheme 10.

$$Fe(II) + O_2 \;\langle\Rightarrow\; Fe(II)\text{-}O_2 \;\langle\rightarrow\; Fe(III)\text{-}O_2^{-\cdot} + RH \rightarrow$$

$$Fe(II) + R^{\cdot} + O_2^{-\cdot} + H^{+}$$

Scheme 10

An analogous scheme could in principle account for a possible contribution to the formation of hydroperoxide catalyzed by native Fe(II)-containing lipoxygenase. However, the lack of evidence for the presence of Fe-bound dioxygen in lipoxygenase essentially rules out such a possibility. Magnetic susceptibility measurements have been carried out by Slappendel et al. (24) in order to investigate the spin and valence state of iron in the native enzyme. Lipoxygenase showed a Curie-dependence over the entire temperature range measured: 40K - 200 K. From this inverse temperature dependence a corrected effective Bohr magneton number of 5.2 was calculated which is characteristic of Fe(II) in a high-spin configuration (S = 2) (24) (Figure 8).

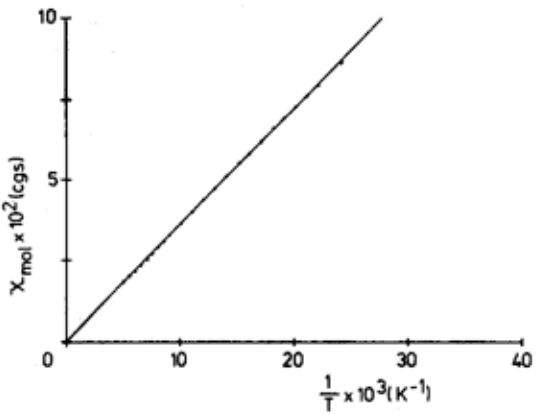


Fig. 8. Temperature-dependent contribution of the magnetic susceptibility of 1.19 mM native lipoxygenase-1, 0.1 M borate (pH 9.0).

Indirect evidence for the high-spin Fe(II)-state came from additional ^{1}H NMR experiments. As with Fe(III)-lipoxygenase before, essentially the same binding experiments with a number of alcohols were carried out with the native protein. A very similar differential line-broadening was observed particularly with butanol-1 and hexanol-1, thus showing that a specific interaction exists between protons of the alcohols and iron. Since low-spin Fe(II) is diamagnetic, these results have led to the conclusion that native lipoxygenase-1 contains high-spin Fe(II).

IX. CONCLUDING REMARKS

Over the years, the investigations on lipoxygenases have yielded significant results on the catalytic mechanism, the structures of the various reaction products and the properties of the protein. However, so far no unique physiological function could be assigned to this type of enzyme in plants. This is by no means a trivial problem because of the reactivity of the primary reaction products (hydroperoxy fatty acids) and the amounts of lipoxygenase present in plant seeds. For example, if one assumes an even distribution of lipoxygenase-1 in a soybean seed, its concentration amounts to at least 20 μM. At first, an even cellular distribution of lipoxygenase may not seem very likely, but it has been established by immunofluorescence labeling of lipoxygenase-1 and -2 in soybeans that the protein is virtually randomly present in the cytosol (62). This finding has been confirmed by immuno-electron microscopy of ultrathin cryosections of soybean seeds in various stages of germination (63).

To a large extent, research on lipoxygenase in plants has been parallelled by research on the so-called alternative respiratory pathway in plants. Since this pathway is insensitive to cyanide it must be different from the normal mitochondrial electron transport chain. As lipoxygenase activity is also not inhibited by cyanide, this enzyme could constitute a part of the cyanide-resistant respiration mechanism. However, on the basis of extensive inhibition experiments in a comparative study of lipoxygenase and the enzymes involved in the alternative respiratory chain, it has been concluded by Peterman and Siedow (64) that the alternative oxidase and lipoxygenase are distinct entities. The activity profile of lipoxygenase during seed germination suggests a role of this enzyme during the very first days of the germination process. Its function cannot be properly assessed without taking into account the presence and function of related enzymes of lipid metabolism like lipases and phospholipases. Furthermore, by its very nature lipoxygenase activity is restricted to the conversion of polyenoic fatty acids. It has been shown that 13S-HPOD can destroy

mitochondrial cytochromes and thus lower the rate of mitochondrial respiration. This event might be a regulatory feature in plant cells because it influences the ATP:ADP ratio. An inhibition of mitochondrial respiration was also established for the lipoxygenase from reticulocytes (65-67). This feature is a highly functional one because the breakdown of mitochondrial membranes is a crucial event in the process of maturation of red blood cells.

Reviewing the literature on the physiological functions and biological effects of prostaglandin and thromboxane synthetases as well as those of mammalian lipoxygenases is beyond the scope of this chapter. The various biological effects of prostaglandins, thromboxanes and leukotrienes include the following: Prostaglandins and thromboxanes affect the contraction and relaxation of smooth muscle tissue, stimulate or prevent the aggregation of blood platelets and have a role in the regulation of gastric secretion. Leukotrienes, in particular leukotriene B_4, are chemotactic for leukocytes and may therefore have a key role in anti-inflammatory processes. Besides, they promote the attachment of leukocytes to the walls of capillary blood vessels. The leukotrienes C_4 and D_4, originally known as the slow-reacting substance(s) of anaphylaxis (SRS-A), are particularly important with respect to their smooth muscle-contracting activity in allergic reactions and most probably are the key factors in asthmatic bronchoconstriction.

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