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REACTIONS CATALYZED BY PLANT LIPOXYGENASES

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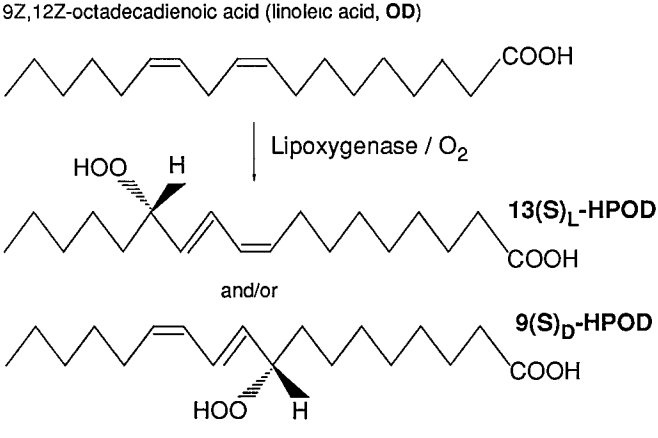
ABSTRACT - Structural and mechanistic aspects of lipoxygenase catalysis are discussed. This enzyme is involved in the formation and conversion of unsaturated fatty acid hydroperoxides. It plays a key role in the biosynthesis of important novel classes of natural compounds *e.g.* the leukotrienes and the lipoxins. Because of the biological roles and effects of these compounds, it is essential to extend our knowledge on this type of enzyme.

1. INTRODUCTION

Lipoxygenases (EC 1.13.11.12) catalyze the dioxygenation of 1Z,4Z-pentadiene moieties of lipid substrates, in particular poly-unsaturated fatty acids like linoleic and arachidonic acids to produce chiral, EZ-conjugated hydroperoxides. These basic features of lipoxygenase action have been summarized in Scheme 1. The enzyme is ubiquitous in both the plant and the animal kingdom. After its discovery in mammalian blood platelets (refs. 1-2), reticulocytes (refs. 3-4), and its role in the biosyntheses of leukotrienes (ref. 5) and lipoxins (ref. 6) had been established, research in this area has grown considerably.



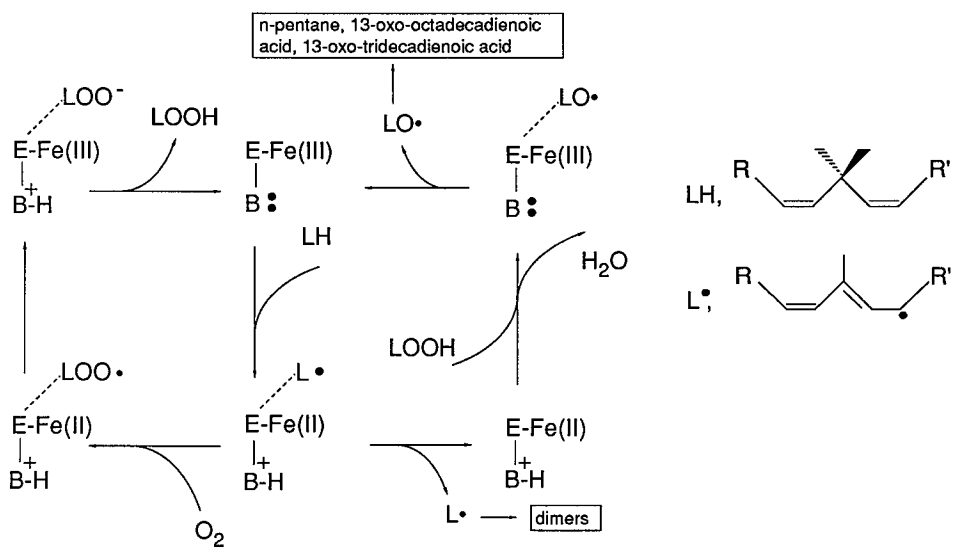
Scheme 1. Dioxygenation of 9Z,12Z-octadecadienoic acid (linoleic acid, OD) by lipoxygenase; regio- and stereospecific formation of EZ-conjugated hydroperoxy-octadecadienoic acids (HPODs).

With the exception of stereochemical control features, the mechanism of hydroperoxide formation has the same initial step as the free radical process of autoxidation of olefins catalyzed by trace amounts of transition metals, i.e. the abstraction of one of the prochiral hydrogen atoms from the central methylene group of the pentadiene system of the substrate. Carrying the analogy to the process of autoxidation one step further would leave the substrate as a carbon-centered free radical to which ground state triplet oxygen may add to form a peroxy free radical. The cycle is formally completed upon the acquisition of a hydrogen atom by this intermediate. Recent reviews focusing on various aspects of lipoxygenase biochemistry are in Refs. 6-11.

2. ENZYME-SUBSTRATE INTERACTIONS

2.1 Role of iron

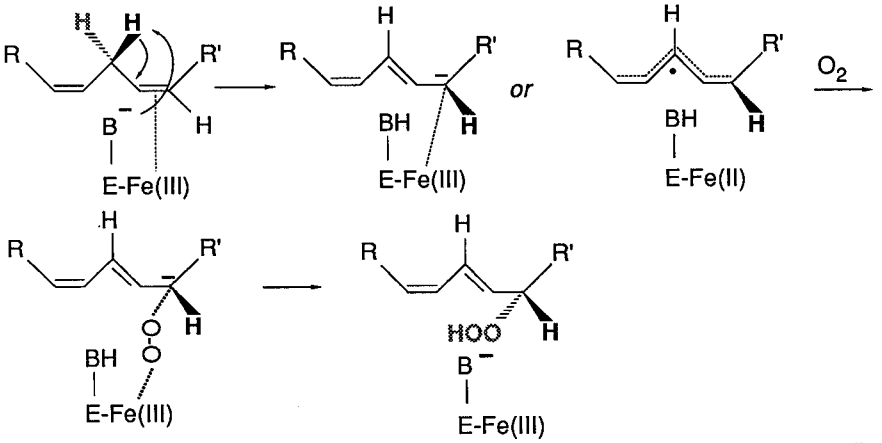
Soybean lipoxygenase-1 is an easily accessible and stable isozyme, that has been the subject of numerous studies. The iso-enzymes from soybeans investigated sofar all contain one mole of iron, which may in its native state, either occur as Fe(III) or Fe(II). The presence of iron in soybean lipoxygenase was first described Roza and Francke (ref. 12) and Chan (ref. 13). Scheme 2 is based upon the low-temperature EPR-investigations by De Groot *et al.* (ref. 14) and Pistorius *et al.* (ref. 15).



Scheme 2. Iron-mediated electron transfer in hydroperoxide (LOOH) formation from an unsaturated lipid substrate (LH) by lipoxygenase (E) *(left)*, anaerobic conversion of LOOH and formation of oxodienes absorbing at 285 nm, n-pentane and fatty acid dimers *(right)*.

Recent work with specific synthetic substrates and a model system for lipoxygenation have led Corey *et al.* (refs. 16-17) to propose a mechanism for the dioxygenation reaction that assigns a crucial role to iron in determining the regio- and stereospecificities of the reaction by the formation of a carbon-

iron σ-bond and subsequent dioxygen insertion (Scheme 3). Initial proton or hydrogen transfer to a basic group of the enzyme may be facilitated by Fe(III).



Scheme 3. Dioxygenation mechanism involving O_2-insertion at an iron-carbon σ-bond (ref. 16).

The native enzyme isolated from mature soybeans contains one mole of EPR-silent iron per mole of enzyme. Upon adding product hydroperoxide (13S-HPOD) a *g*6 signal appears and if the concentration of the protein is sufficiently high, the color of the solution becomes yellow. On adding larger amounts of 13S-HPOD a signal at *g*4.3 emerges with a corresponding loss of intensity by the *g*6 signal. Concomitantly, the color of the solution turns purple. The *g*6 signal stems from high-spin Fe(III) in a ligand field of axial symmetry. Upon thawing, the yellow enzyme can be bleached by anaerobically adding linoleic acid. These oxidation and reduction steps can be repeated several times with a single protein sample. Similar sets of experiments have been carried out using fluorescence, absorption and CD spectroscopy (refs. 18-21).

The reaction scheme (Scheme 2) presents the yellow Fe(III)-species as the active species. Kinetic experiments (refs. 22-27) have shown that the native enzyme needs activation by the product hydroperoxide. The activation step comprises the oxidation of the high-spin Fe(II) of the protein to high-spin Fe(III). Conversely, reversible inactivation can be achieved with chemicals that reduce the enzyme's Fe(III) to Fe(II) like certain N-alkylhydroxylamines (ref. 28) or catechols (ref. 21). This is reflected in the sigmoid nature of progress curves of hydroperoxide formation from linoleic acid and the effects of adding or removing product hydroperoxide on the kinetic lag phase (ref. 29). A quantitative assessment of the Fe(III)-content of various soybean lipoxygenase samples by Slappendel *et al.* (ref. 30) have shown that the native enzyme contains less than 1% of EPR-visible Fe(III), whereas the hydroperoxide-treated species contained 68-89%. Funk *et al.* (ref. 31) have reported on the isolation from immature soybean seeds of a lipoxygenase containing significant amounts (i.e. about 25%) high-spin Fe(III), which seems to suggest that lipoxygenases have an active role during the final phases of maturation.

2.2 Iron binding site

The iron site of soybean lipoxygenase-1 has been probed with a variety of spectroscopic and other techniques including EPR, NMR, EXAFS and magnetic susceptibility. Except for iron no evidence is available indicating the presence of non-amino acid structural elements. The precise way in which iron is coordinated to the polypeptide chain has not yet been elucidated, but some recent EXAFS data point to the involvement of 4 imidazole nitrogens and 2 oxygen atoms derived from carboxylate groups (ref. 32). The proposed model for the architecture consists of a planar structure accommodating 4 imidazole nitrogens and 2 carboxylate-oxygens at the axial positions. This model thus also draws upon EPR-data pointing to an axial ligand symmetry (refs. 14-15) of the iron environment. It should be noted however, that these data have been obtained with the yellow Fe(III)-enzyme. Although EXAFS data cannot always be applied to discriminate between types of atoms, the present evidence virtually excludes sulphur as a ligand of iron.

2.3 Substrate and dioxygen binding

It is by no means certain that iron plays a role in substrate or product binding, but one way of explaining its involvement in the redox-cycle is by assuming that it is sterically close to the fatty acid or, more specifically, to the sites where covalent bonds are to be broken or established. Magnetic susceptibility and EPR-studies (refs. 33-36) have characterized the spin and valence state of the iron and shown that it is highly unlikely that dioxygen is coordinated to the high-spin Fe(II) of the native soybean enzyme. Fluorescence perturbation studies by Finazzi-Agrò *et al.* (ref. 37) have shown that a large hydrophobic binding site consisting of tryptophan residues occurs at about 7Å from the iron. Shibata *et al.* have recently published the complete primary structure of soybean lipoxygenase-1 (ref. 38) as determined from the nucleotide sequence of the complete cDNA. This analysis has afforded a molecular weight of 94,038 based on a total of 838 amino acid residues, 13 of which are tryptophan residues. Applying the algorithm of Hopp and Woods (ref. 39) predicted that 12 out of these 13 residues are in a hydrophobic environment.

EPR- and NMR-studies (refs. 40-41) on the binding of straight-chain monovalent alcohols to lipoxygenase-1 have shown that adding small amounts of these compounds profoundly affects the lineshape of the EPR-signal and that a line broadening of the proton resonances occurs by proton relaxation enhancement through magnetic interaction with iron. The latter effect is seen with both the native and the yellow enzyme. It was found that the affinity of alcohols increased with chain length e.g. the affinity for n-hexanol is about two orders of magnitude higher than for ethanol, whereas t-butanol neither induced a rhombic-to-axial shift of the $g6$ EPR-signal nor caused any line-broadening in the ^{1}H-NMR-spectrum. Of course, line-broadening of protons in close proximity of any paramagnetic center will be greater than that of more remote protons. Such a differential effect was indeed observed upon incubating alcohols with lipoxygenase and it appeared that protons of the C-atom also bearing the OH-group were closest to the iron. Applying the Solomon-Bloembergen equation yielded a value of about 6Å for the distance between iron and the methyl protons of the alcohols (ref. 41). The alcohols did not in any way affect the UV- or CD-spectrum of the protein. In

particular, the absence of any effect on the CD-spectrum implicates that no big conformational changes take place. Earlier studies on the substrate specificity of soybean lipoxygenase-1 (ref. 42) already indicated the importance of the structure of the hydrophobic, methyl-containing part of the molecule.

Two lines of evidence suggest the presence of an essential methionine residue. Zakut *et al.* (ref. 43) found that iodoacetic acid inactivated soybean lipoxygenase in a time and concentration dependent manner. In addition, they found that 1.2 mole of iodoacetate was covalently bound to a methionine residue of a completely inactivated enzyme. Kühn *et al.* (ref. 44) have studied the mechanism of the irreversible inactivation of lipoxygenases from soybeans and reticulocytes by icosatetraynoic acids and concluded that the inactivation is caused by the specific formation of one methionine sulfoxide residue. Methionine sulfoxide formation had previously been shown to cause inactivation of reticulocyte lipoxygenase by 13S-HPOD (ref. 45).

2.4 Product formation

The formation of hydroperoxides is not confined to the type of fatty acid depicted in Scheme 1, i.e. free fatty acids with one or more 1Z,4Z-pentadiene moieties. A detailed study by Brash *et al.* (ref. 46) has shown that phosphatidylcholines (PCs) and some other phospholipids containing either arachidonyl or linoleyl residues can be converted in a regio- and stereospecific way by soybean lipoxygenase-1. The rate of the dioxygenation was found to be about an order of magnitude slower than with the corresponding free fatty acid. If the substrate consisted of a single molecular species like 1-palmitoyl-2-linoleyl-PC only one product was formed, namely 1-palmitoyl-2-(13*S*-hydroperoxy-9Z,11E-octadecadienoyl-)PC.

Another interesting substrate has been investigated by Funk *et al.* (ref. 48). These authors reported that 9E,12Z-octadecadienoic acid is indeed a substrate for soybean lipoxygenase-1, which can be transformed into 13*S*-hydroperoxy-9Z,11E-octadecedienoic acid. Apparently, the enzyme imposes severe steric constraints upon the structure of reaction intermediates, resulting in the formation of a thermodynamically less favorable 9Z-geometry in the product. This finding further demonstrates that the interaction of enzyme and substrate involves the whole of the pentadiene moiety, and not just an allylic part that contains one double bond and the peroxy-carbon. According to Brash *et al.* (ref. 46) the formation of isomeric products is best explained by assuming that the enzyme accepts part of the substrate in a head-tail reversed orientation (*cf.* Egmond *et al.*, ref. 47). A similar approach was recently chosen by Walstra *et al.* (ref. 49) in order to explain the LTA_4-synthase activity of a 12-lipoxygenase from bovine polymorphonuclear leukocytes (PMNs). This enzyme preparation loses its capacity to form 5*S*-HETE from arachidonic acid upon sonication. Instead it produces 12*S*-HETE, though it will not form 5*S*,12*S*-diHETE from 5*S*-HPETE like e.g. 12-lipoxygenase from platelets. When incubated with 5*S*-HPETE, the enzyme preparation from bovine PMNs produces leukotriene A_4-(LTA_4) derived products including LTB_4. In addition, some non-enzymically formed LTB_4-isomers are found. The formation of LTA_4 and the formation of 12*S*-H(P)ETE require the abstraction of the H_{proR} or the H_{proS} from C-10, respectively. As LTA_4-synthase activity is an intrinsic property of 5-lipoxygenases, one would expect the sonicated PMN preparation to produce the 12*R*-HETE

enantiomer. However, 12*S*- stereochemistry was found, which can be explained by assuming a head-tail reversed orientation of the substrate. These authors also point out that substrate inversion on lipoxygenase might be a fairly general phenomenon, which may explain e.g. the formation of 8*S*,15*S*- and 5*S*, 15*S*-diHPETE by soybean lipoxygenase-1 from arachidonic acid (ref. 50). Such an alternate orientation of substrate vs. enzyme has also been put forward by Kühn *et al.* in order to explain the double dioxygenation of arachidonic acid by reticulocyte lipoxygenase (ref. 8).

2.5 Non-specific product formation

Product analysis invariably shows a certain amount of material that seems to have escaped rigid enzymic control during substrate handling and product formation and release. It may be present as a racemic mixture of hydroperoxy- and/or hydroxy fatty acids and the amounts may thus vary with reaction conditions. Furthermore, oxo- and epoxy compounds of varying chain length may be found, as well as low molecular weight hydrocarbons like n-pentane. The reaction scheme published by De Groot *et al.* (ref. 14 and Scheme 2) provides for the possible formation of such compounds by assuming that a certain fraction of the enzyme-fatty acid free radical dissociates thus leaving the enzyme in an inactive Fe(II) state and at the same time delivering a fatty acid free radical. The latter may then be dioxygenated non-specifically to form a peroxy free radical or initiate a free radical chain reaction by abstracting a hydrogen atom from a suitable substrate. Reactivation of the Fe(II)-enzyme by fatty acid hydroperoxide produces alkoxy free radicals that may undergo rearrangements eventually leading to oxo or epoxy compounds. For soybean lipoxygenase-1 the dissociation of enzyme-free radical complexes apparently does not occur to any appreciable extent if molecular dioxygen is present above about 5 μM. The stability of the enzyme-free radical complex may however be different for other enzymes (cf. ref. 51). If anaerobic conditions are deliberately chosen and a mixture of linoleic acid and 13*S*-HPOD is allowed to react with soybean lipoxygenase-1, fatty acid free radicals can be trapped and detected by EPR-spectroscopy (ref. 52). If linoleic acid is not present, 13*S*-HPOD is also converted by soybean lipoxygenase-1 both under anaerobic and aerobic conditions (ref. 52). Under anaerobic conditions substantial amounts of oxodienes are formed in addition to more polar compounds, presumably epoxy-hydroxy fatty acids. Subsequent EPR-studies (ref. 54) have shown that alkoxy and probably acyl-radicals can be trapped under these conditions.

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