Substrates and Products in Lipoxygenase Biocatalysis

G. A. Veldink, W. F. Nieuwenhuizen, M. J. Schilstra and J. F. G. Vliegenthart

Bijvoet Center for Biomolecular Research, Utrecht University, Padualaan 8, NL-3584 CH Utrecht, The Netherlands

Introduction

Lipoxygenases are a group of enzymes with a widespread occurrence in both animals and plants. They specifically dioxygenate unsaturated lipids to form chiral, unsaturated lipid hydroperoxides. The iso-form found in resting soybean seeds has been studied extensively. Like other lipoxygenases, it contains one mol of iron per mol of enzyme which is essential for catalysis. The iron was found to be essential for the activation of the lipid substrate in order to become reactive towards ground state dioxygen.

Lipoxygenase-1 from soybeans is known to accept a fairly wide range of substrates, deviating from naturally occurring substrates like 9Z,12Z-octadecadienoic acid (linoleic acid) and 9Z,12Z,16Z-octadecatrienoic acid (α-linolenic acid)[1]. One such novel substrate is the 9,12-diynoic analogue of linoleic acid, which is converted by lipoxygenase into 11-oxo-9,12-octadecadiynoic acid.

Substrate activation

Generally, the spin barrier between ground state organic molecules (singlet) and ground state dioxygen (triplet) prevents the two types of molecules to react. This also applies to the lipoxygenase-catalysed dioxygenation of *e.g.* linoleic acid to form the hydroperoxide (Fig. 1). In the early work of De Groot *et al.*[2] evidence was found for the formation of carbon-centered free radicals by soybean lipoxygenase-1 (Fig. 2). The radicals were characterized as spin adducts of specifically deuterated or non-deuterated linoleyl radicals and a spin trap. Because the system was anaerobic, a substantial amount of non-oxygenated fatty acid free radical could accumulate. The absence of any super hyperfine structure in the ESR spectra of adducts with a β-^{2}H instead of a β-^{1}H demonstrated that originally an unpaired electron was present at either C-9 or C-13 (Fig. 2). In further studies, evidence was found for the occurrence of intermediate carbon-centered and oxygen-centered free radicals[3-6] during the normal aerobic catalysis by lipoxygenase, thus leaving little room for suggestions involving organo-iron[7] or

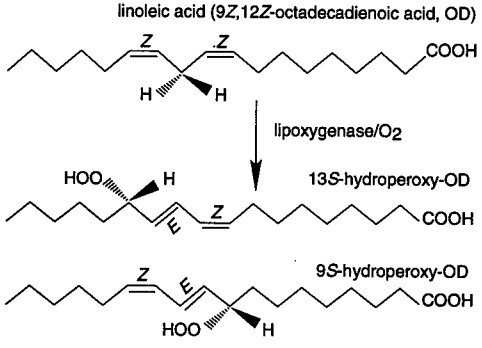

Fig. 1.
Lipoxygenase-catalyzed dioxygenation of linoleic acid leading to *EZ*-conjugated chiral hydroperoxides

carbanion intermediates[8]. The participation of activated dioxygen, in particular in its first excited singlet state was dismissed on the basis of the antarafacial geometry of H-abstraction and dioxygen insertion [9-10]. Furthermore, no evidence could be found to substantiate oxygen binding to native

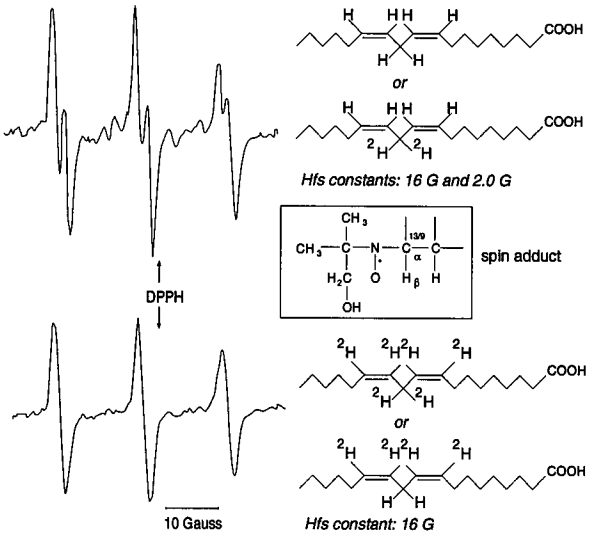

Fig. 2.
Under anaerobic conditions, lipoxygenase-1 is allowed to react with linoleic acid in the presence of a spin trap. The absence of molecular oxygen will allow the release of linoleyl free radical that subsequently reacts with the spin trap. Depending on the structure of the adduct and on the presence or absence of ^2H in the substrate, the resulting ESR-spectrum will or will not show a super hyperfine structure[2].

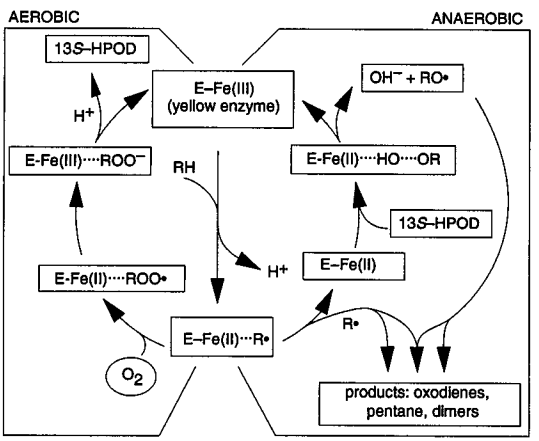

Fig. 3.
General scheme of lipoxygenase catalysis. The left part shows the dioxygenation reaction and the role of iron. The central part is common to both the aerobic and anaerobic cycles (right). Under aerobic conditions at pH 9, soybean lipoxygenase-1 does not produce significant amounts of oxodienes, indicating that O_2 addition to the linoleyl free radical is very efficient. Furthermore, under these conditions, only minute amounts of the enzyme-linoleyl complex actually dissociate, which would make a reoxidation of the iron(II)-enzyme by HPOD necessary. Under anaerobic conditions, each cycle requires the reoxidation of iron(II), which accordingly yields stoichiometric amounts of oxodienes.

iron(II)-containing soybean lipoxygenase-1[11,12]. Therefore, the following scheme (Fig. 3) (*cf.* refs. [3,13]) is most likely to contain the essentials of the catalytic cycle of lipoxygenase-1. In the anaerobic part (Fig. 3, right), the enzyme-fatty acid radical complex is supposed to dissociate, thus releasing the fatty acid radical which in turn reacts with substrate fatty acid to form dimers in a non-specific manner[14-16]. The iron then is in a divalent state, and will be reoxidized by the hydroperoxide to return

to the active iron(III) state. In the process, the hydroperoxide is most likely to be converted into the alkoxy radical, that may rearrange to yield a variety of degradation products (Fig. 4).

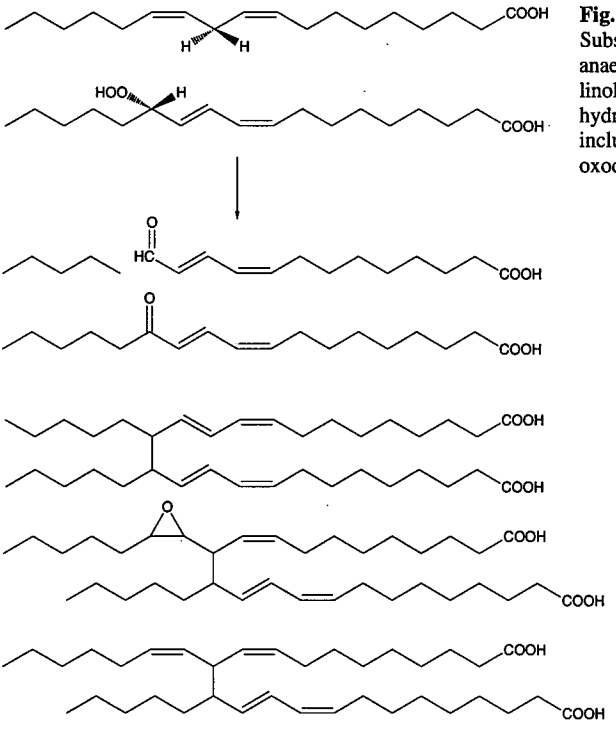

Fig. 4.
Substrates and products of the anaerobic reaction between linoleic acid and linoleic acid hydroperoxide. The products include n-pentane, C-13 and C-18 oxodienes and dimers.

Substrates and Products

Standard *in vitro* substrates for lipoxygenase are naturally occurring polyunsaturated fatty acids with a general structure as given in Fig. 5. The substrate specificity of lipoxygenase towards this type of substrates has been investigated by testing series of fatty acids[9,17-22] with widely varying R-groups. This illustrates the point, *i.e.* while R_1 and/or R_2 were extensively varied, the common element throughout these series is still an intact 1,4-(*E,Z*)-pentadiene system.

Fundamentally different substrates are those that no longer contain the standard 1,4-(*Z,Z*)-pentadiene moiety, *e.g.* Funk *et al.*[23] have found that fatty acids containing 1,4-(*E,Z*)- and 1,4-(*Z,E*)-pentadienyl are actual substrates which are converted into hydroperoxy derivatives. The double bond geometry in the product appears to be strictly controlled by the enzyme (Fig. 6), as is apparent from the E to Z transition of the double bond in the second substrate in Fig. 6. *EZ*-substrates were found to be converted at a fraction of the normal reaction rate. Several other interesting compounds containing modified pentadiene systems have been tried (Fig. 7). Some of these were

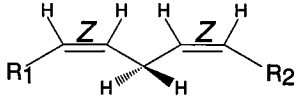

Fig. 5.
Structural element common to standard lipoxygenase-1 substrates.

found to be true substrates, others were inhibitors *e.g.* compounds[24] with sulphur substituting the central methylene group were found to be strong and irreversible inhibitors. Grechkin *et al.*[25] found

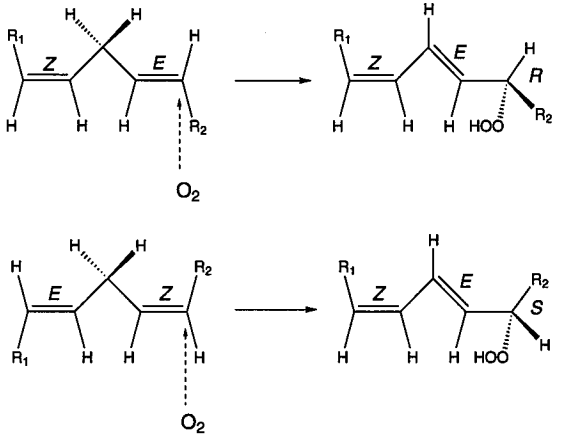

Fig. 6.
ZE- and *EZ* substrates are converted by lipoxygenase into *ZE*-hydroperoxides[23]. The position of the *E*-double bond in the first substrate results in an *R*-configuration at the chiral C-atom of the product hydroperoxide.

that α-ketols like those found by incubating linoleic acid hydroperoxide with hydroperoxide dehydrase from plants, could serve as a substrate for lipoxygenase leading to dioxygen insertion at the outer C-atom of the pseudo-pentadiene system. Experiments by Kühn *et al.*[26], using 12-oxo-oleic acid, demonstrated that also this type of substrate could be handled by the enzyme in a productive manner, yielding mainly the 9,12-dioxo monoenoic fatty acid. According to the authors,

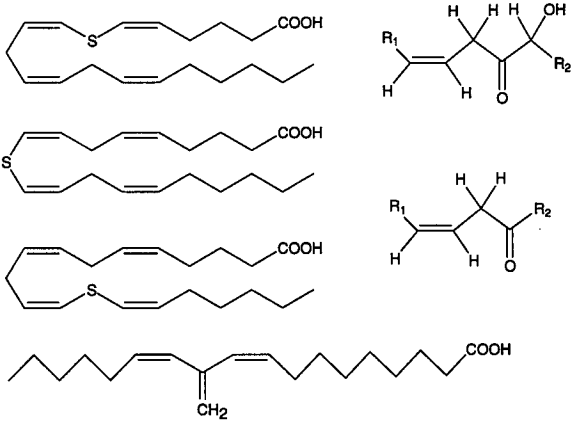

Fig. 7.
The mono-unsaturated α-ketol and oxo-monoene are both substrates for soybean lipoxygenase[25,26]. The 11-methylidene linoleate and the 13-thia-eicosatetraenoate derivatives were found to be irreversible inhibitors of lipoxygenase-1 from soybeans, whereas the other two thia compounds were normal substrates for this enzyme.

the newly formed 9-oxo function is most probably formed from an initial hydroperoxide group. Unsaturated fatty acid hydroperoxides, provided they contain a suitable 1,4-pentadiene system, may function as a substrate in multiple dioxygenation reactions[27,28], *e.g.* leading to lipoxins[29]. Lipoxygenase is also involved in the *dehydration* of hydroperoxides during the formation of the reactive key intermediate LTA_4 or its analogues in the biosynthesis of leukotrienes (Fig. 8) and lipoxins[30]. Another type of dehydration is the formation of oxo compounds from unsaturated hydroperoxides, both under aerobic and anaerobic conditions[15,31-33]. The dehydration modes most

problably have very different mechanisms. For the formation of LTA_4, it was shown that the initial step involves enzymatic H -abstraction from C-10 of ETE, followed by one electron shifts to produce the conjugated 5,6-epoxide and HO [34] (Fig. 8). Widely varying amounts of oxodienes have been reported to accompany the formation of hydroperoxides by lipoxygenase. The amount of oxodiene formed during lipoxygenase catalysis most likely reflects the amount of hydroperoxide needed to reactivate Fe(II)-enzyme, stemming from a dissociation of the Fe(II)—L complex (*cf.* Fig. 3). The decomposition of hydroperoxides by Fe(II) is a well-known non-enzymatic process[35], leading to OH^-, alkoxy free radicals and Fe(III):

$LOOH + Fe^{2+} \rightarrow LO + OH^- + Fe^{3+}$; in subsequent reactions, the LO then rearranges to yield *i.a.* oxodienes.

Downing *et al.*[36] reported the irreversible inhibition of lipoxygenase and prostaglandin synthase catalysis by acetylenic fatty acids that were structurally analogous to normal substrates like arachidonic acid or linoleic acid. The irreversible nature of the inhibition was confirmed by subsequent investigations[24,37,38], but in particular it was noted that the inhibitory action by certain acetylenic fatty acids occurred with remarkable stereospecificity[39]. It was also found to be time-dependent and to require oxygen. On that basis, it was proposed that a rapidly decomposing vinylic hydroperoxide might be the inactivating species[39]. We have synthesized a series of diynoic fatty acids, including the structural

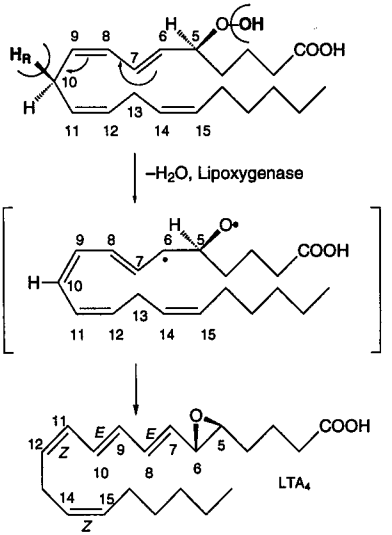

Fig. 8.
Dehydration of 5*S*-HPETE by lipoxygenase is initiated by H_R-abstraction from C-10 of arachidonic acid. Following H-abstraction, epoxide formation is accompanied by varying degrees of 6*R*-oxygenation.

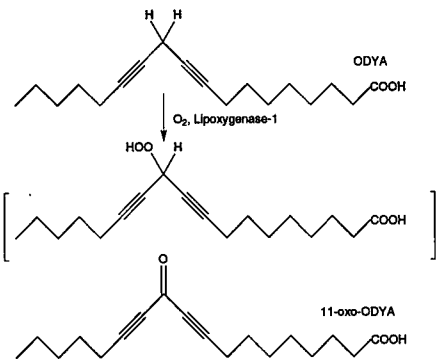

Fig. 9.
In the authors' laboratory, a series of octadecadiynoic acids were synthesized. The linoleate analogue shown here inactivated lipoxygenase-1, while producing the 11-oxodiynoic derivative.

analogue of linoleic acid, 9,12-octadecadiynoic acid. On incubation with soybean lipoxygenase-1, the enzyme was indeed found to inactivate, but in addition a reaction product accumulated: 11-oxo-9,12-octadecadiynoic acid (Fig. 9). This compound *per se* did not inactivate the enzyme. A hypothetical route leading to the 11-oxo compound is given in Fig. 9. Further work is currently being carried out to characterize this pathway, and to identify the inactivating agent.

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