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Recent Progress in the Study on the Mechanism of Action of Soybean Lipoxygenase[1]

Johannes F. G. Vliegenthart,* Gerrit A. Veldink, and Jan Boldingh

Soybean lipoxygenase-1 (EC 1.13.11.12; M_r 98 500) is a mononuclear non-heme iron dioxygenase catalyzing the regio- and stereospecific oxygenation of polyunsaturated fatty acids containing a 1,4-*cis,cis*-pentadiene system. A review is given of some recent results. A new ^{1}H NMR method to analyze the enantiomeric composition of fatty acid hydroperoxides is described. Electron paramagnetic resonance studies of the NO complex of the enzyme indicate that the native enzyme contains Fe(II)–O_2. Kinetic studies of the anaerobic reaction in which both fatty acid hydroperoxides and fatty acids occur as substrates point to a substituted-enzyme mechanism with double substrate inhibition.

Soybean lipoxygenase-1 (EC 1.13.11.12) is a dioxygenase containing 1 mol of non-heme iron per mole of protein (M_r 98 500). The dioxygenase function of this enzyme has been extensively studied. In recent years the enzyme has been characterized with a number of spectroscopic techniques. In addition to its dioxygenase properties, under certain conditions lipoxygenase also displays hydroperoxidase activity. [For reviews, see Axelrod (1974), Veldink et al. (1977), Vliegenthart and Veldink (1977), Eskin et al. (1977).] The present paper summarizes some recent results, mainly from the authors' laboratory.

The lipoxygenase-catalyzed oxygenation of polyunsaturated fatty acids, containing a 1,4-*cis,cis*-pentadiene system, is shown in Figure 1. The soybean enzyme mainly produces the 13-L-hydroperoxide, whereas the corn germ enzyme gives rise to the formation of predominantly the 9-D isomer. Frequently, questions arise as to which molar ratio of the 13 and 9 isomer is produced and to what extent these hydroperoxides are formed in a stereospecific way. Several methods have been proposed for the determination of the ratio of the positional isomers. Most of the literature data are based on mass spectrometry of the hydroxydienoates or hydroxystearates or by thin-layer chromatography (TLC) of ^{14}C-labeled hydroxy fatty acids. At this moment the best method seems to be high-performance liquid chromatography of the hydroxydienoates or the hydroxydienoic acids, followed by integration of the chromatograms (Aoshima, 1977; Verhagen et al., 1978a). This procedure can also be carried out on a preparative scale. The determination of the enantiomeric composition of the positional isomers is more complicated. Two methods are currently in use; the determination of the optical rotation which in view of the low specific rotation of the hydroxy acids has a rather low accuracy. Furthermore, this method requires the availability of standards of high enantiomeric purity.

A second method has been described by Hamberg (1971) and is based on the separation by gas–liquid chromatography of the menthylchloroformate derivatives of compounds obtained after reduction of the hydroperoxy group, followed by ozonolysis and esterification.

Recently, we developed a new and simple nuclear magnetic resonance (NMR) method based on the lanthanide-induced shift of protons in a chiral derivative of hydroxydienoates (Van Os et al., unpublished data). The derivatization is carried out with one of the enantiomers of the acid chloride of α-methoxy-α-(trifluoromethyl)phenylacetic acid (MTPA ester). This gives rise to diastereomers for the D and L hydroxydienoate (Figure 2).

For the determination of the ratio of enantiomers, it is essential that the NMR signals are well resolved. This can be achieved by the addition of a lanthanide shift reagent like Eu(fod)$_3$. The signal of the OCH_3 group of the fatty acid moiety shifts strongly, whereas the OCH_3 signal of the MTPA group broadens and shifts only slightly. Increasing amounts of shift reagent afford a splitting of this signal. When the signal is well separated in two peaks, integration of these peaks gives the molar ratio of the enantiomers (Figure 3). With a spectrometer operating in the Fourier transform mode, less than 1 mg of starting material is sufficient.

MECHANISM OF THE ANAEROBIC REACTION

A few years ago we proposed a scheme for the mechanism of the reactions catalyzed by soybean lipoxygenase-1 (De Groot et al., 1975). Figure 4 summarizes the mech-

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[1]Dedicated to Professor Dr. E. Havinga on the occasion of his 70th birthday.

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$CH_3-(CH_2)_4-CH\overset{c}{=}CH-CH_2-CH\overset{c}{=}CH-(CH_2)_7-COOH$ LH

$CH_3-(CH_2)_4-CH(OOH)-CH\overset{t}{=}CH-CH\overset{c}{=}CH-(CH_2)_7-COOH$ 13-L-LOOH (soybean)

$CH_3-(CH_2)_4-CH\overset{c}{=}CH-CH\overset{t}{=}CH-CH(OOH)-(CH_2)_7-COOH$ 9-D-LOOH (maize germs)

Figure 1. Substrate (LH) and products of lipoxygenase-catalyzed oxygenations.

Figure 2. MTPA (α-methoxy-α-trifluoromethylphenylacetic acid) ester of 13-hydroxy-9-*cis*,11-*trans*-octadecadienoic acid.

Figure 3. Lanthanide-induced shift of OC^1H_3 signals in ^1H NMR by increasing ratios of shift reagent (L_0, $Eu(fod)_3$ and racemic MTPA ester (S_0). In the bottom trace the peaks are well resolved and show the 1:1 enantiomeric composition of the starting material (Van Os et al., 1978).

anism of the anaerobic reaction. The ferric enzyme is reduced by linoleic acid and the ferrous enzyme is then oxidized by the 13-L-hydroperoxylinoleic acid, which leads to the formation of oxodienoic acids absorbing at 285 nm. Combination of linoleic acid radicals with each other or with alkoxy radicals gives rise to the formation of dimers. Previously, we have shown that the oxidation of the ferrous enzyme as well as the reduction of the ferric form can be carried out separately (Egmond et al., 1977). Therefore, any mechanism for the anaerobic reaction involving a compulsory ternary complex of enzyme, linoleic acid, and 13-L-hydroperoxylinoleic acid seems unlikely. In principle this does not exclude the existence of a second substrate binding site. The investigation of the steady-state kinetics of the anaerobic reaction has provided important new mechanistic evidence (Verhagen et al., 1978a). If linoleic acid and 13-L-hydroperoxylinoleic acid compete for one and the same binding site, any inhibition by these two substrates must be linear.

Figure 4. Anaerobic reaction between linoleic and 13-hydroperoxylinoleic acid catalyzed by soybean lipoxygenase-1 (Verhagen et al. 1978a): LH, linoleic acid; LOOH, 13-hydroperoxylinoleic acid; E–Fe(III), ferric enzyme; E–Fe(II), ferrous enzyme.

Figure 5. Concentration ranges of monomers, acid-soaps, and micelles in mixtures of linoleic and 13-L-hydroperoxylinoleic acids in 0.1 M sodium borate buffer (pH 10.0) as a function of the mole fraction of linoleic acid t = 23 °C (Verhagen et al., 1978b).

The physical state of the substrate is an important aspect of any kinetic study which is carried out with lipoxygenase. Lagocki et al. (1976) demonstrated that lipoxygenase-1 reacts exclusively with monomers of linoleic acid. Gatt and Bartfai (1977) have shown that micelle formation of substrates may lead to false interpretations of enzyme kinetic data. However, a wide variety of critical micelle concentration values for linoleic acid have been published, whereas no values at all are available for 13-L-hydroperoxylinoleic nor of mixtures for linoleic acid and hydroperoxide. Verhagen et al. (1978b) studied the aggregation behavior of the substrates and mixtures thereof by surface tension measurements according to Wilhelmy using a thin platinum plate and a Beckman LM 600 electromicrobalance. Various amounts of surface active agents in aqueous solution were added to the solution in a 10-mL circular trough and the surface tension was continuously recorded.

Critical micelle concentration values were obtained by plotting the measured surface tensions vs. the logarithm of the concentrations of the surface active reagent. The concentration at which a deviation from linearity was observed in the curve was defined as the start of aggregation.

Premicellar aggregation must be attributed to the formation of 1:1 dimers of dissociated and undissociated fatty acids or higher aggregates. Figure 5 shows the relation between the concentration ranges of total lipid (i.e., linoleic plus hydroperoxylinoleic acids) and the mole fraction of linoleic acid in which monomers, acid-soap, and micelles are present. Since below pH 10 only small concentration ranges of monomers are available for kinetic measurements, the latter were conducted at pH 10 in

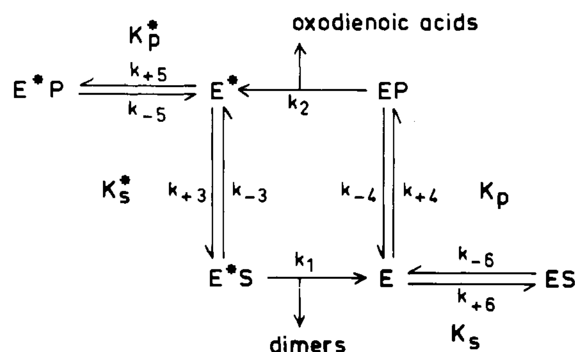

Figure 6. Kinetic scheme for the anaerobic conversion of linoleic acid (S) and hydroperoxylinoleic acid (P). E*:E(FeIII); E:E(FeII).

borate buffer, in the range wherein exclusively monomers occur. The enzyme activity is still optimal at this pH. The anaerobic reaction of lipoxygenase-1 with linoleic acid and 13-L-hydroperoxylinoleic acid proceeds until one of the two substrates is exhausted. The hydroperoxide is thus completely converted if linoleic acid is in excess, whereas linoleic acid is completely consumed when the hydroperoxide prevails. The reaction can be monitored at 285 nm, because regardless of the initial molar ratios of the two substrates throughout the reaction 50% of the consumed hydroperoxide is converted into oxodienoic acids. Substrate inhibition was found to occur for both linoleic acid and the hydroperoxide.

An excellent fit to the experimental data was obtained with a rate equation for a substituted enzyme mechanism (ping pong) with double substrate inhibition (Verhagen et al., 1978a; Figure 6). Obviously the anaerobic experiments cannot provide any straightforward information on a possible binding site for oxygen. Some indications for such a site have been presented by Egmond et al. (1975).

HYDROPEROXIDE CONVERSIONS

Previously, we have shown that lipoxygenase-1 reacts with 13-L-hydroperoxylinoleic acid to afford the ferric enzyme and hitherto unidentified products of the hydroperoxide (Egmond et al., 1977). This ferric enzyme can form a 1:1 complex with another hydroperoxide molecule. Spectroscopically, this complex can easily be recognized, e.g., by its absorption maximum at 570 nm. The complex is labile. It disintegrates into the free ferric enzyme and conversion products of the hydroperoxide one of which was characterized as an epoxy-hydroxy compound (Garssen et al., 1976). On the basis of experiments with ^{18}O-labeled hydroperoxides we could establish that this is a true isomerization reaction. Recently, we found that soybean lipoxygenase-1 reacts with both 13-L- and 9-D-hydroperoxylinoleic acid under anaerobic conditions (Verhagen et al., 1977). However, this reaction proceeds much slower than in the presence of linoleic acid. To obtain reasonable reaction rates 20–40-fold higher enzyme concentrations are necessary.

The UV absorption spectra before and after the conversion of the 13-L- and the 9-D-hydroperoxide are shown in Figure 7. A decrease in absorbance at 234 nm of more than 80% was obtained and a new absorption maximum at 285 nm. Remarkably, the 9-D isomer was found to be a much better substrate than the 13-L-hydroperoxide. A linear relationship between the velocity of the reaction and enzyme concentration was observed, although for the 9-D isomer a deviation from linearity was found at high enzyme concentrations. It is an enzyme-catalyzed reaction since it is abolished by enzyme inactivation and the pH profile coincides with that of other lipoxygenase-catalyzed reactions. In all experiments a kinetic lag phase was observed, which corresponds to the time necessary to consume traces of oxygen still present after deaeration (<2 μM). During this lag phase a reaction takes place between oxygen, hydroperoxide, and enzyme, but the products are unknown. With 13-L-hydroperoxide as substrate, the anaerobic conversion has the following characteristics: (a) no dimeric compounds are formed; (b) a considerable amount of material is converted into a monoenoic epoxyhydroxy fatty acid; (c) ketodienoic acid is formed besides the 13-oxotridecadienoic acid, the cleavage product in the normal anaerobic reaction (Garssen et al., 1971, 1972). Evidently, these carbonyl compounds are responsible for the absorption at 285 nm. With the 9-D isomer as substrate, no chain cleavage occurs. One oxodiene is formed besides an epoxyhydroxy compound. Linoleic acid stimulates the conversion of the 13-L isomer 1500 fold, whereas it has no effect on the reaction rate of the 9-D-hydroperoxide conversion. This explains the observation by Garssen et al. (1972) that the 9-D isomer is virtually no substrate in the anaerobic reaction. The mechanism of this new anaerobic reaction is still subject of speculation as is its possible physiological significance.

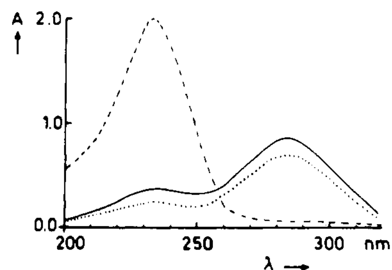

Figure 7. UV spectra before (- -) and after the anaerobic conversion of 13-L-hydroperoxylinoleic acid (80 μM) (—) and 9-D-hydroperoxylinoleic acid (80 μM) (···) by soybean lipoxygenase-1 (1.0 μM).

CATALYTIC ROLE OF IRON

From fluorescence spectroscopy, it might be inferred that tryptophan residues are not too far removed from the active site since fluorescence quenching occurs upon reaction between enzyme and 13-L-hydroperoxide (Finazzi-Agrò et al., 1973). Modification by means of hydrogen peroxide or diethyl pyrocarbonate leads to inactivation of the enzyme, probably via reaction with a histidine residue.

Some years ago we proposed that the abstraction of hydrogen requires the iron to be in a ferric state (De Groot et al., 1975). In this step trivalent iron is reduced to a ferrous state. Both in the aerobic and anaerobic cycle an electron from iron is donated back to keep the cycles going. Since the native form is EPR silent but shows normal activity toward linoleic acid, we suggested that this form is the diamagnetic [ferrous:oxygen/ferric:superoxo] complex $Fe(II)-O_2 \leftrightarrow Fe(III)-O_2^-$. Recent experiments with nitric oxide indeed substantiated this view (Galpin et al., 1978).

Nitric oxide is a paramagnetic molecule whose bonding structure is very similar to that of molecular oxygen. A typical EPR spectrum of the lipoxygenase–NO complex at pH 7.0 and 15 K is shown in Figure 8. The signal at $g = 4$ follows Curie's law on cooling to 3.7 K.

The signal at $g = 4$ is quite unique in biochemical systems. It must be ascribed to high spin ferric iron since it is inconceivable that it should belong to a nitroxide radical. Therefore it must be concluded that an electron has been transferred from the iron atom in the native enzyme to an NO molecule. The formation of the native enzyme–NO complex is reversible by evacuation. The yellow color and typical EPR signal are lost and over 90%

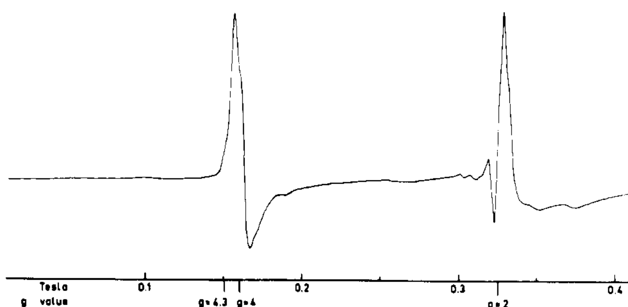

Figure 8. EPR spectrum after the addition of NO gas to anaerobic native lipoxygenase (85 mg/mL) in 0.1 M sodium phosphate (pH 7.0). Microwave frequency 9.105 GHz. Power 0.5 mW. Receiver gain 10^3, temperature 15.3 K.

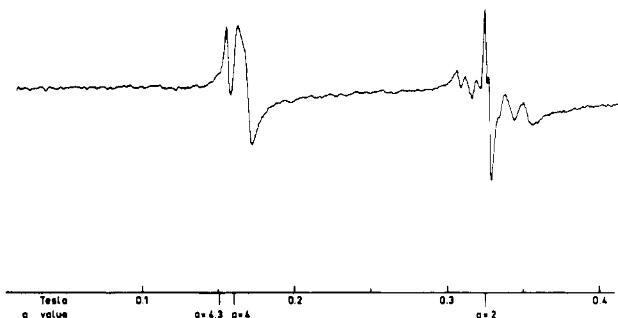

Figure 9. As in Figure 8 but with the addition of 13-hydroperoxylinoleic acid in a 2:1 ratio to enzyme. Conditions as in Figure 8 except receiver gain = 5×10^3 (Galpin et al., 1978).

of the activity in the normal aerobic reaction is recovered.

Addition of linoleic acid in large excess caused no significant change in the signal near $g = 4$. However, the addition of 13-L-hydroperoxide to the enzyme–NO complex results in a substantial decrease in the signal near g = 4 (Figure 9). If the 13-LOOH was added before treatment with NO the usual signal near $g = 6$ (De Groot et al., 1975) disappeared completely and only a small signal near $g = 4$ was observed. Apparently a ferric–NO complex is formed which is EPR silent. It seems therefore quite reasonable to conclude that (a) the anaerobic native enzyme is a ferrous species and (b) the aerobic species indeed contains an iron–oxygen complex.

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Determination of Phorate and Its Metabolites by Mixed-Phase Gas Chromatography

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Recent advances in mixed-phase gas chromatography are developed and applied to the determination of phorate and its metabolites. Phases which were taken into account are OV-101, OV-17, OV-225, and Apolar-5CP.

Phorate (*O,O*-diethyl *S*-[(ethylthio)methyl] phosphorodithioate) (I) is used extensively as an insecticide for the protection of a wide variety of crops. Due to its high toxicity to mammals (LD_{50} to rats 1.5 mg/kg) there is a particular need for the monitoring of its residues. This is especially true where such crops as tomatoes, lettuce, carrots, and cabbages are concerned, which are consumable in their raw state and where their residues are not therefore destroyed through boiling. It has become evident that such monitoring should not be confined to phorate, as is often done, but extended to include its three major (II–IV) and two minor (V–VI) metabolites as well (Figure 1). While, for example, the conversion of phorate to its sulfoxide in plant tissue (Saunders and Getzin, 1973), by soil bacteria (Higgins and Burns, 1975) or even during the normal methods of extraction (Brown, 1975), is relatively rapid, its subsequent oxidation and breakdown is known to be

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