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LIPOXYGENASE-CATALYSED OXIDATION OF LINOLEIC ACID

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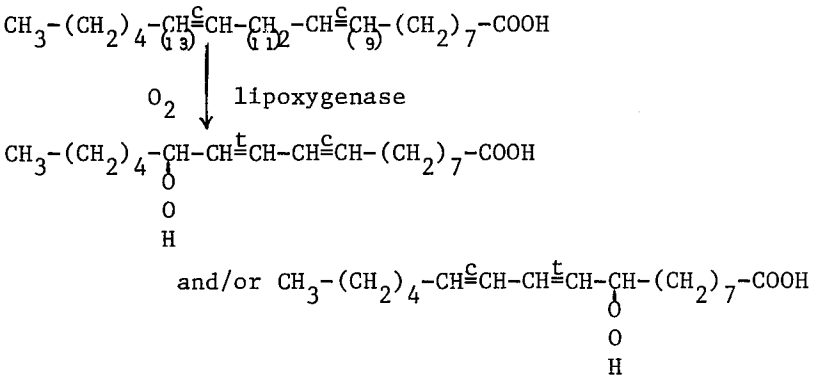
INTRODUCTION

Lipoxygenase (linoleate:oxygen oxidoreductase, EC 1.13.11.12) is a dioxygenase containing non-heme iron. It catalyzes the conversion of poly-unsaturated fatty acids having a 1,4-pentadiene system into conjugated hydroperoxy fatty acids (Scheme 1)



Scheme 1. The oxygenation of cis-9, cis-12-octadecadienoic acid by lipoxygenase.

The formation of the hydroperoxides occurs stereospecifically provided the reaction conditions (pH, temperature, fatty acid and

oxygen concentrations) are properly chosen. The enzyme has been found in a great number of plant species (1 - 3) while its presence has only recently been established in some animal tissues (4 - 9). The richest sources of lipoxygenase are the seeds of Leguminosae e.g. soybeans and peas. Plant lipoxygenases may differ in substrate and product specificities, pH dependence, sensitiveness to inhibitors, stability, amino acid composition and molecular weight. For comprehensive reviews see refs. 3, 10 - 16.

In soybeans several iso-enzymes have been found. Lipoxygenase-1 (the "theorell"-iso-enzyme) is the most thoroughly investigated species and it has the following characteristics. The enzyme consists of a single polypeptide chain with a molecular weight of approximately 100 000 and it contains 1 gatom of iron/mol. With regard to the amino acid composition it has to be mentioned that the enzyme contains 5 sulfhydryl groups but no disulfide bridges. In the native enzyme the iron is epr-silent and the optical spectrum only shows the protein absorption band with a maximum at 280 nm at pH 9.0.

SPECIFICITY

Among the poly-unsaturated fatty acids linoleic acid is the best substrate for lipoxygenase-1. At 0°C and optimum pH (9 - 10) linoleic acid is converted into predominantly 13-L-hydroxyperoxy-9-cis, 11-trans-octadecadienoic acid. In addition, a small amount of 9-hydroperoxy-10-trans, 12-cis-octadecadienoic acid is formed. Other lipoxygenases shown varying product specificities which may lead to different ratios of the positional and stereo-isomers. For the analysis of the products formed several methods are available. The regiospecificity can adequately be determined by HPLC of the hydroperoxy fatty acids as such or of suitable derivatives (17). Recently, we developed a new and simple NMR-method to establish the stereospecificity based on the lanthanide-induced shift of protons in a chiral derivative of hydroxy-octadecadienoates (18, 19). For a few enzymes the results are summarized in Table 1.

Table I

Enzyme	pH	13 : 9	$13L_S$:$13D_R$	$9L_R$:$9D_S$
Soybean-1	9.0	97.5 : 2.5	97 : 3	39 : 61
Soybean-1	6.6	77 : 23	94 : 6	8 : 92
Soybean-2	9.0	35 : 65	87 : 13	78 : 22
Soybean-2	6.6	25 : 75	50 : 50	55 : 45
Pea	9.0	41 : 59	91 : 9	62 : 38
Pea	6.6	33 : 67	50 : 50	50 : 50
Corn germs	6.6	14 : 86	79 : 21	6 : 94

*13-hydroxyperoxy-9-cis,11-trans-octadecadienoic acid,9-hydroperoxy-10-trans,12-cis-octadecadienoic acid.

THE EFFECT OF H_2O_2 ON SOYBEAN LIPOXYGENASE-1

It has been shown that soybean lipoxygenase-1 is irreversibly inactivated by H_2O_2 (27). H_2O_2 and 13-L-hydroperoxylinoleic acid cause similar types of quenching of the protein fluorescence (28). Both reagents also have in common their effects on the epr spectra of lipoxygenase-1 (29). The CD spectra (Fig. 1) of lipoxygenase samples treated with either H_2O_2 or 13-L-hydroperoxylinoleic acid appear to provide the only spectroscopic data to distinguish the enzyme species, which differ so greatly in catalytic properties (30). Possibly, only the iron chromophoric group is attacked by treatment of the enzyme with H_2O_2 which ultimately leads to a destruction of the iron complex in the enzyme (Fig. 1). This suggestion is supported by the findings that substrate analogues like linolelaidic acid or conjugated linoleic acid are capable of protecting the enzyme against attack by H_2O_2 (27).

THE EFFECT OF 4-NITROCATECHOL ON SOYBEAN LIPOXYGENASE-1

Several catechol derivatives e.g. 4-nitrocatechol; 1,2-dihydroxybenzene-3,5-disulphonate (Tiron); catechol; caffeic acid and protocatechualdehyde have an inhibitory effect on the enzyme (31). On the basis of epr data it could be concluded that binding of these inhibitors to Fe(III) occurs. A subsequent study with 4-nitrocatechol revealed that the inhibition follows a rather complex pattern consisting of a reversible and an irreversible part. Fe (III)-soybean lipoxygenase-1 gives a green-colored 1 : 1 complex with 4-nitrocatechol (32) at pH 7. It has absorption maxima at 385 nm and 650 nm. 4-Nitrocatechol can be displaced from the green complex by 13-L-hydroperoxylinoleic acid, which then yields the purple complex between Fe(III)-lipoxygenase and product hydroperoxide. This illustrates that under these conditions the formation of the 4-nitrocatechol/Fe(III)-lipoxygenase-1 complex is reversible. Studies on the steady-state kinetics of the oxygenation of linoleic acid showed that the inhibition has a non-competitive nature with an inhibition constant $K^*_{4\text{-NC}} = 16.3\ \mu M$. However, due to the slow establishment of the complex equilibrium the inhibition pattern appears as pseudo-non-competitive (Fig. 2).

On prolonged incubation of Fe(III)-lipoxygenase-1 and 4-nitrocatechol the green complex converts into a brown species. It was found that this conversion is coupled with a change in the nature of the inhibition from reversible to irreversible (Fig. 3).

It should be noted that complex formation between the native enzyme and 4-nitrocatechol could not be demonstrated. Therefore, any

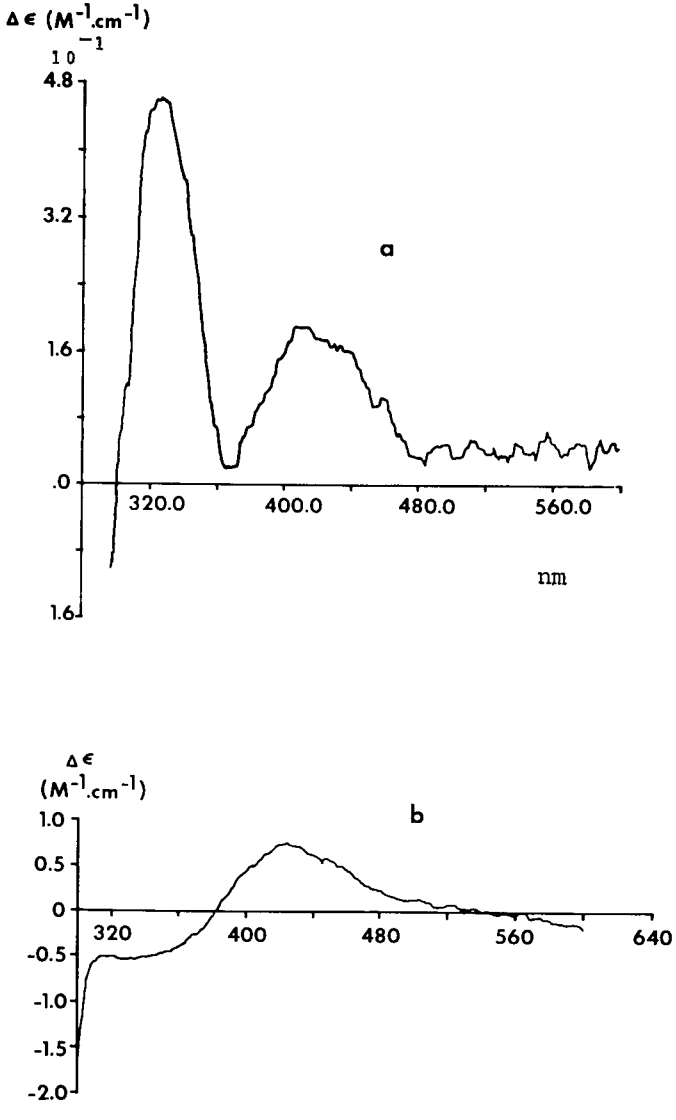


Fig. 1a. CD spectrum of 280 μM lipoxygenase-1 treated with an equimolar amount of H_2O_2. Buffer : 0.1 M sodiumborate pH 9.0, t=0°C.

b. CD spectrum of 162 μM lipoxygenase-1 treated with an equimolar amount of 13-L-ROOH. Buffer : 0.1 M sodiumborate pH 9.0, t=0°C.

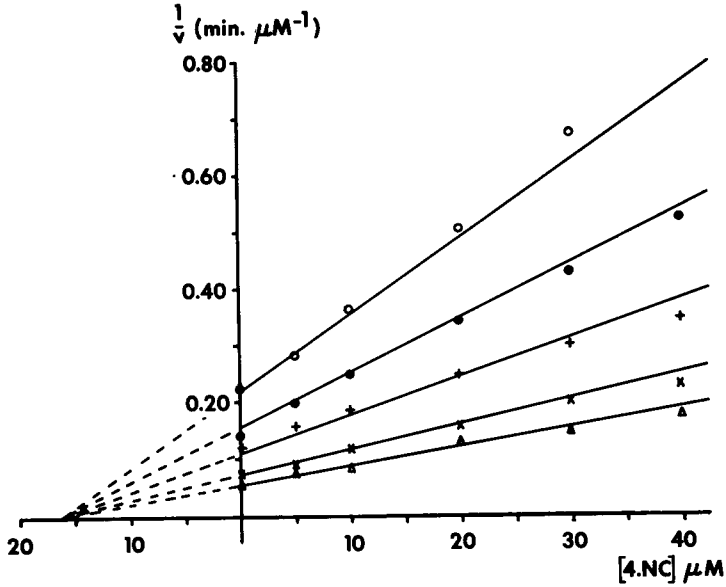


Fig. 2. Single reciprocal plot of the initial oxygenation rate (v) as a function of the 4NC concentration. Enzyme concentration: 2 nM, pH 8.0, t=25°C. The computed values are represented by the solid lines. Linoleic acid concentrations: o——o, 5 μM, ●——●, 7.5 μM. +——+, 10 μM, x——x, 15 μM and Δ——Δ, 20 μM.

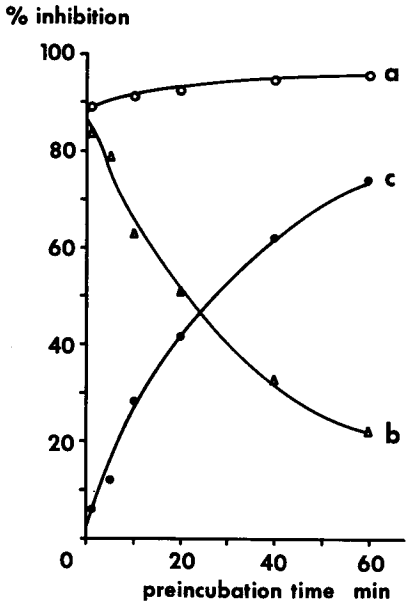


Fig. 3. Inhibition of Fe(III)-lipoxygenase-1 by 4NC.
a) inhibition of 20 nM Fe(III)-lipoxygenase-1 upon preincubation with 300 μM 4NC.
b) reversible part of the inhibition, obtained from measurements of the activity of 50-fold diluted samples of the preincubation mixture, mentioned under a). The diluted samples were allowed to stand for 5 minutes for the establishment of the complex equilibrium.
c) calculated irrervsible part of the inhibition.

inhibition by 4-nitrocatechol of the oxygenation of linoleic acid found in reactions starting with native enzyme has to be attributed to the complex formation with the yellow Fe(III)-enzyme, evidently occurring as a key intermediate in the catalytic cycle.

THE EFFECT OF ORGANIC MERCURIALS ON SOYBEAN LIPOXYGENASE-1

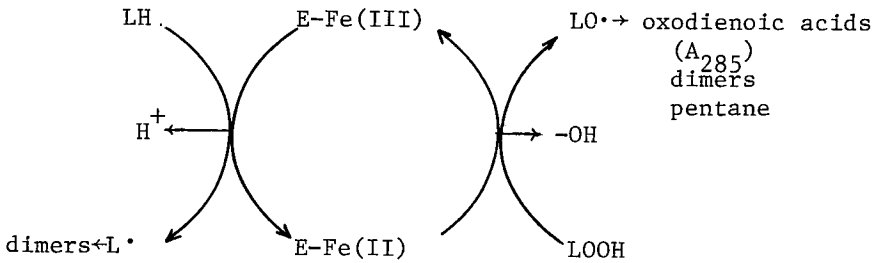
Relatively few investigators have studied the involvement of certain amino acids in the catalytic cycle (27, 33 - 37). The possibility of a participation of sulfydryl groups in lipoxygenase-catalysis has been excluded on the basis of the absence of effects of pCMB and NEM on the enzymic activity. However, it could well

be that the steric requirements of these reagents are too high to effect a modification in the native enzyme. We therefore carried out modification studies with a different type of sulfhydryl-modifying reagent. viz. CH_3HgX and $HOCH_2CH_2HgX$. It was found that three out of five free sulfhydryl groups of lipoxygenase-1 reacted easily with these substances.

This modification has an interesting influence on the catalytic properties of the enzyme. *i*) Kinetic measurements showed that the catalytic constant for the oxygenation of linoleic acid is reduced by 50%. Surprisingly, the affinity of linoleic acid towards lipoxygenase-1 remains unchanged: K_m = 3.3 μM. This value is much lower than those reported previously (38 - 41) which might have been due to the presence of a kinetic lag phase. *ii*) the kinetic lag phase in reactions with methylmercury-modified enzyme is considerably longer than with native enzyme. *iii*) the regio- and stereospecificities of the oxygenation are significantly lower. *iiii*) the activity as well as the other properties of the enzyme can completely be restored upon treatment with NaHS (42).

THE ANAEROBIC REACTION

The most widely known catalytic activity of lipoxygenase is the dioxygenation of poly-unsaturated fatty acids like linoleic and linolenic acids (Scheme 1). However, lipoxygenase is also capable of converting fatty acids and fatty acid hydroperoxides in the complete absence of molecular oxygen (43 - 44). This reaction leads to the formation of oxodienoic acids, n-pentane and a mixture of dimers. The mechanism of this reaction can be represented as follows (Scheme 3).



Scheme 3

Also under anaerobic conditions the enzyme contains iron alternatingly in the Fe(III) and Fe(II) states. Both substrates i.e. the fatty acid and the fatty acid hydroperoxide give rise to the formation of radicals. Subsequent reactions of these primary radicals are responsible for the complex mixture of products. The

two halves of the cycle given above can be carried out separately: the Fe(III)-enzyme can be reduced by linoleic acid under anaerobic conditions, and the Fe(II)-enzyme can be oxidized by 13-L-hydroperoxylinoleic acid (21, 45). These findings exclude a mechanism in which the enzyme, linoleic acid and 13-L-hydroperoxylinoleic acid form a compulsary ternary complex. A study of the steady-state kinetics (17) has shown that the simplest mechanism that fits the experimental data is a substituted enzyme ('ping-pong') mechanism with double substrate inhibition.

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