On the Mechanism of Lipoxygenase Catalysis

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INTRODUCTION

Lipoxygenase (linoleate : oxygen oxido-reductase, EC 1.13.11.12), a dioxygenase containing non-heme iron, catalyzes the hydroperoxidation of polyunsaturated fatty acids which possess a 1,4-cis,cis-pentadiene system. Products are optically active, cis,trans-conjugated hydroperoxides [1-9]. The occurrence of this enzyme is not restricted to plants, but it is also present in animal tissue [10-17]. Its presence has been reported in legume seeds (soybeans, peas, horse beans, peanuts, alfalfa), cereals (wheat, rye, barley), corn (germs), oats, flaxseed, potatoes, tomatoes, watermelon seedlings, dimorphoteca sinuata and green algae (chlorella pyrenoidosa). Incubation of linoleic acid with lipoxygenase results in the formation of a mixture of 9- and 13-hydroperoxylinoleic acids (Fig. 1). The molar ratio and the optical activity of both positional isomers depends on the type of lipoxygenase and the pH of the incubation mixture.

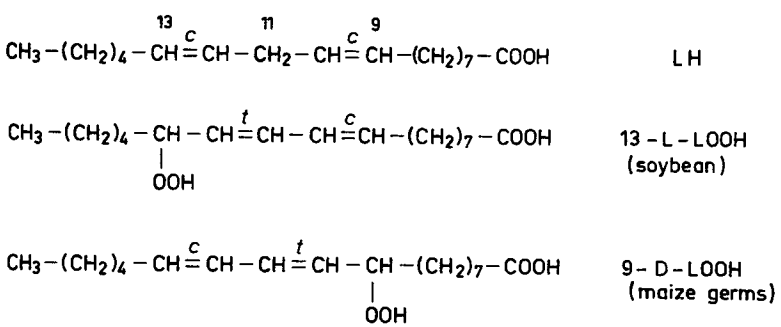

Fig. 1. The oxygenation of linoleic acid (LH) by lipoxygenase.

Soybean lipoxygenase-1 was the first lipoxygenase which was obtained in a highly purified, crystalline form [18]. Several relatively simple procedures are described for its isolation and purification [19]. The pH optimum of the enzyme is 9.0. With linoleic acid as substrate predominantly 13-L-hydroperoxylinoleic acid is formed, in addition to a few percent of 9-hydroperoxylinoleic acid, wherein the 9-D isomer prevails [22]. The native enzyme is colourless, has a molecular weight of approximately 100,000 Daltons, and contains one atom of iron per molecule. Two additional forms of the enzyme have been demonstrated [20,21] viz: the yellow ferric enzyme and the blue ferric enzyme-product complex. From low temperature EPR spectroscopy it appeared that the native enzyme is EPR silent, whereas the EPR spectra of the yellow and blue species show signals characteristic for high-spin ferric ions (Fig. 2).

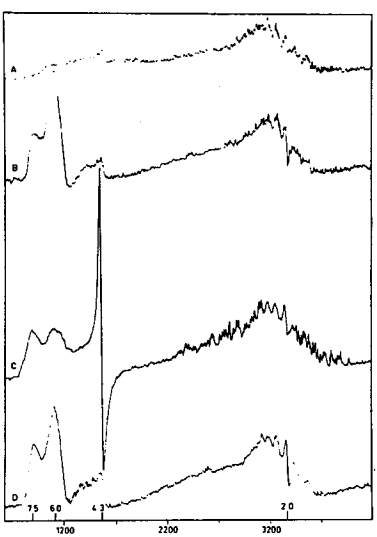

Fig. 2 Effect of 13-ROOH on the EPR spectrum of lipoxygenase-1. (a) 300 µl of a lipoxygenase solution(30 mg/ml); (b) 8 µl of a hydroperoxide solution (11.4 mM) was added to 300 µl of a lipoxygenase solution (30 mg/ml). Final concentrations: 0.29 mM and 0.30 mM; (c) another 16 µl of 11.4 mM hydroperoxide solution was added to the reaction mixture. Final concentrations: 0.28 mM and 0.84 mM, respectively; (d) the same reaction mixture as described under (c) after 1 hr at 0°C. Microwave frequency, 9.307 GHz.

The yellow enzyme species is obtained from the native enzyme by treatment with one equivalent of 13-L-hydroperoxylinoleic acid. The addition of a molar excess of 13-L-hydroperoxylinoleic acid results in the formation of the blue complex. The latter is rather unstable and slowly reverts to the yellow form. During this transition the hydroperoxide is converted mainly into threo-11-hydroxy-12,13-trans-epoxy, 9-cis-octadecenoic acid [23].

THE OXYGENATION REACTION

In the oxygenation of linoleic acid by soybean lipoxygenase-1 formally three steps can be distinguished:

1. abstraction of the C-11 hydrogen
2. conjugation of the double bonds
3. attachment of oxygen

The hydrogen abstraction proceeds stereospecifically and is the rate-limiting step of the reaction [24]. The hydrogen abstraction and oxygen insertion take place from opposite sides of the plane through the pentadiene system. This stereochemical relationship excludes the involvement of singlet oxygen. In spite of this evidence the suggestion for a possible role of singlet oxygen is repeatedly made in literature. A clue to the type of hydrogen species, which is abstracted by the enzyme gives the so called anaerobic reaction.

ANAEROBIC REACTION

When an incubation is carried out with soybean lipoxygenase-1 at pH 9.0 and linoleic acid in a closed system using a molar excess of linoleic acid over the available amount of oxygen, hydroperoxides are formed until the oxygen has been consumed. Subsequently a reaction takes place wherein both linoleic acid and 13-L-hydroperoxylinoleic acid function as substrates. This anaerobic reaction is characterized by the formation of a strong chromophore absorbing at 285 nm. Besides the oxodienoic acids responsible for this chromophore dimers are formed derived from linoleic acid and 13-L-hydroperoxylinoleic acid [25-27]. By applying the water-soluble spin trap 2-methyl-2-nitrosopropanol it could be demonstrated that in this reaction linoleic acid radicals are formed [28]. It is then

obvious to suggest that linoleic acid radicals are formed by the abstraction of hydrogen radicals. The anaerobic reaction can be formalized as given in Fig. 3.

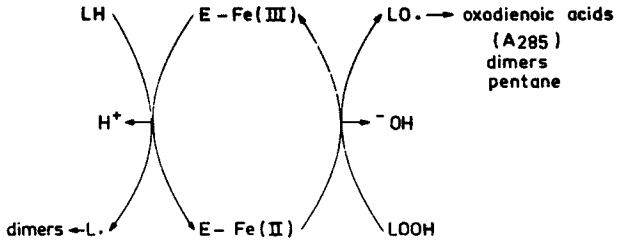

Fig. 3. Proposed mechanism for the anaerobic reaction of soybean lipoxygenase-1 with linoleic acid (LH) and 13-hydroperoxylinoleic acid (LOOH).

The two electron transferring steps (i.e. the reduction of the ferric enzyme by linoleic acid and the oxydation of the ferrous enzyme by hydroperoxide) can be carried out separately [29].

AEROBIC REACTION

On extrapolating the findings of the anaerobic reaction to the aerobic situation it becomes likely that the abstraction of hydrogen proceeds via a radical mechanism. Furthermore it is reasonable to propose that also under aerobic conditions a kind of ferric species is involved in the hydrogen abstraction. The proposed reaction pathway is summarized in Fig. 4. However, at first sight the involvement of a ferric species is difficult to reconcile with the apparent diamagnetic nature of iron in the native enzyme. Two alternatives have to be taken into consideration:

a) the native enzyme needs to be activated to a fully active ferric species, e.g. the yellow enzyme

b) the native enzyme as such is capable of forming hydroperoxides as well (Fig. 5).

Fig. 4. Proposed oxygenation mechanism of linoleic acid (LH) by a ferric enzyme.

$$E\text{-}Fe^{III} \xrightarrow[O_2]{LH} E\text{-}Fe^{II}\text{-}LOO\cdot + H^{\oplus}$$

$$E\text{-}FE^{II}\text{-}LOO\cdot \longrightarrow E\text{-}Fe^{III} + LOO^{\ominus}$$

$$E\text{-}Fe^{II} + O_2 \rightleftharpoons E\text{-}Fe^{II}\text{-}O_2 \leftrightarrow E\text{-}Fe^{III}\text{-}O_2^- \xrightarrow{LH} LOOH + Fe^{II}$$

Fig. 5 Proposed oxygenation mechanism of linoleic acid (LH) by a diamagnetic enzyme.

In an attempt to discriminate between these possibilities native and performed yellow enzyme were incubated under identical conditions with linoleic acid [21]. No differences were observed. By consequence, either both enzyme species are equally active or the conversion from one into the other has reached a steady state before the actual measurement could be made. The conversions can be formalized in the following equations

$$E\ Fe^{II}\text{-}O_2 + ROOH \longrightarrow E\ Fe^{III} + \text{products}$$

$$E\ Fe^{III} + RH \longrightarrow E\ Fe^{II} + \text{products}$$

ROLE OF IRON

4-Nitrocatechol has been found to be an inhibitor for a variety of non-heme iron dioxygenases [30]. Preincubation of the yellow (Fe^{III}) lipoxygenase with 4-nitrocatechol results in an almost complete inhibition. In a similar experiment with the native enzyme hardly any inhibition was observed. The complex formation between ferric enzyme and 4-nitrocatechol is relatively slow, leading to a pseudo non-competitive type of inhibition.

To investigate further the valence state of iron in the EPR silent native enzyme experiments with nitric oxyde were carried out. NO was added to a deoxygenated sample of the native enzyme. Subsequently, the low temperature EPR spectrum was recorded [31] (Fig. 6). Interestingly, an iron signal at g = 4 was observed, which is unique for biochemical systems. The conclusion must be that an electron has been transferred from the iron atom in the native enzyme to a NO molecule. Because the bonding structure of NO closely resembles that of molecular oxygen, it is tempting to suggest that the native enzyme under aerobic conditions has co-ordinated an oxygen molecule.

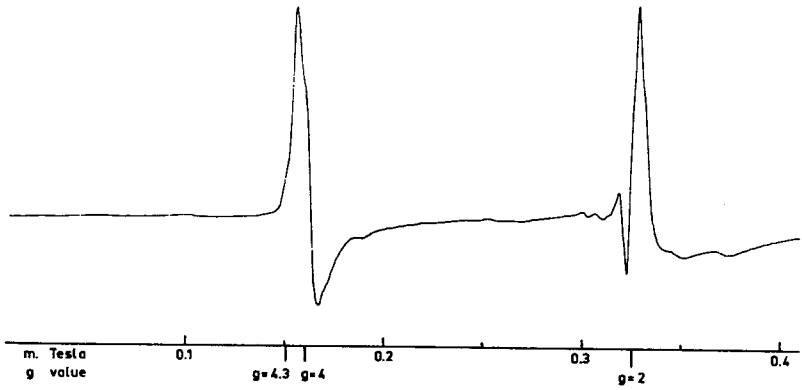

Fig. 6. EPR spectrum after the addition of NO gas to anaerobic native lipoxygenase.

ACKNOWLEDGEMENTS

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DISCUSSION

PEISACH: I'm interested in the reaction between NO and your enzyme because this seems to be a new type of chemistry that's appearing in various laboratories. Specifically, it was observed by Salerno that the addition of NO to Fe(II) EDTA produced a new resonance heretofore never observed with g-values suggestive of spin 3/2 Fe(III) in solution. This is a rather unusual species. When NO is added to your enzyme, you report an EPR signal which could also be interpreted in terms of spin 3/2. What are the optical characteristics of the NO enzyme, and how do they compare to that of the peroxide addition product?

VLIEGENTHART: They are slightly different, but they have some similarity to the yellow enzyme. So we think that it is indeed the ferric form that we are dealing with, the electron going from ferrous Fe to NO.

SPIRO: How intense is the absorption at 330 nm?

VLIEGENTHART: Absorbancy = 1200 M^{-1} cm^{-1} at 300 nm.

MASON: Which of the four states of iron in the cycle that you postulate is the yellow form? Is it radical containing, or is it the enzyme without radical?

VLIEGENTHART: The yellow enzyme can be prepared completely free from other forms. It is possible to convert the native enzyme to it with hydroperoxide. A chemical reaction occurs; all products can be removed, and there is a free ferric enzyme. The enzyme that you start with is EPR silent. The yellow enzyme has high spin ferric iron.

MASON: What is the evidence for the existence of the ferrous - alkyl free radical state?

VLIEGENTHART: If you carry out the reduction of the yellow enzyme anaerobically with linoleic acid in the presence of a radical scavenger, it is possible to detect it by EPR.

COON: Is anything known about the function of soybean lipoxygenase?

VLIEGENTHART: No. The enzyme occurs in relatively high amounts in soybeans and in other seeds, but no reasonable suggestions have been made so far with respect to its physiological function. It is present in the dormant seeds. After about 40 hours of germination, the enzyme goes down. It may be that if it has any role, it is exerted during the early phase of germination.

WALLING: As I understand it then, this enzyme does two things in a row: first it makes the hydroperoxide and then it reacts with it. Chemically, how do you picture this first step in which H• is removed from linoleic acid?

VLIEGENTHART: We think that iron is directly involved, or via a mediator, in the abstraction of the hydrogen atoms. As soon as you have H• the reaction goes on smoothly.

WALLING: One way of picturing it would be to simply take an electron out of the double bond, to give a radical cation which would lose a proton. But this takes a very high oxidation-reduction potential.

PEISACH: A possible clue to the puzzle--can you convert the native enzyme to the yellow enzyme by adding ferricyanide?

VLIEGENTHART: No. It doesn't go.

PEISACH: Oh, it doesn't go! That's a clue to the redox potential.

VLIEGENTHART: Let us not say that it is in principle impossible to oxidize the iron; but at least it doesn't react with the reagent.

SPIRO: Does the reduced enzyme react with O_2?

VLIEGENTHART: We think the reduced enzyme is a complex of Fe and O_2.

SPIRO: I am talking about the color that is formed.

VLIEGENTHART: The native enzyme is colorless, but we think that there is a difference between the native enzyme under aerobic and anaerobic conditions.

SPIRO: The colorless enzyme is the anaerobic one, is that right?

VLIEGENTHART: That doesn't matter. The native enzyme is colorless under both aerobic and anaerobic conditions.

SPIRO: You think there is an O_2 complex without any color?

VLIEGENTHART: Yes, why not?

SPIRO: I would be surprised if the oxygen were close to ferrous ion without generating the chromophore.

PEISACH: I'm at the point that since the NO reacts with ferrous enzyme and generates a color, one would certainly think that CO or O_2 would generate a colored species as well.

VLIEGENTHART: There is no color whatsoever with CO.

MASON: Can the O_2 be pumped off the resting form of the enzyme?

VLIEGENTHART: I think this can be done, yes. We are doing some experiments trying to quantify it, to prove our hypothesis that it is a complex of Fe and O_2.

MASON: Maybe that's the complex that makes the first alkyl free radical. When you react the enzyme with lipid peroxide, under aerobic conditions, the enzyme goes through yellow and purple forms. Do you get the same results under anaerobic conditions?

VLIEGENTHART: No, if it is carried out under anaerobic conditions, the products are far better defined. For example, going from purple to yellow enzyme, if the natural conversion takes place then an expoyhydroxy compound can be isolated as the only major product, only if it is carried out under anaerobic conditions. When oxygen is present, more polar products are formed. That's pretty reasonable because the reaction proceeds by radical intermediates, and these radical intermediates react with O_2.

FEE: If you start with resting or yellow enzyme and add an excess of LOOH to that, isn't that when you go to the purple compound?

VLIEGENTHART: If it is a molar excess of LOOH, then you go directly to the purple form, via the yellow intermediate. The first equivalent of hydroperoxide is necessary to get the yellow form plus decomposition products of hydroperoxide, and

the main component in that mixture is the ketodiene. The yellow enzyme with an excess of hydroperoxide gives a blue complex. This blue complex is relatively unstable and decomposes into yellow enzyme and conversion products of the hydroperoxide. Under these conditions, the main product is an epoxyhydro compound.

FEE: I just want to understand: does the purple compound depend upon the presence of molecular oxygen?

VLIEGENTHART: No. Nothing to do with it. It is as stable under aerobic as under anaerobic conditions.

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