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DEMONSTRATION BY EPR SPECTROSCOPY OF THE FUNCTIONAL ROLE OF IRON IN SOYBEAN LIPOXYGENASE-1

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Summary

1. The EPR spectrum at 15°K of soybean lipoxygenase-1 in borate buffer pH 9.0 has been studied in relation to the presence of substrate (linoleic acid), product (13-L-hydroperoxylinoleic acid) and oxygen.

2. The addition of 13-L-hydroperoxylinoleic acid to lipoxygenase-1 at pH 9.0 gives rise to the appearance of EPR lines at $g = 7.5$, 6.2, 5.9 and 2.0, and an increased signal at $g = 4.3$.

3. In view of the effect of the end product on both the kinetic lag period of the aerobic reaction and the fluorescence of the enzyme, it is concluded that 13-L-hydroperoxylinoleic acid is required for the activation of soybean lipoxygenase-1. Thus it is proposed that the enzyme with iron in the ferric state is the active species.

4. A reaction scheme is presented in which the enzyme alternatingly exists in the ferric and ferrous states for both the aerobic and anaerobic reaction.

Introduction

Lipoxygenase (linoleate: oxygen oxidoreductase, EC 1.13.11.12) is widely distributed in the plant kingdom. In the presence of molecular oxygen the enzyme catalyses the oxidation of polyunsaturated fatty acids, which contain a 1,4-*cis*,*cis*-pentadiene system. Soybean lipoxygenase-1 converts linoleic acid mainly into 13-L-hydroperoxylinoleic acid at pH 9.0 [1]. Several details of the reaction mechanism are known: The rate-determining and probably first step is the removal of the 11-pro-L_S(n-8) hydrogen [2]. The stereospecific introduction of oxygen then proceeds antarafacially with respect to the removed hydrogen [3].

The product hydroperoxide plays a peculiar role in the reaction, since the

induction period, observed when soybean lipoxygenase-1 is incubated with hydroperoxide-free linoleic acid, can only be abolished by addition of 13-L-hydroperoxylinoleic acid [4,5], whereas, 9-D-hydroperoxylinoleic acid is ineffective [6]. A further indication for the interaction of soybean lipoxygenase-1 and 13-L-hydroperoxylinoleic acid stems from investigations by Finazzi-Agrò et al. [7]. They showed that the fluorescence of the enzyme at 328 nm on excitation at 280 nm is quenched by replacing oxygen in the solution by argon, and that a further quenching is obtained by the addition of 13-L-hydroperoxylinoleic acid. One mole of the hydroperoxide per mole of enzyme already causes the maximum effect.

Furthermore, soybean lipoxygenase-1 is capable of converting 13-L-hydroperoxylinoleic acid, provided that substrate linoleic acid is present, and that oxygen is excluded [8]. In this anaerobic reaction oxodienoic acids, *n*-pentane and dimeric fatty acids [9] are formed. The involvement of linoleic acid radicals in this reaction makes it very probable that at least the anaerobic reaction proceeds along a free-radical mechanism [10].

A new feature has been added to our knowledge of lipoxygenase by the demonstration of the presence of one mole of iron per mole of enzyme [7,11–13], although its role in the enzyme catalysis is still unknown.

The present paper reports on EPR measurements of lipoxygenase, incubated under aerobic and anaerobic conditions with substrate, product or both. The results lend support to the view that iron plays an essential role in the enzymic activities of soybean lipoxygenase-1.

Materials and Methods

Lipoxygenase-1 was isolated from soybeans according to the method of Finazzi-Agrò et al. [7] and was kindly supplied by the Unilever Research Laboratories, Vlaardingen/Duiven, The Netherlands. Iron content: 0.90 gatom Fe per mole of enzyme (mol. wt 98 600) as determined by atomic absorption spectrometry. Content of copper and manganese: less than 0.1 gatom per mole of enzyme.

The lipoxygenase solution was brought at pH 9.0 by washing the solution with 0.2 M borate buffer pH 9.0 through a Diaflo UM 20E membrane (Amicon).

Linoleic acid was a gift from Unilever Research Laboratories, Vlaardingen/Duiven, The Netherlands (purity >99%).

13-L-hydroperoxylinoleic acid was prepared by incubating linoleic acid with soybean lipoxygenase-1 in a 0.2 M borate buffer pH 9.0 [14]. The samples contained a few percent of 9-hydroperoxylinoleic acid.

All reagents used for EPR measurements were dissolved in 0.2 M borate buffer pH 9.0 (oxygen concentration 240 μM at room temperature).

Anaerobiosis was achieved by repeated evacuation and flushing with N_2 gas washed through an alkaline pyrogallol column containing catalytic amounts of iron and copper ions [15,16].

Spectra were recorded using a Varian E-9 EPR spectrometer with 100 Kcycles/s field modulation (cf. ref. 16), at a microwave power of 4 mW. All measurements were carried out at 15°K, modulation amplitude of 10 G and at the same gain.

Results

Fig. 1 shows the EPR spectrum at 15°K of lipoxygenase. The signal at g = 4.3, known to be characteristic of the high-spin state of ferric ions in a ligand field of rhombic symmetry [17], is relatively low in intensity. Probably it stems from contaminating iron. Chemically it has been demonstrated that the purified enzyme still contains traces of copper and manganese, giving rise to the complex signals around g = 2. Removal of oxygen by evacuation has no effect on the EPR spectrum.

Since 13-L-hydroperoxylinoleic acid affects both the kinetic lag phase of the aerobic reaction and the fluorescence of the enzyme, we first investigated the effect of this hydroperoxide on the EPR spectrum of lipoxygenase-1. Under aerobic conditions (Fig. 2, spectrum a) addition of 13-L-hydroperoxylinoleic acid to lipoxygenase results in the appearance of EPR lines at g = 7.5, 6.2 and 5.9. The latter line is only observed as a shoulder on the resonance at g = 6.2. Furthermore, the spectrum shows an increase of the intensity of the signal at g = 4.3 and the appearance of a free radical type of signal at g = 2.0.

In the absence of oxygen a similar spectrum is obtained (Fig. 2, spectrum b), but the intensities of the resonances at g = 7.5, 5.9 and 2.0 are considerably lower than under aerobic conditions (cf. Fig. 2, spectrum a).

Fig. 3 shows the EPR spectrum after the incubation of linoleic acid with lipoxygenase under aerobic conditions. The signal at g = 4.3 is significantly increased and lines at g = 7.5, 6.2, 5.9 and 2.0 are observed. Under anaerobic conditions, however, no effect of linoleic acid on the EPR spectrum of lipoxygenase (cf. Fig. 1) is observed, suggesting that the 13-L-hydroperoxide is responsible for the appearance of the EPR signals.

When the anaerobic reaction is carried out in the presence of an excess of linoleic acid with respect to 13-L-hydroperoxylinoleic acid (molar ratio, 4 : 1) the EPR spectrum (Fig. 4, spectrum a) is identical to that of lipoxygenase as

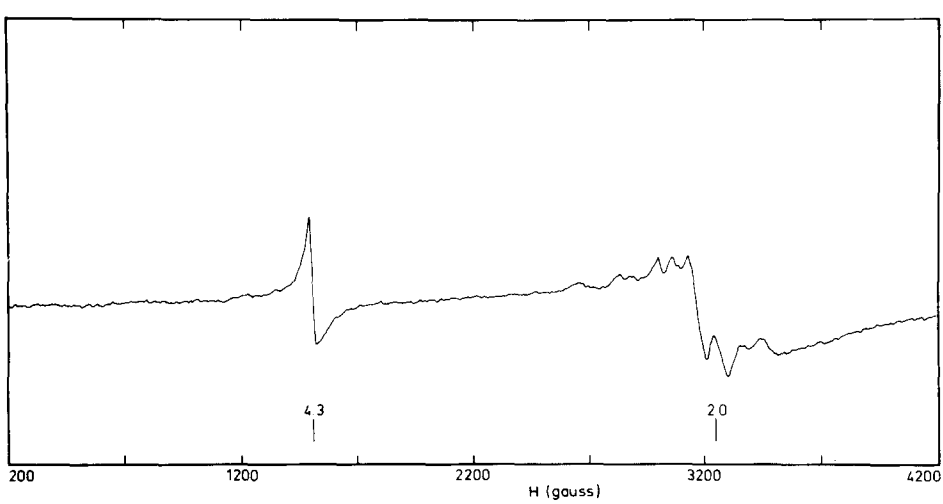

Fig. 1. EPR spectrum of soybean lipoxygenase-1. Protein concentration, 26 mg/ml in borate buffer pH 9.0. Microwave frequency, 9.087 GHz.

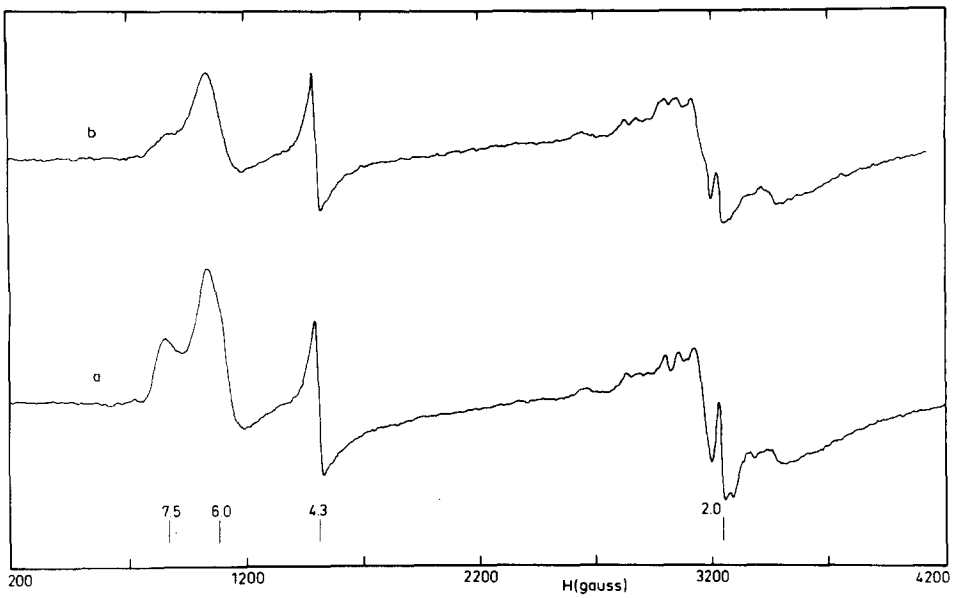

Fig. 2. Effect of 13-L-hydroperoxylinoleic acid on the EPR spectrum of lipoxygenase: a, under aerobic conditions 15 μl of a 5.67 mM hydroperoxide solution was added to 300 μl of a lipoxygenase solution (26 mg/ml). Final concentrations: 0.27 mM and 0.25 mM respectively in borate buffer pH 9.0. Microwave frequency, 9.079 GHz; b, under anaerobic conditions 20 μl of a 5.67 mM hydroperoxide solution was added to 400 μl of a lipoxygenase solution (26 mg/ml). Final concentrations: 0.27 mM and 0.25 mM respectively in borate buffer pH 9.0. Microwave frequency, 9.086 GHz.

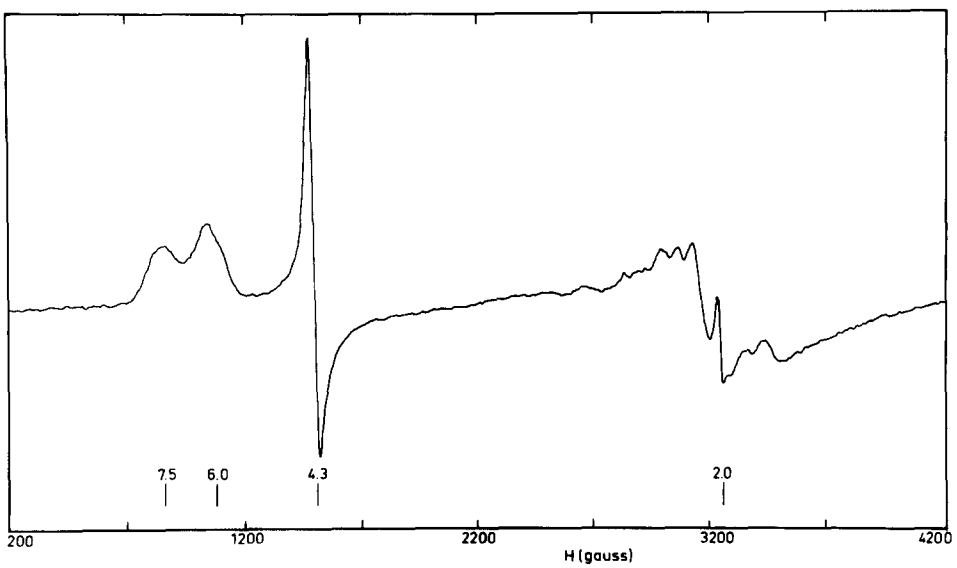

Fig. 3. EPR spectrum of the incubation mixture of 300 μl of a lipoxygenase solution (26 mg/ml) and 100 μl of a 5.67 mM linoleic acid solution under aerobic conditions. Final concentrations: 0.19 mM and 1.42 mM respectively in borate buffer pH 9.0. Microwave frequency, 9.087 GHz.

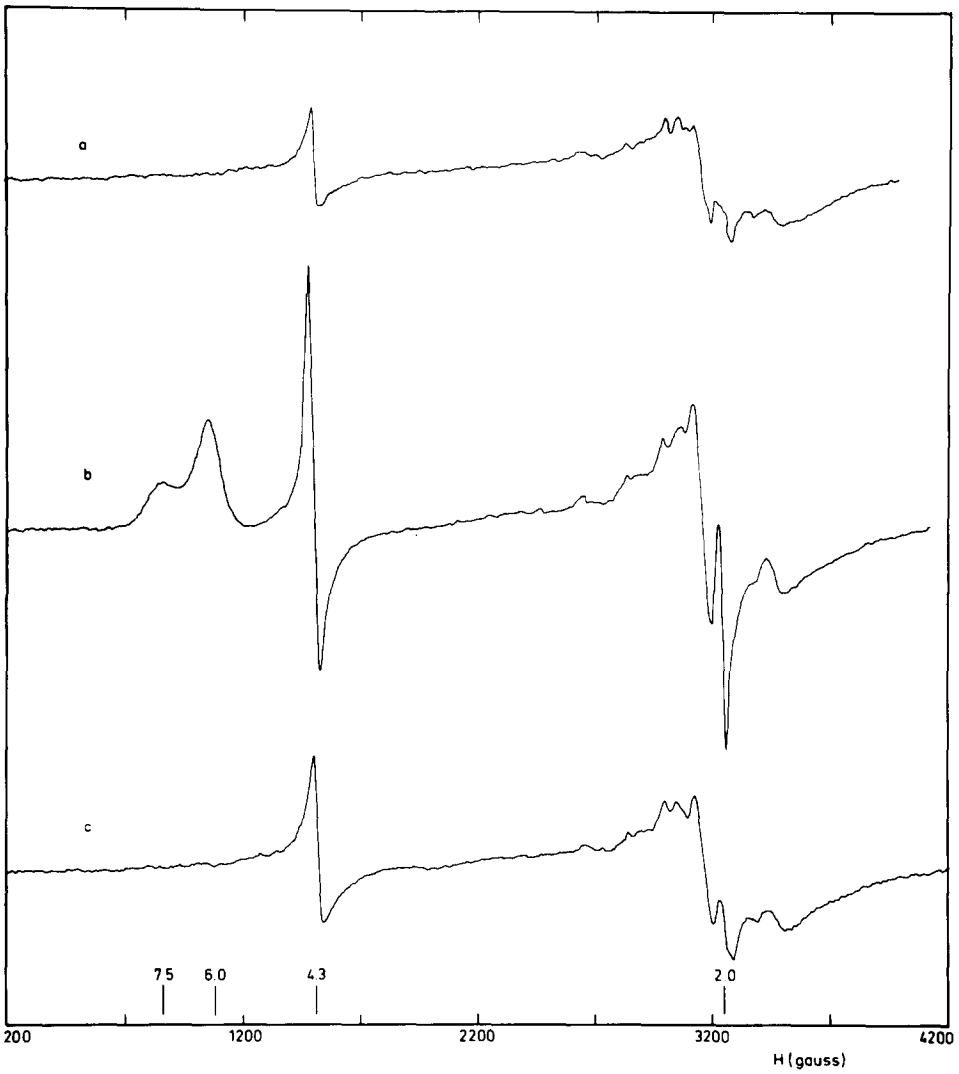

Fig. 4. EPR spectra of the successive phases during the cycling between anaerobic and aerobic reaction conditions: a, the anaerobic incubation of 400 µl of a lipoxygenase solution (26 mg/ml), 25 µl of a hydroperoxide solution (5.67 mM) and 100 µl of a linoleic acid solution (5.67 mM), final concentrations: 0.20 mM, 0.27 mM and 1.08 mM, respectively; b, after bubbling oxygen through the sample for about one minute; c, after the addition of 150 µl of a linoleic acid solution (5.67 mM) under strictly anaerobic conditions. After each measurement the sample was thawed and brought at room temperature for another addition. Microwave frequency, 9.082 GHz.

such (cf. Fig. 1). This is due to the complete consumption of the product hydroperoxide. After the conversion of the remaining linoleic acid into 13-L-hydroperoxylinoleic acid by thawing the sample and subsequent bubbling of oxygen through the incubation mixture, the EPR spectrum shows lines at g = 7.5, 6.2, 5.9 and 2.0 (Fig. 4, spectrum b) and an intensified signal at g = 4.3. When the incubation mixture is again freed of oxygen and a 1.5 molar excess of linoleic acid is added anaerobically, the hydroperoxide is again removed en-

zymically, resulting in the disappearance from the EPR spectrum of the signals at g = 7.5, 6.2, 5.9 and 2.0 and a decrease of the signal at g = 4.3 (Fig. 4c).

The whole sequence of reactions has been repeated giving the same results.

Discussion

Previously, it has been demonstrated that iron is a constituent of lipoxygenase [7,11—13]. The present EPR study provides evidence that high-spin ferric iron, with an EPR signal at g = 4.3 as well as other paramagnetic species with EPR lines at g = 7.5, 6.2 and 5.9 are involved in the enzymic reaction. Although in the EPR spectrum the signals around g = 6 point to the presence of an iron—porphyrin system, the optical absorption spectrum does not show any features characteristic of a hemoprotein [7]. Furthermore no prosthetic group could be removed from the enzyme by treatment with acetone—HCl. Therefore it is likely, that the iron atom is coordinated to amino acid residues of the polypeptide chain. Since iron is the only metal constituent of the enzyme, the signals around g = 6 are most likely due to iron. It should be noted, however, that in biological systems no comparable resonances for non-heme iron compounds have been described. The present data do not allow an unambiguous assignment of the spin states of the iron. On theoretical grounds [18—21] it is unlikely that the signals stem from low-spin iron. The effect of oxygen (cf. Fig. 2) on the intensities of the resonances at g = 6.2 and those at g = 7.5 and 5.9 indicates that at least two paramagnetic species are present. Interestingly, the radical type of signal at g = 2.0 is also dependent on the presence of oxygen.

In the native resting enzyme one of the ligands of iron is dioxygen [7], as was proposed on basis of the effect of hydroperoxide and/or oxygen on the fluorescence behaviour of the enzyme. It is firmly established [4,5,7], that soybean lipoxygenase-1 interacts with 13-L-hydroperoxylinoleic acid, the product of the enzymic oxygenation of linoleic acid. Our EPR experiments show that the product reacts with the enzyme both in the absence and presence of oxygen giving rise to paramagnetic ferric species. Since the hydroperoxide abolishes the kinetic lag phase of the aerobic reaction [4,5], the ferric species is considered to be the enzymically active form. Hence, it can be concluded that the enzyme is activated by its own product. The present observations, together with previous interpretations of the aerobic [2,3,5] and anaerobic reaction [8—10], may adequately be presented in the following scheme (Fig. 5).

The reaction of the native resting enzyme ($E—Fe^{2+}—O_2$) with one equivalent of the hydroperoxide yields the active form of the enzyme. In this step it is proposed that a one-electron transfer takes place from iron to either oxygen or to the hydroperoxide. The complexed oxygen is released, as the superoxide anion or as excited oxygen [7]. The hydroperoxide, utilized for the activation is converted into yet unidentified products. It is conceivable that the conversion of the hydroperoxide is due to a reaction with the released oxygen.

The activated enzyme ($E—Fe^{3+}$) is reduced to the ferrous state by the substrate fatty acid (e.g. linoleic acid) with concomitant formation of the fatty acid free-radical [3,10]. In the aerobic cycle, oxygen then combines stereospecifically [2,22] with the fatty acid free-radical—enzyme complex which is

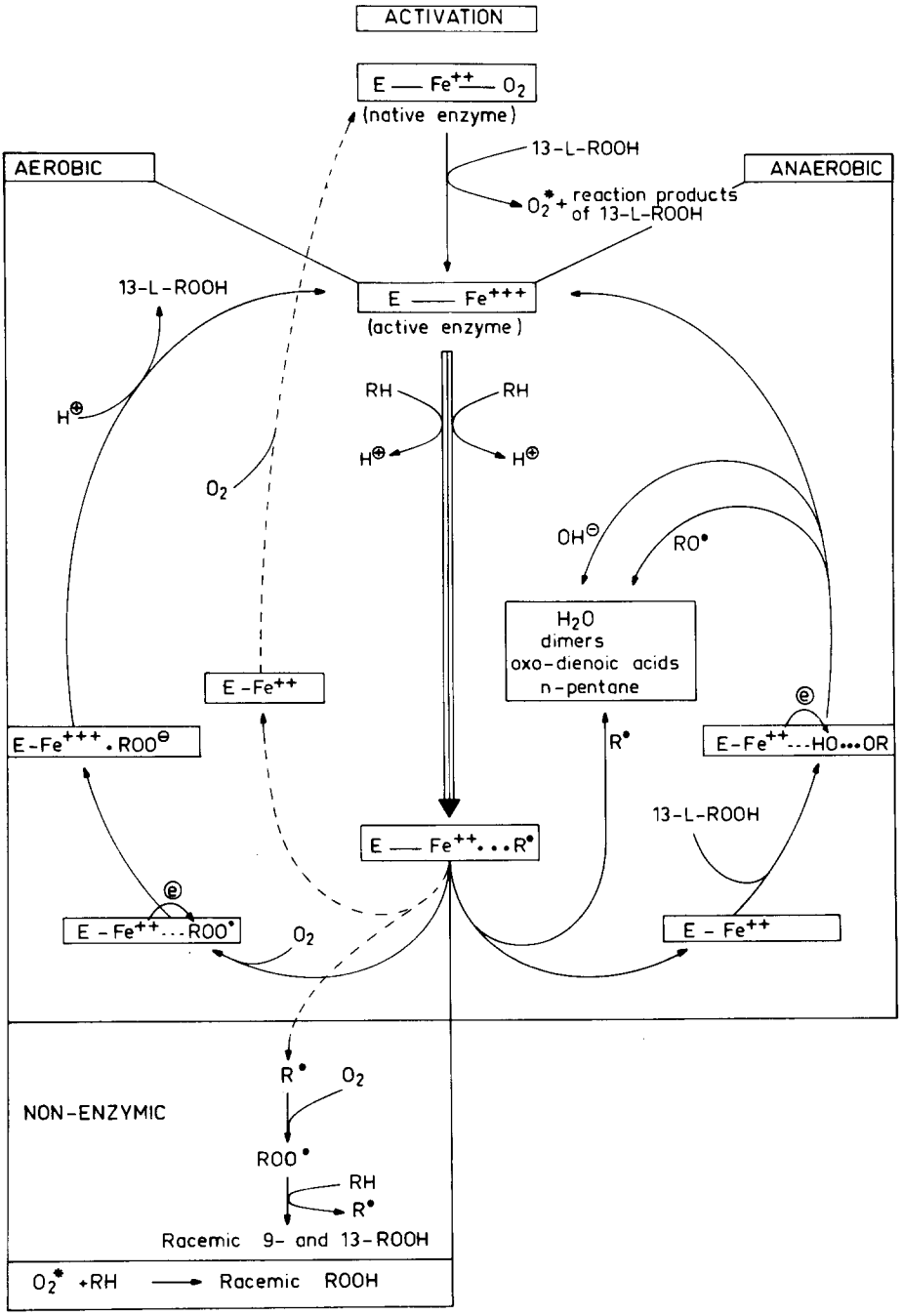

Fig. 5. Proposed reaction scheme for the activation of soybean lipoxygenase-1 and for the catalytic activities at pH 9.0 under aerobic and anaerobic conditions. RH = fatty acid.

probably followed by a one-electron transfer from iron to the peroxy-radical. After protonation of the peroxy-anion the free active enzyme ($E—Fe^{3+}$) is regenerated and thus the aerobic cycle is completed.

Under anaerobic conditions, in the presence of both substrate (fatty acid) and product (hydroperoxide) it is proposed that a dissociation of the fatty acid free-radical—enzyme complex takes place, leading to the free enzyme in its ferrous state ($E—Fe^{2+}$). The latter enzyme species is oxidized to the active ferric state ($E—Fe^{3+}$) by the product (hydroperoxide) under the formation of alkoxy free-radicals and hydroxyl ions. The end products of the anaerobic reaction are derived from further reactions of fatty acid free-radicals, alkoxy free-radicals and fatty acid [9].

As has been shown (Fig. 4) the anaerobic reaction proceeds to a stage, in which the enzyme is in the ferrous or ferric state, depending on the relative amounts of fatty acid and hydroperoxide added. These observations can be readily reconciled with the proposed anaerobic cycle (Fig. 5). After completion of the anaerobic reaction the enzyme is present in the ferric state when the reaction was carried out with an excess of hydroperoxide; however, the enzyme occurs in the ferrous state in the case that linoleic acid prevails.

During the oxygenation of linoleic acid by soybean lipoxygenase-1 a few percent of racemic 13-hydroperoxide and 9-hydroperoxide are formed [22]. This side reaction, observed under aerobic conditions can partly be explained by dissociation, to a small extent, of the fatty acid free-radical—enzyme complex followed by aspecific oxygenation of the fatty acid free-radical. By consequence a proportional additional amount of hydroperoxide will be needed for the regeneration of the active enzyme. Another part of the racemic hydroperoxides might originate from the aspecific oxidation of linoleic acid with activated oxygen, generated during activation of the enzyme.

Note added in proof (received November 25, 1974)

After this manuscript was in preparation, Pistorius and Axelrod [23] reported some preliminary EPR data on soybean lipoxygenase-1.

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