



Understanding the radical mechanism of lipoxygenases using ^{31}P NMR spin trapping

Luca Zoia^c, Raffaella Perazzini^d, Claudia Crestini^d, Dimitris S. Argyropoulos^{a,b,*}

^a Organic Chemistry of Wood Components Laboratory, Department of Forest Biomaterials, North Carolina State University, NC 123321, USA

^b Department of Chemistry, University of Helsinki, PO Box 55, 00014, Finland

^c Dipartimento di Scienze dell'Ambiente e del Territorio, Università degli Studi di Milano-Bicocca, Piazza della Scienza 1, 20126 Milano, Italy

^d Dipartimento di Scienze e Tecnologie Chimiche, Università di Tor Vergata, Via della ricerca Scientifica, 00133 Roma, Italy

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ABSTRACT

In this paper, we use our quantitative ^{31}P NMR spin trapping methods, already developed for simple oxygen- and carbon-centered radicals, to understand the radical intermediates generated by enzymatic systems and more specifically lipoxygenases. Our methodology rests on the fact that free radicals react with the nitroxide phosphorus compound, 5-diisopropoxy-phosphoryl-5-methyl-1-pyrroline-*N*-oxide (DIP-PMPO), to form stable radical adducts, which are suitably detected and accurately quantified using ^{31}P NMR in the presence of a phosphorus containing internal standard. This system was thus applied to better understand the mechanism of enzymatic oxidation of linoleic acid by soybean lipoxygenases-1 (LOX). The total amount of radicals trapped by DIPPMPO was detected by ^{31}P NMR at different experimental conditions. In particular the effect of dioxygen concentration on the amount of radicals being trapped was studied. At low dioxygen concentration, a huge increase of radicals trapped was observed with respect to the amount of radicals being trapped at normal dioxygen concentrations.

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1. Introduction

Lipoxygenases (LOXs) form a heterogeneous family of lipid peroxidizing enzymes that catalyze dioxygenation of polyunsaturated fatty acids (PUFA) to their corresponding hydroperoxy derivatives.¹ In animal tissues, the most common substrate encountered is the arachidonic acid (C20:4), which is dioxygenated by lipoxygenases into precursors of products involved into inflammatory processes,² cell membranes maturation,³ cancer metastases,^{4,5} atherogenesis^{6,7} and osteoporosis.⁸ The role of plant lipoxygenases, whose main substrates are linoleic (C18:2) and linolenic acids (C18:3), is not very fully elucidated, although they are implied in processes such as senescence or plant response to wounding.⁹

A single non-heme iron is present in each enzyme and it exists in two oxidation states: Fe(II) and Fe(III).¹⁰ According to the current working mechanism,^{10,11} the native enzyme is inactive in the Fe(II) form. When treated with an equimolar amount of product, the iron is oxidized to the Fe(III) form, resulting in an active enzyme. The ferric form can then catalyze the abstraction of a hydrogen from the bis-allylic carbon atom of the substrate from 11 position of linoleic acid, in a stereospecific manner, yielding a pentadienyl radical complexed with the ferrous enzyme.

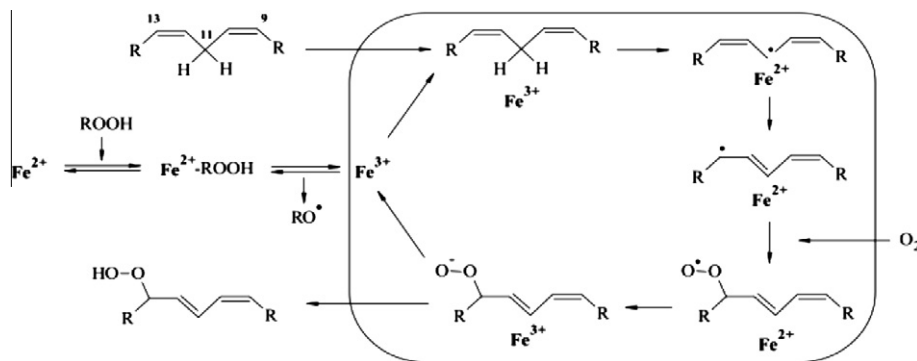
Bimolecular oxygen is then inserted to the pentadienyl radical, through a channel in the lipoxygenase, which leads to the formation of the hydroperoxide product and the reoxidation of the cofactor to the ferric form (Scheme 1).

Although the LOX-reaction involves the formation of radical intermediates it may not be considered an effective source of free radicals as most of the intermediates remain enzyme bound. However, under certain conditions a considerable portion of radicals may escape the active site leaving the enzyme in the inactive form Fe(II).^{12,13} These observations were based on the change in LOXs regioselectivity at different dioxygen concentrations. In fact, under most conditions, soybean lipoxygenase-1 is highly specific for the position at which the dioxygen is inserted (position 13). However, this specificity can be greatly influenced by the dioxygen concentration.¹³

In order to obtain direct evidence of the increase in the radical escaping mechanism from the enzyme active site at different dioxygen concentrations, a spin trap technique could be applied. Using the 5-diisopropoxy-phosphoryl-5-methyl-1-pyrroline-*N*-oxide (DIPPMPO) as spin trap, the presence of phosphorus allows for the use of phosphorus nuclear magnetic resonance (^{31}P NMR) spectrometry to investigate the detailed chemistry of the radical reaction. ^{31}P NMR could be exploited to perform quantitative analyses, in the presence of a suitable internal standard.¹⁴ Early qualitative work showed that the chemical shift of the ^{31}P atom depends on the nature of the adducts.^{15,16}

* Corresponding author. Tel.: +1 919 515 7708; fax: +1 919 515 6302.

E-mail address: dsargyro@ncsu.edu (D.S. Argyropoulos).



Scheme 1. Partial reaction mechanism for soybean-1 lipoxygenase.

In the present study we have attempted to determine and explain the influence of dioxygen concentration on soybean lipoxygenase-1 radical generation. The dioxygen concentration in a reaction solution is a function of two parameters: the initial dioxygen concentration and the rate of consumption. As such we have systematically varied the initial and subsequent oxygenation conditions (argon headspace, air bubbling) and have been able to determine and quantify the radical intermediates involved under the different conditions.

2. Experimental procedures

2.1. Chemicals

Soybean lipoxygenase-1 (type I-B, activity 150,000 U/mg), linoleic acid and all chemicals were purchased from Sigma (St. Louis, MO, USA) and used as received. DIPPMPO was synthesized as describe below. All reagents and buffers were prepared using Millipore MilliQ deionized water ($\rho = 18 \text{ M}\Omega \text{ cm}$).

2.2. Synthesis of DIPPMPO

DIPPMPO (5-diisopropoxyphosphoryl-5-methyl-1-pyrroline-*N*-oxide) was synthesized according to the literature.¹⁷ The spin trap was stored under argon at -78°C .

2.3. Linoleic acid oxidation by lipoxygenase

The linoleic acid oxidation was carried out at room temperature at three different experimental conditions: in an atmosphere of air with continuous bubbling and magnetic stirring, in an idle atmosphere without stirring and in a closed reaction vial whose headspace was purged with argon. Briefly, in a glass reaction vial equipped with a screw cap (inner volume 70 mL), different amounts of linoleic acid (3.25, 5.0, 6.3 and 13 μL) were dissolved in 65 mL of 0.1 M carbonate buffer (pH 9.0) containing 0.01 M of EDTA (ethylenediaminetetracetic acid), corresponding to 160, 247, 311 and 642 μM concentration, respectively. In order to completely dissolve the substrate, 2–3 drops of a NaOH 5% were added. For the spin trapping experiments, 5 mg of DIPPMPO were added to the mixture. Finally different amounts of soybean lipoxygenase-1 were added (9.0, 12.5, 17.5, 36 and 90 nM). After 30 min the solution was acidified with a 1.0 M HCl solution to pH 4 and extracted three times with chloroform. Then the organic layer was recovered, dried with Na_2SO_4 and evaporated under reduce pressure. The residue was dissolved in CDCl_3 for ^{31}P NMR analyses.

2.4. LOX reaction analysis

The LOX reaction was followed spectro photometrically by measuring the increase in absorbance at 234 nm, using a ϵ of

$25,000 \text{ M}^{-1} \text{ cm}^{-1}$. For photometric measurements a Hewlett Packard 8453E spectrophotometer was used.

2.5. ^{31}P NMR spectra

^{31}P NMR spectra were acquired on a Bruker-300 spectrometer (operating at 121.49 MHz). The chemical shifts reported are relative to external orthophosphoric acid (85%). All spectra were acquired with proton decoupling. The total number of scans for all experiments was 256–1024 with an acquisition time of 1.60 s. Trimethylphosphate was used as the internal standard for quantification and added to the sample prior to measurement. The results reported are means \pm SD, for three individual experiments.

3. Results and discussion

Soybean lipoxygenase-1 shares good sequence identity with mammalian lipoxygenases, and its X-rays crystal structure reveals similar details with that of the rabbit 12/15-lipoxygenase. For this reason, soybean lipoxygenase-1 has been used in place of the mammalian enzyme.^{18–20} Qian et al.^{21–23} examined the free radical generation in the reaction between soybean lipoxygenase-1 and linoleic acid by using nitron spin trapping including DMPO (5,5'-dimethyl-1-pyrroline-*N*-oxide) and POBN (α -(4-pyridyl-1-oxide)-*N*-*t*-butylnitron). Koshiishi et al.²⁴ have studied the same reaction in the presence of CmP (3-carbamoyl-2,2,5,5-tetramethyl-pyrrolidine-*N*-oxyl), a well-examined and convenient nitroxyl radical, as an indicator for redox balance and free radical reactions. In the present study, we used DIPPMPO (5-diisopropoxy-phosphoryl-5-methyl-1-pyrroline-*N*-oxide) as the spin trapping system to study the radicals involved in the linoleic acid oxidation catalyzed by lipoxygenase.

3.1. Identification of lipid-derived radical adducts

As shown on Figure 1, the radical specie (R^\bullet) reacts with the double bond of the nitron spin trap DIPPMPO (a) to form the paramagnetic adduct (b). The species (b) decays with time via different reactions (unimolecular and/or bimolecular processes) such as oxidation, reduction, dismutation and rearrangement. In many cases the products of the decomposition reactions are the diamagnetic corresponding hydroxylamine (c) and nitron (d).

These species are suitably detected and quantified using ^{31}P NMR in the presence of phosphorus containing internal standard. Moreover, the chemical shift of the phosphorus atom is related to the nature of the radical being trapped.^{14–16}

Figure 2 shows acquired typical ^{31}P NMR spectra obtained after the DIPPMPO trapping reaction that was carried out under a series of different oxygenation conditions. The experiments were run: (A)

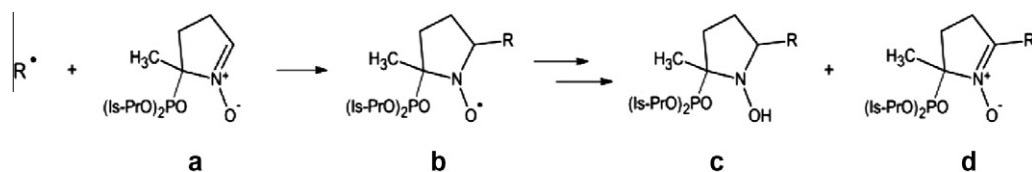


Figure 1. The general trapping chemistry of the DIPPMPO spin trap system.

in a glass vial with air saturated buffer whose head space was filled with argon, (B) in air without stirring, (C) with air bubbling and stirring. The initial dioxygen concentrations (approximately 260 μM) were in any case smaller than the substrate concentration (642 μM) and the amount of lipoxygenase was 36.0 nM in all the experiments. Under conditions (A) the dioxygen is limited, assuming a 1:1 stoichiometric reaction between dioxygen and linoleic acid, while the unreacted linoleic acid remained in solution. The amount of the resulting hydroperoxides, detected by UV, correlates with the initially dissolved dioxygen concentration in the reaction mixture (data not shown). Under conditions (B), the dioxygen concentration at some point of the reaction becomes limited due to the low dioxygen dissolution rate in the absence of stirring. This in turn affected the consumption of linoleic acid.¹³ Under conditions (C) the dioxygen concentration is not limited relative to the substrate linoleic acid since there was a continuous supply of air in the system. In this case a complete depletion of the substrate was detected by UV and all the linoleic acid was converted in the corresponding hydroperoxide.

In the ^{31}P NMR spectra of Figure 2 the large signal at 22.2 ppm is due to the original and unreacted spin trap. In the case of linoleic acid oxidation under conditions (A), the ^{31}P NMR spectrum showed the presence of different adducts at 23.2 and 29.5 ppm (Fig. 2A). On the basis of the chemical shift values, the adducts have been interpreted as carbon-centered radicals.^{14–16} As reported by Khramtsov et al.,²⁵ during the trapping of methyl radicals with DEPMPO, the ^{31}P NMR spectrum showed the presence of three radical adduct decomposition products at: 24.5, 30.8 and 32.3 ppm, respectively. The peak at 24.5, due to the similarity with the original spin trap (DEPMPO 23.7 ppm) was related to the nitronium product decay (1), while the peaks at 30.8 and 32.3 ppm were assigned to the two stereoisomeric hydroxylamines (2). In our work, the trapped methyl radical with DIPPMPO resulting in the nitronium form was detected at 23.1 ppm¹⁴ and other studies confirmed the radical

adduct structure by GC–MS.²⁶ In view of this data, the peak at 23.2 ppm in the linoleic/lipoxygenase oxidation system was related to the nitronium form (1) of the radical adduct decay products (Fig. 3).

In fact the nitronium is shifted by 1.0 ppm downfield from the parent spin trap, as observed for the methyl radical (0.8 for DEPMPO and 0.9 ppm for DIPPMPO).^{14,25} In the same manner the peak observed at 29.5 ppm was related to the hydroxylamine degradation product (2). The difference between these adducts and the parent spin trap (7.3 ppm downfield) matches with the difference between the DEPMPO parent spin trap and the hydroxylamine of the methyl radical adduct (7.1 and 8.6, respectively, for the two stereoisomers). Moreover, the peak related to the hydroxylamine appears broad, possibly indicating the presence of different diastereoisomers.

During linoleic acid oxidation catalyzed by lipoxygenase, the enzyme reacts with the substrate by hydrogen abstraction from the bisallylic methylene position, as reported by De Groot et al.¹⁰ The enzyme/substrate radical complex (LOX-R \cdot) may react with molecular dioxygen to form an enzyme/peroxyl radical complex (LOX-ROO \cdot). This complex is stabilized during the catalytic cycle via an intracomplex electron transfer process, which reduces the radical to the corresponding anion (ROO $^-$). Alternatively, under anoxic conditions, the LOX-R \cdot could decay liberating the inactive enzyme and the substrate radical R \cdot . The carbon-centered radicals, shown in Figure 3, are thus trapped by DIPPMPO and the nitroxide radical adducts, after decomposition reactions, are detected by ^{31}P NMR.

During reaction under conditions (B) (air without stirring) we detected similar radical adducts at 23.2 and 29.5 ppm. Moreover, additional signals were detected at 17.0 and 18.0 ppm (Fig. 2B). We interpreted these data as adducts formed by the trapping of oxygen-centered radicals (3) and (4). In actual fact, during the lipoxygenase cycle, the LOX-ROO \cdot complex, could decay releasing peroxyl radicals (ROO \cdot) in solution. The peroxyl radicals could be trapped by DIPPMPO. Furthermore, the peroxyl radicals could also be generated in solution via a stereo random oxygenation of the carbon centered radical R \cdot that has escaped from the lipoxygenase active site. As reported by Argyropoulos et al.,¹⁴ the HOO \cdot radical adducts were trapped with DIPPMPO and detected by ^{31}P NMR with their chemical shifts being at 16.9 and 17.1 ppm. The trapping reaction is shown in Figure 4.

Similar radical adducts have been reported by Reis et al. and detected by LC–MS.^{27,28} The authors have studied the radicals involved in the Fenton system oxidation of linoleic acid using DMPO as a spin trap. Using LC–MS they detected the formation of carbon- and oxygen-centered radical of linoleic acid. Partial confirmation of our conclusion was also established via the ^{31}P NMR spectra obtained under reaction conditions (C) (Fig. 2C). In the presence of a continuous stream of air and stirring, the spin trap DIPPMPO was able to trap only oxygen-centered radicals.

3.2. Quantification of radical adducts

The presence of an internal standard in our reactions offered the possibility to measure the radical concentrations being trapped by

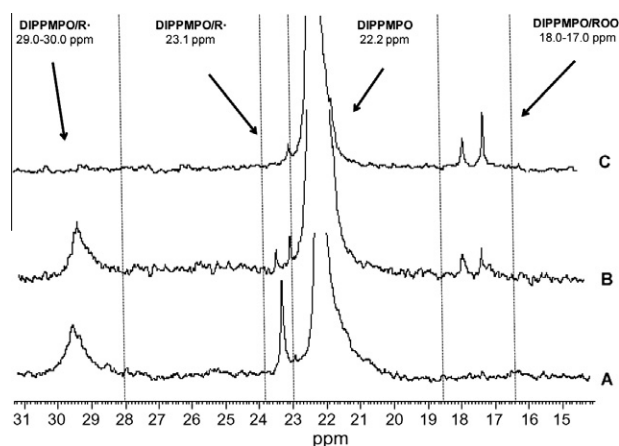


Figure 2. ^{31}P NMR spectra of DIPPMPO after reaction with linoleic acid in the presence of lipoxygenase. (A) In a glass vial with air saturated buffer and head space filled with argon; (B) in air without stirring; and (C) with a continuous air stream and stirring.

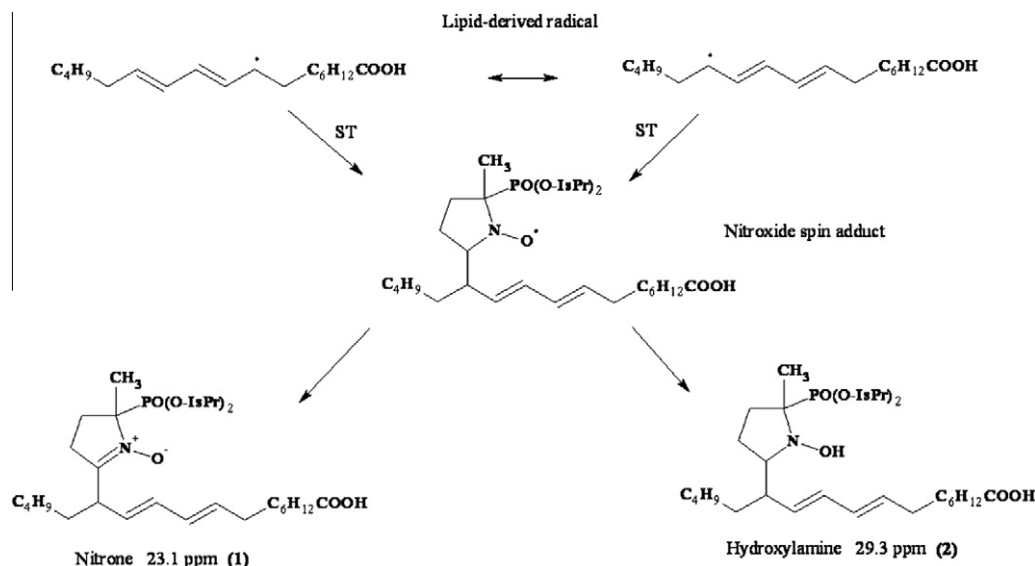


Figure 3. The trapping reaction of the lipid-derived carbon-centered radicals from linoleic acid/lipoxygenase system and the various decomposition reactions of the spin adducts with DIPPMPPO. For clarity the structure of the adducts are reported only for the 13 stereoisomers.

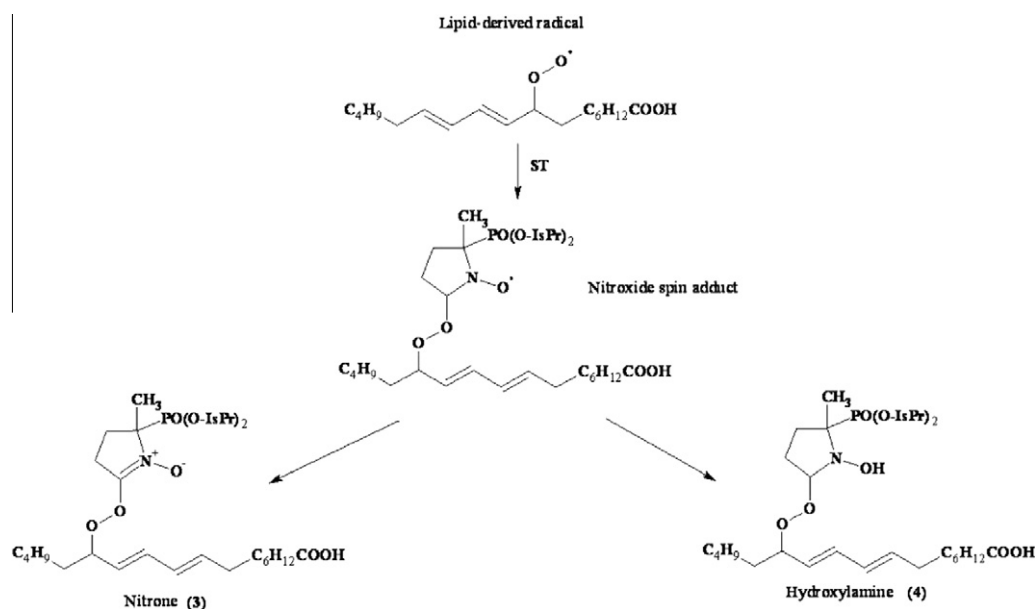


Figure 4. The trapping reaction of the lipid-derived oxygen-centered radicals from linoleic acid/lipoxygenase system and their relative decomposition reactions of the spin adducts with DIPPMPPO. For clarity, the structure of the adducts reported is only for the 13 stereoisomers.

DIPPMPPO. Consequently we explored the effect of the experimental conditions on the relative amount of free radicals present in solution. Initially one may compare the amount of radicals trapped by DIPPMPPO in the LOX/linoleic acid system under the three conditions examined (A), (B) and (C). The data of Figure 5 shows how these three different environments affected the total amount of the trapped radicals.

Furthermore, ^{31}P NMR spin trapping was applied in order to understand how the dioxygen concentration affected the lipoxygenase reactivity. To study this effect, the amount of radical adducts formed under two different oxygenation conditions was compared. The first series of experiments were carried out in an atmosphere of air and under vigorous stirring. This system permitted the maintenance of a constant dioxygen concentration (water saturated with air, containing approximately 260 μM of dioxygen). The second

series of experiments were conducted with the medium saturated with air but in a sealed reaction vessel whose head space was filled with argon. Under the conditions of the first series of experiments the dioxygen was not limited and the reaction occurred until the complete depletion of the linoleic acid substrate (examined by UV-vis measurements). In the second series of experiments the dioxygen was limited, being consumed by the linoleic acid oxidation reaction. Therefore, depending on the starting linoleic acid concentration (160, 247, 311 and 642 μM), a condition was eventually reached being varied from normoxic (linoleic acid 160 μM) to anoxic (linoleic acid 642 μM). The amount of lipoxygenase was 9.0, 12.5, 17.5 and 36.0 nM, respectively, in a constant ratio respect to the substrate. The amounts of total radicals being trapped and detected for these experiments are shown in Figure 6. The amount of radicals trapped by DIPPMPPO in the lipoxygenase/linoleic acid

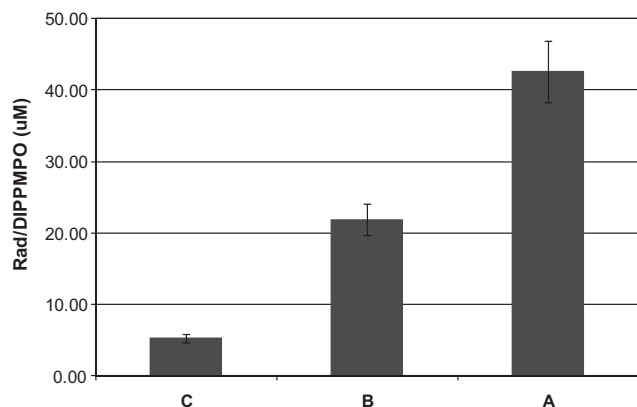


Figure 5. The effect of different experimental conditions on the total amounts of radical adducts: (A) in a glass vial with air saturated buffer and head space filled with argon; (B) in air without stirring; and (C) with a continuous air stream and stirring.

system under normoxic conditions (gray bars) was in all cases lower than ipoxic conditions (black bars). Thus, the increasing of the linoleic concentration increased the amount of radicals being trapped.

As reported by Berry et al.,¹³ when the dioxygen is not limited with respect to the linoleic acid substrate, the specificity of the lipoxygenase oxidation reaction is high: the hydroperoxy-octadecadienoic acid (HPOD) 13:9 isomer ratio was close to the generally reported 95:5. When the reaction buffer was bubbled with N₂ before the reaction, LOX was found to catalyze the oxygenation reaction with a specificity lower than that observed under higher dioxygen concentrations (13-HPOD/9-HPOD = 55:45). According to the mechanism reported in Scheme 1, it has been hypothesized that the complex enzyme–linoleic acid radical could dissociate instead of associating with O₂, leading to a release of radicals in solution. Under normal dioxygen concentrations (air bubbling), the amount of radicals released is low. As shown in Figure 6 (grey bar), the amount of radicals trapped was linearly correlated with the amount of starting linoleic acid, and the maximum radical adducts measured was 8.6 μM. The enzyme cycle is complete, with the formation of 13-HPOD as the major oxidation product. The percentage of radical adducts (2.7, 3.3, 5.3 and 8.7 μM) with respect to the initial linoleic acid (160, 247, 311 and 642 μM) is a constant value around 1.5%. These data are in agreement with the observed

specificity of 95:5. Under anoxic conditions (argon head-space), the amount of trapped radicals was found to be increased in a very significant way (Fig. 6, black bars). At a linoleic acid concentration of 160 μM the total amount of radicals trapped was found to be similar to those obtained under normoxic conditions. The amount of trapped radicals, however, at an initial linoleic acid concentration of 642 μM, was increased by about ten fold (8.6 vs 73.3 μM).

Assuming a stoichiometric ratio of 1:1 dioxygen and linoleic acid consumption, with an initial linoleic acid concentration of 160 μM and 247 μM, respectively, the dioxygen (260 μM) was not limiting during the reaction. At 311 and 642 μM initial linoleic acid concentration, during the reaction, the dioxygen started to be limiting. In this condition the amount of radicals released in solution and trapped increased. At the end of these reactions, the amount of radicals trapped was measured to be around the 12% of the starting substrate. Again this observation is in agreement with the observed reduction of specificity at low dioxygen concentration.¹³

Qualitative spectral observations showed that the radical adducts trapped under normoxic conditions were mainly oxygen-centered radicals (ROO[•]) while under anoxic conditions and at high linoleic acid concentrations they were mainly carbon-centered radicals. All these data are in agreement with the following qualitative mechanistic description: under normal oxygenation conditions the LOX system is a weak radical generator (low amount of R[•] could escape from the active site and it reacts with dioxygen in non stereospecific way, leading to the formation of oxygen-centered radicals trapped by DIPPMPPO). When the dioxygen starts to be limited the amount of radicals escaping the active site is higher. These radicals are then trapped mainly as carbon-centered radicals R[•] due to the low concentration of dioxygen.

In an effort to better integrate our knowledge, similar experiments were conducted at a constant LOX concentration (17.5 nM) (Fig. 7). As in the previous series of data the amount of radicals trapped increased when the linoleic acid concentration varied from lower (160 and 247 μM) to higher (311 and 642 μM) concentration with respect to the dioxygen (260 μM). In this series of experiments the radical adducts detected are not related to the initial linoleic acid concentration. This is because when the dioxygen is not limited the amount of radicals trapped was found to be around 7.0 μM, while at the end of reaction, when the dioxygen is depleted the radicals trapped were approximately constant (38.0 and 35.0 μM, respectively).

These data are indicative that the amount of radicals trapped may be related to the concentration of the complex LOX/Linoleic (Scheme 1). In order to confirm this hypothesis, we carried out a series of experiments under argon head-space conditions at a high

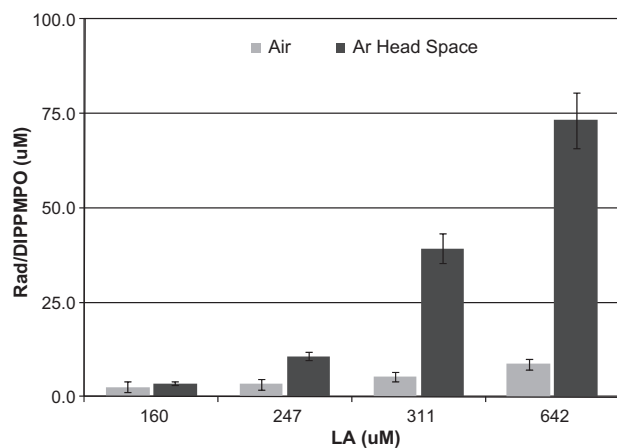


Figure 6. Radical adduct formation under normoxic conditions (with a continuous air stream and stirring, gray bars) and under anoxic conditions (in a glass vial with air saturated buffer and head space filled with argon, black bar).

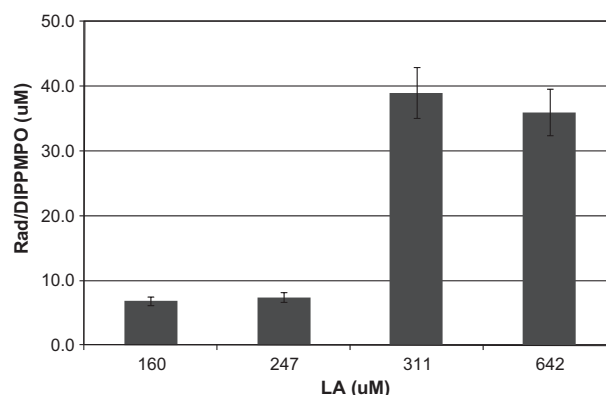


Figure 7. Total radicals trapped under argon head-space conditions at different initial linoleic acid concentrations and at a constant LOX concentration.

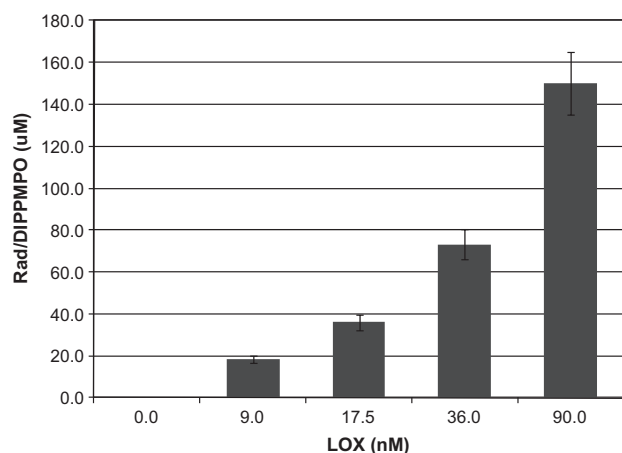


Figure 8. Total radicals trapped under argon head-space conditions at a constant initial linoleic acid concentration (642 μM) and at different LOX concentrations.

initial concentration of linoleic acid (642 μM) aimed to reach an anoxic condition during the reaction, under different LOX concentrations (Fig. 8). The data indicated that the amount of radicals trapped was linearly correlated with the LOX concentration.

By taking into account all the above data we arrived at the conclusion that the total amount of radicals generated is linearly related to the LOX concentration. Furthermore, if one considers separately the data from the experiments where the dioxygen concentration was not limited throughout the reaction versus the experiments where the dioxygen concentration was completely depleted, the correlations showed two different slopes (Fig. 9).

Overall our data can be rationalized by considering that during the linoleic acid oxidation the enzyme exists as a complex with the allyl radical. Under normal conditions the dioxygen is inserted in position 13 leading to the formation of the linoleic acid hydroperoxide. Part of the complex could then release the radical in solution. This natural decay is described quantitatively by the linear coefficient 0.24. When the dioxygen concentration is low, the release of the radical becomes more important as a reaction pathway. This reactivity is quantitatively describes by the linear coefficient 1.65 (Fig. 9).

Moreover, the literature accounts²⁹ on the fact that the enzyme, when releases the radical in solution becomes inactive and the iron is in the form Fe(II). Consequently, the enzyme needs to be reactivated by formation of Fe(III) and this could occur by reaction with a hydroperoxide specie (Scheme 1).

To confirm this hypothesis, we carried out the following experiment: linoleic acid was oxidized with lipoxygenase with air bubbling and stirring, until the complete depletion of the starting

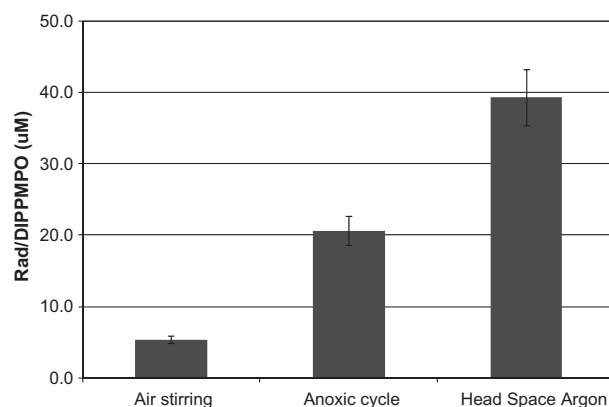


Figure 10. Amount of radical trapped under different conditions: in air with bubbling and stirring; during the anoxic reactivation cycle; in argon head-space condition.

material occurred (as verified by UV-vis). The reaction medium was then purged with argon and kept under a constant stream of argon. Thus an aliquot of linoleic acid was finally added together with the spin trap. After 2 h the total amount of radicals trapped was determined using ³¹P NMR. Qualitatively the radical detected were of the same type as on all previous experiments. Quantitatively, however, the data varied as shown in Figure 10, where we put in comparison the amount of radical trapped in air stirring oxidation, in argon head space and during the above describe anoxic reactivation cycle.

These experiments demonstrated that the radicals can be also generated from the linoleic acid/lipoxygenase system in complete absence of dioxygen. As such in the experiments when the initial dioxygen concentrations were smaller than the substrate concentration, the reaction could show three distinct phases in term of radical generation:

- A dioxygen consumption phase due the enzymatic reaction, where the dioxygen concentration is not limited and the radicals generated by the system are low. In this condition the DIPPMPPO is able to trap a limited amount of oxygen-centered radicals in agreement with the observed high lipoxygenase specificity.¹³
- A second phase where the dioxygen concentration becomes limiting and the amount of radical trapped increases. This higher amount of released radicals (mainly carbon-centered) is correlated to the previous observed decrease in the enzymatic specificity.¹³
- A third phase where the dioxygen concentration is ~ 0, and the enzyme in the presence of both product and residual substrate is able to generate radicals until its complete deactivation.²⁸

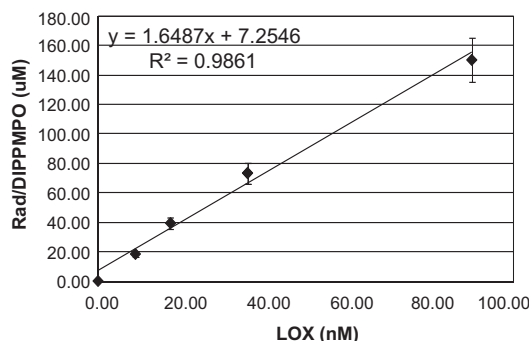
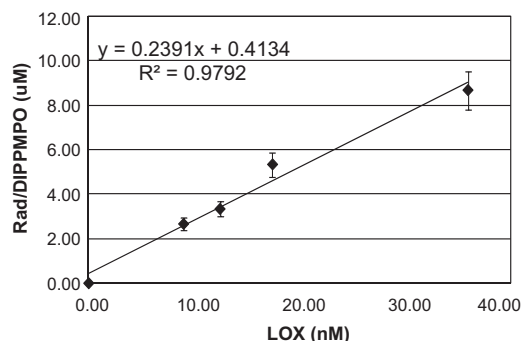


Figure 9. Linear correlations observed between the amounts of total radicals trapped as a function of LOX concentration for anoxic (left) and normoxic conditions (right).

The present set of experiments suggests the relevance of the DIP-PMPO spin trapping system toward understanding the chemistry and the radical intermediates involved in the linoleic acid/lipoxygenase reaction. This spin trapping system allows the quantification of the radicals involved and their correlation with the amount of dioxxygen present. In this effort we report that under certain conditions, the LOX system may generate a considerable amount of free radicals and the release of these intermediates may increase the multiplicity of the LOX-induced secondary reactions.

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