CHEMILUMINESCENCE DURING LIPOXYGENASE-CATALYSED OXYGENATION OF LINOLEIC ACID

Gerrit A. Veldink, G. Jan Garssen, Steven Slappendel, Johannes F.G. Vliegenthart and Jan Boldingh

Laboratory of Organic Chemistry, University of Utrecht, Croesestraat 79, Utrecht, The Netherlands

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SUMMARY

Contrary to earlier observations (7) the present investigation shows that light emission from the lipoxygenase-catalysed oxygenation of linoleic acid can be readily measured in the absence of luminol with standard liquid scintillation counting equipment. The quenching effect of superoxide dismutase suggests superoxide ($O_2^{\cdot-}$) to play a key role in this process.

INTRODUCTION

Lipoxygenase (EC. 1.13.11.12), a dioxygenase containing non-heme iron catalyses the conversion of unsaturated fatty acids containing a 1,4-cis, cis pentadiene system into the corresponding conjugated hydroperoxy fatty acids by means of molecular oxygen. Under strictly anaerobic conditions however, the enzyme is capable of converting the product hydroperoxide in the presence substrate fatty acids. In this anaerobic reaction a complex mixture of oxodienoic acids, n-pentane and fatty acid dimers is formed. For the enzymic oxygenation of unsaturated fatty acid, it has been proposed by several authors that activated oxygen species (e.g. $^1\Delta_g$ O_2 or $O_2^{\cdot-}$) are involved. With regard to the mechanism of this reaction several reports on the possible occurrence of activated oxygen species during lipoxygenase catalysis have recently appeared (1-4). Following cooxidation studies of singlet oxygen scavengers (5) the luminescence from the lipoxygenase-catalysed oxygenation of linoleic acid has been attributed to singlet oxygen. This has stimulated research to answer questions regarding the substrate specificity of superoxide dismutase (6-8). The present paper shows that luminescence occurs only during the aerobic stage of the lipoxygenase reaction. The luminescence yield is maximal after consumption of about 95% of the available oxygen. It is strongly enhanced by luminol but quenched by superoxide dismutase.

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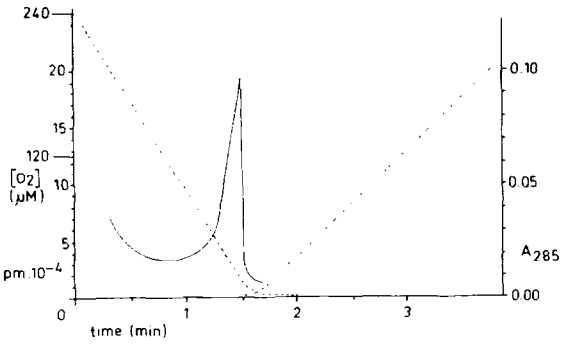


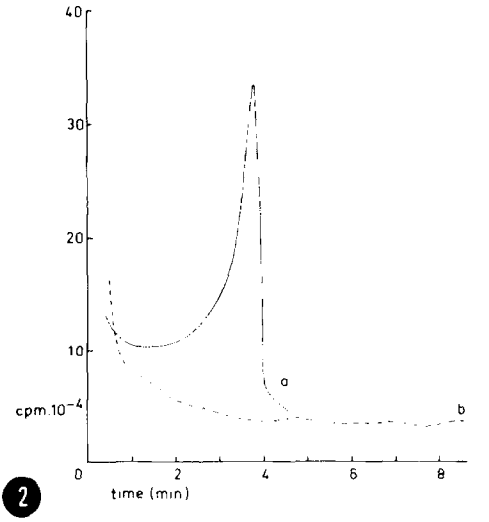


Fig. 1. Oxygen concentration (---), light emission (solid line) and absorbance change at 285 nm vs. time. Reactants: linoleic acid (1.85 mM), lipoxygenase (5×10^{-9}M), initial oxygen concentration: 240 μM, luminol (20 μM). 0.1 M borate buffer, pH 9.0

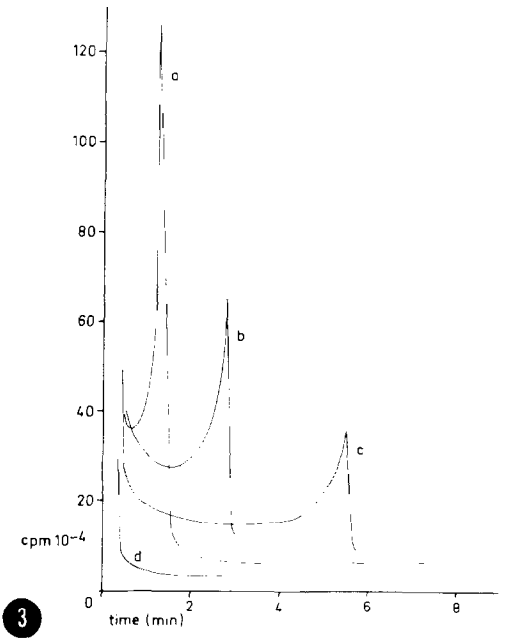
Fig. 2. Luminescence as a function of substrate concentration. Curve a) linoleic acid 540 μM; b) linoleic acid 180 μM. Initial O_2 concentration 240 μM. No luminol.

MATERIALS AND METHODS

Soybean lipoxygenase I was prepared as described previously (9) (specific activity 124 μmol O_2/min/mg). Linoleic acid (purity > 99%) was a gift from Unilever Research Laboratories, Vlaardingen/Duiven. Bovine superoxide dismutase was a gift from Dr. A. Finazzi-Agrò. Luminescence was measured in an air-tight scintillation vial with a Packard 2425 Liquid Scintillation Spectrometer with the coincidence circuitry switched off. Lipoxygenase activity was measured either spectrophotometrically at 234 nm with a Unicam SP 1800 instrument or polarographically in a Gilson Oxygraph equipped with a Clark oxygen electrode. Anaerobic reaction rates were measured at 285 nm. All experiments were carried out at 14°C.

RESULTS

Fig. 1 shows the time course of the formation of hydroperoxide, the chemiluminescence and after depletion of oxygen the onset of the anaerobic reaction. When the oxygen concentration has reached a level of 15 μM the luminescence increases rapidly, then reaches a sharp maximum and returns instantaneously to the noise level. From the absorbance at 285 nm it can be seen that the anaerobic conversion of linoleic acid hydroperoxides and



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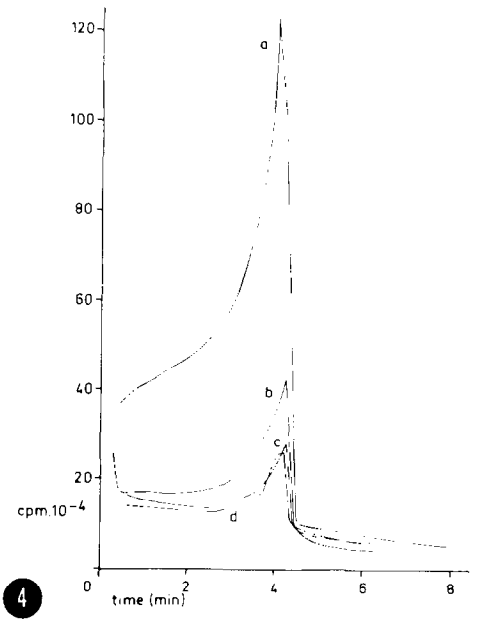

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Fig. 3. Effect of lipoxygenase concentrations on the time course of luminescence. Curve a) 5.2×10^{-8}M; b) 2.6×10^{-8}M; c) 1.3×10^{-8}M; d) Noise level. No luminol.

Fig. 4. Effect of superoxide dismutase (SOD) on the chemiluminescence. Curve a) no SOD; b) 1.2×10^{-8}M SOD; c) 6×10^{-8}M SOD; d) 1.2×10^{-7}M SOD. No luminol.

linoleic acid starts at an oxygen concentration below 5 µM. Thus, gradual increase and the sharp decline of luminescence just precede the onset of the anaerobic reaction.

Fig. 2 shows the effect of varying linoleic acid concentrations. At substrate concentrations below the oxygen concentration (240 µM) there is no significant light emission in contrast to the effect of a substrate concentration well above 240 µM. Increasing the enzyme concentration at a sufficiently high substrate concentration (> 240 µM) brings about a proportional decrease in time required to attain the luminescence maximum (Fig. 3).

In Fig. 4 the effect of superoxide dismutase on the light emission from the lipoxygenase/linoleic acid system is shown. Small amounts (70 units/ml) already cause an almost complete quenching of the luminescence which is indicative of the involvement of $O_2^{\cdot-}$ in the light-emitting process.

The addition of catalase had no effect while carbonate (up to 0.25 M)

did not stimulate the emission of light as has recently been reported for xanthine oxidase (10).

DISCUSSION

The presence of oxygen is obligatory for light emission, however the nature of the emitting species is still unknown. The cooxidation of compounds regarded to be specific for reaction with singlet molecular oxygen during lipoxygenase catalysis has been taken as evidence for the enzymic production of this activated oxygen species (5).

However, a reinvestigation of the structures of the cooxidized compounds has shown that oxidation of unsaturated fatty acids as catalysed by lipoxygenase does not mimic oxidation by singlet molecular oxygen (11). In fact, Teng and Smith (12, 13) concluded on the basis of the cooxidation products of cholesterol during lipoxygenase catalysis that involvement of singlet oxygen is highly unlikely. Therefore, the evidence for the production of singlet oxygen by lipoxygenase seems rather poor. Moreover, the antarafacial way in which oxygen reacts with the substrate is inconsistent with the participation of singlet oxygen (14, 17). The quenching effect of superoxide dismutase does, however, imply that $O_2^{\cdot-}$ plays a key role in the process of light emission.

Electron paramagnetic resonance (EPR) studies on lipoxygenase (18, 19, 20) have shown that the iron in the native enzyme is probably diamagnetic. Moreover, preincubation of the native enzyme with 4-nitrocatechol, a strong chelator for Fe(III), has no inhibitory effect (21). The EPR-studies (18, 19, 20) have already provided substantial evidence for the formation of an Fe(III)-species from the reaction between the native enzyme and 13-L-hydroperoxylinoleic acid. If the native enzyme has molecular oxygen bound to the iron-atom then a reaction of the following type (scheme 1) may be the origin of the superoxide which was shown to be involved in the chemilumi-

$$\text{E-Fe(II)}\ldots O_2 \leftrightarrow \text{E-Fe(III)}\ldots O_2^- \xrightarrow{\text{ROOH}} \text{E-Fe(III)} + O_2^{\cdot-} + \text{decomp. prod.}$$

Scheme 1.

nescence process. The amounts of superoxide dismutase used neither affected the initial rate nor the extent of the oxygentation reaction. By consequence, free superoxide is unlikely to occur as an intermediate during hydroperoxide formation. The time course of the luminescence shows that

the intensity increases concomitantly with the hydroperoxide formation. In conclusion these results demonstrate that chemiluminescence of the lipoxygenase/linoleic acid/oxygen systems in the absence of luminol can be readily measured (cf. 7) with a standard liquid scintillation spectrometer and $O_2^{\cdot}$ is involved in this process.

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