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CHANGES IN THE FLUORESCENCE AND ABSORBANCE OF LIPOXYGENASE-1 INDUCED BY 13-L_S-HYDROPEROXYLINOLEIC ACID AND LINOLEIC ACID

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Summary

1. The addition of 13-L_S-hydroperoxylinoleic acid to lipoxygenase-1 (linoleate: oxygen oxidoreductase EC 1.13.11.12) from soybeans at pH 9 and 25°C causes a quenching of the fluorescence of the enzyme at 328 nm when exciited at 280 nm and gives rise to an increase of the absorbance of the enzyme in the 300 nm to 450 nm region.

2. In the absence of O_2, addition of linoleic acid to enzyme treated with 13-L_S-hydroperoxylinoleic acid, causes an increase of the fluorescence at 328 nm and a decrease of the absorbance in the 300 nm to 450 nm region.

3. The fluorescence changes are suggested to be directly coupled to the absorbance changes via a non-radioactive energy transfer process.

4. It is proposed that the observed fluorescence and absorbance changes are related to changes in the formal charge of iron in the protein.

Introduction

Lipoxygenase-1 from soybeans (linoleate:O_2 oxidoreductase, EC 1.13.11.12) is a non-heme [1,2] iron protein (1 Fe/mol of enzyme [3,4]) of high molecular weight:(M_r = 98 600) [1]. At pH 9.0 the enzyme catalyses the conversion of linoleic acid into 13-L_S-hydroperoxylinoleic acid (13-ROOH) in the presence of O_2 [5]. The oxygenation reaction involves the abstraction of the 11 $L_{pro\,S}$ H atom from linoleic acid [6], which is the rate-limiting step at saturating O_2 concentrations [7]. Indirect evidence [6,8] suggested, that the

Abbreviation: 13-ROOH, 13-L_S-hydroperoxylinoleic acid.

reaction is more likely to proceed via a radical mechanism than via singlet $(^1\Delta_g)\,O_2$.

Recently De Groot et al. [2] demonstrated, that lipoxygenase-1 produces linoleic acid radicals under anaerobic conditions. However, the anaerobic reaction only proceeds in the presence of 13-ROOH [9—11]. Since 13-ROOH is also converted into oxodienoic fatty acids, *n*-pentane [9—12] and fatty acid dimers together with linoleic acid, lipoxygenase-1 can be considered as a peroxidase under these conditions.

The recent detection of iron in lipoxygenase-1 [1,3,4], which was previously considered to be a non-metal enzyme [13], stimulated research on the role of the iron in the enzymic reaction. Pistorius and Axelrod [14] suggested, that lipoxygenase-1 contains ferric iron. However, EPR studies by De Groot et al. [15] revealed, that in the native enzyme iron is present in an EPR-silent (ferrous) state. The involvement of iron in the reaction mechanism was demonstrated by the observation [15], that 13-ROOH converts the iron in the protein into the ferric state. When linoleic acid was added to the ferric enzyme in excess over both 13-ROOH and O_2 present in the incubation medium, the ferric EPR signals disappeared, suggesting that linoleic acid counteracts the effect of 13-ROOH under anaerobic conditions.

Finazzi-Agrò et al. [1] studied the interaction between 13-ROOH and lipoxygenase-1 by fluorescence spectroscopy and observed, that either in the absence or in the presence of O_2 the intrinsic fluorescence of the enzyme is quenched by the addition of a stoichiometric amount of 13-ROOH to the enzyme. The quenching could not be attributed to a change in the absorbance of the enzyme, since at the range of enzyme concentrations studied (approx. 10^{-5} M) no absorbance changes due to the addition of 13-ROOH were detectable. The present paper describes a more detailed study of the interaction between 13-ROOH and lipoxygenase-1 and also between linoleic acid and the enzyme at higher enzyme concentrations (approx. 10^{-4} M).

Materials and Methods

Lipoxygenase-1 was isolated from soybeans and purified according to Finazzi-Agrò et al. [1].

Linoleic acid was a gift from the Unilever Research Laboratories, Vlaardingen/Duiven, The Netherlands (purity 99% by gas-liquid chromatography). A fresh solution of linoleic acid in 0.1 M Tris · HCl, pH 9.0, contained less than 0.2% linoleic acid hydroperoxides, based on a molar extinction coefficient of the hydroperoxides at 234 nm, being 25 000 $l \cdot M^{-1} \cdot cm^{-1}$ [16]. 13-ROOH was prepared from linoleic acid with lipoxygenase-1 at pH 9.0 and 0°C, according to Veldink et al. [17]. The 13-ROOH contained approx. 10% of racemic linoleic acid hydroperoxidase. [1-^{14}C] Linoleic acid was purchased from The Radiochemical Centre, Amersham, U.K. (specific activity: 54 Ci/mol). (1-^{14}C)-labelled 13-ROOH was prepared from the labelled linoleic acid after dilution with unlabelled linoleic acid (specific activity 10 Ci/mol).

Thin-layer chromatography was carried out on 0.25 mm silica gel plates (20 × 20 cm. DC silica gel 60F254, E. Merck, Darmstadt, G.F.R.); solvent system: light petroleum (bp. 60—80°C)/diethylether, (3 : 2, v/v). Radioactivity

was scanned with a Berthold Dunnschicht scanner II model LB 2723; detection gas: methane.

Fluorescence experiments were performed with a FICA model 55 L spectrofluorimeter. This instrument corrects the spectra in terms of incident energy. Absorbance spectra were recorded with a Beckman DB-GT grating spectrophotometer. Anaerobiosis was obtained essentially as described previously [1].

Results

(1) Effect of 13-ROOH on lipoxygenase-1

The influence of 13-ROOH on the fluorescence and absorbance of lipoxygenase-1 under aerobic conditions at pH 9.0 and 25°C is shown in Figs 1a and 1b, respectively. The hydroperoxide was generated in situ by adding small amounts of linoleic acid to the enzyme. Previously it has been shown [1], that in air saturated solutions the addition of linoleic acid or 13-ROOH produces the same quenching of the enzyme fluorescence. However, under anaerobic conditions only 13-ROOH affects the fluorescence of the enzyme. From these results it was concluded [1], that either in the absence or in the presence of O_2 only 13-ROOH quenches the fluorescence of the enzyme. In the range of enzyme concentrations studied the maximum fluorescence quenching is obtained, when a stoichiometric amount of linoleic acid or 13-ROOH is added. As is shown in Fig. 2, the maximum absorbance change at 330 nm is obtained when approximately a stoichiometric amount of linoleic acid is added to the enzyme.

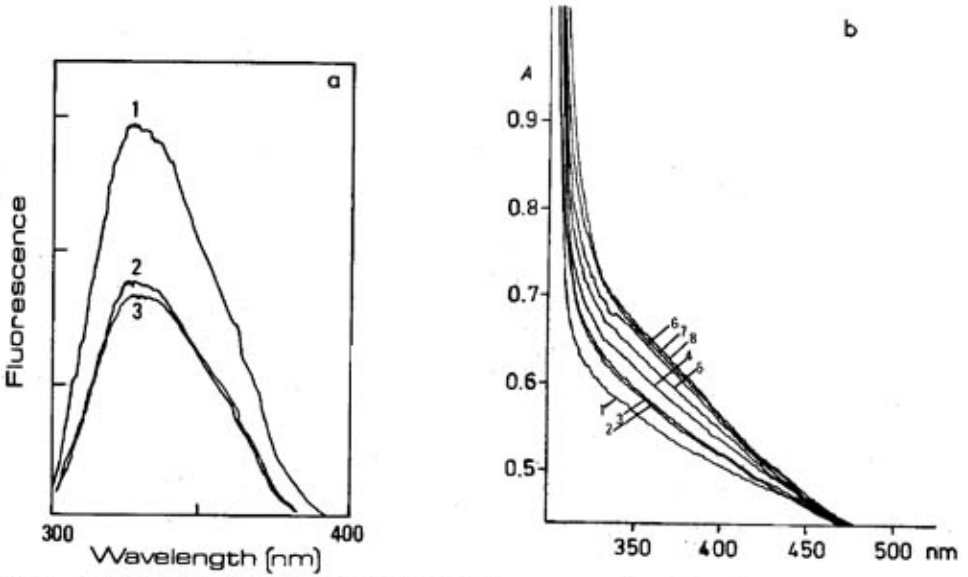

Fig. 1. Effect of 13-ROOH or linoleic acid on the fluorescence (a) and absorbance (b) of lipoxygenase-1 under aerobic conditions at 25°C in 0.1 M Tris · HCl, pH 9.0; O_2 concentration $2.4 \cdot 10^{-4}$ M. (a) $5 \cdot 10^{-6}$ M lipoxygenase (1); $5 \cdot 10^{-6}$ M linoleic acid added (2); $2 \cdot 10^{-5}$ M linoleic acid added (3). $8 \cdot 10^{-5}$ M lipoxygenase (1); concn of linoleic acid added: $8.5 \cdot 10^{-6}$ M (2); $1.7 \cdot 10^{-5}$ M (3); $2.6 \cdot 10^{-5}$ M (4); $4.3 \cdot 10^{-5}$ M (5); $6.0 \cdot 10^{-5}$ M (6); $8.5 \cdot 10^{-5}$ M (7); $1.7 \cdot 10^{-4}$ M (8).

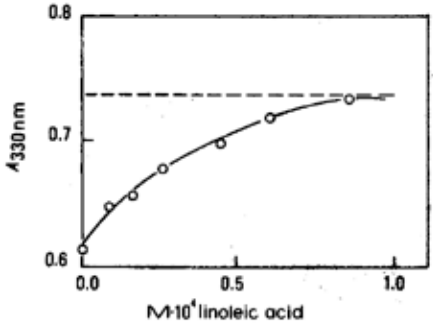

Fig. 2. Absorbance change at 330 nm induced by the aerobic addition of linoleic acid (conditions see Fig. 1b).

(2) Effect of H_2O_2 on lipoxygenase-1

In order to obtain more information about the type of interaction between 13-ROOH and lipoxygenase-1, also the effect of H_2O_2 on the fluorescence and absorbance of the enzyme was investigated. Small amounts of H_2O_2 were added to the enzyme at pH 9.0 and 25°C and immediately after mixing the fluorescence (Fig. 3a) and absorbance (Fig. 3b) of the enzyme were recorded. Comparison of Figs 1a, b and 3a, b, respectively, shows that the effects of 13-ROOH and H_2O_2 are qualitatively similar. However, at an enzyme con-

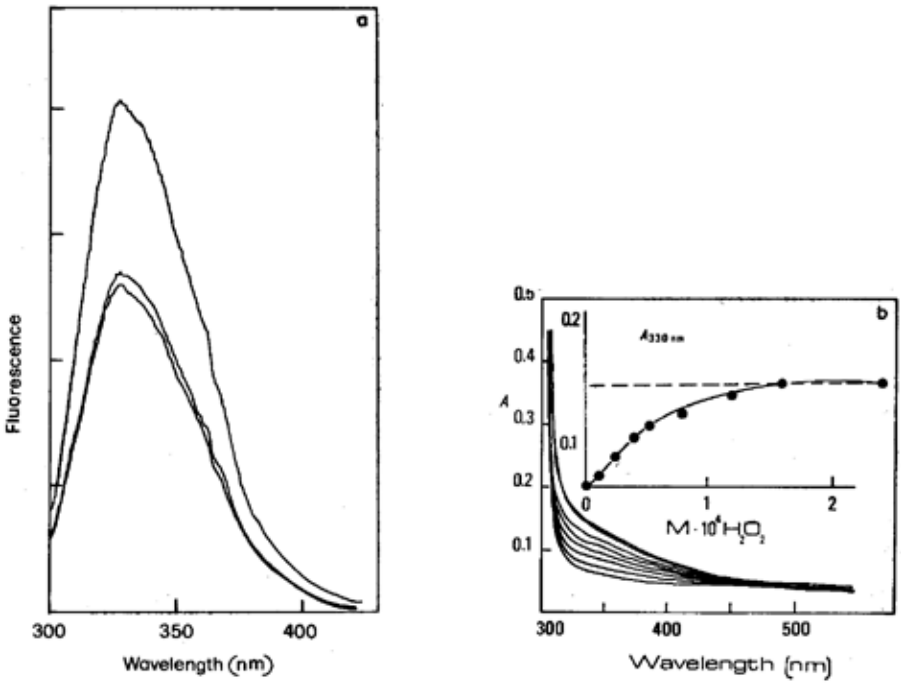

Fig. 3. Effect of H_2O_2 on the fluorescence (a) and absorbance (b) of lipoxygenase-1 under aerobic conditions at 25°C in 0.1 M Tris · HCl, pH 9.0. (a) $1.6 \cdot 10^{-6}$ M lipoxygenase-1 (upper curve); concentrations of H_2O_2 added: $4 \cdot 10^{-5}$ M and $8 \cdot 10^{-5}$ M (lowest curve). (b) $4.6 \cdot 10^{-5}$ M lipoxygenase; concentrations of H_2O_2 added are shown in the inserted figure.

centration $4.6 \cdot 10^{-5}$ M a 4-fold molar excess of H_2O_2 over 13-ROOH is required to obtain the maximum effect (see Figs 2 and 3b). It must be noted, that H_2O_2 (unlike 13-ROOH) strongly impairs the catalytic activity of the enzyme. At $3.5 \cdot 10^{-4}$ M H_2O_2 the activity of 10^{-8} M lipoxygenase-1 at pH 9.0 is completely destroyed.

(3) Conversion of 13-ROOH

The nature of the interaction between 13-ROOH and lipoxygenase-1 was further studied by investigating, whether the fluorescence and absorbance changes of the enzyme induced by 13-ROOH are accompanied by a conversion of the latter. Therefore, $2 \cdot 10^{-5}$ M lipoxygenase-1 was incubated with an equimolar amount of (1-^{14}C)-labelled 13-ROOH, either in the absence or in the presence of O_2 at pH 9.0 and 25°C. 1 min after mixing the reactants the medium was acidified to pH 2 with 2 M HCl and rapidly thereafter extracted with cold diethylether. The extracts were washed once with water, treated with diazomethane at 0°C and analyzed via thin-layer chromatography. Approximately 90% of the hydroperoxide was found to be converted by the enzyme both under aerobic and anaerobic conditions. Although the extent of the conversion of 13-ROOH is not influenced by O_2, under anaerobic conditions probably only one compound is formed, while in the presence of O_2 several products are formed. The identity of the final products formed remains to be elucidated.

(4) Effect of linoleic acid

As was mentioned before, linoleic acid itself does not change the fluorescence or absorbance of the native enzyme. However, an effect of linoleic acid on the fluorescence and absorbance of the enzyme does occur, if the fatty acid is added under anaerobic conditions to enzyme, which is previously treated with a stoichiometric amount of 13-ROOH. The resulting fluorescence an

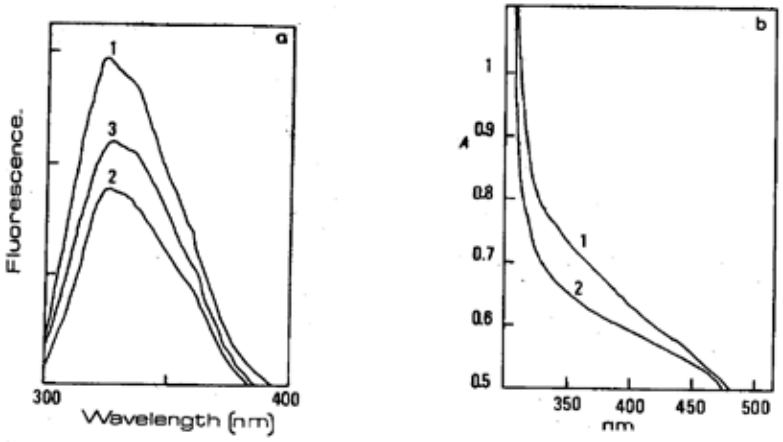

Fig. 4. Effect of linoleic acid on the fluorescence (a) and absorbance (b) of lipoxygenase-1, which was previously treated with a stoichiometric amount of 13-ROOH at 25°C in 0.1 M Tris · HCl, pH 9.0 anaerobically. (a) $5 \cdot 10^{-6}$ M lipoxygenase (1); $5 \cdot 10^{-6}$ M 13-ROOH added anaerobically (2); $1.0 \cdot 10^{-5}$ M linoleic acid added anaerobically (3). (b) $8 \cdot 10^{-5}$ M lipoxygenase, treated with $8 \cdot 10^{-5}$ M 13-ROOH anaerobically (1); $1.6 \cdot 10^{-4}$ M linoleic acid added anaerobically (2).

absorbance spectra of the enzyme are shown in Figs 4a and 4b, respectively. Fig. 4a shows, that addition of 13-ROOH quenches the fluorescence, while subsequent addition of linoleic acid under anaerobic conditions partially restores the fluorescence of the enzyme. Fig. 4b shows the absorbance spectra of the enzyme before and after addition of linoleic acid. The addition of more than a 2-fold molar excess of linoleic acid over enzyme did not result in further changes. Approximately 40% of the fluorescence at 328 nm and 70% of the absorbance at 330 nm of the untreated enzyme were obtained. The apparent inability of linoleic acid to fully counteract the effect of 13-ROOH may have several causes: (i) the secondary products formed in this anaerobic process absorp in the 280-330 nm region, (ii) the enzyme is partly inactivated by intermediary radicals, giving rise to a decrease of the fluorescence yield or (iii) linoleic acid reacts slowly with the enzyme. Repeated recordings of the fluorescence and absorbance spectra gave identical results, while no loss of activity was observed. This leaves possibility (i) as the most likely source of interference with the results.

When linoleic acid is added anaerobically in excess over enzyme and 13-ROOH, subsequent addition of O_2 by shaking in air causes a quenching of the fluorescence at 328 nm and an increase of the absorbance at 330 nm. The fluorescence at 328 nm is quenched to a value, lower than that shown in curve 2 (Fig. 4a), probably due to possibility (i), while approx. the same or a slightly higher absorbance at 330 nm is obtained.

Discussion

Figs 1a and b clearly show, that 13-ROOH affects both the fluorescence and absorbance of lipoxygenase-1 at pH 9.0. The absorption band, just in the tail of the ultraviolet absorption of the protein, previously remained unnoticed because of its rather low molar extinction coefficient (ϵ_{330} is approx. $1.6 \cdot 10^3$ $l \cdot M^{-1} \cdot cm^{-1}$). However, this extinction coefficient is sufficiently high to attribute the observed fluorescence quenching to a non-radiative energy transfer between the tryptophans [1,18] and the chromophore responsible for this absorption [19].

Considering the parallels with the EPR experiments by De Groot et al. [15], one is tempted to speculate, that the chromophore formed upon treatment with 13-ROOH or H_2O_2 (Fig. 3b) stems from the oxidized enzyme, the iron being in the ferric state. It is worth noting, that apoazurin, which can bind one ferric iron atom per molecule, shows an identical absorption band and a similar fluorescence quenching when the ferric iron is bound to the protein [20].

As a result of the oxidation of lipoxygenase-1, 13-ROOH and H_2O_2 are converted probably as shown below:

$$\text{13-ROOH} + E(Fe^{II}) \rightarrow \text{13-RO}^{\cdot} + OH^- + E(Fe^{III}) \quad \text{(a)}$$

$$H_2O_2 + E(Fe^{II}) \rightarrow OH^{\cdot} + OH^- + E(Fe^{III}) \quad \text{(b)}$$

The incubations with (1-^{14}C)-labelled 13-ROOH showed, that the hydroperoxide is converted into one main compound under anaerobic conditions. In the

presence of O_2 more final products were found, probably due to nonenzymic oxygenation of the intermediary alkoxy radicals ($RO^{\cdot}$) [9—11]. The inactivation of the enzyme by H_2O_2 is likely to be due to the formation of $OH^{\cdot}$ radicals (b) which may modify amino acids in the protein.

According to De Groot et al. [15], linoleic acid (RH) converts the ferric iron in the protein into the ferrous state via:

$$RH + E(Fe^{III}) \rightarrow R^{\cdot} + H^{+} + E(Fe^{II}) \qquad (c)$$

We propose, that the spectral changes (Figs 4a,b) occuring upon anaerobic addition of linoleic acid to an enzyme, which was treated with 13-ROOH, are concomitant with the previously reported disappearance of the ferric EPR signals. Research is in progress to measure the rates of the changes in the fluorescence and absorbance of the enzyme, brought about by 13-ROOH and linoleic acid.

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References

1 Finazzi Agrò, A., Avigliano, L., Veldink, G.A., Vliegenthart, J.F.G. and Boldingh, J. (1973) Biochim. Biophys. Acta 326, 462—470
2 De Groot, J.J.M.C., Garssen, G.J., Vliegenthart, J.F.G. and Boldingh, J. (1973) Biochim. Biophys. Acta 326, 279—284
3 Chan, H.W.-S. (1973) Biochim. Biophys. Acta 327, 32—35
4 Roza, M. and Francke, A. (1973) Biochim. Biophys. Acta 327, 24—31
5 Hamberg, M. and Samuelsson, B. (1967) J. Biol. Chem. 242, 5329—5335
6 Egmond, M.R., Vliegenthart, J.F.G. and Boldingh, J. (1972) Biochem. Biophys. Res. Comm. 48,1055—1060
7 Egmond, M.R., Vliegenthart, J.F.G. and Boldingh, J. (1973) Biochem. Biophys. Res. Commun. 54, 1178—1184
8 Teng, J.I. and Smith, L.L. (1973) J. Am. Chem. Soc. 95, 4060—4061
9 Garssen, G.J., Vliegenthart, J.F.G. and Boldingh, J. (1971) Biochem. J. 122, 327—332
10 Garssen, G.J., Vliegenthart, J.F.G. and Boldingh, J. (1972) Biochem. J. 130, 435—442
11 Garssen, G.J., (1972) Thesis University of Utrecht, The Netherlands
12 Johns, E.B., Pattee, H.E. and Singleton, J.A. (1973) J. Agric. Food Chem. 21, 570—573
13 Tappel, A.L. (1963) in 'The Enzymes' VIII, 2nd edn, P.D. Boyer, H. Lardy and K. Myrbäck edits., Acad. Press. N.Y., pp. 275—283
14 Pistorius, E.K. and Axelrod, B. (1974) J. Biol. Chem. 249, 3183—3186
15 De Groot, J.J.M.C., Veldink, G.A., Vliegenthart, J.F.G., Boldingh, J., Wever, R. and van Gelder, B.F. (1975) Biochim. Biophys. Acta 377, 71—79
16 Johnston, A.E., Zilch, K.T., Selke, E. and Dutton, H.J. (1961) J. Am. Oil Chem. Soc. 38, 367—371
17 Veldink, G.A., Garssen, G.J., Vliegenthart, J.F.G. and Boldingh, J. (1972) Biochem. Biophys. Res. Commun. 47, 22—26
18 Stevens, F.C., Brown, D.M. and Smith, E.L. (1970) Arch. Biochem. Biophys. 136, 413—421
19 Förster, T. (1966) Modern Quantum Chem., Istanbul Lects., Acad. Press. N.Y.
20 Kanazawa, K., Mori, T., and Matsushita, S. (1973) J. Nutr. Sci. Vitaminol. 19, 263—275