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THE INFLUENCE OF OXYGEN ON THE FLUORESCENCE OF LIPOXYGENASE

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SUMMARY

1. Soybean lipoxygenase (EC 1.13.1.13) shows fluorescence at 328 nm on excitation at 280 nm.

2. The fluorescence emission is quenched for 18–20% by replacing the oxygen in the solution by argon.

3. Addition of one equivalent of product (linoleate hydroperoxide) to a solution of lipoxygenase causes a further quenching of the fluorescence which is possibly caused by a more complete removal of oxygen from the enzyme.

4. Denatured lipoxygenase shows a weak fluorescence emission at 345 nm which is not affected by oxygen, substrate or product.

5. Lipoxygenase fluorescence is due to the relatively large number of tryptophans in the molecule which should be in a non-polar region of the molecule.

6. The mechanism of fluorescence enhancement of lipoxygenase induced by oxygen is discussed in relation to similar observations previously described for fluorescent molecules contained in a solid polyvinyl matrix.

INTRODUCTION

Lipoxygenase (linoleate: oxygen oxido-reductase, EC 1.13.1.13) is present in various seeds. Its physiological role is not fully understood. It catalyses the formation of hydroperoxides from unsaturated fatty acids and molecular oxygen[1]. Furthermore the enzyme is capable of oxidizing, in the presence of substrate fatty acid and product, a large number of substances, *e.g.* carotene, luminol, cytochrome *c*, diphenylisobenzofuran, tetracyclone[2,3] (unpublished results) and cholesterol[4]. Since the oxidation of diphenylisobenzofuran and tetracyclone is generally attributed to singlet oxygen[5], Chan[2] proposed that the enzyme could produce this reactive form of oxygen during its catalytic action. The cooxidation of cholesterol is thought to proceed along a radical mechanism[4]. However, an appropriate mechanism for the oxidation of tetracyclone in the lipoxygenase reaction is still lacking[4,6].

In this paper we present evidence on the binding of oxygen to lipoxygenase. The fluorescence behaviour suggests a novel way of interaction between oxygen and a protein.

MATERIALS AND METHODS

Reagent grade chemicals were used without further purification. Linoleic acid was a gift from Unilever Research Laboratories, Vlaardingen/Duiven, The Netherlands (99.9% pure, by gas–liquid chromatography).

Linoleic acid hydroperoxides were prepared with soybean lipoxygenase-1 at pH 9.0 according to Veldink *et al.*[7].

Lipoxygenase-1 was prepared as follows: 200 g of soybeans were swollen with 200 ml of redestilled water for 52 h at 4 °C in the dark. After homogenisation in a Sorvall Omnimixer the mixture was centrifuged twice at $800 \times g$ to remove fat and large cell particles. The resulting solution was brought to 60% saturation with solid $(NH_4)_2SO_4$. After centrifugation the precipitate was transferred to a dialysis tube and dialysed against a 0.1 M NH_4HCO_3 solution until the precipitate was completely dissolved. The solution was brought on a Sephadex G-100 column (80 cm × 5 cm) which was eluted with 0.1 M NH_4HCO_3. The active fractions were pooled and concentrated by ultrafiltration in a 400-ml Amicon ultrafiltration cell through a Diaflo UM 20 E membrane.

By the same ultrafiltration technique the NH_4HCO_3 solution was gradually replaced by a 0.05 M sodium acetate buffer, pH 5.5, containing 0.05 M NaCl. After buffer exchange the protein solution was fractionated on a CM-Sephadex C-50 column (70 cm × 2.6 cm) equilibrated with 0.05 M sodium acetate buffer containing 0.05 M NaCl. Elution was then performed with 0.05 M sodium acetate containing a linear gradient of 0.05–0.3 M NaCl. Active fractions were combined and checked for purity by polyacrylamide gel electrophoresis. A yield of 200 mg of homogeneous enzyme was obtained. The specific activity was 78 μmoles O_2/min per mg protein as determined polarographically in a Gilson Oxygraph ($1.8 \cdot 10^{-3}$ M linoleate, pH 9.0, 25 °C). The enzyme contained 0.90 gatom of Fe per mole of enzyme (mol. wt 98600) as determined by atomic absorption spectrometry. Chemical determination of Fe by the method of Van de Bogart and Beinert[8] yielded 0.78 g atom of Fe per mole of enzyme. Fluorescence experiments were performed with a Turner model 210 or a FICA model 55 L spectrofluorimeter. Both instruments give corrected spectra in terms of incident energy.

The experiments were conducted at 20 °C. Anaerobiosis was obtained by evacuation followed by refilling with 99.9% pure argon. This cycle was usually repeated four times.

Additions of substrate or product in anaerobiosis were made with a microsyringe through the rubber sealcap of the cuvette. Quantum yield determinations were done by integrating the areas under the fluorescence curves obtained from a protein sample and from a solution of tryptophan under the same conditions. The solutions had the same absorbances at the excitation wavelength. The quantum yield of tryptophan was taken to be 0.2 (ref. 9).

Fluorescence polarization experiments were conducted with the FICA spectrofluorimeter using a 25-Å bandwidth for excitation and a 75-Å bandwidth for emission.

Phosphorescence experiments were conducted with an Aminco–Bowman spectrofluorimeter equipped with an Aminco–Kiers phosphoroscope at liquid N_2 temperature. Difference absorption spectra were recorded with a Beckman DK-2A ratio-recording spectrophotometer using a four-cuvettes assembly.

Circular dichroism experiments were made with a Cary 60 spectropolarimeter equipped with a CD 6001 attachment. The optical dissymmetry was recorded as $[\theta]$ (molar ellipticity).

RESULTS

Fluorescence experiments

The fluorescence of the protein at 20 °C in the presence of air is presented in Fig. 1. The maximum emission is at 328 nm and the maximum excitation at 280 nm. No fine structure is evident on both the excitation and emission spectra. No difference was seen on excitation at 295 nm, indicating the absence of tyrosine contribution to the fluorescence.

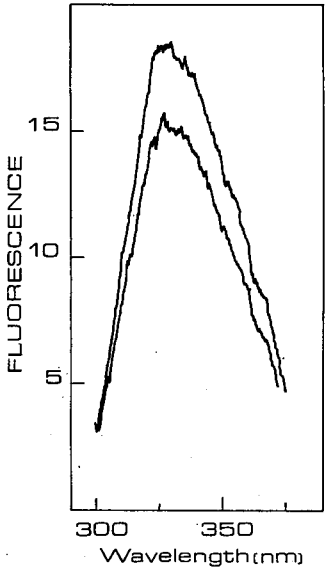


Fig. 1. Effect of oxygen removal on lipoxygenase fluorescence. Top curve: $1.7 \cdot 10^{-6}$ M lipoxygenase in 0.1 M sodium acetate. Bottom curve: the same after four cycles of evacuation followed by refilling with argon. Excitation wavelength: 280 nm.

As shown in the same figure the removal of oxygen causes a decrease in fluorescence of 18–20 % without any change in the shape of the spectrum. The decrease of the fluorescence is roughly proportional to the residual oxygen since samples with a less complete anaerobiosis showed a lower quenching effect. The quenching process is slowly reversible on opening to air of the cuvettes which contained the evacuated samples. After several hours the enzyme solutions regained their original fluorescence.

Apparently, the protein was not modified by the evacuation since the enzymic activities of aerated and evacuated samples proved to be identical.

The process is fully reversible and can be repeated several times with the same enzyme sample without affecting the enzymic activity. The quantum yield of the fluorescence was found to be 0.15 by comparing it with free tryptophan. This value was calculated by subtracting the contribution of tyrosines to the overall absorbance of the protein at 280 nm on the basis of the amino acid composition[12].

An attempt to increase the rate of reoxygenation of the protein by bubbling pure O_2 through the solution failed because of denaturation of the protein due to foam formation. Impure commercial preparations of lipoxygenase showed a fluorescence decrease on evacuation which corresponded with their specific enzymic activities.

Fluorescence polarization experiments

Fig. 2 shows the fluorescence polarization curves obtained for lipoxygenase in the presence and absence of air, respectively. Both show very similar polarization values. The fluorescence excitation polarization is roughly similar to that of pure tryptophan. Despite a scatter of the experimental points the fluorescence emission polarization seems to be constant over the whole range.

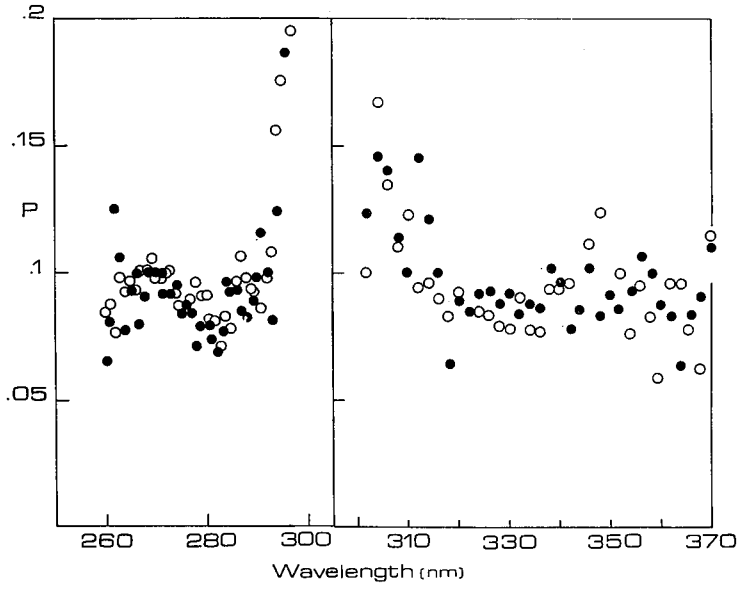


Fig. 2. Fluorescence polarization of lipoxygenase. Right: fluorescence polarization of absorption spectrum: emission 328 nm. Left: fluorescence polarization of emission spectrum: excitation 280 nm. ○, in the presence of air. ●, in the absence of air.

Phosphorescence experiments

The phosphorescence spectra of lipoxygenase are given in Fig. 3. The shape of the phosphorescence curve is typical for proteins containing tryptophan. In this case oxygen removal caused an increase of phosphorescence.

Opening of the sample tube to air was followed by the return of the phosphorescence to the original level.

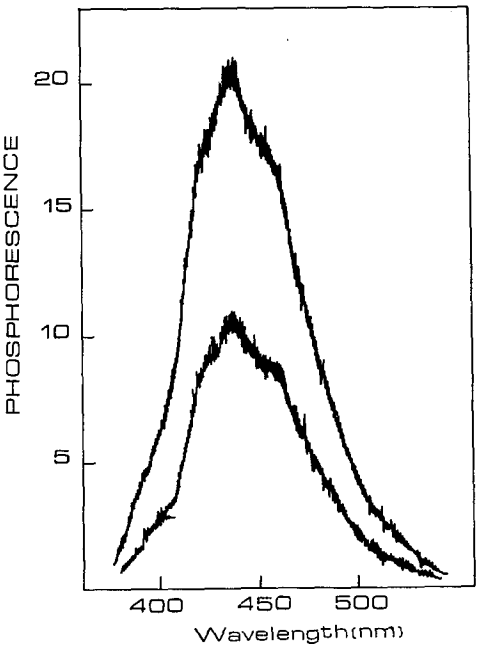


Fig. 3. Phosphorescence spectrum of lipoxygenase. Bottom curve: $8 \cdot 10^{-6}$ M lipoxygenase in 0.1 M sodium acetate–ethylene glycol (1:1, v/v). Top curve: the same after four evacuation cycles. Excitation wavelength: 280 nm.

Effect of substrate and product on lipoxygenase fluorescence

The addition of linoleate to an air-saturated lipoxygenase solution resulted in a quenching of the fluorescence (Fig. 4). The maximum effect was obtained when one mole of linoleate had been added to one mole of enzyme.

The addition of linoleate hydroperoxide also caused a decrease of the fluorescence to the same extent.

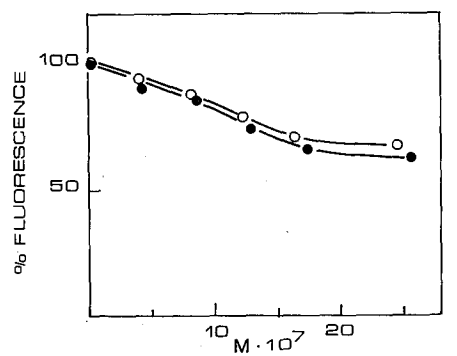


Fig. 4. Fluorescence quenching by linoleate and linoleate hydroperoxide. To a solution containing $1.7 \cdot 10^{-6}$ M lipoxygenase in 0.1 M sodium acetate small aliquots of linoleate (○) or its hydroperoxide (●) dissolved in ethanol were added.

All fluorescence measurements were made immediately after the addition of either substrate or product.

In the absence of air, the addition of an excess of linoleate unexpectedly resulted in a small decrease of fluorescence (Fig. 5). In this experiment the linoleate had been added as an alcoholic solution (4 μl to 2 ml) which had not been freed from oxygen. The addition of a small further amount of air saturated alcohol (10 μl) alone

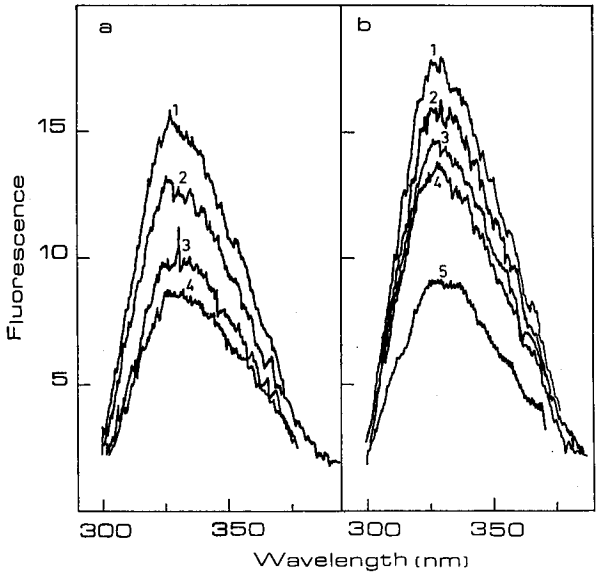
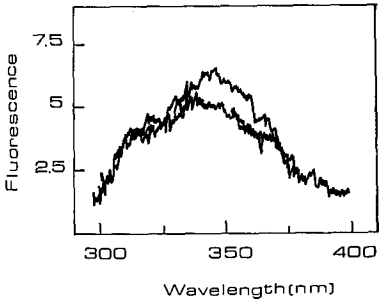


Fig. 5. Quenching effect of linoleate and linoleate hydroperoxide in the absence of air. (a) $1.3 \cdot 10^{-6}$ M lipoxygenase in 0.1 M sodium acetate as such (Curve 1). The evacuation (Curve 2) and the addition of $4.5 \cdot 10^{-5}$ M linoleate dissolved in 4 μl of ethanol (Curve 3) both produced a fluorescence quenching. The subsequent addition of 10 μl of ethanol produced a further decrease (Curve 4). The addition of more alcohol did not give a further fluorescence change. (b) $1.6 \cdot 10^{-6}$ M lipoxygenase in 0.1 M sodium acetate. From the top: (1) as such; (2) after two cycles of evacuation and refilling with argon; (3) after two more cycles; (4) in the presence of $4 \cdot 10^{-7}$ M linoleate hydroperoxide dissolved in ethanol; (5) in the presence of $1.6 \cdot 10^{-6}$ M hydroperoxide. Further addition of hydroperoxide or of ethanol alone had no effect.

induced the maximum quenching, whereas the addition of alcohol to an enzyme solution without linoleate being present had no quenching effect. On the other hand the addition of one mole or more of linoleate hydroperoxide in the absence of air resulted in the maximum quenching as had been observed in the aerobic incubation. These findings indicate that the quenching is due to linoleate hydroperoxide. The phosphorescence yield of lipoxygenase is not quenched by the presence of linoleate hydroperoxide. Denatured lipoxygenase showed fluorescence at a higher wavelength with a maximum at 345 nm. This fluorescence was not affected by the presence or absence of oxygen, whereas the addition of linoleate hydroperoxide (Fig. 6) caused a small increase. The evacuation performed after the addition of linoleate to a solution of active enzyme (Fig. 7) caused a further decrease of fluorescence identical to that produced by evacuation in the absence of hydroperoxide.



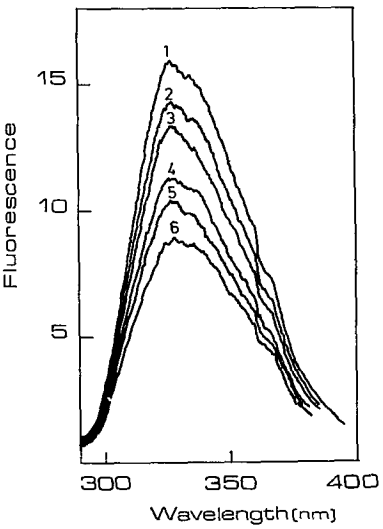


Fig. 6. Fluorescence of denatured lipoxygenase in the presence of linoleate hydroperoxide. A solution containing $5.8 \cdot 10^{-7}$ M lipoxygenase was kept for 10 min at 100 °C. After centrifugation the fluorescence spectrum was recorded (bottom curve). The addition of $1.3 \cdot 10^{-6}$ M linoleate hydroperoxide gave a small increase of fluorescence.

Fig. 7. Additivity of oxygen and linoleate effects on fluorescence. $7 \cdot 10^{-7}$ M lipoxygenase dissolved in 0.1 M sodium acetate. From the top: (1) as such; (2) in the presence of $1.6 \cdot 10^{-7}$ M linoleate; (3) in the presence of $3.2 \cdot 10^{-7}$ M linoleate; (4) in the presence of $6.4 \cdot 10^{-7}$ M linoleate; (5) in the presence of $12.8 \cdot 10^{-7}$ M linoleate; (6) same as (5) after evacuation.

Difference absorption spectra

No difference absorption spectrum in the region 360–250 nm was induced by substituting Ar for O_2 in solutions containing lipoxygenase.

Furthermore the protein is colourless; it does not show any absorbance up to a concentration of $3 \cdot 10^{-4}$ M in the 400–310-nm region. Absorption spectra, recorded either in the presence or absence of linoleate hydroperoxide did not show any significant difference in the 360–280-nm region.

Circular dichroism experiments

The circular dichroism spectrum of soybean lipoxygenase at a concentration of $3.5 \cdot 10^{-5}$ M showed a positive band at 280 nm ($\Delta\varepsilon$ approx. 10) related to aromatic amino acids (tryptophans *plus* tyrosines) the shape and intensity of which were not affected by evacuation. Furthermore, no difference was observed after oxygen removal in the 360–250-nm region.

DISCUSSION

The fluorescence experiments seem to indicate the presence of an oxygenated and a deoxygenated form of lipoxygenase. A partial fluorescence quenching of lipoxygenase can be induced by substituting Ar for O_2 and a further quenching is obtained by adding stoichiometric amounts of linoleate hydroperoxide. Neither effect can be explained in simple terms like energy transfer or collisional quenching. In fact no

absorbance change was seen in concomitance with the fluorescence quenching. Furthermore, no change was seen after evacuation in the circular dichroism spectrum and in the fluorescence polarization. The latter result points to an invariance of the lifetime of the singlet excited state in the absence of conformational changes[10], which are excluded on the basis of the CD experiments. Similar results have been reported by Bolton *et al.*[11] who observed a fluorescence enhancement by molecular oxygen with fluorescent molecules like anthracene, chrysene and dibenzanthracene contained in a polyvinyl chloride matrix. In their experimental conditions molecular oxygen was found to induce a 10% increase of fluorescence concomitant with a decrease of phosphorescence. This effect was attributed to the formation of singlet oxygen *via* annihilation of the triplet state. The singlet oxygen may then increase the population of the first excited singlet state thereby also increasing the fluorescence yield. The fluorescence enhancement was found by these authors to occur only in a rigid matrix and was a delayed fluorescence. It was proposed by Chan[2] and afterwards confirmed by one of us (Finazzi-Agrò, A., unpublished) that lipoxygenase might form singlet oxygen in the course of its catalytic action.

This finding is consistent with observations made on other oxygenases (Finazzi-Agrò, A., unpublished), where the presence of this reactive form of oxygen or of a species which can generate it (superoxide anions) was demonstrated.

The quenching of fluorescence caused by addition of one equivalent of linoleate in the presence of oxygen or of its hydroperoxide could be due to a more thorough depletion of the bound oxygen which cannot be accomplished by evacuation.

The slow rate of reoxygenation and the small amounts of linoleate or hydroperoxide needed to achieve the maximum fluorescence quenching might suggest that the active form of lipoxygenase during catalysis is a low fluorescent species.

It should be pointed out that the quenching observed after addition of either substrate or product is always caused by the product which might be interpreted as stripping of (active) oxygen from the enzyme by the hydroperoxide.

The mechanism of fluorescence quenching in lipoxygenase requires further investigation also in view of the 20 tryptophans present in the protein[12]. Recently, Nagami[13] reported on the involvement of a tryptophan residue in the binding of Fe (III) to pyrocatechase. The tryptophans present in lipoxygenase probably are in a non-polar environment in view of the wavelength of emission. The shape of the fluorescence spectra is not affected in the quenched samples which could mean that either the tryptophans are equivalent in terms of polarity or differently solvated tryptophans have a much lower quantum yield.

It was demonstrated previously[14] that linoleate decreases the apparent affinity of lipoxygenase for oxygen. Michaelis constants for oxygen are $3 \cdot 10^{-4}$ M at $7.2 \cdot 10^{-3}$ M linoleate and $3 \cdot 10^{-5}$ M at $3.6 \cdot 10^{-4}$ M linoleate, respectively.

Therefore, it is possible that the binding constant for oxygen is very high in the absence of linoleate hydroperoxide. If the quenching process induced by linoleate hydroperoxide indeed reflects the further removal of oxygen from the enzyme this oxygen could be in the singlet state and thus oxidize unspecifically part of the linoleate and other compounds present in the solution.

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