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# PROPERTIES OF A COMPLEX OF Fe(III)-SOYBEAN LIPOXYGENASE-1 AND 4-NITROCATECHOL

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## Summary

Fe(III)-soybean lipoxygenase-1 yields with 4-nitrocatechol a green coloured 1 : 1 complex, which shows at pH 7.0 absorption maxima at 385 nm and 650 nm. The formation of this complex is reversible. The circular dichroism spectrum of the complex of Fe(III)-lipoxygenase-1 and 4-nitrocatechol has a positive band at around 380 nm and a negative band at around 450 nm and is significantly different from that of the Fe(III)-enzyme as such.

4-Nitrocatechol can be displaced from the green complex by 13-L-hydroperoxy-*cis*-9,*trans*-11-octadecadienoic acid, resulting in the formation of the blue complex between the Fe(III)-enzyme and 13-L-hydroperoxy-*cis*-9,*trans*-11-octadecadienoic acid both under aerobic and anaerobic conditions. Also linoleic acid competes with 4-nitrocatechol for the binding site on the Fe(III)-enzyme, as can be demonstrated under anaerobic conditions, ultimately leading to reduction of the Fe(III)-enzyme.

The oxygenation of linoleic acid by Fe(III)-lipoxygenase-1 is inhibited by 4-nitrocatechol. From steady-state kinetics a non-competitive inhibition pattern is obtained. Probably it has to be considered as pseudo non-competitive because of the slow establishment of the complex equilibrium. An inhibition constant ($K^*_{4NC}$) of 16.3 $\mu$M is found. On prolonged incubation of Fe(III)-lipoxygenase-1 and 4-nitrocatechol the green complex converts into a brown species. This conversion is found to be coupled with a change in the nature of the inhibition from reversible to irreversible.

A complex between native lipoxygenase-1 and 4-nitrocatechol is found to be unlikely.

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Abbreviations: 13-L-ROOH, 13-L-hydroperoxy-*cis*-9,*trans*-11-octadecadienoic acid; E*-4NC, complex of Fe(III)-lipoxygenase-1 with 4-nitrocatechol.

## Introduction

Native lipoxygenase-1 from soybeans (linoleate:oxygen oxidoreductase, EC 1.13.11.12), which is a mononuclear dioxygenase containing non-heme iron, is converted by 13-L-ROOH into yellow Fe(III)-enzyme [1]. Addition of 13-L-ROOH to the Fe(III)-enzyme results in the formation of a blue complex with specific spectral features [1,2].

Tyson [3] showed that 4-nitrocatechol can inhibit the oxygenation of linoleic acid by soybean lipoxygenase. Recently Galpin et al. [4] showed by EPR measurements that 4-nitrocatechol and various other catechols form a complex with the $Fe^{3+}$ in yellow soybean lipoxygenase-1. They reported that the formation of the complex was apparently irreversible.

In the present paper we describe a more detailed investigation on the binding of 4-nitrocatechol to yellow Fe(III)-lipoxygenase-1.

## Materials and Methods

Lipoxygenase-1 was isolated from soybeans and purified as described before [4]. Linoleic acid was purchased from Lipid Supplies (St. Andrews University, St. Andrews, U.K.). 4-Nitrocatechol was supplied by J.T. Baker. 13-L-Hydroperoxy-*cis*-9,*trans*-11-octadecadienoic acid was prepared and purified according to Verhagen et al. [5]. The yellow Fe(III)-enzyme was prepared at 0°C immediately before use by incubation of native lipoxygenase-1 with 13-L-ROOH at a 1 : 1.1 molar ratio either at pH 7.0 or pH 8.0. For CD spectroscopic measurements the Fe(III)-enzyme was freed from reaction products by gel filtration (Sephadex G-25).

Absorption spectra were recorded on a Cary 118C spectrophotometer equipped with thermostated cuvette holders, containing 1 cm pathlength cells. CD spectra were recorded on a Dichrograph II (Jouan) as described before [2].

In air-saturated sodium phosphate buffer (0.1 M, pH 8.0) rates of oxygenation of linoleic acid were measured spectrophotometrically by recording the increase of absorbance at 235 nm at 25°C (1 cm pathlength cuvette). The inhibition constant was determined by measuring initial velocities of the oxygenation of linoleic acid by 2.0 nM Fe(III)-enzyme at various concentrations of linoleic acid and 4-nitrocatechol. Reactions were started by adding an appropriate amount of a solution of 12.5 mM linoleic acid in 1.0 M $NH_4OH$ to a solution of yellow enzyme and 4-nitrocatechol, which was preincubated for 5 min.

## Results and Discussion

*Formation of the Fe(III)-lipoxygenase-1-4-nitrocatechol complex*

Upon incubation of yellow Fe(III)-lipoxygenase-1 in 0.1 M sodium phosphate buffer (pH 7.0) at 0°C with an equimolar amount of 4-nitrocatechol, the colour of the solution turns into green. The absorption spectrum shows maxima at 385 and 650 nm (Fig. 1). From titration of yellow enzyme with 4-nitrocatechol, as recorded by difference spectroscopy, it was concluded that in the green complex 1 mol 4-nitrocatechol is bound per mol enzyme. The green

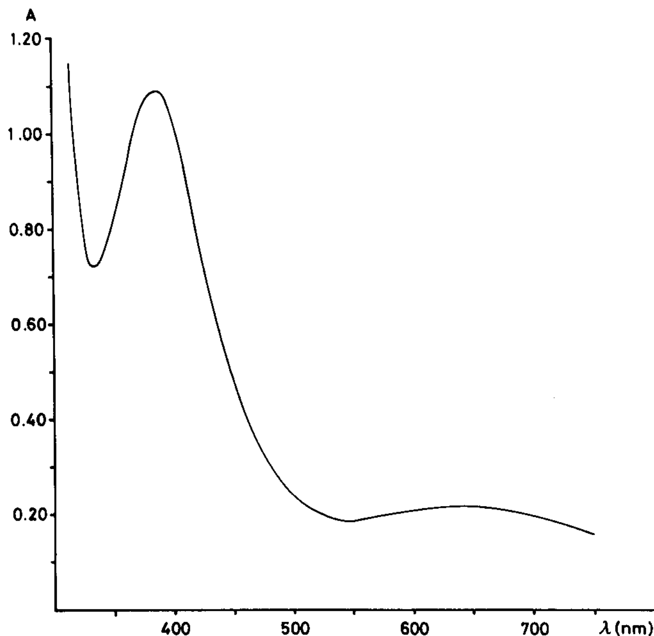



Fig. 1. Absorption spectrum of the E*-4NC complex formed from 100 μM Fe(III)-lipoxygenase-1 and 100 μM 4-nitrocatechol in 0.1 M sodium phosphate buffer (pH 7.0) at 0°C. With these concentrations of reactants the development of the green colour was completed within 10 min.

$E^*$-4NC complex can be formed in the pH range between 4.5 and 9.0. Increasing the pH of the solution causes a shift of the absorption maximum from 380 nm at pH 4.5 to 430 nm at pH 9.0, whereas the maximum at 650 nm remains unaffected. These absorption maxima are characteristic for a Fe(III)-4-nitrocatechol complex as is apparent from the absorption spectrum of a 1 : 1 mixture of $FeCl_3$ and 4-nitrocatechol at pH 3.5 [3]. It should be noted that the green complex of inorganic $Fe^{3+}$ with 4-nitrocatechol can only be formed between pH 3.0 and 4.5. The formation of the $E^*$-4NC complex could also be demonstrated by titrating a solution of 4-nitrocatechol at pH 7.0 with yellow lipoxygenase-1. The successive spectra show an isosbestic point at 400 nm (Fig. 2). To further characterize the green $E^*$-4NC complex, the interaction of 4-nitrocatechol with the yellow enzyme was investigated by CD spectroscopy. As reported before, purified Fe(III)-lipoxygenase-1 shows a negative dichroic band at around 345 nm and a positive band at around 425 nm [2]. The CD spectrum of a solution of equimolar amounts of yellow lipoxygenase-1 and 4-nitrocatechol (pH 7.0) shows a positive and a negative dichroic band around 380 and 450 nm, respectively. Comparison of the CD spectra of the $E^*$-4NC complex and yellow Fe(III)-lipoxygenase-1 (Fig. 3) suggests that binding of 4-nitrocatechol to the Fe(III)-enzyme gives a change in the ligand symmetry around the $Fe^{3+}$ in the yellow enzyme [2].

The possibility of a reversible formation of the $E^*$-4NC complex was studied by diluting a concentrated solution of green complex (pH 7.0) and recording of the absorbance at 650 nm. A few minutes after dilution the maximum decrease

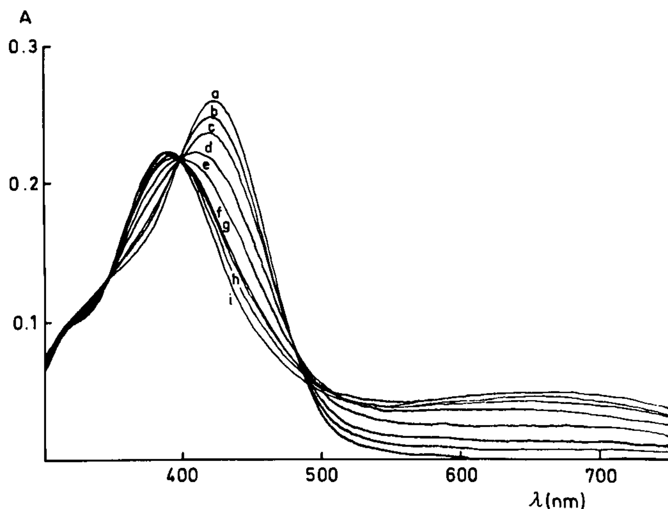



Fig. 2. Titration of 4-nitrocatechol with Fe(III)-lipoxygenase-1 at 20° C. Spectrum a is from 1 ml 30 μM 4-nitrocatechol in 0.1 M sodium phosphate buffer, pH 7.0. To this solution were added small volumes of 300 μM Fe(III)-lipoxygenase-1. Final concentrations of Fe(III)-lipoxygenase-1: b–i, 3.0, 6.0, 12.0, 18.0, 24.0, 27.0, 30.0 and 38.0 μM, respectively.

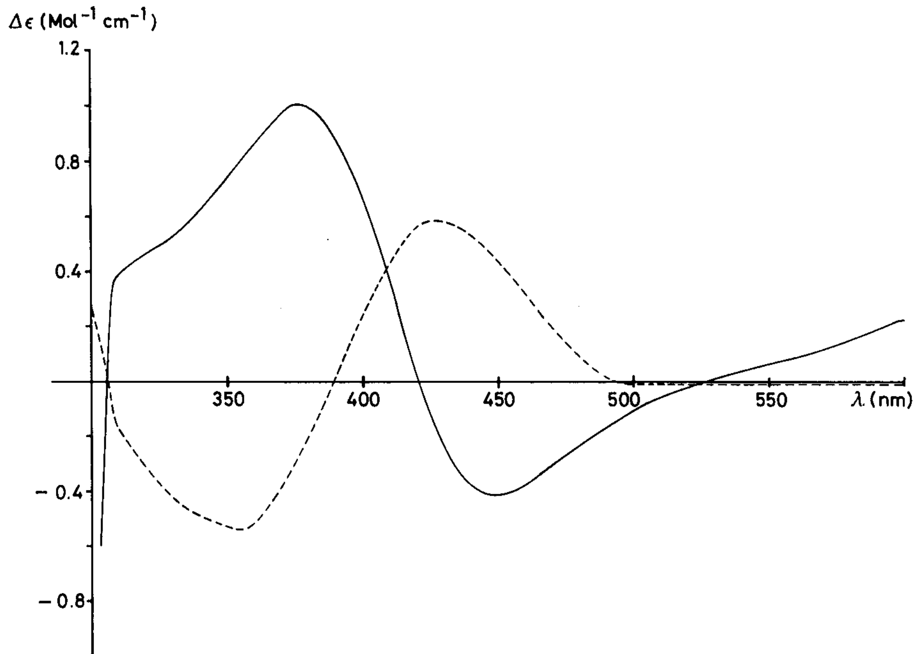



Fig. 3. CD spectra of 150 μM Fe(III)-lipoxygenase-1 with 150 μM 4-nitrocatechol in 0.1 M sodium phosphate buffer, pH 7.0 (———), recorded 10 min after mixing enzyme and 4-nitrocatechol ($t$ = 0° C) and of 150 μM Fe(III)-lipoxygenase-1 (- - - - - -).

of the absorbance at 650 nm was larger than could be expected on the basis of the dilution factor as such. Therefore it must be concluded that the formation of the green E*-4NC complex is reversible. The dissociation constant of the E*-4NC complex was estimated by measuring the absorbance at 650 nm of solutions of 120 μM yellow Fe(III)-enzyme in 0.1 M sodium phosphate buffer (pH 8.0) * to which various amounts of 4-nitrocatechol were added. After addition of 4-nitrocatechol to the enzyme solution it took several minutes before the maximum absorbance at 650 nm was reached. Upon titrating a solution of 120 μM Fe(III)-lipoxygenase-1 no further increase of the absorbance at 650 nm was observed at concentrations of 4-nitrocatechol over 2 mM. From this absorbance the extinction coefficient was calculated to be 2123 $M^{-1} \cdot cm^{-1}$. Using this value a dissociation constant ($K^*_{4NC}$) of 10.2 ± 0.6 μM (S.D.) for the E*-4NC complex was found.

The green E*-4NC complex was found to be rather unstable, especially at higher pH, as was apparent from the change in colour of the E*-4NC solution from green into brown (absorption maximum at 435 nm, shoulder at around 475 nm) (Fig. 4). From the decrease of the absorbance at 650 nm conversion rate constants were calculated to be $145 \cdot 10^{-5}$ $s^{-1}$ at pH 9.0, $16 \cdot 10^{-5}$ $s^{-1}$ at pH 8.0 and $3 \cdot 10^{-5}$ $s^{-1}$ at pH 7.0 ($t$ = 25°C). Possibly, oxidation of 4-nitrocatechol by Fe(III)-lipoxygenase-1 is responsible for the observed conversion.

Garssen et al. [7] reported that under anaerobic conditions both linoleic acid and 13-L-ROOH are converted by soybean lipoxygenase-1 in coupled reactions. Recently, Verhagen et al. [5] showed that in the anaerobic reaction only one substrate binding site is present in lipoxygenase-1, which alternately binds 13-L-ROOH and linoleic acid. In the present study the effect of 13-L-ROOH and of linoleic acid on the binding of 4-nitrocatechol to the Fe(III)-enzyme was investigated. It was found that 13-L-ROOH competes with 4-nitrocatechol for the binding site at the yellow enzyme. This was demonstrated by adding 13-L-ROOH to an oxygen-free solution of the E*-4NC complex (pH 7.0) which resulted in a shift of the absorption maximum from 650 to 580 nm, the latter being the absorption maximum of the blue complex of Fe(III)-lipoxygenase-1 and 13-L-ROOH [1,2]. Under anaerobic conditions, displacement of 4-nitrocatechol from the E*-4NC complex by linoleic acid is expected to lead ultimately to the reduction of the Fe(III)-enzyme. Addition of a molar excess of linoleic acid to an oxygen-free solution of E*-4NC complex (pH 7.0) causes the complete disappearance of the green colour ($A_{650}$) and the recovery of the unperturbed spectrum of 4-nitrocatechol. To show that concomitantly with the disappearance of the green colour the Fe(III)-enzyme is reduced by linoleic acid, the solution was flushed with oxygen. This leads to the formation of hydroperoxy-fatty acids and the reappearance of the green colour. The disappearance of the green colour of the E*-4NC complex under anaerobic conditions points to a competition between linoleic acid and 4-nitrocatechol for the binding site at the yellow enzyme. It has to be noted that also under aerobic conditions a competition between 13-L-ROOH and 4-nitrocatechol is

* The experiments were carried out at pH 8.0 to be able to compare the calculated dissociation constant with the value to be obtained from kinetic measurements. pH 8.0 is a compromise between the pH-dependent stability of the E*-4NC complex (see text) and the availability of a reasonable concentration range of linoleic acid in monomeric form for the kinetic experiments [6].

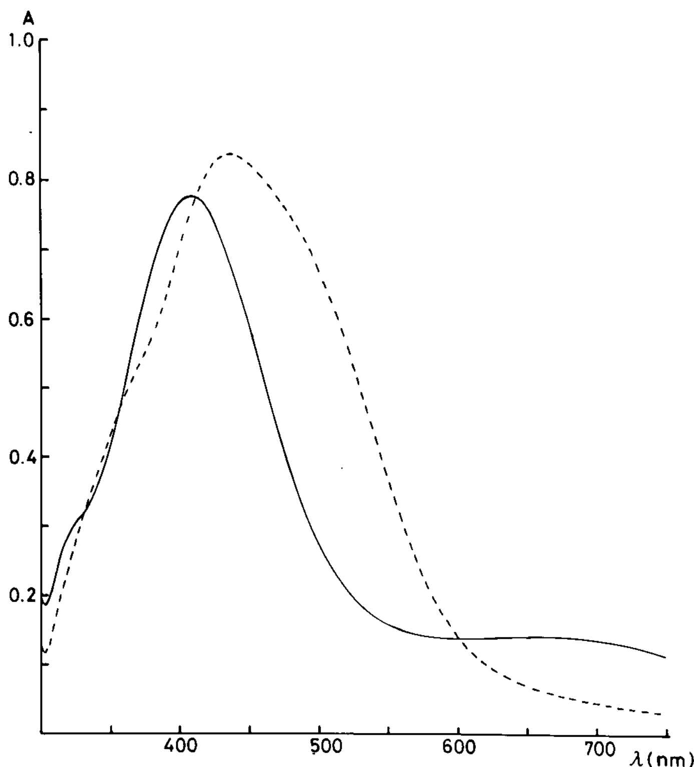



Fig. 4. Absorption spectra of 100 μM Fe(III)-lipoxygenase-1 with 100 μM 4-nitrocatechol in 0.1 M sodium phosphate buffer (pH 8.0) at 25° C (———) and of the same solution after 3 h at 25° C (- - - - -).

found, whereas competition between linoleic acid and 4-nitrocatechol cannot be observed in a similar experiment because the substrate fatty acid is rapidly converted into the hydroperoxide.

*Effect of 4-nitrocatechol on the oxygenation of linoleic acid by Fe(III)-lipoxygenase-1*

In view of the characteristics of the binding of 4-nitrocatechol to yellow Fe(III)-lipoxygenase-1, kinetics of the inhibition by 4-nitrocatechol of the lipoxygenase-catalyzed oxygenation of linoleic acid were studied. The following aspects were taken into consideration: viz. the reversibility of the inhibition (and the rate of the establishment of the $E^*$-4NC complex equilibrium) and the type of inhibition.

*Reversibility.* In a typical experiment 20 nM Fe(III)-lipoxygenase-1 was incubated with 300 μM 4-nitrocatechol in 0.1 M sodium phosphate buffer (pH 8.0) at 25°C. After various time intervals aliquots of the incubation mixture were taken in which the activity was determined directly or after 50-fold dilution. In the assays the final linoleic acid concentration was 20 μM in 0.1 M sodium phosphate buffer, pH 8.0. An almost complete inhibition (91%) in the undiluted sample was reached within 5 min. After dilution the activity could be

completely recovered only if the equilibrium of the $E^*$-4NC complex was allowed to re-establish, which took about 5 min. The inhibition is reversible and characterized by a slow establishment of the complex equilibrium, a feature which is not unusual for high-affinity inhibitors [8]. Although the inhibition in the undiluted samples did not increase significantly after incubation of Fe(III)-lipoxygenase-1 and 4-nitrocatechol for longer than 5 min it appeared that the recovery of the activity upon dilution decreased. From Fig. 5 it follows that during the incubation of Fe(III)-enzyme and 4-nitrocatechol the type of inhibition slowly changes from reversible to irreversible. This change in the nature of the inhibition was found to be directly coupled with the already mentioned conversion of the green $E^*$-4NC complex into a brown species.

*Type of inhibition.* To get insight into the mechanism of the inhibition by 4-nitrocatechol of the oxygenation of linoleic acid by Fe(III)-lipoxygenase-1, initial velocities were measured at various concentrations of linoleic acid and 4-nitrocatechol. Experimental conditions are described under Materials and Methods. On plotting the reciprocal values of the initial rates of hydroperoxide formation vs. the concentration of 4-nitrocatechol it was found that the straight lines, representing various concentrations of linoleic acid, have a common point of intersection on the negative abscissa (Fig. 6). This is characteristic for a non-competitive inhibition for which the following rate equation

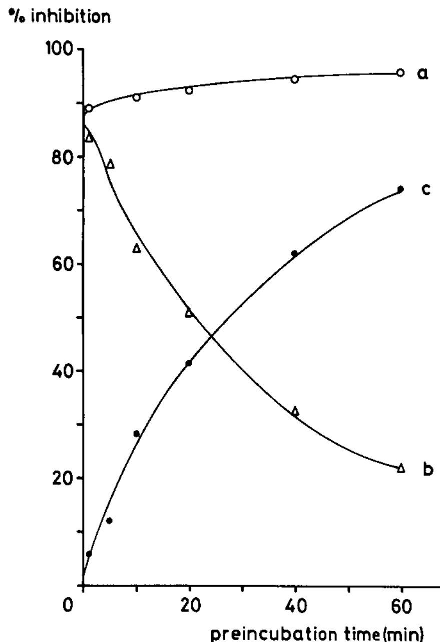



Fig. 5. Inhibition of Fe(III)-lipoxygenase -1 by 4-nitrocatechol (a) Inhibition of 20 nM Fe(III)-lipoxygenase-1 upon preincubation with 300 μM 4-nitrocatechol. (b) Reversible part of the inhibition, obtained from measurements of the activity of 50-fold diluted samples of the preincubation mixture, mentioned under (a). The diluted samples were allowed to stand for 5 min for the establishment of the complex equilibrium. (c) Calculated irreversible part of the inhibition.

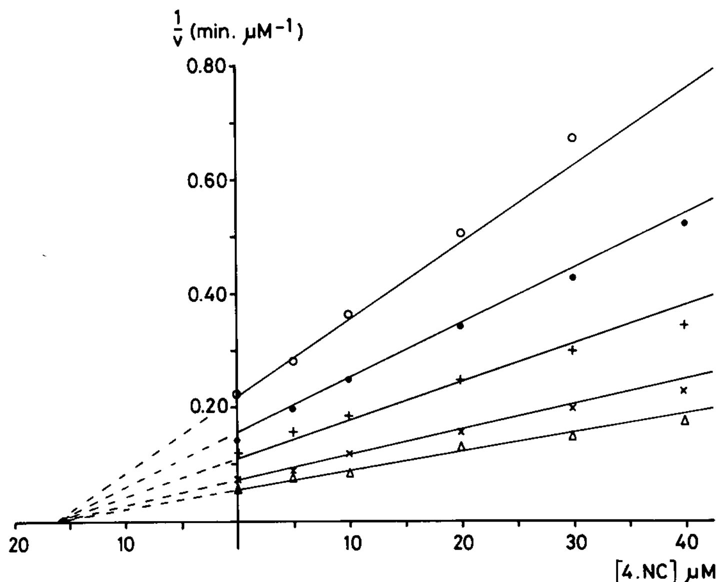


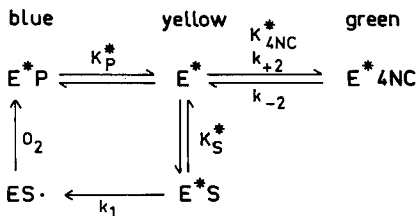

Fig. 6. Single-reciprocal plot of the initial oxygenation rate ($v$) as a function of the 4-nitrocatechol concentration. Enzyme concentration: 2 nM, pH 8.0, $t$ = 25°C. ———, computed values. Linoleic acid concentrations: ○———○, 5 μM; ●———●, 7.5 μM; +———+, 10 μM; X———X, 15 μM and △———△, 20 μM.

can be derived:

$$\frac{1}{v} = \frac{1}{V}\left[\frac{K_m^{*S}}{[S]}\left(1 + \frac{[4NC]}{K_{4NC}^*}\right) + 1 + \frac{[4NC]}{K_{4NC}^*}\right]$$

S represents the substrate linoleic acid and $K_{4NC}^*$ the inhibition constant for 4-nitrocatechol (4NC) (i.e. the dissociation constant for the E*-4NC complex). The experimental data were fitted to the given rate equation by means of an iterative least-squares method. A best-fit value of 16.3 ± 0.6 μM (S.D.) was found for the inhibition constant $K_{4NC}^*$ which agrees reasonable with the dissociation constant of 10.2 μM, estimated from the direct titration of Fe(III)-enzyme with 4-nitrocatechol.

The non-competitive type of inhibition of 4-nitrocatechol on the oxygenation of linoleic acid by Fe(III)-lipoxygenase-1 does not necessarily lead to the conclusion that two sites are present on the enzyme under aerobic conditions. Although several investigators [9—11] proposed that under aerobic conditions two sites on the enzyme are involved in the conversion of linoleic acid, viz. the binding site for linoleic acid and a regulatory site for 13-L-ROOH, it is worthwhile to consider the possibility of a pseudo non-competitive inhibition. As mentioned before, with a high-affinity inhibitor in combination with a slow establishment of the enzyme-inhibitor complex equilibrium, a competitive inhibition may appear non-competitive [8]. This can occur if the rate constant $k_{-2}$ (Scheme I) of the dissociation of the E*-4NC complex is much smaller than the catalytic constant of the oxygenation of linoleic acid by the enzyme ($k_{cat}$ = 309 $s^{-1}$) [5]. The rate constant ($k_{+2}$) of the formation of the green complex from Fe(III)-lipoxygenase-1 and 4-nitrocatechol was found to be 347 $M^{-1} \cdot s^{-1}$.




Scheme I. E*, Fe(III)-lipoxygenase-1, E, native lipoxygenase-1; S, linoleic acid; P, 13-L-hydroperoxy-*cis*, 9,*trans*-11-octadecadienoic acid; 4NC, 4-nitrocatechol.

Using this value and a $K^*_{4NC} = (k_{-2}/k_{+2})$ of 16.3 μM a first-order rate constant $k_{-2}$ of $5.7 \cdot 10^{-3}$ $s^{-1}$ was calculated. Consequently addition of linoleic acid to a solution of E*-4NC will not considerably perturb the complex equilibrium during the first stage of the oxygenation. Therefore the observed inhibition can adequately be explained in terms of a pseudo non-competitive model.

*Effect of 4-nitrocatechol on native lipoxygenase-1*

The existence of a complex between native lipoxygenase-1 and 4-nitrocatechol is unlikely because of the following observations. From Fe(III)-lipoxygenase-1 and 4-nitrocatechol the same amount of E*-4NC is formed both in the presence and in the absence of native enzyme. Furthermore titration of 4-nitrocatechol with native lipoxygenase-1 at pH 8.0, 7.0 or 4.5 did not result in any perturbation of the absorption spectrum of 4-nitrocatechol. Although the oxygenation of linoleic acid by native lipoxygenase-1 was found to be inhibited in the presence of 4-nitrocatechol, this probably represents a complexation by 4-nitrocatechol of the Fe(III)-enzyme, which was shown to be rapidly formed from the native enzyme during the oxygenation of linoleic acid [12].

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