Hydroperoxyfatty acids inactivate the reticulocyte lipoxygenase independently of a hydroperoxidase reaction

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From a comparison of $9D_S$-HPODE and $13L_S$-HPODE and their methyl esters as substrates and inactivating agents of reticulocyte lipoxygenase it is concluded that the compounds inactivate the enzyme independently of any hydroperoxidase reaction. The protective effect of 4-nitrocatechol indicates the formation of Fe(III) complexes of the enzyme with the hydroperoxyfatty acid compounds prior to inactivation.

Lipoxygenase *Enzyme inactivation* *Hydroperoxidase reaction* *Hydroperoxyfatty acid*

1. INTRODUCTION

Hydroperoxyfatty acids, products of lipoxygenase-catalyzed oxygenation, inactivate the reticulocyte lipoxygenase under both aerobic and anaerobic conditions [1]. The inactivation is paralleled by the conversion of one methionine residue, presumably located in the active center of the enzyme, to its sulfoxide [2]. It was hypothesized that the inactivation proceeds by way of a hydroperoxidase reaction. The present study was designed to investigate the connection between the inactivation and hydroperoxidase reactions. With this in mind, $9D_S$-HPODE and $13L_S$-HPODE were compared as substrates and inactivating reagents. It had been demonstrated previously that $9D_S$-HPODE was a substrate neither in the aerobic lipoxygenase nor in the anaerobic linoleic-acid-dependent hydroperoxidase reactions of soybean lipoxygenase-1 [3]. On the other hand, it was found to be a much better substrate than $13L_S$-HPODE in a linoleic acid-independent hydroperoxidase reaction. The mechanism of this type of reaction is unclarified; it may possibly proceed similarly to that described for heme peroxidase, with the formation of an iron complex with the hydroperoxy group of its substrate. In this case one would expect that Fe(III)-complexing agents may compete with the hydroperoxyfatty acid and thus protect the enzyme from inactivation. 4-Nitrocatechol has been described as an inhibitor of soybean lipoxygenase-1 and forms a distinct complex with the enzyme. 4-Nitrocatechol also forms a complex with the reticulocyte enzyme and inhibits it (unpublished).

Abbreviations: 9-HPODE, 9-hydroperoxy-10,12*(E,Z)*-octadecadienoic acid; 13-HPODE, 13-hydroperoxy-9,11*(Z,E)*-octadecadienoic acid

2. MATERIALS AND METHODS

Lipoxygenase was isolated from rabbit reticulocytes and purified according to [4]. Soybean lipoxygenase-1 was obtained from Sigma (St. Louis) and further purified by isoelectric focusing. Linoleic acid (>95%) was purchased from Fluka (Switzerland). 4-Nitrocatechol was obtained from Sigma.

$13L_S$-HPODE and $9D_S$-HPODE were prepared by aerobic incubation of linoleic acid at 2°C with soybean lipoxygenase-1 at pH 9.0 [5] and wheat lipoxygenase at pH 6.8, respectively, according to [6]. The racemic mixture of 13- and 9-hydroperoxyoctadecadienoic acids was prepared by oxygenation of linoleic acid at 2°C with pea lipoxygenase

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(isoenzyme 1) at pH 6.8 according to [7]. The hydroperoxyfatty acids were purified by silica gel column chromatography and checked for purity by straight phase HPLC [8]. The chemical structure of the compounds was confirmed by co-chromatography in straight phase HPLC, by UV and IR spectroscopy and gas chromatography/mass spectroscopy of the corresponding hydroxy derivatives obtained after triphenylphosphine reduction. The configurations of the chiral centers were determined by means of chiral phase HPLC [8]. The concentration of the hydroperoxides was determined spectrophotometrically by measuring the absorption at 234 nm using an ϵ of 2.5×10^4 $M^{-1} \cdot cm^{-1}$ [9].

Lipoxygenase activity was assayed by the method of Holman [10] measuring the increase in absorbance at 234 nm at 2°C in oxygen-saturated potassium phosphate buffer (0.2 M, pH 7.8) containing 0.2% sodium cholate and 0.530 mM linoleic acid. The anaerobic hydroperoxidase activity of the reticulocyte lipoxygenase in the presence and absence of linoleic acid was assayed by the change in absorption at 234 or 285 nm with a Unicam SP 1750 spectrophotometer. All assays were performed in an oxygen-free argon atmosphere. The anaerobic inhibition of the reticulocyte lipoxygenase by 4-nitrocatechol was determined by measuring the initial velocity of oxygenation of the preformed Fe(III)-enzyme prepared at 2°C immediately before use by incubation of native reticulocyte lipoxygenase with $13L_S$-HPODE at a 1:1.1 molar ratio at pH 7.8.

The molarity of reticulocyte lipoxygenase solutions was calculated from the catalytic activity using a molar catalytic activity of the enzyme of 20 s^{-1}.

3. RESULTS

In fig.1 are presented the effects of various concentrations of $9D_S$-HPODE and the $13L_S$ isomer on the extent of inactivations of reticulocyte lipoxygenase after a fixed time. It may be seen that both compounds exerted comparable effects. However, a somewhat more extensive inactivation was observed with $9D_S$-HPODE at lower concentrations.

In fig.2 the two isomers are compared as substrates in hydroperoxidase reactions under anaerobic conditions. Similarly to soybean lipoxygenase-1, $9D_S$-HPODE was found to be a good substrate in a linoleic acid-independent hydroperoxidase reaction. The rate of its disappearance was not stimulated by the addition of linoleic acid, even though a somewhat higher proportion of ketodienes was formed in its presence (fig.2A). In contrast, the consumption of $13L_S$-HPODE, which was practically zero in the absence of linoleic acid, was greatly stimulated by its addition (fig.2B).

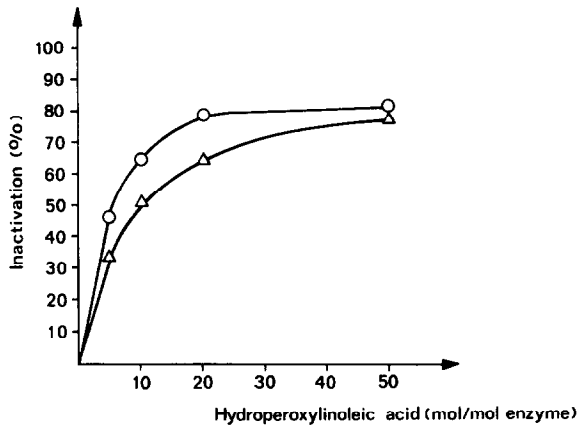

Fig.1. Titration curves of the anaerobic inactivation of reticulocyte lipoxygenase by $13L_S$-HPODE and $9D_S$-HPODE. 11.4 nM lipoxygenase (catalytic activity 0.225 $nmol \cdot s^{-1}$) and varying amounts of the $13L_S$-isomer (Δ—Δ) and $9D_S$-isomer (○—○) were preincubated at 37°C for 30 s in the assay medium without linoleic acid. The residual activity was assayed in the aerobic spectrophotometric lipoxygenase assay at 2°C.

To study the effect of positional and steric isomerism the methyl esters of $9D_S$-HPODE and $13L_S$-HPODE and the racemic mixtures of 9-HPODE and 13-HPODE were tested both for their potency as inactivators and as substrates of hydroperoxidase activity of reticulocyte lipoxygenase. The methyl esters inactivated the enzyme to about the same extent (fig.3). However, in contrast to the free acid, the methyl ester of $9D_S$-HPODE was no longer a substrate in the linoleic acid-independent hydroperoxidase reaction. The methyl ester of $13L_S$-HPODE on the other hand behaved like the free acid in both the presence and absence of linoleic acid (not shown).

Neither the rates of inactivation nor those of the hydroperoxidase reactions of the racemic mixtures of 9- or 13-HPODE differed significantly from

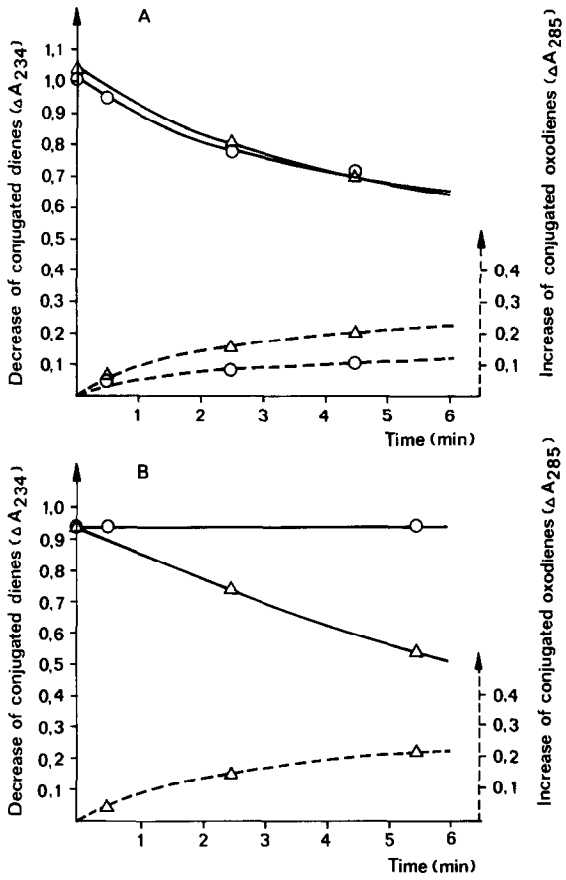

Fig.2. Anaerobic conversion of 13LS-HPODe and 9DS-HPODE in the absence and presence of linoleic acid at 2°C. (A) 24.6 nM lipoxygenase (catalytic activity 0.49 nmol·s^{-1}) was added to the anaerobic assay medium containing 40 μM 9DS-HPODE ((○—○) or the 9DS isomer and 120 μM linoleic acid (△—△). The absorbance was recorded at 234 nm (○—○, △—△) and 285 nm (○---○, △---△). (B) See (A) with 13LS-HPODE.

those of the pure 9DS-HPODE or 13LS-HPODE, respectively.

To test the assumption that the hydroperoxyfatty acids form complexes with the Fe(III) of reticulocyte lipoxygenase as a precondition of the inactivation, the effects of 4-nitrocatechol on the inactivation by 13LS-HPODE were studied. The results of one representative experiment are listed in table 1. All tests were performed under anaerobic conditions to circumvent as much as possible irreversible secondary inactivation of the 4-nitrocatechol-enzyme complex, which has been observed [11].

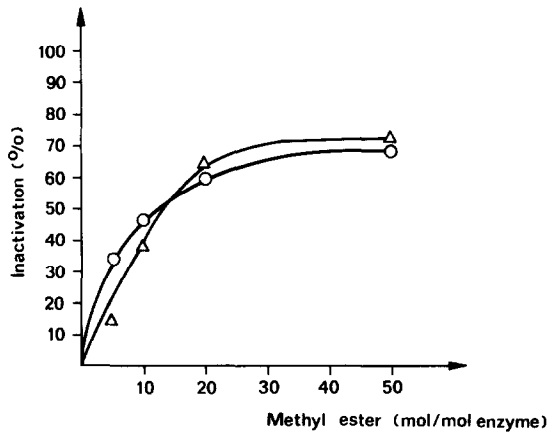

Fig.3. Titration curves of the anaerobic inactivation of reticulocyte lipoxygenase by methyl-13LS-HPODE and methyl-9DS-HPODE. 6.6 nM (catalytic activity 0.13 nmol·s^{-1}) lipoxygenase and varying amounts of methyl ester of the 13LS-isomer (△—△) and the 9DS-isomer (○—○) were preincubated at 30°C for 2 min in the assay medium without linoleic acid. The residual activity was assayed in the aerobic spectrophotometric lipoxygenase assay at 2°C.

The results on samples 3 and 4 demonstrate the inactivation of the enzyme by 4-nitrocatechol at 2 and 37°C, respectively. The extent of reactivation of the enzyme produced by displacement of the 4-nitrocatechol by an excess of 13LS-HPODE is shown on sample 5. The enzyme was nearly completely irreversibly inactivated by a small excess of 13LS-HPODE at 37°C as indicated by the result on sample 7 but not at 2°C (sample 6). The crucial evidence for the protective effect of 4-nitrocatechol is demonstrated on sample 9. Addition of a large excess of 13LS-HPODE at 2°C to a sample of enzyme which had been previously incubated at 37°C with both 4-nitrocatechol and lower amount of 13LS-HPODE, which by itself would have caused irreversible inactivation (see sample 6), restored activity, to the extent of 55% of the initial rate, or 70% of the rate after inhibition by nitrocatechol (see sample 5).

4. DISCUSSION

The results presented yield clear-cut evidence along two lines:

Table 1

The protective effect of 4-nitrocatechol against inactivation of reticulocyte lipoxygenase by 13Ls-HPODE at 37°C

Addition	Temperature (°C)	Lipoxygenase catalytic activity (nmol HPODE · s^{-1})	Inhibition (%)
(1) None	2	0.110	15.2
(2) None	37	0.093	92.2
(3) 4-Nitrocatechol	2	0.008	92.2
(4) 4-Nitrocatechol	37	0.020	81.8
(5) Addition of 13Ls-HPODE to sample 3	2	0.086	21.9
(6) 13Ls-HPODE	2	0.093	15.2
(7) 13Ls-HPODE	37	0.010	90.9
(8) Addition of 13Ls-HPODE to sample 4	37	0.026	75.8
(9) Addition of 13Ls-HPODE to sample 8	2	0.060	45.5

5.5 nM lipoxygenase and 6.05 nM 13Ls-HPODE were preincubated at 2°C for 1 min to convert the enzyme into the Fe(III) form. 10 μM 4-nitrocatechol was added and the reaction mixture was incubated at 2°C for 1 min. 8.4 nM 13Ls-HPODE was added to the preformed Fe(III)-enzyme (sample 6 and 7) and the 4-nitrocatechol-inhibited Fe(III)-lipoxygenase (sample 8). Samples 4, 7 and 8 were incubated for 3 min at 37°C. Samples 5 and 9 were cooled to 2°C, and 30 μM 13Ls-HPODE was added and incubated for 2 min at this temperature. All samples were added after the treatments to the aerobic spectrophotometric lipoxygenase assay at 2°C

(i) The data prove a dissociation between the hydroperoxidase reactions on the one hand and the inactivations of the lipoxygenase on the other. This conclusion is supported by two circumstances. Firstly, the inactivation by 9Ds-HPODE, which does not take part in the classic hydroperoxidase reaction with linoleic acid as hydrogen donor, rules out the relevance of its reaction scheme [12] for the mechanism of inactivation. Secondly, even the hydrogen-donor-independent hydroperoxidase reaction is excluded, since the methyl ester of 9Ds-HPODE, which is not a substrate of this type of reaction, inactivates the enzyme. Thus one is led to assume that the hydroperoxyfatty acids act in a direct manner, independent of any enzymatic catalytic mechanism.

(ii) A second conclusion can be drawn from the protective effect of 4-nitrocatechol on the inactivation of reticulocyte lipoxygenase by hydroperoxy acids. The inactivation must involve the formation of a Fe(III) complex of the enzyme with the inactivating compounds. It is conceivable that the inactivation as well as the selective oxidation of one methionine result from a homolytic O-O bond cleavage of the Fe(III) complex with the hydroperoxy group of the fatty acid.

The data on the effects of positional isomers and racemic mixtures of hydroperoxylinoleic acid do not permit an unequivocal answer as to whether positional or steric factors affect the inactivation and presumably the complexation of the enzyme. At any rate the effects, if any, appear to be small.

The demonstration of an inactivating effect of the methyl esters of hydroperoxylinoleic acid appears to be of biological relevance. Inactivation of reticulocyte lipoxygenase occurs during its interaction with mitochondria or electron transport particles without obligatory release of free fatty acids. It may be assumed that the hydroperoxyfatty acid-containing phospholipids of the mitochondria inactivate the enzyme in the same way as the methyl esters of the hydroperoxyfatty acids.

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