

Mechanism of Lipoxygenase Inactivation by the Linoleic Acid Analogue Octadeca-9,12-diyenoic Acid

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ABSTRACT: During the irreversible inactivation of soybean Fe(III)-lipoxygenase [Fe(III)-LOX] by octadeca-9,12-diyenoic acid (ODYA), significant quantities of 11-oxooctadeca-9,12-diyenoic acid (11-oxo-ODYA) are formed [Nieuwenhuizen, W. F., et al. (1995) *Biochemistry* 34, 10538–10545]. To elucidate the inactivation mechanism, a quantitative study into the relationship between the inactivation and 11-oxo-ODYA formation was carried out. The following observations were made. (1) LOX (0.84 μ M) was completely inactivated by 10 to 80 μ M ODYA. However, at ODYA concentrations greater than 100 μ M, LOX was only partially inactivated, and there was no inactivation at all at ODYA concentrations above 750 μ M. The average number of turnovers in which 11-oxo-ODYA was formed increased from 1.2 to 12 when the ODYA concentration increased from 1 to 50 μ M and then decreased again to 1.2 at 1000 μ M ODYA. (2) The enzyme that was not irreversibly inactivated by ODYA was in the Fe(III) form at ODYA concentrations below 10 μ M but in the Fe(II) form at ODYA concentrations greater than 100 μ M. (3) In the presence of 750 μ M ODYA and 25 μ M 13(*S*)-hydroperoxy-9Z,11E-octadecadienoic acid, all of the enzyme was inactivated. On the basis of these results, it is proposed that the dioxygenation product of ODYA is 11-hydroperoxyoctadeca-9,12-diyenoic acid (11-HP-ODYA), which can convert Fe(II)-LOX into its Fe(III) form. However, 11-HP-ODYA is converted into 11-oxo-ODYA, which cannot perform the oxidation. It is proposed that the inactivating agent is either 11-HP-ODYA or the 11-peroxy-octadeca-9,12-diyenoic acid radical (11-peroxy-ODYA radical), formed from the ODYA radical and O₂. The oxidation of Fe(II)-LOX into its Fe(III) form as well as the inactivation of Fe(III)-LOX is competitively inhibited by ODYA.

Lipoxygenases (EC 1.13.11.12) catalyze the conversion of 1Z,4Z-pentadiene systems in polyenoic fatty acids into 1-hydroperoxy-2E,4Z-pentadiene systems [for reviews, see Kühn et al. (1986), Schewe et al. (1986), Gardner (1991), and Veldink and Vliegthart (1991)]. Lipoxygenases are widespread among eukaryotes. The function of lipoxygenases in plants is only partly understood. However, lipoxygenase-1 from soybeans, which shares many important characteristics with other lipoxygenases, is widely used as a model enzyme, because it is available in quantities that allow detailed mechanistic and physical studies. In mammals, the product hydroperoxides play an important role in the immune response and in inflammatory processes. Therefore, there is considerable pharmaceutical interest in compounds that inhibit the lipoxygenase reaction (Nuhn et al., 1991; Ford-Hutchinson et al., 1994).

Triple-bond analogues of polyunsaturated fatty acids have been known as inactivators of lipoxygenase since 1965, and their inactivating potential has been investigated by a number of authors (Blain & Shearer, 1965; Downing et al., 1970, 1972; Hammerström, 1977; Corey & Munroe, 1982; Corey & Park, 1982; Kühn et al., 1984, 1991; Shieh et al., 1985; Corey, 1987; Borel et al., 1993; Nieuwenhuizen et al., 1995). It was shown in several of these studies that triple-bond analogues are enzymatically converted by lipoxygenase. A

conversion product of eicosa-5,8,11,14-tetraynoic acid (ETYA)¹ was observed, but not further identified (Kühn et al., 1984). Shieh et al. (1985) found that lipoxygenase converted 11Z-eicosa-11-en-14-ynoic acid (EEYA) into 11-hydroperoxy-12E-eicosa-12-en-14-ynoic acid (11-HP-EEYA) and 11-oxo-12E-eicosa-12-en-14-ynoic acid (11-oxo-EEYA). The product 11-HP-EEYA appeared to decompose non-enzymatically into 11-oxo-EEYA. We observed that octadeca-9,12-diyenoic acid (ODYA) was converted into 11-oxooctadeca-9,12-diyenoic acid (11-oxo-ODYA) upon treatment with Fe(III)-lipoxygenase (Nieuwenhuizen et al., 1995).

During the conversion of the triple-bond fatty acids, lipoxygenase is irreversibly inactivated, but it is not clear at which stage of the reaction the inactivation takes place. It has been firmly established that inactivation occurs only in the presence of O₂ (Corey & Park, 1982; Kühn et al., 1984; Shieh et al., 1985; Nieuwenhuizen et al., 1995). Furthermore, only Fe(III)-lipoxygenase is susceptible to inactivation by ODYA, whereas Fe(II)-lipoxygenase is hardly affected by this inhibitor (Nieuwenhuizen et al., 1995). Linoleic acid

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¹ Abbreviations: EEYA, 11Z-eicosa-11-en-14-ynoic acid; ETYA, eicosa-5,8,11,14-tetraynoic acid; GC/MS, gas chromatography/spectrometry; 11-HP-EEYA, 11-hydroperoxy-12E-eicosa-12-en-14-ynoic acid; 13-HP-OD, 13(*S*)-hydroperoxy-9Z,11E-octadecadienoic acid; 11-HP-ODYA, 11-hydroperoxyoctadeca-9,12-diyenoic acid; 11-oxo-ODYA, 11-oxooctadeca-9,12-diyenoic acid; 11-oxy-ODYA•, 11-oxyoctadeca-9,12-diyenoic acid free radical; 11-peroxy-ODYA•, 11-peroxyoctadeca-9,12-diyenoic acid free radical; ODYA, octadeca-9,12-diyenoic acid; ODYA•, octadeca-9,12-diyenoic acid free radical.

protects lipoxygenase against inactivation by EDTA (Kühn et al., 1984). Hydroxyl radicals, superoxide anions, and H_2O_2 are not involved in the inactivation (Kühn et al., 1984). 11-oxo-ODTA binds covalently to solvent-exposed amino groups of the protein, but this modification did not cause inactivation either (Nieuwenhuizen et al., 1995). However, Shieh et al. (1985) observed that one of the conversion products of EDTA, 11-HP-EDTA, can irreversibly inactivate lipoxygenase.

Kühn et al. (1984) analyzed their kinetic data on the basis of a suicide mechanism in which inactivation of the enzyme occurs effectively immediately after the enzymatic conversion of the triple-bond analogue. However, their mechanism does not explain the formation of significant amounts of reaction product during the inactivation. Shieh et al. (1985) used the same basic model to rationalize their data but allowed for dissociation and rebinding of the product. Neither of these mechanisms accounts for the role of the redox state of the lipoxygenase iron cofactor in the process of inactivation.

Here, we present the results of a detailed mechanistic study of the reaction between ODTA and soybean lipoxygenase-1 to further elucidate the relationship between 11-oxo-ODTA formation and lipoxygenase inactivation and to assess the nature of the inactivating species.

MATERIALS AND METHODS

Materials. The purification of lipoxygenase from soybeans (White Hilum) has been described previously (Finazzi-Agrò et al., 1973). The protein concentration in the lipoxygenase preparation was calculated from the absorbance at 280 nm, using an ϵ_{280} of $1.6 \times 10^5 \text{ L} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$. The purified lipoxygenase was stored at 15 mg/mL in a 0.05 M sodium acetate buffer, pH 5.5, containing 134 g/L ammonium sulfate. Fe(III)-lipoxygenase was prepared by mixing the purified Fe(II)-lipoxygenase (typically 1 mL of 15 mg/mL) with a 5-fold molar excess of 13(*S*)-hydroperoxy-9 Z ,11 E -octadecadienoic acid (13-HPOD) and dialyzing the solution $3 \times 45 \text{ min}$ against $3 \times 400 \text{ mL}$ of a 0.1 M sodium borate buffer, pH 10, at 4 °C. After this treatment, the specific activity of the enzyme preparation was approximately $90 \mu\text{mol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$. Linoleic acid (octadeca-9 Z ,12 Z -dienoic acid, 99% pure) was obtained from Sigma. The preparation and purification of 13-HPOD, octadeca-9,12-dienoic acid (ODTA), and 11-oxooctadeca-9,12-dienoic acid (11-oxo-ODTA) have been described elsewhere [13-HPOD: Schilstra et al., 1992; (11-oxo-)ODTA: Nieuwenhuizen et al., 1995]. Linoleic acid (300 mM in methanol, Merck, gradient grade), 13-HPOD (200 mM in methanol), ODTA (173 mg/mL, 625 mM, in methanol), and 11-oxo-ODTA (1 mM in methanol) were stored at -20 °C.

Methods. Incubations of lipoxygenase with ODTA were carried out at room temperature in 0.1 M sodium borate buffer, pH 10. The total volume of the incubation mixtures was 1.2 mL, and the lipoxygenase concentration was typically 0.14 mg/mL unless stated otherwise. ODTA (in methanol) was added to the incubation mixtures in volumes of 6 μL . Where applicable, 13-HPOD was added to the incubation mixtures in volumes of 12 μL . The final methanol concentration was 0.6% in all incubation mixtures. Measurements of the amount of reaction product (11-oxo-ODTA) and of the lipoxygenase activity were performed

after 5–7 h of incubation, unless stated otherwise. Duplicate measurements were performed 30–60 min after the first measurement, so as to ensure that no significant changes in residual activities and 11-oxo-ODTA concentrations occurred at the time of the measurement. The amount of 11-oxo-ODTA that was produced during the incubation of lipoxygenase and ODTA was estimated from the absorptions at 258 and 340 nm as $[11\text{-oxo-ODTA}] = \epsilon_{258}(A_{258} + 0.58A_{340})$ (Nieuwenhuizen et al., 1995) as measured on a Hewlett Packard 8452A diode array spectrophotometer.

Lipoxygenase activity was assayed at 25 °C in 0.1 M sodium borate buffer, pH 10, using a Hi-Tech Scientific SF-51 stopped-flow apparatus, as described previously (Schilstra et al., 1994). Two hundred fifty microliters of the incubation mixture was diluted with 5 mL 0.1 M of sodium borate buffer, pH 10, and stopped-flow mixed in a 1:1 ratio with a solution of 100 mM linoleic acid. The concentrations in the observation chamber were 50 μM linoleic acid, typically 3.5 $\mu\text{g/mL}$ lipoxygenase (active and inactivated) and 0–25 μM ODTA. At this linoleic acid concentration, the dioxygenation reactions reach steady state after approximately 2 s. The redox state of lipoxygenase at the start of the reaction is apparent from the shape of the curve during the pre-steady-state phase. Reactions initiated with Fe(II)-lipoxygenase show a pre-steady-state lag phase, whereas reactions initiated with Fe(III)-lipoxygenase show a burst. Reactions that are initiated with Fe(III)-lipoxygenase may also show a rate increase after the burst phase (a “secondary” lag period). Owing to the occasional dissociation of the Fe(II)-lipoxygenase–linoleic acid radical complex, a small fraction of Fe(II)-lipoxygenase is constantly being formed during the reaction. The Fe(III) enzyme is regenerated by the reaction product 13-HPOD. At steady state, the regeneration rate equals the rate at which the Fe(II)-lipoxygenase–linoleic acid radical dissociates. At high 13-HPOD concentrations, the regeneration rate is high, there is a large steady-state fraction of Fe(III)-lipoxygenase present, and hence the 13-HPOD production rate is high. Under the conditions of the test reaction (initial linoleic acid concentration 50 μM) the steady-state rate is maximum at about 5 μM 13-HPOD. At concentrations of active lipoxygenase (i.e., lipoxygenase that has not been irreversibly inactivated) higher than 10 nM, steady state is reached relatively late in the reaction. Under these conditions, the shapes of the curves are dominated by the pre-steady-state events. At lower lipoxygenase concentrations, however, the 13-HPOD production rate is low, and steady state is reached some time before the concentration of 13-HPOD is 5 μM . As a result, the curves will show a secondary lag period, regardless of the initial redox state of the enzyme (Schilstra et al., 1992, 1993, 1994). The maximum rate that is reached after the pre-steady-state period (i.e., the rate at the end of the secondary lag period, $r_{50\mu\text{M}}$) was used as a measure for the active lipoxygenase concentration. Measured values in the control samples (no ODTA, no additional 13-HPOD) were, typically, $4.5 \pm 0.1 \mu\text{M} \cdot \text{s}^{-1}$ at the beginning of the incubation and $2.0 \pm 0.1 \mu\text{M} \cdot \text{s}^{-1}$ after 4 h of incubation. The background inactivation is most likely due to the prolonged exposure of the enzyme to a temperature of 25 °C in 0.1 M sodium borate buffer, pH 10. The above rates correspond to active lipoxygenase concentrations in the incubation mixtures of 0.84 μM at the start and 0.4 μM after 4 h of incubation [using the simple Michaelis–Menten approximation of lipoxygenase kinetics

and assuming that $k_{\text{cat}} = 300 \text{ s}^{-1}$ and $K_m = 20 \mu\text{M}$ (Schilstra et al., 1992)]. The maximum concentration of ODA in the activity tests was $25 \mu\text{M}$. Control experiments, in which 150 nM lipoxygenase was stopped-flow mixed with a solution of $100 \mu\text{M}$ linoleic acid and $50 \mu\text{M}$ ODA, showed that the presence of $25 \mu\text{M}$ ODA reduces $r_{50\mu\text{M}}$ by less than 10%. Therefore, no attempts were made to remove the excess of ODA from the test mixtures or to correct the observed rates for the effect of ODA.

RESULTS

General. Fe(II)-lipoxygenase is sometimes described as the "inactive" form of the enzyme, because, unlike Fe(III)-lipoxygenase, it is incapable of carrying out hydrogen abstraction, the first step in the dioxygenation of polyunsaturated fatty acids. Fe(II)-lipoxygenase can be oxidized to the active Fe(III) form by 13-HPOD. However, we note that throughout this paper we have restricted the terms inactive and inactivated to describe a lipoxygenase species whose enzymatic activity is *irreversibly* lost, i.e., is *not* restored by 13-HPOD.

Product Formation and Lipoxygenase Inactivation at Various ODA Concentrations. In order to determine the stoichiometry of the reaction between ODA and lipoxygenase, Fe(III)-lipoxygenase ($0.84 \mu\text{M}$) was incubated with increasing concentrations (0 – $15 \mu\text{M}$) of ODA. After 5 h of incubation the concentration of 11-oxo-ODA and the residual lipoxygenase activity were measured.

Figure 1 shows that, at ODA concentrations up to $8 \mu\text{M}$, all ODA was converted into 11-oxo-ODA. At higher ODA concentrations the conversion was incomplete.

The relationship between the ODA concentration and the concentration of active lipoxygenase at the end of the reaction was nonlinear. In the ODA concentration range between 1 and $10 \mu\text{M}$, the amount of ODA required for the inactivation of 1 mol of lipoxygenase increased from 2 to 12 mol, with the concomitant production of 2–10 mol of 11-oxo-ODA (Figure 1B).

These findings are not compatible with a suicide mechanism in which inactivation takes place during or immediately after the enzymatic conversion of the substrate. In such a model, the molar ratio of product to inactivated enzyme is predicted to be constant, even if the inactivation occurred in a fraction of the enzymatic turnovers only.

Figure 2 shows the effects of ODA concentrations up to 1 mM on 11-oxo-ODA formation. The product formation reached a maximum at 30 – $50 \mu\text{M}$ ODA. At higher ODA concentrations, the amount of 11-oxo-ODA in the incubation mixtures decreased as the ODA concentration increased. At the highest ODA concentrations, the amount of 11-oxo-ODA had decreased to approximately 5% of the amount that was formed at $40 \mu\text{M}$ ODA. GC/MS analysis of the reaction mixtures revealed no evidence for reaction products other than 11-oxo-ODA [see Nieuwenhuizen et al. (1995)].

The residual lipoxygenase activity in the mixtures containing 10 – $80 \mu\text{M}$ ODA was less than 1% of the controls (no ODA). However, as the ODA concentration was increased above $80 \mu\text{M}$, the residual activity also increased, until, at the highest ODA concentrations, no inactivation was observed.

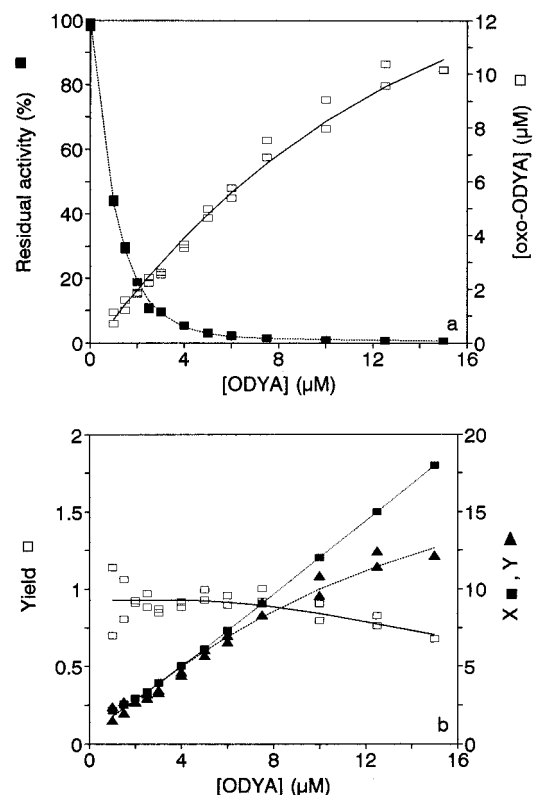


FIGURE 1: Relationship between the inactivation of lipoxygenase and the production of 11-oxo-ODA at ODA concentrations up to $15 \mu\text{M}$. (A, top) Residual lipoxygenase activity (■) and concentration of 11-oxo-ODA (□) after 5–6 h of incubation of $0.84 \mu\text{M}$ Fe(III)-lipoxygenase with ODA in 0.1 M sodium borate buffer, pH 10 at room temperature. (B, bottom) 11-oxo-ODA yield ($= [11\text{-oxo-ODA}]/[\text{ODA}]$, □), X, the number of moles of ODA required for the inactivation of 1 mol of lipoxygenase (■), and Y, the number of moles of 11-oxo-ODA produced during the inactivation of one molecule of lipoxygenase (▲). $X = [\text{ODA}]/[\text{E}_{\text{inact}}]$, and $Y = [11\text{-oxo-ODA}]/[\text{E}_{\text{inact}}]$. The concentration of inactivated lipoxygenase, $[\text{E}_{\text{inact}}]$, is equal to $[\text{E}_{\text{tot}}] \times \{1 - (\text{residual activity})/100\}$, where $[\text{E}_{\text{tot}}]$ is the total lipoxygenase concentration ($0.84 \mu\text{M}$).

Although lipoxygenase was not fully inactivated at ODA concentrations above $80 \mu\text{M}$, high ODA concentrations appeared to have a significant effect on the redox state of the enzyme. In Figure 2B, the progress curves that were obtained in four representative activity tests are displayed, together with their first derivatives (representing the 13-HPOD production rates). Curves 1 (control, no ODA) and 2 ($5 \mu\text{M}$ ODA) both have pre-steady-state burst phases of about 2 s. The burst phases are most clearly seen as an initial negative slope (a rate decrease) in the first derivative curves 1r and 2r. The progress curves of all samples that contained up to $7.5 \mu\text{M}$ ODA showed similar pre-steady-state burst phases. The presence of a pre-steady-state burst phase in these reaction progress curves indicates that most of the lipoxygenase in the corresponding samples was still in the Fe(III) form. However, curves 3 and 3r ($125 \mu\text{M}$ ODA), and 4 and 4r ($750 \mu\text{M}$ ODA), and all other curves of samples that were incubated with more than $80 \mu\text{M}$ ODA, showed no burst phase at all, but an initial rate increase. The absence of a burst indicates that the reaction was initiated with Fe(II)-lipoxygenase (Schilstra et al., 1994). Apparently, incubation of Fe(III)-lipoxygenase with ODA concentrations higher than $80 \mu\text{M}$ results in reduction of the iron cofactor to Fe(II). Curve 2 also shows a pronounced rate increase immediately

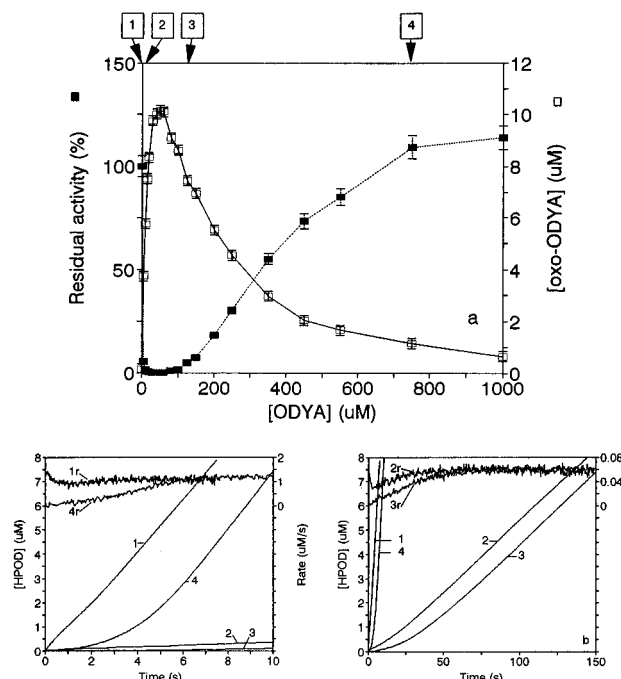


FIGURE 2: Relationship between the inactivation of lipoxygenase and the production of 11-oxo-ODYA at ODA concentrations up to 1 mM. (A, top) Residual lipoxygenase activity (■) and concentration of 11-oxo-ODYA (□); concentrations as in Figure 1. Square blocked numbers on top of panel A indicate that the reaction progress curves of the corresponding test reactions are shown in panel B. (B, bottom) Reaction progress curves obtained in the activity tests (curves 1, 2, 3, 4, left axes), and their first derivatives (i.e., 13-HPOD production rates, curves 1r, 2r, 3r, 4r, right axes) on two different time scales. Incubations with 0 (curves 1, 1r), 5 (curves 2, 2r), 125 (curves 3, 3r), and 750 (curves 4, 4r) μM ODA. Test conditions: 50 μM linoleic acid + 20 nM lipoxygenase (active plus inactivated) in 0.1 M sodium borate buffer, pH 10, 25 °C.

after the burst phase, but this secondary lag period is not related to the initial redox state of lipoxygenase (see Materials and Methods).

The experiments described above lead us to the following conclusions. At ODA concentrations below 10 μM, the reactions stop when all ODA has been converted, and the remaining lipoxygenase is in the Fe(III) form. At ODA concentrations greater than 80 μM, the reactions terminate when all lipoxygenase is reduced to its Fe(II) form, which is insensitive to the remaining ODA in the solution. Only at ODA concentrations between 10 and 80 μM do the reactions stop because all the lipoxygenase is inactivated.

Effect of 13-HPOD. To investigate whether (re-)oxidation of Fe(II)-lipoxygenase by 13-HPOD enhances 11-oxo-ODYA formation and enzyme inactivation, Fe(II)-lipoxygenase (1.5 μM) was incubated with increasing amounts of 13-HPOD (0–53 μM) for 2 min. Then ODA was added to final concentrations of 75 or 750 μM. In the mixtures that contained 75 μM ODA and 3.3 μM 13-HPOD or more, the concentration of active lipoxygenase had decreased to less than 0.05% of its original value after 5 h of incubation (Figure 3). The 11-oxo-ODYA concentration detected in these samples was 20 μM. This implies that approximately 13 mol of 11-oxo-ODYA had been formed during the inactivation of 1 mol of lipoxygenase. In the samples containing 750 μM ODA, complete inactivation (<0.1% active lipoxygenase) was achieved only at the two highest 13-HPOD concentrations (27 and 53 μM). However, the

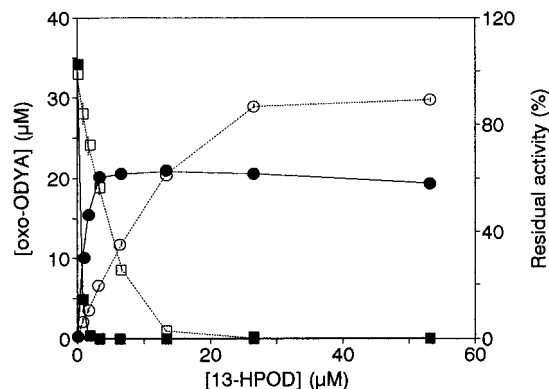


FIGURE 3: Effect of 13-HPOD on inactivation of lipoxygenase and 11-oxo-ODYA formation. Residual lipoxygenase activity (squares) and 11-oxo-ODYA concentrations (circles) after approximately 5 h of incubation of 1.5 μM Fe(II)-lipoxygenase with 75 μM (closed symbols) or 750 μM ODA (open symbols) in 0.1 M sodium borate buffer, pH 10, at room temperature.

concentration of 11-oxo-ODYA in these mixtures was significantly higher (30 μM) than in the 75 μM ODA mixtures; under these conditions, 20 mol of 11-oxo-ODYA is formed during the inactivation of 1 mol of lipoxygenase. On the basis of these observations, the following conclusions may be drawn. (1) Lipoxygenase can be irreversibly inactivated by high concentrations of ODA, provided that an agent such as 13-HPOD, capable of reoxidizing Fe(II)- to Fe(III)-lipoxygenase, is present. (2) The higher the concentration of ODA in the incubation mixtures, the more 13-HPOD is required for complete irreversible inactivation of lipoxygenase. (3) The maximum number of turnovers increases with the ODA concentration.

Effect of 13-HPOD under Anaerobic Conditions. Several authors have reported that, in the absence of O₂, lipoxygenase is not susceptible to inactivation by triple-bond substrate analogues (Corey & Park, 1982; Kühn et al., 1984). In order to investigate whether ODA can stimulate the peroxidase activity of lipoxygenase under anaerobic conditions, we incubated 40 μM ODA or 40 μM linoleic acid with 80 μM 13-HPOD and 1 μM Fe(II)-lipoxygenase in 0.1 M sodium borate buffer, pH 10, at 25 °C in the absence of O₂ and monitored the changes in the absorbances at 234 nm (indicative of the disappearance of 13-HPOD) and 280 nm (indicative of the appearance of the products of the peroxidase reaction). The reaction with ODA was approximately 30 times slower than the reaction with linoleic acid, but in both experiments A_{234} decreased from 2 to 0.9, and A_{280} increased from 0.2 to 1.9. Lipoxygenase remained fully active in both experiments. It is concluded, therefore, that ODA is capable of reducing Fe(III)- to Fe(II)-lipoxygenase in the absence of O₂.

DISCUSSION

In a previous study (Nieuwenhuizen et al., 1995) we demonstrated that, in the reaction between soybean lipoxygenase-1 and ODA, lipoxygenase can undergo some 10 catalytic cycles before it is irreversibly inactivated. The product of this reaction, 11-oxo-ODYA, does not by itself inactivate the enzyme. The aim of the study presented here was to further clarify the mechanism of the reaction in which 11-oxo-ODYA is formed, to unravel the relationship between product formation and inactivation, and hence to identify the inactivating species.

Table 1: Postulated Short-Lived Intermediates in the Reaction between Lipoyxygenase and O₂YA

short-lived intermediate	molecular structure	formation
ODYA•	C ₅ H ₁₁ C≡C(CH•)C≡CC ₇ H ₁₄ CO ₂ H	upon dissociation of Fe(II)-LOX-ODYA• complex
11-peroxy-ODYA•	C ₅ H ₁₁ C≡C(CHO ₂ •)C≡CC ₇ H ₁₄ CO ₂ H	upon reaction between O ₂ YA• and O ₂
11-HP-ODYA	C ₅ H ₁₁ C≡C(CHO ₂ H)C≡CC ₇ H ₁₄ CO ₂ H	upon enzymatic dioxygenation of O ₂ YA
11-oxo-ODYA•	C ₅ H ₁₁ C≡C(CHO•)C≡CC ₇ H ₁₄ CO ₂ H	upon oxidation of Fe(II)-LOX by 11-HP-ODYA

Mechanism of 11-oxo-ODYA Formation. The conversion of O₂YA into 11-oxo-ODYA, catalyzed by soybean lipoyxygenase-1, shares some important characteristics with the dioxygenation of linoleic acid. In both reactions the enzyme shuttles between its Fe(III)- and Fe(II) forms, and product formation requires equimolar amounts of lipid substrate and O₂. However, the two processes differ in a number of aspects. First, the main reaction product of linoleic acid dioxygenation is 13-HPOD, a hydroperoxide, whereas the final product in the reaction with O₂YA is a ketone. Secondly, dioxygenation of linoleic acid does not stop before all of the substrate has been converted, and, at the end of the reaction, lipoyxygenase is invariably in the Fe(III) form. However, complete conversion, with lipoyxygenase in the Fe(III) form at the end of the reaction, is observed only at O₂YA concentrations smaller than 7.5 μ M. Reactions with O₂YA concentrations greater than 80 μ M stop before complete conversion of O₂YA has taken place, and the residual active enzyme is Fe(II)-lipoyxygenase.

Because of the similarities between the dioxygenation of linoleic acid and the lipoyxygenase-catalyzed formation of 11-oxo-ODYA, it is highly likely that the primary reaction steps in the formation of 11-oxo-ODYA (Nieuwenhuizen et al., 1995) are identical to those in the lipoyxygenase-catalyzed formation of 13-HPOD from linoleic acid, namely, hydrogen abstraction step leading to radical formation and reduction of Fe(III)- to Fe(II)-lipoyxygenase. In the O₂-insertion step, reoxidation of the iron cofactor is accompanied by the formation of a putative intermediate reaction product, 11-hydroperoxy-octadeca-9,12-diynoic acid (11-HP-ODYA). In contrast to 13-HPOD, which is stable, 11-HP-ODYA is further converted into 11-oxo-ODYA and H₂O. Shieh et al. (1985) found that the main product in the reaction between lipoyxygenase and EEYA, a lipoyxygenase inhibitor containing a penta-1Z-en-4-ynoic system, was a hydroperoxide compound, which metabolized slowly into the corresponding ketone. This is another indication for the occurrence of the unstable 11-HP-ODYA intermediate in the formation of 11-oxo-ODYA.

Fe(II)-lipoyxygenase is formed when an Fe(II)-lipoyxygenase-ODYA• complex dissociates before O₂ insertion has occurred. We propose that Fe(II)-lipoyxygenase is converted back into its Fe(III) form by 11-HP-ODYA, formed in a previous catalytic cycle. O₂YA and the unstable 11-HP-ODYA compete for binding to Fe(II)-lipoyxygenase. At low O₂YA concentrations, reoxidation is sufficiently fast to maintain most of the enzyme in its Fe(III) form, but, at higher O₂YA concentrations, the reoxidation process is hindered, and at very high O₂YA concentrations the reoxidation is blocked. This explains why, at high O₂YA concentrations, the residual active lipoyxygenase is in the Fe(II) form, and why there is little 11-oxo-ODYA formation under those conditions.

The Inactivation Process. The main difference between the lipoyxygenase catalysed conversions of linoleic acid into

13-HPOD on the one hand, and of O₂YA into 11-oxo-ODYA on the other, is the extent to which lipoyxygenase is inactivated. Models in which the inactivation occurs during the O₂-insertion step are ruled out, because the molar ratio of 11-oxo-ODYA to inactivated lipoyxygenase is not constant (see Results). The fact that the number of O₂YA molecules that is required for the inactivation increases with the O₂YA concentration indicates that O₂YA competes with the inactivating agent for the site where inactivation occurs. Competition between O₂YA and an agent that causes irreversible inactivation will only occur if the inactivating agent exists free in solution, and if its lifetime is finite. In that case, the chance that the inactivating species binds to lipoyxygenase decreases when the O₂YA concentration increases. Short-lived compounds that may be present in the reaction mixture are listed in Table 1. Since lipoyxygenase remains active during the anaerobic reaction between 13-HPOD and O₂YA, one can exclude that the O₂YA radical functions as the inactivating agent. Shieh et al. (1985) found that the hydroperoxide HP-EEYA was an inactivator of lipoyxygenase. Our results support a model in which 11-HP-ODYA functions not only as a stimulant but also as an inactivating agent. However, a mechanism in which the 11-(per)oxy-ODYA radical acts as the inactivator cannot be excluded. Work is in progress to distinguish between these possibilities.

This study has shown that inactivation of lipoyxygenase is caused by a conversion product of O₂YA that, for a limited amount of time, exists free in solution. O₂YA itself, however, inhibits the inactivating process. It is, therefore, highly likely that isolated conversion products of O₂YA and other triple-bond analogues of polyunsaturated fatty acids will be more effective as lipoyxygenase inhibitors than the triple-bond analogues themselves (cf. Shieh et al., 1985).

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