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On the Mechanism of Action of Soybean Lipoxygenase-1

A Stopped-Flow Kinetic Study of the Formation and Conversion of Yellow and Purple Enzyme Species

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1. The conversion of the native iron-containing lipoxygenase-1 into yellow and purple ferric enzyme species by 13-L-hydroperoxy-9-*cis*,11-*trans*-octadecadienoic acid (R-13-OOH) was studied by measuring the absorbance changes at 330 nm and 580 nm, respectively in the stopped-flow apparatus. The pseudo-first-order rate constant for this reaction was found to be 50 s^{-1} at 4.4 °C, either in the presence or absence of O_2. This rate constant is lower than the one for R-13-OOH formation from linoleic acid and O_2 catalysed by lipoxygenase-1: k_{cat} = 232 s^{-1} at 4.4 °C.

2. The coloured ferric enzymes are formed only in the presence of R-13-OOH; therefore the formation of the coloured ferric enzymes from oxygen, linoleic acid and the native enzyme indicates that the latter is active even before it is converted into the ferric forms.

3. Under anaerobic conditions the yellow and purple enzyme species are rapidly converted into colourless ferrous enzyme species by linoleic acid at a rate which is faster than the formation of the coloured enzymes from R-13-OOH and the native ferrous enzyme.

4. Both the aerobic formation of the coloured ferric enzymes and the anaerobic bleaching of these enzymes occur more slowly when [11-$^2H_2$]linoleic acid instead of unlabeled linoleic acid is used. The kinetic deuterium isotope effect as estimated from the rates of aerobic formation of R-13-OOH is approximately 9, whereas in the anaerobic conversion of R-13-OOH and deuterated linoleic acid a value of 1.1 is found. This demonstrates that H abstraction from linoleic acid determines the rate of the aerobic formation but not of the anaerobic conversion of R-13-OOH.

5. The rate constant for the anaerobic conversion of [11-$^2H_2$]linoleic acid by the yellow ferric enzyme species, $k(^2H)$ = 30 s^{-1} at 25 °C, is close to the rate constant for the overall oxygenation reaction of the deuterated substrate, $k_{cat}(^2H)$ = 32 s^{-1}. For unlabelled linoleic acid the rate constants of the anaerobic H-abstraction step and the overall oxygenation reaction were found to be 111 s^{-1} and 290 s^{-1}, respectively at 25 °C. The former rate constant is probably affected by substrate inhibition.

6. Since the native ferrous enzyme is capable of catalysing H abstraction from linoleic acid under aerobic but not under anaerobic conditions, while the yellow ferric enzyme is active either in the absence or presence of O_2, it is proposed that O_2 reversibly converts the ferrous enzyme species into a ferric state.

Lipoxygenase-1 from soybeans (lineolate : O_2 oxido-reductase) catalyses the conversion of linoleic acid into 13-L$_S$-hydroperoxy-linoleic acid (R-13-ROOH) [1]. The enzyme (M_r approx. 10^5) contains EPR-silent non-heme iron (1 mol/mol of enzyme [2, 3]) and is colourless. A yellow-coloured enzyme species is formed (broad absorption band, ε_{max} at 330 nm = 1600 $M^{-1} \cdot cm^{-1}$) upon addition of an equimolar amount of R-13-OOH to the native enzyme [4]. Formation of this absorption band gives rise to a quenching of the tryptophan fluorescence of the enzyme at 328 nm [5]. The yellow enzyme species shows EPR signals around g = 6 [6]. Further addition of R-13-OOH changes the colour of the enzyme from yellow into purple [7]. The purple enzyme form has an absorption band at 580 nm in addition to one at 330 nm (ε at 580 nm = 1000 $M^{-1} \cdot cm^{-1}$) and shows a strong EPR signal at g = 4.3, while the signals around g = 6 are reduced in intensity [7]. This enzyme species consists of a complex of R-13-

Abbreviations. EPR, electron paramagnetic resonance; R-13-OOH, 13-L-hydroperoxy-9-*cis*,11-*trans*-octadecadienoic acid.

Enzyme. Lipoxygenase or lineolate : oxygen oxidoreductase (EC 1.13.11.12).

OOH and the yellow enzyme [7]. The complex is unstable at room temperature and shows a slow isomerization of the bound R-13-OOH into *threo*-11-hydroxy,*trans*-12,13-epoxy,9-*cis*-octadecenoic acid [8]. Both oxygen atoms are retained in this product to a high extent without exchange with solvent water. The yellow enzyme species is regenerated after complete conversion of R-13-OOH. The rate of the isomerization reaction is insignificant with respect to the rate of formation of R-13-OOH. The native colourless enzyme and the yellow enzyme species appeared to have identical catalytic activities, when incubated with linoleic acid and O_2 under steady-state conditions [9]. Both enzyme species are inhibited by excess linoleic acid, while substrate inhibition is reversed by the product, R-13-OOH [9,10]. The latter result explains the typically curved progress curves observed when R-13-OOH formation or O_2 uptake is followed at high levels of the fatty acid substrate and provides additional evidence for the formation of a relatively stable enzyme · R-13-OOH complex.

In the absence of O_2 the activity of the native colourless enzyme is lost. However, the yellow enzyme species is still able to convert linoleic acid [4,6]. In this process the yellow enzyme is converted into a colourless EPR-silent form.

When R-13-OOH is added to this enzyme, the yellow enzyme species is regenerated [6]. It was found, that lipoxygenase-1 converts linoleic acid and R-13-OOH under anaerobic conditions into fatty acid dimers, oxodienoic acids and *n*-pentane [11]. By using the radical trapping method, the intermediary formation of linoleic acid radicals could be demonstrated [12]. Very recently Aoshima *et al.* [13] reported on the kinetics of the aerobic conversion of the native lipoxygenase-1 by R-13-OOH as studied by the fluorescence stopped-flow technique. They confirmed previous evidence of the formation of an enzyme · R-13-OOH complex, which then decomposes to yield the low fluorescent yellow enzyme species and unknown products derived from R-13-OOH. A comparison of the rate of this conversion with the catalytic activity of the enzyme was not made.

In order to study the relevance of the formation and conversion of the different enzyme species during catalytic turnover, the kinetics of their interconversions were investigated by the stopped-flow technique.

MATERIALS AND METHODS

Linoleic acid (purity higher than 99%) and [11-$^2H_2$]linoleic acid (purity 98%; ^2H content at C-11 96%) were kindly provided by the Unilever Research Laboratories (Vlaardingen/Duiven, The Netherlands). Preparation of R-13-OOH was carried out as described previously [9, 14]. Lipoxygenase-1 was isolated from soybeans and purified according to Finazzi-Agrò *et al.* [5]. The yellow ferric enzyme species was prepared by the addition of a slight molar excess of R-13-OOH to the native enzyme at pH 9.5 and 0 °C. The yellow enzyme was either used as such or was purified from the reaction products and unconverted R-13-OOH by column chromatography on Sephadex G-25 (fine) (column dimensions: 80 × 1.5 cm).

Stopped-flow experiments were carried out with a Durrum single-beam apparatus (light pathway 2 cm). Recorder traces were stored in a Biomation transient recorder model 802 (Biomation, Palo Alto, Cal., U.S.A.). Calculations on the raw data were carried out with a Hewlett Packard calculator model 9830 A, equipped with a model 9863 A punched tape reader and a model 9862 A plotter. For slow absorbance changes relative to the sweep time 100 data points per recorder trace were found to be sufficient; more rapid changes were monitored on the basis of 1000 data points per trace. All experiments were carried out in 0.1 M sodium borate buffer, pH 9.5.

RESULTS AND DISCUSSION

Formation of the Yellow Enzyme Species from R-13-OOH and the Native Colourless Enzyme

At high concentrations of R-13-OOH the formation of the yellow enzyme species appeared to be too fast for stopped-flow analysis at 25 °C. Therefore the native enzyme was incubated with a range of R-13-OOH concentrations at lower temperatures (approx. 4 °C). Typical examples of the formation of the yellow enzyme species at 4.4 °C are shown in Fig. 1. This figure demonstrates that the reaction traces are essentially identical under aerobic and anaerobic conditions. Thus the reaction of R-13-OOH with the native EPR-silent enzyme species is not affected by O_2 as far as the formation of the yellow enzyme species is concerned.

The insert in Fig. 1 shows a semilogarithmic plot of the absorbance change *vs* time for trace (a) of Fig. 1 and confirms the conclusion by Aoshima *et al.* [13] that the reaction follows pseudo-first-order kinetics. The correlation coefficient is 0.99 for the least-squares fit to the straight line shown. The rate constants for traces (a) and (b) are $25 \pm 0.5\ s^{-1}$ and $22 \pm 0.5\ s^{-1}$, respectively. Indeed at higher concentrations of R-13-OOH higher rate constants were found (*cf.* [13]).

For the suggested reaction sequence (Scheme I):

$$E(FeII) + R\text{-}13\text{-}OOH \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E(FeII) \cdot R\text{-}13\text{-}OOH \xrightarrow{k_2} E(FeIII) + P$$

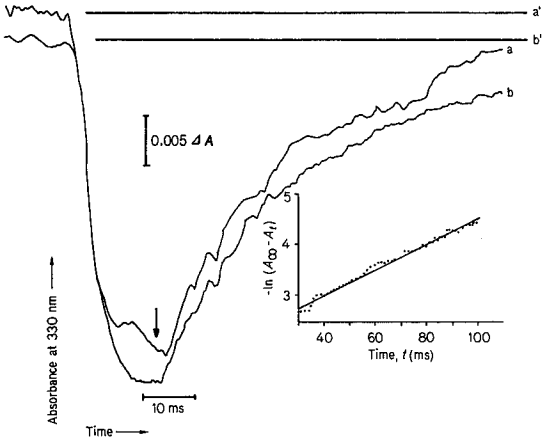


Fig. 1. *Absorbance changes at 330 nm resulting upon mixing of R-13-OOH (30 μM) with the native lipoxygenase-1 (25 μM) at 4.4 °C in the absence (trace a) and presence (trace b) of O_2 (240 μM).* The rapid initial drop in the absorbance is due to the introduction of fresh material; the flow stops at the time indicated by the arrow. The final absorbance is given by the straight lines a' and b' respectively. The traces contain 100 data points per full sweep. Insert: the negative logarithm of the absorbance change plotted *vs* time

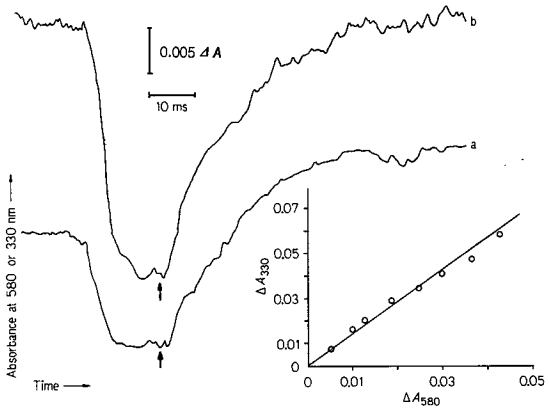


Fig. 2. *Absorbance changes at 580 nm (trace a) and 330 nm (trace b) after mixing of R-13-OOH (100 μM) with the native enzyme (25 μM) at 4.4 °C in the absence of O_2.* 100 points per full sweep; flow stops at arrow. Insert: absorbance change at 330 nm plotted *vs* the change at 580 nm

where k_1 and k_{-1} are $\gg k_2$ and P represents the final products formed from R-13-OOH (not identified, *cf.* [4]); k_2 and k_{-1}/k_1 were found in our experiments to be $50 \pm 2\ s^{-1}$ and $20 \pm 2\ \mu M$, respectively. It has to be mentioned that at R-13-OOH concentrations higher than 100 μM the semilogarithmic plots of the reaction traces fitted less well to straight lines. If for example at 500 μM R-13-OOH only the first half of the reaction trace is plotted in a semilogarithmic plot, then a slightly higher value for k_2 is obtained, being $61 \pm 3\ s^{-1}$ (correlation coefficient for 50% conversion is 0.98). Under these conditions formation of the yellow enzyme species may be more complicated than suggested above.

Formation of the Purple Enzyme Species from R-13-OOH and the Native Colourless Enzyme Species

In Fig. 2 the formation of the purple enzyme species is shown, monitored at 580 nm (trace a). For comparison the absorbance change at 330 nm under identical conditions is also shown (trace b). The purple enzyme species appears to be formed concomitantly with the yellow enzyme, as can be observed in the insert in Fig. 2.

At lower R-13-OOH concentrations (Fig. 3) the two coloured enzyme species are first concomitantly formed, but the absorbance at 580 nm levels off before all yellow enzyme is formed (see also insert in Fig. 3).

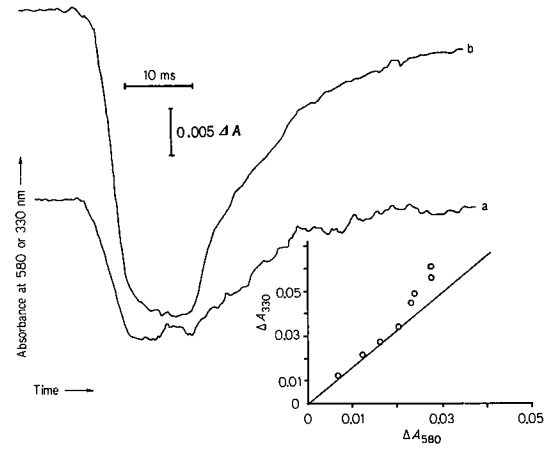


Fig. 3. *Absorbance changes at 580 nm and 330 nm (traces a and b, respectively) resulting upon mixing of 50 μM R-13-OOH with 25 μM enzyme at 4.4 °C in the absence of O_2.* 100 points per full sweep; flow stops at arrow. Insert: absorbance change at 330 nm plotted *vs* the change at 580 nm

Formation of the Purple Enzyme Species from R-13-OOH and the Yellow Enzyme Species

The results in the preceding paragraph suggest that the purple enzyme species is very rapidly formed, once the native enzyme species is converted into the yellow form by R-13-OOH and excess R-13-OOH is still present. This sequence of events was checked by mixing R-13-OOH and pre-formed yellow enzyme in the stopped-flow apparatus. Indeed under these conditions formation of the purple enzyme species pro-

ceeds too rapidly for stopped-flow analysis (*i.e.* within a few milliseconds). At approximately 4 °C the purple enzyme species is fairly stable. For 25 μM purple enzyme in the presence of 30 μM free R-13-OOH the half life of the isomerization reaction is about 30 min. Thus, the reactions of R-13-OOH with lipoxygenase-1 (neglecting possible complications at high R-13-OOH concentrations) can be summarized as follows (Scheme II):

$$\underset{\text{colourless, EPR silent}}{E(FeII)} + \text{R-13-OOH} \underset{k_1}{\overset{k_{-1}}{\rightleftharpoons}} \underset{\text{colourless}}{E(FeII)\cdot\text{R-13-OOH}} \xrightarrow[k_2]{} \underset{\text{yellow, EPR at } g=6}{E(FeIII)} + P$$

$$\underset{\text{yellow, EPR at } g=6}{E(FeIII)} + \text{R-13-OOH} \underset{k_3}{\overset{k_{-3}}{\rightleftharpoons}} \underset{\text{purple, EPR at } g=4.3}{E(FeIII)\cdot\text{R-13-OOH}} \xrightarrow[k_4]{} \underset{\text{yellow, EPR at } g=6}{E(FeIII)} + Q$$

where P and Q represent the product(s) derived from R-13-OOH in the oxidation step (*cf.* [4]) and the epoxy, hydroxy fatty acid [11], respectively. At 4.4 °C (± 0.2 °C) the affinity constant of the yellow enzyme species for R-13-OOH (*i.e.* the ratio k_{-3}/k_3) was calculated from the hydroperoxide concentration dependence and found to be 10 ± 2 μM, which is somewhat less than the affinity constant of the native enzyme for R-13-OOH (k_{-1}/k_1 is approx. 20 μM). The constant k_4 was estimated to be about 0.02 min^{-1} at 4.4 °C. In order to compare the rates of the above processes with the rate of R-13-OOH formation from linoleic acid and O_2, steady-state rates of R-13-OOH formation were measured spectrophotometrically (ε_{max} = 25000 $M^{-1} \cdot cm^{-1}$ at 234 nm) at 3 nM enzyme concentration. Formation of R-13-OOH cannot be followed spectrophotometrically at high enzyme concentrations because the enzyme strongly absorbs in the region from 210–250 nm (ε at 230 nm is higher than 10^5 $M^{-1} \cdot cm^{-1}$).

At 4.4 °C the rate constant k_{cat}, obtained from the maximum rate of R-13-OOH formation, and the apparent affinity constant for linoleic acid were found to be 232 s^{-1} and 75 μM, respectively. In agreement with Tappel *et al.* [15] the effect of temperature on the activity of the enzyme was found to be rather small (activation energy is approx. 3.8 kcal/mol or 15.9 kJ/mol, *cf.* Tappel *et al.*: 4.3 kcal/mol or 18.0 kJ/mol). The temperature effect on the apparent affinity constant for linoleic acid is more profound (ΔH° = − 9.5 kcal/mol or − 39.7 kJ/mol).

Thus, at 4.4 °C the rate constant found for the formation of the yellow enzyme species (k_2 = approx. 50 s^{-1}) is smaller than the rate constant of R-13-OOH formation (k_{cat} = 232 s^{-1}). An important question to be solved, because of divergent hypotheses on the reaction mechanism of lipoxygenase-1, is whether the native enzyme itself catalyzes the formation of R-13-OOH or has to be converted into the yellow form for activation. In the latter case R-13-OOH is a compulsory activator for the enzyme, while in the former it is not. Previous experiments at low enzyme concentrations indicated that the catalytic activities of the native enzyme and the yellow enzyme species were identical [9]; however, a rapid conversion of the native enzyme into the yellow form could not be excluded. Therefore, the catalytic activity of the native enzyme was studied by monitoring the formation of the yellow and purple enzyme species starting from linoleic acid, O_2 and native enzyme.

Formation of Yellow Enzyme from Linoleic Acid, O_2 and Native Enzyme

In Fig. 4 reaction traces are shown of the formation of the yellow enzyme species at 25 °C for a range of linoleic acid concentrations, which are all below the initial oxygen concentration. The initial concentration of R-13-OOH is less than 0.1 % of the concentration of linoleic acid. The formation of the yellow enzyme under these conditions demonstrates that the native lipoxygenase-1 is indeed catalytically active. Generally, the yellow enzyme species is formed more rapidly from the native enzyme and R-13-OOH than from the native enzyme and linoleic acid plus O_2, at identical concentrations of the fatty acid compounds (*e.g.* compare the traces in Fig. 1, 2 or 3 obtained at 4.4 °C and those in Fig. 4 obtained at 25 °C). Thus the rate of formation of R-13-OOH affects the rate of conversion of the native enzyme into the yellow form. It is also possible that linoleic acid inhibits the reaction of R-13-OOH with the native enzyme species. However, the yellow enzyme species is formed faster at higher concentrations of linoleic acid (Fig. 4A). At linoleic acid concentrations above approximately 50 μM the initial rate of R-13-OOH formation is affected by substrate inhibition [9, 10, 16]. This inhibition is competitively reversed by R-13-OOH. The slow initial rate of R-13-OOH formation is also reflected in the reaction traces monitored at 330 nm (Fig. 4B).

Formation of the Purple Enzyme Species from Linoleic Acid, O_2 and the Native Enzyme Species

In Fig. 5 the absorbance changes at 580 nm and 330 nm are shown which result upon incubation of the native enzyme with linoleic acid and O_2 at 25 °C.

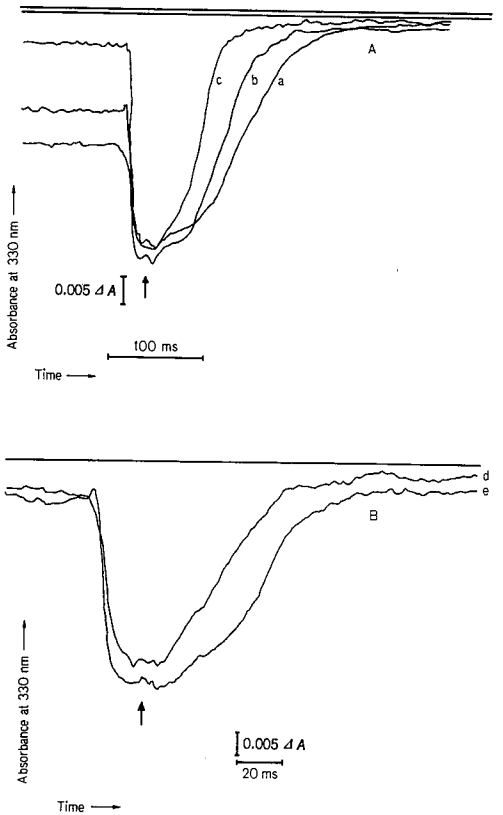


Fig. 4. *Formation of the yellow enzyme (330 nm) species upon mixing of linoleic acid, O_2 and the native lipoxygenase-1 at 25 °C, for several concentrations of linoleic acid.* 100 data points per full sweep for all; flow stops at arrow. (A) Full sweep time 500 ms, linoleic acid: 30 (a), 60 (b), 230 (c) μM; (B) full sweep time 200 ms, linoleic acid: 120 (d), 200 (e) μM

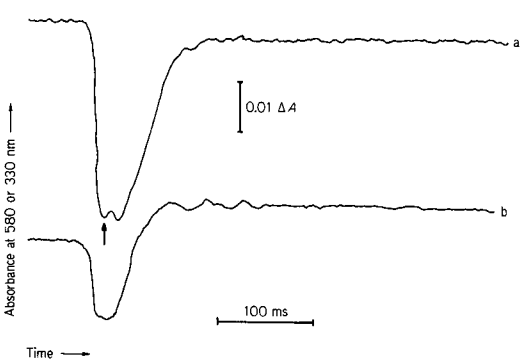
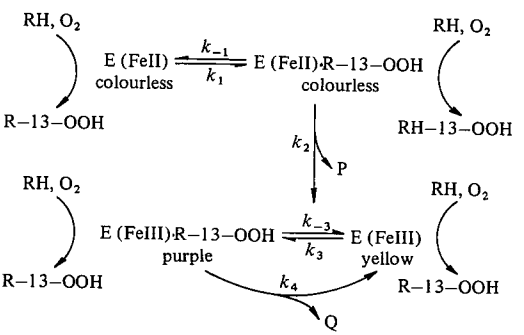


Fig. 5. *Formation of the yellow (a) and purple (b) enzyme species upon mixing of linoleic acid (200 μM), O_2 (240 μM) and the native enzyme at 25 °C.* 100 data points per full sweep; flow stops at arrow

As was shown in Fig. 2 and 3 the yellow and purple enzyme species appear to be concomitantly formed. This result demonstrates that the net formation of R-13-OOH is sufficiently fast to saturate the newly formed yellow enzyme species with R-13-OOH under the conditions used. At 25 °C the purple enzyme species is converted within several minutes to the yellow enzyme form *via* the isomerization of R-13-OOH. This reaction is too slow to interfere with the rapid formation of the purple enzyme species. In summary the results shown in the preceding and this section demonstrate that the yellow enzyme species does not have to be formed before R-13-OOH formation can start, nor is R-13-OOH formation completed before formation of the yellow ferric enzyme species has started. A simplified scheme of these events is shown below (Scheme III):



where RH represents linoleic acid. Complications such as substrate inhibition and reversal of this inhibition by R-13-OOH are not included in the scheme. All enzyme species catalyse the conversion of linoleic acid and O_2 [9].

The activity of the native enzyme species could not be measured directly. The reaction traces in Fig. 4 suggest that the activity of the native enzyme is close to or less than the activity of the yellow enzyme.

Aerobic Incubations of Native Enzyme at High Linoleic Acid Concentrations

In these experiments anaerobiosis is reached during the conversion of linoleic acid. The absorbance change at 330 nm is shown in Fig. 6, where reaction traces are compared for incubations with linoleic acid at concentrations slightly below and above the O_2 concentration. In Fig. 7 and 8 the absorbance changes at 330 nm and 580 nm are shown, respectively, for a range of linoleic acid concentrations which are all above the initial O_2 concentration. These reaction traces demonstrate that the yellow and purple enzyme species are rapidly bleached, once the systems becomes anaerobic. Maximum bleaching is obtained when the concentration of linoleic acid is at least

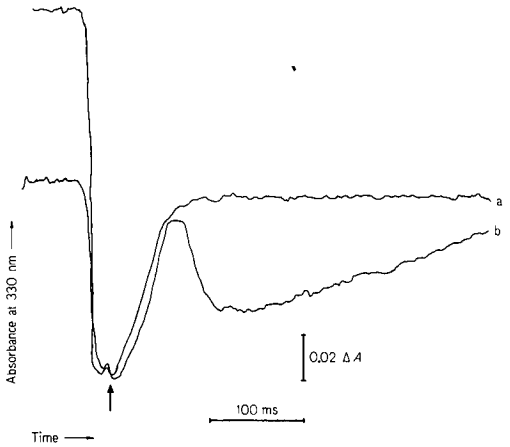


Fig. 6. *Incubations with linoleic acid at a concentration slightly below (trace a) and above (trace b) the O_2 concentration (240 μM) added to the native enzyme species at 25 °C.* The absorbance at 330 nm decreases sharply at the moment that all O_2 is converted (see text); the traces contain 100 data points per full sweep; the flow stops at the time indicated by the arrow

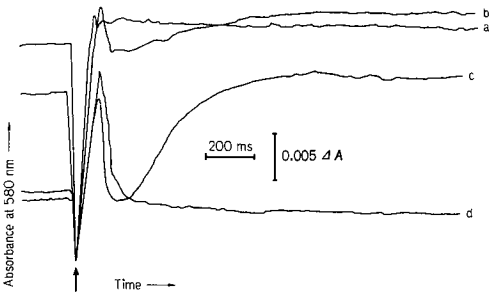


Fig. 8. *Formation and conversion (580 nm) of the purple enzyme species at (a) 200, (b) 250, (c) 275 and (d) 500 μM linoleic acid added in excess over O_2 to the native enzyme (25 μM) species at 25 °C.* The traces contain 1000 data points per full sweep; the arrow indicates the moment that the flow stops

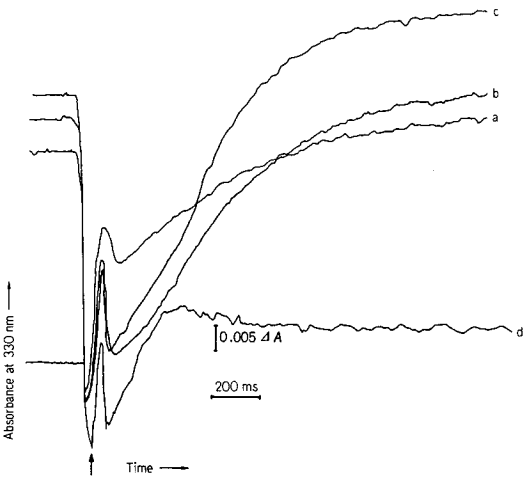


Fig. 7. *Formation and conversion (330 nm) of the yellow enzyme species at (a) 225, (b) 250, (c) 275 and (d) 500 μM linoleic acid added in excess over the dissolved O_2 (220 μM) to the native enzyme (25 μM) at 25 °C.* When all O_2 is consumed another chromophore than the ferric enzyme species complicates the traces at 330 nm (see text); the traces contain 1000 data points per full sweep; the flow stops at the arrow

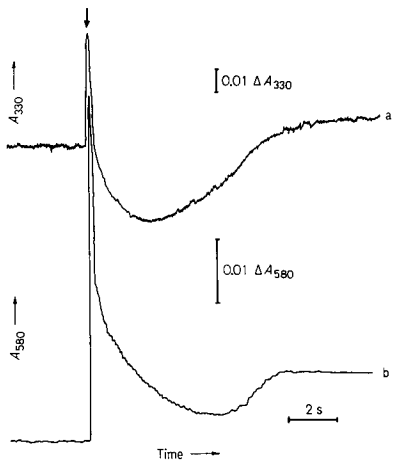


Fig. 9. *Absorbance changes at 330 nm (trace a) and 580 nm (trace b) resulting upon incubation of pre-formed yellow enzyme species (25 μM) with 440 μM linoleic acid and 220 μM O_2 at 7 °C.* The traces consist of 1000 data points per full sweep; the arrow indicates the moment that the flow stops

equimolar with the enzyme concentration at the moment that all O_2 is consumed (*cf.* [4]).

The finding that all O_2 (approx. 240 μM) has reacted before the native enzyme species is completely converted (total enzyme 25 μM) confirms the conclusion reached above that under these conditions formation of the yellow and purple enzyme species are slower than R-13-OOH formation.

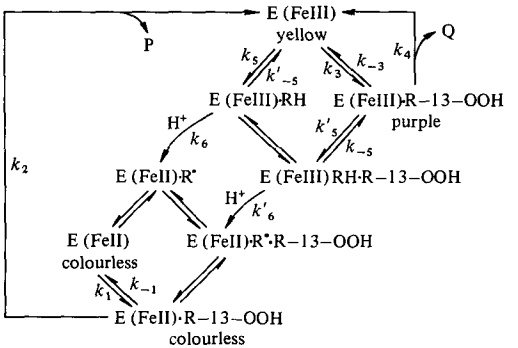
The rapid decrease of the absorbance at 330 nm and 580 nm is followed by a slower increase of the absorbance for all traces shown, except trace (d) in Fig. 7. This slow absorbance increase at 330 nm only

is due to the formation of another chromophore (see next section).

Aerobic Incubations of Yellow Enzyme at High Linoleic Acid Concentrations

Since O_2 consumption was found to be too fast for complete conversion of the native enzyme into the yellow and purple forms, the same type of experiments was carried out starting with preformed yellow enzyme species. In Fig. 9 two reaction traces are shown, monitored at 330 nm and 580 nm, respectively, at 7 °C. Now, the full bleaching steps of both coloured ferric enzyme species can be observed. The formation of the purple enzyme species in the aerobic phase of the reaction occurs within the mixing time of the stopped-flow instrument (*i.e.* a few milliseconds). After the rapid bleaching step the absorbance at 330 nm and also the absorbance at 580 nm slowly increase again. Note that the lowering of the temperature from 25 °C to 7 °C drastically reduces the rates of these slow anaerobic processes (compare Fig. 7 and 8 with Fig. 9).

The reaction scheme proposed for the events taking place under anaerobic conditions is shown below (Scheme IV):



Thus, the coloured ferric enzyme species formed during the aerobic phase of the reaction are rapidly converted under anaerobic conditions into the colourless ferrous enzymes *via* steps k_6 and k'_6. Linoleic acid (RH) is converted in these steps into a radical (R·) [4, 12]. Reoxidation of the ferrous enzyme species then occurs *via* step k_2 by the action of R-13-OOH. The anaerobic conversion of both linoleic acid and R-13-OOH finally leads to the formation of fatty acid dimers, oxodienoic acids and *n*-pentane [11, 17].

The oxodienoic acids, which represent about 40% of all products formed [17], exhibit an absorption maximum at 285 nm ($\varepsilon_{285} = 25000\ M^{-1} \cdot cm^{-1}$). The additional absorption observed in the reaction traces monitored at 330 nm is most probably due to the formation of these compounds (ε at 330 nm is approx. $10^3\ M^{-1} \cdot cm^{-1}$). Comparison of the absorbance changes at 330 nm and 580 nm then demonstrates that lipoxygenase-1 mainly remains in the reduced form during the anaerobic conversion of linoleic acid and R-13-OOH, *i.e.* reduction by linoleic acid proceeds faster than oxidation by R-13-OOH. The final oxidation state of the enzyme depends on the relative concentrations of linoleic acid and R-13-OOH. If R-13-OOH was present in excess over linoleic acid at the moment that all O_2 was consumed, then the oxidized form of the enzyme slowly reappears during catalysis. If on the other hand linoleic acid was in excess over R-13-OOH, then the enzyme resides in the reduced form throughout the reaction (see *e.g.* Fig. 8 trace d).

Incubation of Native Enzyme with [11-$^2H_2$]Linoleic Acid

Steady-state experiments indicated that conversion of [11-$^2H_2$]linoleic acid into [11-^{2}H]R-13-OOH shows a strong kinetic isotope effect, $v_H/v_{^2H}$ being approximately 9. It was concluded from these experiments that *pro-S* H abstraction from C-11 of linoleic acids is the rate-determining step in the overall oxygenation reaction [18, 19]. The H-abstraction step can be observed under anaerobic conditions by the bleaching of the coloured ferric enzyme species (steps k_6 and k'_6, Scheme IV). This step and also the aerobic formation of R-13-OOH were found to be faster than the formation of the yellow enzyme species (step k_2 in the absence or presence of O_2). Incubations of the native enzyme species with the deuterated linoleic acid would provide important additional information about the reaction mechanism of lipoxygenase-1, since the presence of the deuterium label would only affect the conversion of the fatty acid substrate. The formation of the yellow and purple enzyme species from the native enzyme and deuterated linoleic acid is shown in Fig. 10. Comparison of the time scales of these reaction traces with those for unlabelled linoleic acid already indicates that formation of the coloured ferric enzymes is about 10-fold slower for the deuterated acid. For traces (a1) and (a2) the whole reaction takes place under aerobic conditions, while the reaction medium becomes anaerobic for traces (b1) and (b2) and (c1) and (c2); (the absorbance changes at 330 nm and 580 nm are numbered 1 an 2, respectively).

In contrast to the incubations with unlabelled linoleic acid the purple enzyme is formed more slowly than the yellow enzyme species. This probably indicates that the net formation of R-13-OOH is not sufficiently fast to saturate the yellow enzyme species with R-13-OOH. Another effect of the deuterium label may be that the apparent affinity for R-13-OOH is decreased.

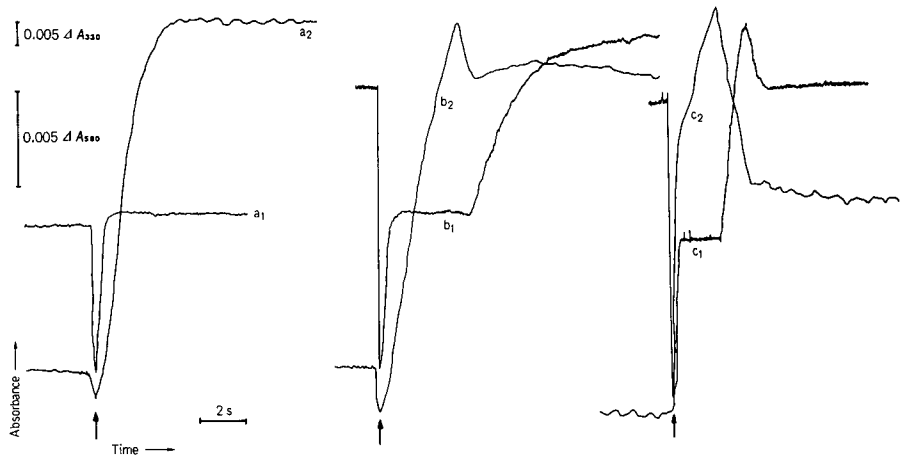


Fig. 10. *Formation and conversion of the yellow enzyme (traces a1, b1, c1) and purple enzyme species (traces a2, b2, c2) starting from 25 μM native enzyme, 195 μM O_2 and [$^2H_2$]linoleic acid at 120 μM (traces a), 200 μM (traces b) and 240 μM (traces c) respectively.* The traces contain 1000 data points per full sweep; the flow stops at the times indicated by the arrows

The moment anaerobiosis is reached is indicated in traces (b2) and (c2) by the decrease of the absorbance at 580 nm. As is shown by traces (b1) and (c1) the native enzyme is converted into the yellow enzyme species long before all O_2 is consumed. A striking difference with the incubations with unlabelled linoleic acid is the apparent absence of a rapid bleaching of the yellow enzyme species when anaerobiosis is reached. Instead, the absorbance at 330 nm increases which is due to the formation of oxodienoic acids in the anaerobic reaction.

This result suggests that the enzyme mainly remains in the oxidized (ferric) state during the anaerobic conversion of R-13-OOH and the deuterated linoleic acid. As a consequence the decrease of the absorbance at 580 nm must represent the conversion of R-13-OOH in the anaerobic reaction, the extent of which depends on the concentration of the substrate which is present at the lowest concentration (*e.g.* compare traces b2 and c2). Since in experiments (b) and (c) R-13-OOH is present in excess over linoleic acid the enzyme mainly remains in the oxodized state during the whole anaerobic reaction. The final drop of the absorbance at 330 nm (trace c1) at the moment all linoleic acid has been converted (see trace c2) indicates that some of the products formed in the anaerobic reaction are unstable. This absorbance decrease was also observed in experiments with unlabelled linoleic acid (not shown in Fig. 7 because this relatively slow process takes place after 2 s). In Fig. 11 the absorbance changes at 330 nm and 580 nm are compared for incubations with deuterated and unlabelled linoleic acid, respectively, added in excess over O_2 under identical experimental conditions. The final absorbances at 330 nm and 580 nm are identical. The purple enzyme species then decomposes *via* the slow isomerization of R-13-OOH (step k_4 in Scheme IV) to yield the yellow ferric enzyme species.

For unlabelled linoleic acid the H-abstraction step (k_6 and k_6' in Scheme IV) was found to be fast relative to the rate of conversion of R-13-OOH (step k_2), leaving the enzyme mainly in the ferrous state during the anaerobic reaction. On the other hand, the rate of ^2H abstraction from the deuterated linoleic acid is close to, or slower than the rate of R-13-OOH conversion, for the enzyme mainly remains in the ferric state after its formation under aerobic conditions.

The effect of the ^2H label on the formation of the anaerobic reaction products is also evident from the partial absorbance of the oxodienoic acids at 330 nm. In the initial stages of the anaerobic reaction process the absorbance increase at 330 nm is mainly due to the formation of the oxodienoic acids (see Fig. 11). The apparent rates of formation of these acids, obtained from the slopes of the reaction traces in the initial stages of the anaerobic reactions were found to be approx. 62.5 $\mu M \cdot s^{-1}$ and 67.5 $\mu M \cdot s^{-1}$ (ε_{330} = 1000 $M^{-1} \cdot cm^{-1}$) for deuterium-labelled and unlabelled substrates, respectively. This negligibly small kinetic isotope effect ($v_H/v_{^2H}$ = 1.1) confirms the above conclusion that conversion of R-13-OOH and not the H-abstraction step determines the rate of the anaerobic reaction. Furthermore, this result shows that conversion of deuterium-labelled linoleic acid is not so slow that it affects the rate of product formation. Combined with the finding that the enzyme mainly remains in the ferric state under the conditions

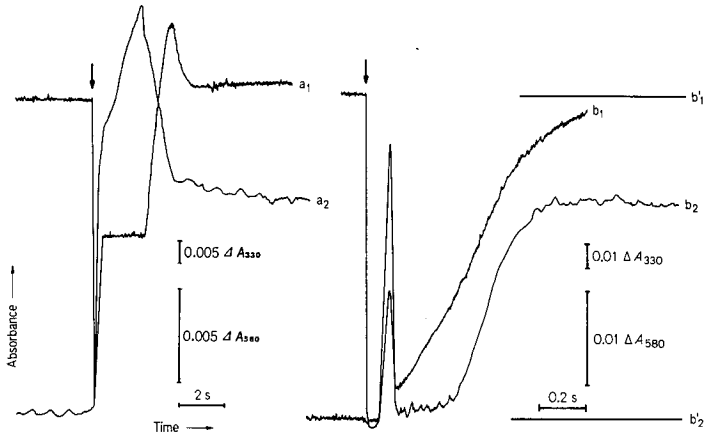
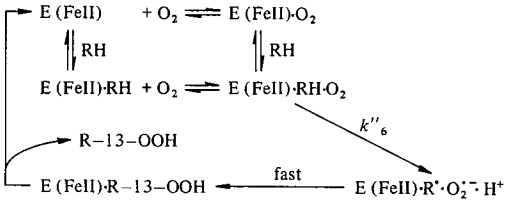


Fig. 11. *A comparison of the absorbance changes at 330 nm (traces a1 and b1) and 580 nm (traces a2 and b2) for incubations starting with 25 μM lipoxygenase-1, 195 μM O_2 and either 340 μM [$^2H_2$]linoleic acid (a) or 340 μM unlabelled linoleic acid (b).* The traces contain 1000 data points per full sweep; the arrows indicate the moments that the flow stops; the straight horizontal lines show the final absorbances (b'1, b'2)

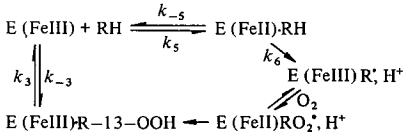
used, it is concluded that the rates of conversion of deuterated linoleic acid and R-13-OOH are nearly identical.

The mechanism of the anaerobic process as shown in Scheme IV indicates that the slow oxidation of the ferrous enzyme species into the ferric state by R-13-OOH is essential for activity. Indeed the ferrous enzyme is not able to convert linoleic acid in the absence of both R-13-OOH and O_2 [20]. However, in the presence of O_2 the oxidation of the enzyme by R-13-OOH is not essential for activity, as shown above. As the H-abstraction step is only catalysed by ferric species, it must be concluded that O_2 is able to convert the native ferrous enzyme into a ferric species, presumably the ferric-superoxide complex. The reaction mechanism proposed for the aerobic conversion of linoleic acid into R-13-OOH catalysed by the native ferrous enzyme species is shown below (Scheme V).



The slowest step in Scheme V is the abstraction of H from linoleic acid (step k_6'). Subsequent oxygenation of the intermediary linoleic acid radical by O_2^- is considered to be very fast.

After conversion of the native enzyme into the yellow ferric state by R-13-OOH the oxygenation reaction may proceed as shown below in Scheme VI.



The experiments described in the third section of Results suggest that even at low enzyme concentrations the yellow enzyme species will be formed within a few seconds after mixing of the reactants provided that the formation of R-13-OOH from linoleic acid is sufficiently rapid (*i.e.* not affected by substrate inhibition). The intermediary linoleic acid radical formed in this process is converted by (ground state) O_2 into a peroxyradical which then takes up an electron from the enzyme to form the (purple) ferric enzyme-R-13-OOH complex. Also the oxygenation reaction *via* (ground state) O_2 must be very fast.

The reliability of the proposed mechanisms was further investigated by comparing the rate of R-13-OOH formation with the rate of the anaerobic conversion of linoleic acid by the yellow enzyme species in the absence of R-13-OOH (step k_6 in Scheme IV).

Aerobic and Anaerobic Incubations of the Yellow Enzyme Species with Linoleic Acid and [11-$^2H_2$]Linoleic Acid

The bleaching of the yellow enzyme species by linoleic acid and [11-$^2H_2$]linoleic acid under anaerobic conditions at 25 °C is shown in Fig. 12 and 13, respectively. The reactions follow pseudo-first-order kinetics between about 10–74% conversion of the yellow enzyme species, as is shown by the semilogarithmic plots of the absorbance changes (see inserts in Fig. 12 and 13). These results are in line with the mechanism

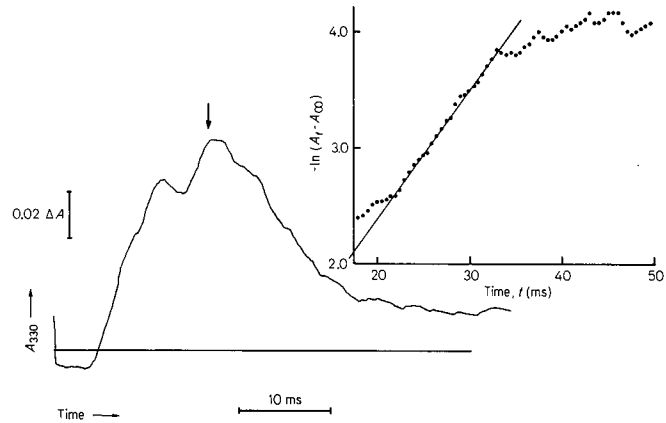


Fig. 12. *Anaerobic conversion of the yellow enzyme species (30 μM) by 300 μM linoleic acid at 25 °C.* The straight horizontal line gives the final absorbance at 330 nm. The trace contains 100 data points per full sweep; the arrow indicates the moment that the flow stops. Insert: logarithmic plot of the absorbance change at 330 nm *vs* the incubation time

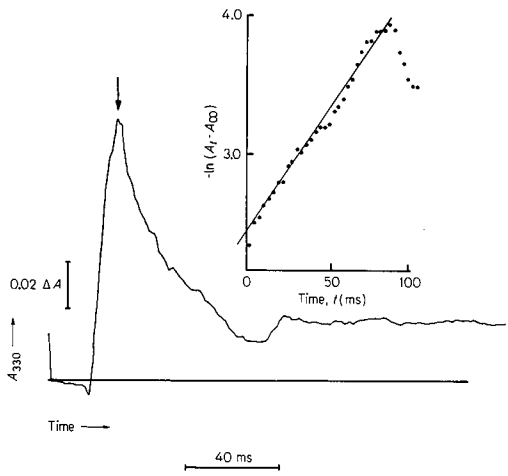


Fig. 13. *Anaerobic conversion of the yellow enzyme species (30 μM) by 300 μM [11-$^2H_2$]linoleic acid at 25 °C.* The trace contains 100 data points per full sweep; the arrow indicates the moment that the flow stops. The final absorbance is given by the straight horizontal line. Also in this experiment the intermediary formation of another chromophore than the enzyme seems to complicate the reaction trace (compare Fig. 12). Insert: logarithmic plot of the absorbance change *vs* time

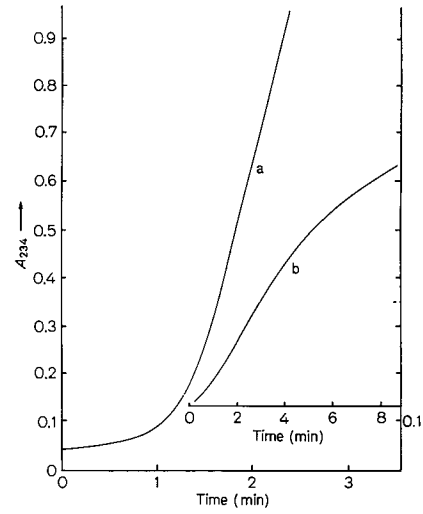


Fig. 14. *Reaction traces of the formation of R-13-OOH from linoleic acid (a) and [11-$^2H_2$]linoleic acid (b), respectively, monitored at 234 nm at 25 °C.* The reactions are started by additions of the yellow enzyme species (final concentrations 3.4 nM) to air-saturated solutions (O_2: 240 μM) of the fatty acid substrates (both at 300 μM). No R-13-OOH is present at the start of the reactions

as proposed in Scheme IV. The rate constants k_6(H) $= 111 \pm 3\ s^{-1}$ and $k_6(^2H) = 30 \pm 1\ s^{-1}$ for unlabelled and deuterium-labelled linoleic acid, respectively, were obtained from the slopes of the straight lines fitted to the data points of the semilogarithmic plots between 10–75% conversion of the yellow enzyme species.

Since H abstraction is the slowest step in the overall oxygenation reaction, the above rate constants k_6 must be close to the rate constants for the overall reactions. The latter constants derived from the maximum rates of R-13-OOH formation by the yellow enzyme species at 25 °C, are $290 \pm 4\ s^{-1}$ and $32 \pm 3\ s^{-1}$ for unlabelled and deuterium-labelled linoleic acid, respectively, when substrate inhibition is not taken into account. For deuterium-labelled linoleic acid the rate constants agree very well, while k_6(H) for unlabelled linoleic acid is approximately

40% of the overall reaction rate constant. The latter discrepancy probably arises from substrate inhibition. The effect of substrate inhibition on the rates of R-13-OOH formation is shown in Fig. 14 for unlabelled and deuterium-labelled linoleic acid, respectively. For both substrates the reaction traces are initially curved upwards. This is caused by R-13-OOH formed during the incubations and is only observed when the rates are influenced by substrate inhibition [9,10,16]. The rates appear to be influenced more for unlabelled linoleic acid as substrate than for deuterated linoleic acid: the initial rate is about 36 times lower than the highest rate for unlabelled linoleic acid, while the initial and highest rates only differ by a factor 2 for the deuterium-labelled linoleic acid. In agreement with previous results it was found that the substrate inhibition is not completely reversed by R-13-OOH [9]. The highest rates are 172 s^{-1} and 27 s^{-1} for unlabelled and deuterated linoleic acid, respectively. The final downward curvature in the trace for deuterium-labelled linoleic acid (Fig. 14b) is probably due to the self-catalysed destruction of lipoxygenase-1 as was described by Lands *et al.* [10,16].

In summary, the results in this section confirm the finding that H abstraction from linoleic acid is the rate-determining step in the overall oxygenation reaction catalysed by the yellow ferric enzyme species.

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