Steady-State Kinetics of Lipoxygenase Oxygenation of Unsaturated Fatty Acids†

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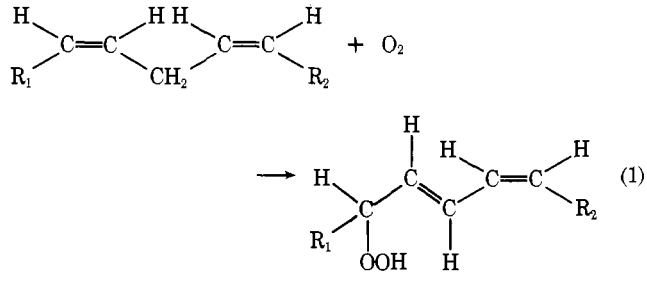
ABSTRACT: The oxygenation of linoleate and arachidonate catalyzed by soybean lipoxygenase is subject to competitive product inhibition. For normal conditions, there is an additional inhibition due to product that causes the reaction to cease before completion. This process is reversible upon addition of further substrate and is proposed to be a chemical (reversible) change of the enzyme. At very low enzyme concentrations, inactivation or adsorption of enzyme on the vessel surface (or "wall effect") is significant, leading to even lower rates and percent completions. In the very early stages of a typical catalyzed reaction, a lag, or induction period, occurs. It was previously known that this lag is eliminated by product hydroperoxide—and not by the corresponding alcohol. The hydroperoxide elimination of the lag is inhibited by the alcohol. It is proposed that this is a chemical activation of the enzyme to produce a catalytically functional form.

Lipoxygenase (EC 1.13.11.12) catalyzes the oxygenation of 1,4-*cis,cis*-pentadiene units on long-chain fatty acids to 1-hydroperoxy-2,4-*trans,cis*-pentadienes (eq 1).



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This addition of molecular oxygen (Dolev et al., 1967) involves removal of the central bisallylic hydrogen in the rate-limiting step (Hamberg and Samuelsson, 1967; Egmond et al., 1973) and the probable involvement of one iron atom on the enzyme (Chan, 1973; Roza and Francke, 1973; Pistorius and Axelrod, 1973, 1974). Recent studies have begun to make progress on the pathway of this chemically interesting reaction (DeGroot et al., 1972; Johns et al., 1973; Pistorius and Axelrod, 1974; DeGroot et al., 1975a,b); in this paper we report solely on aspects of the steady-state kinetics.

The turnover kinetics with a number of appropriate substrates have been studied (Tappel et al., 1952; Koch, 1968; Allen, 1968; Yasumoto et al., 1970; Ben Aziz et al., 1970; Smith and Lands, 1972) using enzyme of varying purity and containing (in many cases) more than one isozyme. Lipoxygenase has recently become available in much higher purity in both senses (Christopher et al., 1970, 1972; Verhue and Francke, 1972). It has been generally reported (Haining and Axelrod, 1958; Dillard et al., 1961; Zimmerman, 1968; Lands et al., 1971; Smith and Lands, 1972) that: (a) lipoxygenase shows a lag (induction) period upon its addition to aerated substrate solution (attributed to a required product activation by Smith and Lands (1972)); (b) the turnover reaction ceases before completion (attributed by the same workers to enzyme destruction during, and by, the catalytic process); and (c) the maximal rate is then affected by this enzyme depletion.

As part of a mechanistic study we have had to look at the turnover kinetics and have confirmed the pre-steady-state product activation, but found that the limited extents of reaction and decreases in maximal rate during reaction at normal ($>10^{-8}$ M) enzyme concentrations are due to competitive product inhibition and a *reversible* inactivation by product rather than enzyme destruction. At low ($\leq 2 \times 10^{-9}$ M) enzyme, inactivation of lipoxygenase on the surface of the reaction vessel occurs. The results lead us to a reinterpretation of the role of product in this reaction.

Experimental Section

Materials. Lipoxygenase was Sigma, Grade I, of specific activity 80 000 (σ_{80}) or (for most of the work) 156 000 (σ_{156}) EU mg^{-1} (i.e., 8 or 15.6 μmol min^{-1} mg^{-1} in the standard assay). The latter material was essentially pure lipoxygenase-1 isozyme (Christopher et al., 1972) by Sephadex chromatography, electrophoresis, and pH dependence. For convenience we have taken the molecular weight as 100 000 (Stevens et al., 1970; Christopher et al., 1970) and, only in statements of concentration, assumed pure enzyme. All specific activities are in mg^{-1} protein (easily converted by using 10^5 mg/mmol, so that 10^{-8} M is 10^{-3} mg/ml in this report).

Linoleic (99+%), arachidonic (99+%), and [1-^{14}C]arachidonic (94+% cis; radiopurity 99%; 58 mCi/mmol) acids were from Applied Science. Purity was ascertained on TLC,[1] and in some cases substrates were further purified on a silica gel column eluted with 1% acetic acid in redistilled hexane. Buffers were made from commercial best grade materials in twice-distilled water.

13-Hydroperoxy-*cis,trans*-9,11-octadecadienoic acid was produced enzymatically. Linoleic acid (200 μM) in 1 l. of pH 9.0 borate buffer (0.2 M, containing 3.8% ethanol) was stirred magnetically at room temperature in air, and 10-mg samples of crystalline lipoxygenase were added at half-hour intervals for 90 min. After stirring overnight at 0 °C, the mixture was acidified to pH 1.5 and thrice extracted with 300 ml of ether. The ethereal extract was dried, the solvent was removed, and

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Abbreviations used: TLC, thin-layer chromatography; GC, gas chromatography; cmc, critical micelle concentration; ESR, electron spin resonance.

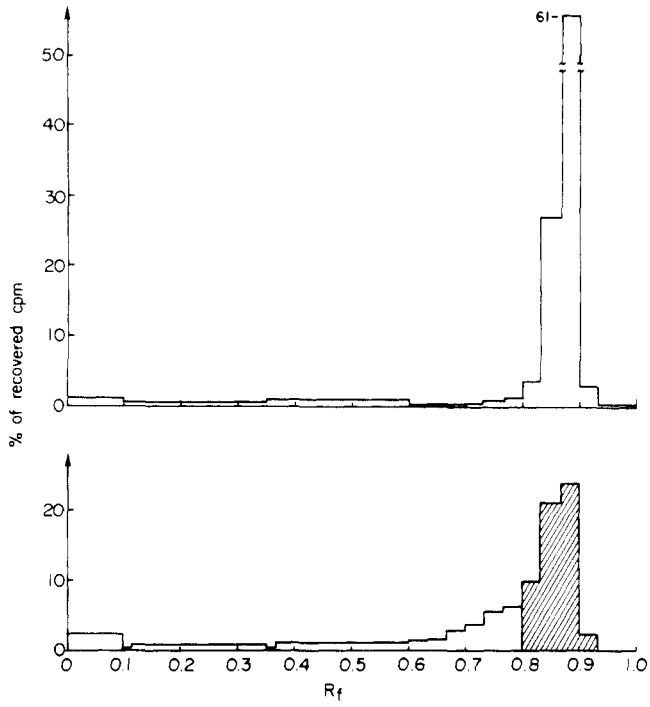

FIGURE 1: Recovery of radioactivity from TLC of [1-^{14}C]arachidonic acid. See text for procedure. Top half: control with no enzyme. Bottom half: after reaction with lipoxygenase. Shaded area in bottom is arachidonate region.

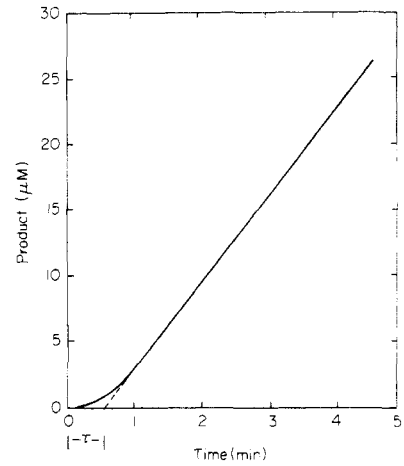

FIGURE 2: Measurement (by uv) of the lag period (τ) in the oxygenation of arachidonic acid (66 μM) by lipoxygenase (1×10^{-8} M), borate buffer (0.2 M, pH 9.0), 25 °C.

the residue was dissolved in 25 ml of ethanol and stored under argon at 5 °C. Titration for hydroperoxide (I^- in acidic 2-propanol vs. $S_2O_3^{2-}$ (Wagner et al., 1947)) showed the solution to be 7.90 mM, assuming C_{18} hydroperoxydienoic acid.

To prepare 13-hydroxy-*cis,trans*-9,11-octadecadienoic acid, the hydroperoxide (10 mg, 32 μmol) in 4 ml of ethanol was reacted with 25 mg (0.66 mmol) of $NaBH_4$ at 0 °C for 10 min, then one additional hour at 25 °C (Hamberg and Samuelsson, 1967). After filtration the iodimetric hydroperoxide content was zero.

The value of $\Delta\epsilon$ for linoleate going to conjugated diene (and also a check on the purity) was determined via isomerization by potassium *tert*-butoxide (0.768 M) in *tert*-butyl alcohol at 88 °C for 10.5 h under argon (Sreenivasan and Brown, 1956). Various concentrations of linoleic acid (and blanks) were run, appropriately diluted with ethanol, and spectra recorded at 234 nm. The $\Delta\epsilon$ was $2.78 \pm 0.11 \times 10^4$ M^{-1} cm^{-1} (lit. 2.80×10^4 M^{-1} cm^{-1} (Tappel et al., 1952; Privett et al., 1955)); GC (10% Apiezon L (60/80 Chrom Acid), 255 °C) showed one peak; and silica gel TLC with the upper phase of ethyl acetate–water–2,2,4-trimethylpentane–acetic acid (11:10:5:2) as eluent gave one spot.

Using 10^{-7} M lipoxygenase to convert linoleate to termination of reaction, the total absorbance change at 234 nm varied linearly with initial substrate concentration (from 10 to 100 μM), showing that Beer's law is obeyed.

Methods. Initially, substrate stock was in ethanol–buffer (6:10, v/v) with or without Tween 20 (Allen, 1968). This method had no apparent advantage over simply using ethanol and maintaining constant organic solvent in the reaction by using different stock solutions and additional ethanol when needed. While Tween helps solubilize substrate, it inhibits the enzyme and was not used in most of the experiments. Ethanol solutions were stored under argon at 5 °C and kept less than 1 week. Most recently acetonitrile has proved very useful.

Uv assays were performed in quartz cells (usually 1-cm paths) at 234 nm on a Cary 14 or Beckman DB-GT (both at 25.0 ± 0.1 °C). Initial rates were used unless otherwise stated, and $\Delta\epsilon_{234}$ was taken as 2.80×10^4 M^{-1} cm^{-1}. Reactions were usually initiated by a small (25–100 μl) aliquot of fresh enzyme stock added to aerated (~10 s) buffered substrate. A YSI Model 4004 Clark O_2 electrode (with home-made control and monitor and a Speedomax H recorder) was in a 3.0-ml volume thermostated glass reaction vessel with magnetic stirrer.

To test for remaining substrate at normal enzyme concentrations, [1-^{14}C]arachidonic acid (1.65×10^{-4} M, 29.8×10^3 cpm) was reacted (1.0 ml total) with lipoxygenase (σ_{156}) to cessation (typical 45% apparent conversion), and a control was carried through with no enzyme. Reaction mixture was acidified with HCl (pH <2) and extracted three times with $CHCl_3$. The concentrated organic phase was applied to a 5 × 20 cm TLC plate and developed to 17 cm in the solvent described. Plates were zone scraped and fractions counted in a toluene–Triton X-100 (2:1, v/v) scintillation fluid made from Concifluor (Mallinkrodt). Figure 1 shows that 57% of the material in the reaction (shaded area) vs. >96% in the control was in the arachidonic acid region. Fatty acid hydroperoxides decompose on TLC at room temperature, accounting for the product spread in Figure 1 (Nugteren and Hazelhof, 1973); TLC at −30 °C is reported to show one sharp product peak. Substrate oxidation or peroxide breakdown produces material of lower R_f (more polar) than arachidonate.

Results

It was previously shown (Allen, 1968), and confirmed here, that organic solvent (1–5%) significantly affects the kinetics (increasing ethanol or acetonitrile causes decreasing rates), as does detergent above about 0.02%. Our standard assay mixture was 1.5% (v/v) ethanol with no detergent in 0.2 M borate buffer at pH 9.0 and 25 °C, unless otherwise noted.

Kinetic Lag and Product Activation. Figure 2 shows a typical run exhibiting a lag period. Our observations concerning this phenomenon agreed with those of Lands et al. (1971) and Smith and Lands (1972), indicating removal of this lag by addition of product hydroperoxide. One series of new experiments demonstrates (confirming the earlier conclusion) that the hydroperoxy group is required for activation.

Linoleyl hydroperoxide (synthesized enzymatically) was isolated and chemically reduced with $NaBH_4$. The alcohol was isolated and then tested as a potential activator. *Not only did the alcohol not eliminate the lag (Table I), but it competitively*

TABLE I: Effect of Linoleyl Hydroperoxide (Product of Linoleate Oxygenation by Lipoxygenase) and Linoleyl Alcohol (Its Reduction Product) on the Kinetic Lag Period Shown in Lipoxygenase Oxygenation of Linoleate.[a]

ROH[b] (μM)	ROOH[c] (μM)	Lag Period (s)[d]
		60
	7.5	0
25		105
38		150
48		270
6	7.5	0
14	7.5	3
25	7.5	5
38	7.5	10
48	7.5	20

[a] Linoleate (100 μM) and 8×10^{-8} M lipoxygenase in air saturated 0.2 M borate, pH 9.0 at 25 °C; followed by uv (234 nm). In these runs ethanol varied from 2 to 5% and showed inhibition of V_{max} but no effect on the lag. [b] 13-Hydroxy-9,11-octadecadienoic acid. [c] 13-Hydroperoxy-9,11-octadecadienoic acid. [d] Measured as in Figure 2.

inhibited the activation by hydroperoxide. This direct result using the alcohol, anticipated by the experiment of Lands et al. (1971) in which glutathione and glutathione peroxidase were added, leaves no doubt about the requirement for the hydroperoxide group.

Turnover Rates. Normal Michaelis-Menten behavior from 5 μM to 1.1 mM linoleate is shown for initial rates measured when low levels (3-5 μM) of product are added to initial reaction mixtures. For linoleate, K_m = 25.0 μM and V_{max} = 11.8 μmol min^{-1} mg^{-1} E (Sigma I lipoxygenase, 80 000 EU/mg) or V_{max} = 18 μmol min^{-1} mg^{-1} E (Sigma I lipoxygenase, 156 000 EU/mg). The reaction was first order in enzyme from 7.5 to 200 $\times 10^{-9}$ M enzyme. This preparation is almost pure lipoxygenase-1 (see Experimental Section), and a brief examination agreed with prior work on this isozyme (maximum rate at pH 9.0).

Both O_2 uptake and conjugated diene uv absorbance were followed for many reactions under a wide variety of conditions, agreeing to ±4% (one O_2 consumed per diene produced) for rates and extent of completions.

Stoichiometry-Extent of Reaction. Under virtually no set of conditions did complete formation of diene hydroperoxide by uv or stoichiometric O_2 uptake occur (noted in many previous studies also). The effect does not involve O_2 depletion (see below). Smith and Lands (1972) concluded that enzyme was destroyed during the reaction, in that under their conditions addition of further enzyme (but not substrate) to a spent reaction mixture caused more conversion of substrate. Our *initial* experiments did not agree with this, in that only more substrate, but not enzyme, caused further reaction.

Careful reexamination revealed that, at $\geq 10^{-8}$ M lipoxygenase, active enzyme is still present upon cessation of reaction with either linoleate or arachidonate (typical experiments in Figure 3 and later tables), while at $\leq 10^{-9}$ M enzyme no active enzyme is left and reaction stops even earlier (Figure 4). The earlier workers' results were at less than 10^{-8} M, assuming pure enzyme, but actually less than 10^{-9} M, given the specific activities of their material at that time (ca. 1.5 μmol min^{-1} mg^{-1}) and our enzyme (10-15 μmol min^{-1} mg^{-1}).

At low concentrations a combination of effects operates,

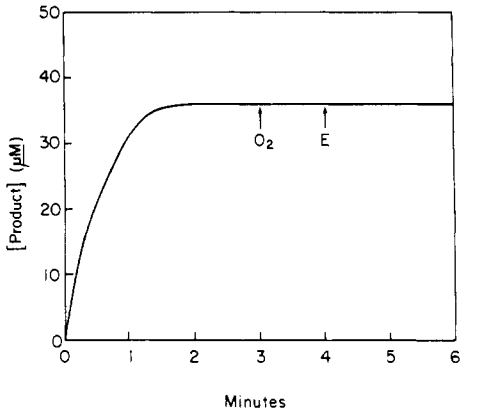

FIGURE 3: Reaction of linoleic acid (50 μM) in air with lipoxygenase (6.1×10^{-8} M) in borate buffer (0.2 M, pH 9.0) at 25 °C. After cessation (at ca. 61% completion), additional oxygen (by bubbling) and enzyme (same amount as original) were added as indicated to attempt to increase extent. Reaction monitored by uv at 234 nm.

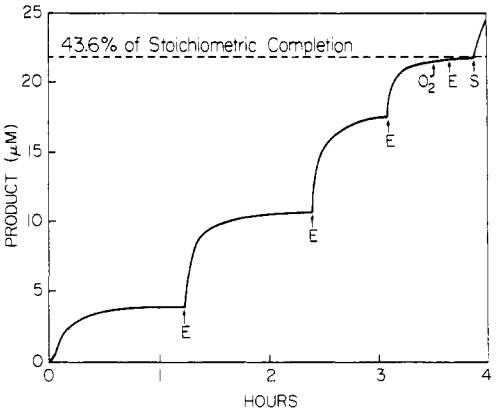

FIGURE 4: Same as in Figure 3, but at 1×10^{-9} M lipoxygenase. It was not possible to achieve 100% diene formation or O_2 consumption.

while at higher ("normal") levels of enzyme the situation is a case of product inhibition and subsequent reversible product deactivation. These will be discussed below, but it was first important to examine certain obvious (and largely experimental) potential causes.

The linoleic acid used was pure by TLC (single spot) and GC (99% one peak). It could be converted to the conjugated diene quantitatively, giving $\Delta\epsilon_{234}$ 2.78×10^4 M^{-1} cm^{-1}, in agreement with the literature (see Experimental Section). With [1-^{14}C]arachidonate as substrate, upon cessation of reaction the starting material not converted to product was recovered unchanged after reaction had ceased (see Experimental Section). Thus neither impure substrate nor side reactions are responsible for the low completions.

Calculations and measurements of O_2 concentrations in air-saturated buffer [(S) can be $\ll(O_2)$], reaerations of reaction mixtures (no additional reaction), and runs with pure O_2 (same yields) all clearly showed that O_2 depletion was not the cause of incomplete reaction.

It is unlikely that micelle formation by the substrate fatty acids is responsible for the effects noted. (a) The cmc for linoleate (Allen, 1968; Orthoefer and Dugan, 1973) is within the concentration range of the double-reciprocal plots, and there is no discontinuity. This clearly shows lack of discrimination (interesting of itself) on the part of the enzyme. (b) Both well above and below the cmc of linoleate, at constant enzyme, the percent completions are reasonably constant as long as $(O_2) > (S)$.

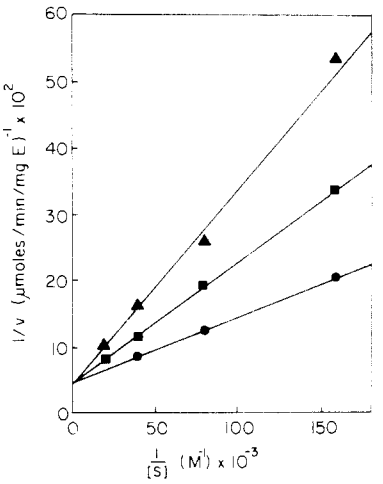

FIGURE 5: Double-reciprocal plots of product inhibition by linoleyl hydroperoxide [(●) no product; (■) 25 μM; and (▲) 50 μM product] in reaction of varying linoleate concentrations with lipoxygenase (3.0–15.0 × 10^{-8} M) in borate buffer (0.2 M, pH 9.0) containing 1.2% (v/v) ethanol at 25 °C. Reactions monitored by O_2 consumption.

Phenomenologically, it appears that at high (normal) enzyme either an equilibrium is attained or an inhibitor is formed during the reaction, while at low enzyme this is accompanied by a destruction of enzyme causing even lower completion percentages.

Normal (10^{-8} M) Enzyme. We have ruled out an equilibrium situation both by thermodynamic calculation and by experiment. Using (Ingold, 1969) D(C=C)$_2$CH—H = +84, D(RO$_2$—H) = +90, and D(C=CC=CCH—OO·) = +15, gives $\Delta H^\circ = -21$ *kcal/mol.* Overall, two molecules go to one molecule, and there is certainly an entropy decrease, but likely not more than ca. 20 eu (ΔS° for $H_2 + O_2 \rightarrow H_2O_2$(g) is ca. −25 eu). The result is then $\Delta G^\circ \simeq -15$ *kcal/mol.* In order to entropically compensate for the exothermicity to give an equilibrium constant of about 10 ($\Delta G^\circ = -1.4$), ΔS° would have to be improbably high, about −70 eu.

The experiment was designed to examine the ratio (P)/(S)(O_2) after adding increasing amounts of linoleate (S) to freshly ceased reaction mixtures. The last column in Table II shows that regular additional reaction occurs to extents too high to be accommodated by an equilibrium situation. The rate decreases with each run, but the (P)/(S) ratios are not at all constant. Again, additional O_2 has no effect. These data suggest product inhibition, in that specific activity and extents of reaction both decrease with increasing (P)/(S) ratio.

By having relatively high initial product, it was possible to show that solely the *presence of product* caused these effects. Table III shows data for two substrates in which the runs in parts labeled ii are the rates and extents of reactions with *fresh substrate and enzyme* added to spent reaction mixtures (from the i parts) after denaturation of all residual enzyme. Runs in 2 were controls identical with ii runs in substrate and enzyme concentrations. The results reinforce the conclusion that product inhibition is functioning here, but during denaturation there was undoubtedly some decomposition of product, and in the second runs we have a potentially complex reaction mixture.

Finally, linoleyl hydroperoxide, prepared enzymatically and isolated as described in the Experimental Section, was used in a classical inhibition experiment. The Lineweaver–Burke plots shown in Figure 5 (by O_2 uptake) exhibit competitive inhibition behavior. A Dixon plot of $1/v$ vs. (P) gave K_p (or K_i) = 37.5 μM.

TABLE II: Effect on Rate and Conversion of Repeated Additions of Substrate (Only) to Ceased Reaction Mixtures.[a]

S_{init}[b] (μM)	P_{init}[c] (μM)	V_{init} (μmol min^{-1} mg^{-1})	% Conversion at End	(P)/(S) Final
50	0	9.4	50	1.0
58	25	8.2	49	1.8
62	53	6.6	44	2.3
67	80	4.7	37	2.5[d]

[a] The first run had the indicated concentration of linoleate (S) in borate (0.2 M) buffer at pH 9.0 with 1.5% v/v ethanol and 1 × 10^{-7} M lipoxygenase. Upon cessation of reaction, the solution was aerated, additional linoleate added, and reaction monitored. This was done three times; followed by uv (234 nm). [b] The initial concentration of linoleate as calculated (except for the first run) from that remaining from the reaction just above it and the fresh substrate added (in each case an additional 32–33 μM amount). [c] Calculated from the conversion in the previous run (one line above). [d] Addition of more *enzyme* produced no further reaction (as a check).

TABLE III: Product Inhibition and Substrate and Enzyme Readdition after Denaturation.[a]

	S_{init}[b] (μM)	P_{init}[c] (μM)	V_{init} (μmol min^{-1} mg^{-1})	% Conversion at End
	Arachidonate[f]			
(1) (i)	100		25.4	47
(ii)	153[d]	47	13.5	22
(2)	153[e]		21.0	42
	Linoleate[g]			
(1) (i)	100		13.3	60
(ii)	140[d]	60	7.0	14
(2)	140[e]		12.6	74

[a] Lipoxygenase (1 × 10^{-7} M) in the usual buffer with 0.57% (runs 1i) or 1.14% (v/v) (runs 1ii and 2) acetonitrile. Substrate and product concentrations are initial values. Runs followed at 234 nm. The runs labeled 1 were one cell and reaction solution used first directly (i) and then after denaturation of enzyme from the first run (ii). See footnote *d*. [b] Total substrate concentration, including, for the runs labeled ii, that left from the run labeled i plus the fresh substrate. [c] Product concentration for the two runs labeled ii calculated from the percent completion of the runs labeled i. [d] These two reactions (ii) were the solutions of the previous runs (i) which had been capped and heated for 10 min at 60 °C and then cooled to 25 °C. Fresh enzyme (10^{-7} M) was added, giving no reaction (as expected). Additional substrate then gave the V_{init} and percent conversions shown. [e] These were arranged to have the same $(S)_0$, $(E)_0$ (active) and acetonitrile as the ii runs, so are directly comparable but for the P in the ii runs. [f] Triplicate experiment. [g] Single experiment.

Low ($\lesssim 10^{-9}$ M) Enzyme. Figure 4 and many similar experiments with both linoleate and arachidonate show that at low enzyme concentration the percent completion is considerably less than that at normal concentration due to irreversible depletion of active enzyme. This result, which is in agreement with that of Smith and Lands (1972), directly contrasts to that at $\geq 10^{-8}$ M lipoxygenase.

This effect is largely, or entirely, due to adsorption or inactivation of enzyme on the walls of the reaction vessel. One quartz uv cell was used for repeated fresh runs to cessation (Table IV) with no rinsing between runs (enzyme remaining after draining the cell from the prior run, which had very low

TABLE IV: Repeated Reactions of Linoleate with Lipoxygenase at Low Enzyme Concentration in One Cell with No Rinsing.[a,b]

V_{init} (μmol min^{-1} mg^{-1})	% Conversion
2.4	7.4
2.6	7.7
3.1	10.7
2.8	11.2
3.6	16.4
4.7	22
4.5	22
4.4	27

[a] Each assay contained 200 μM linoleate and 1.7×10^{-9} M lipoxygenase at 0.2 M borate, pH 9.0, 25 °C, in a 3-ml volume in a quartz, 1-cm cuvette, and was monitored at 234 nm. The same cell was used in each experiment after simply pouring out the previous contents (previous line of table). The trace of remaining enzyme gave no reaction with fresh substrate. Repeated seven times as shown. [b] At normal lipoxygenase ($>10^{-8}$ M), the V_{init} = 14 μmol min^{-1} mg^{-1} and percent conversion >60%.

enzyme, was insufficient to cause reaction with fresh substrate solution). It is obvious from Table IV that the uv cell becomes "conditioned", giving progressively higher rates with percent completions in repeated runs.

In another experiment, 10^{-6} μM or higher lipoxygenase was allowed to sit for a day in a uv cell which was then emptied and rinsed with linoleate in buffer until active enzyme was gone (usually three rinses). Addition of fresh enzyme (2×10^{-9} M in the cell) produced a threefold rate and three- to fourfold extent increase over an identical reaction in a cleaned cell. A precoat of denatured enzyme (1 μM, heated at 70 °C for 10 min) gave the same increases.

In a third type of experiment, an intermediate concentration of lipoxygenase (5×10^{-9} M) was left in one cell (A) while a run was performed at the same concentration in a different cell (B) with 100 μM linoleate. When the reaction in cell B had ceased (V_{init} = 18.9 μmol min^{-1} mg^{-1} E, 43% conversion), substrate (to be 100 μM) was added to cell A, giving V_{init} = 3.2 μmol min^{-1} mg^{-1} E, 18% conversion, showing a dramatic decrease in active enzyme. When concentrated enzyme (5×10^{-6} M, same buffer) was used in an analogous experiment, the result (V_{init} = 17.5 μmol min^{-1} mg^{-1}, % conversion >50%) showed that inactivation occurs only in very dilute solution. Little diminution in rate occurred when 2×10^{-8} M lipoxygenase was left alone in a cell for an hour.

Smith and Lands (1972) noted a constant ratio of (initial rate)/(extent reaction) as lipoxygenase concentration was varied in this low range, their kinetic derivation accounting for this. Indeed, the wall effect does also. Since initial rate and surface inactivation are both first-order in enzyme, the percent completion and initial rate are both linear functions of initial enzyme, and the ratio is constant until the enzyme saturates the wall sites (obviously $>5 \times 10^{-9}$ M).

Discussion

Two irregularities of the turnover kinetics of soybean lipoxygenase catalyzed oxygenation were explored: the lag and the limited percent completion with accompanying rate retardation.

At $\lesssim 10^{-9}$ M lipoxygenase a surface inactivation of enzyme occurs in our quartz uv cells; ceased reaction mixtures at less than complete conversion go further when additional enzyme is added. Precoating experiments, repeated runs in the same vessel, and enzyme incubations in the absence of substrate also show that this is a wall effect. The inactivation is not dependent on substrate turnover but rather is a physical inactivation of enzyme.

Approximately 10^{-9} M lipoxygenase was used by Smith and Lands (1972) in experiments purportedly showing catalytic enzyme destruction. In the tests for purely physical inactivation by S, P, and O_2 (Figures 2 and 3 of Smith and Lands (1972)), the enzyme concentration was, unfortunately, much higher than in their kinetic assays and showed much slower apparent losses (apparently $(E)_0$ was high so that aliquots diluted into reaction mixtures would be at the usual levels of enzyme). These workers interpreted the slower loss of activity at higher enzyme concentration as an apparent zero-order component to the enzyme destruction, so that more concentrated enzyme would last longer. The experiments described here show that this interpretation is incorrect; the zero-order component is in all probability a saturation of the deactivating cell surface sites. Hence, we do not normally observe enzyme inactivation at higher concentrations.

Others have noted a wall effect with lipoxygenase. Allen (1968) showed an adherence, or "plating out", of purified enzyme upon pipetting it in dilute (but not concentrated) solution. Earlier, Ames and King (1966) noted anomalously slow rates with low enzyme levels, but attributed this to impure substrate. The data reported here are readily explained by physical deactivation, with no need to postulate an inactivating $[E{\cdot}S{\cdot}O_2]$ intermediate.

Our results on the initial lag are in agreement with the conclusion of Smith and Lands (1972) that the lag is abolished by product hydroperoxide. They found that neither H_2O_2 nor n-$C_8H_{17}OOH$ eliminated the lag (the latter may have had some effect, but only at concentrations high enough to inhibit V_{max}). Glutathione plus glutathione peroxidase (neither separately) caused increasing lags, presumably due to reduction of product hydroperoxide in situ. We have shown that the chemically produced 13-alcohol of the product 13-hydroperoxy-9,11-octadecadienoic acid not only fails to diminish the lag but inhibits hydroperoxy product from abolishing it. We take this as evidence for competition at a binding site, so that hydroperoxide can no longer activate. It is clear that the long-chain specificity *and* chemical hydroperoxide functionality are crucial to action here.

Product inhibition of turnover is evident at normal lipoxygenase concentrations. A thorough analysis eliminated impure substrate, improper or incorrectly calibrated O_2 or uv assay, side reactions of substrate, product reactions, equilibrium, medium or surface effects, consumption of O_2, and irreversible inactivation of enzyme as reasons for lowered rates and incomplete conversion. Competitive product inhibition of initial rates was demonstrated by direct experiments using isolated product from linoleate, with K_p = 37.5 μM, compared with 25 μM for $K_{m(app)}$ for linoleate as substrate (quite similar values given that a cis-to-trans and one-position shift of olefin has taken place).

The data show that not only is the product a competitive inhibitor but it also *reversibly* deactivates the enzyme in a time-dependent process. Reactions at normal $(E)_0$ cease too soon, and the initial rates of the later runs of Table II are slower than those calculated from Figure 5. Yet, the substrate is still present, and addition of O_2 or fresh enzyme causes no further reaction; addition of more substrate does. From Table II this is not a simple function of (P)/(S), but rather is a function of

(S), (P), and the ratio. The rapid decline and cessation of rate are not due primarily to an intermediate in the reaction (as proposed by Smith and Lands) because the effect is very large when product is present initially (Table III) with fresh enzyme.

It seems that P can compete with S for the active site (initial rate competitive inhibition) and then, for active enzyme, can cause a reversible deactivation of the enzyme. We shall return to this topic below.

The competitive product inhibition is not very unusual in enzyme systems in vitro. For reactions which involve a change in charge type (e.g., amide or ester hydrolysis at pH >5, phosphorylations, etc.) or size (e.g., nucleases or lysozyme acting on small oligomers) product(s) bind poorly. But for many others, including as trivial but common cases those proceeding to equilibrium with K_e's near unity, products are sufficiently like substrates to inhibit as reaction proceeds (e.g., carboxypeptidase and C-exoproteases in general and many other enzymes). Occasionally product binds well (α-imino acid to D-amino-acid oxidase) but reacts further upon liberation (in this example, to give α-keto acid). Indeed, this is the simplest feedback control, relieved only by the next metabolic transformation.

Finally, we return to the elimination of the lag by product and the reversible inactivation during catalysis. The earlier workers (Smith and Lands, 1972) proposed that there is a separate product binding site, and that each catalytic event in turnover requires substrate, oxygen, and product. A mechanism was proposed that involved reaction of the hydroperoxide group to give an intermediate hydrotetroxide (an unknown species).

We would like to propose here a perhaps simpler explanation that includes both the lag and reversible inactivation and is in accord with all available information. It is difficult to reconcile the demonstrated product inhibition with a product requirement in turnover unless there are two separate binding sites (a matter of speculation at this time). Indeed, it is only certain that the product activation functions at the early stages of the reaction. A product requirement during turnover has not only not been demonstrated but seems countermanded by the inactivation.

The alternative proposal is that the *hydroperoxide product initially activates the enzyme* by binding and reacting at the active site to produce activated enzyme. This activated enzyme would then function in the normal turnover without intervention of product. The activation is likely a redox reaction between hydroperoxide and the Fe atom (well-known chemistry) to produce the active form. It has recently been found (DeGroot et al., 1975b) that ESR signals and visible spectral peaks appear upon addition of small quantities of product to native (inactive) lipoxygenase, and further changes occur with excess product. These are reversible with substrate. The exact nature of the chemistry is a matter of speculation at this time because the state of iron in the enzyme is not known (Gibian and Galaway, 1976), but the first change was proposed to be ferrous to ferric. The reversible deactivation in the presence of larger amounts of product could involve a further change of the redox state of the enzyme to another inactive form, that in this case is reversible by substrate.

The current kinetic work shows that under normal conditions lipoxygenase is *chemically* activated by its product, that product binds well to the substrate site (competitively inhibiting), and that it also then reacts to reversibly inactivate the enzyme.

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