Lipoxygenase Reaction Mechanism: Demonstration That Hydrogen Abstraction from Substrate Precedes Dioxygen Binding during Catalytic Turnover†

Michael H. Glickman‡ and Judith P. Klinman*

Department of Chemistry, University of California, Berkeley, California 94720

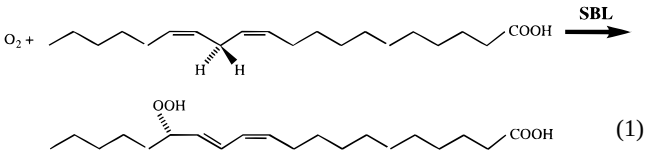
Received April 24, 1996; Revised Manuscript Received July 12, 1996⊗

ABSTRACT: Molecular oxygen is generally unreactive toward covalent bonds, due to spin conservation rules; a major role for oxygen-utilizing enzymes is, therefore, to activate dioxygen through a change in electronic configuration. In an effort to understand how lipoxygenase catalyzes lipid hydroperoxidation under conditions of catalytic turnover, kinetic deuterium isotope effects have been measured as a function of oxygen concentration. The properties of oxygen binding to lipoxygenase have also been pursued. The results presented herein show that, under steady state conditions, atmospheric oxygen enters the reaction pathway only after abstraction of hydrogen from substrate. Furthermore, it has not been possible to detect any form of lipoxygenase capable of binding molecular oxygen in the absence of activated substrate. We propose that molecular oxygen is not productively bound by lipoxygenase but rather interacts directly with the substrate radical lipoxygenase to form the hydroperoxyl radical of linoleate. A mechanism involving substrate activation, instead of the more familiar oxygen activation pathway, is a unique mechanism for a metallo-oxygenase.

A major question regarding the utilization of molecular oxygen in chemical and biological reactions is the nature of oxygen activation. While oxidation reactions are generally thermodynamically favorable, molecular oxygen is kinetically unreactive toward most biological compounds. The limitations on spontaneous reactions of atmospheric oxygen can be explained by spin conservation rules. Oxygen with two electrons in its highest energetic state can exist in two electronic configurations: singlet (electron spins paired) and triplet (unpaired). While covalent diatomic bonds are singlet and would react readily with singlet oxygen, only triplet dioxygen remains in the atmosphere. As a rule, enzymes utilize dioxygen by first activating oxygen to overcome the barrier described by spin conservation rules (Feig & Lippard, 1994; Que, 1980). An alternative strategy for the introduction of molecular oxygen would be to catalyze homolytic cleavage of a covalent bond. Organic radicals thus produced could spontaneously react with triplet molecular oxygen while maintaining spin conservation.

Lipoxygenase is a non-heme iron dioxygenase that catalyzes the conversion of unsaturated fatty acids to hydroperoxides. Lipoxygenase was discovered in plants over 60 years ago (Andre & Hou, 1932) and now is known to be found in cyanobacteria, fungi, algae, plants, and numerous vertebrates [for reviews, see Gardner (1991), Schewe *et al.* (1986), Siedow (1991), and Veldink and Vleigenthart (1984)]. Plant lipoxygenases aid in germination by catalyzing initial steps of fatty acid turnover (Gardner, 1991; Siedow, 1991) and have been proposed to participate in the synthesis of fatty acid-derived wound response and pest deterrent products such as jasmonic acid (Gardner, 1991; Siedow, 1991). The role of mammalian lipoxygenase is to catalyze the first committed step in the cascade converting arachidonic acid to lipoxins and leukotrienes, hormones with a role in inflammation of the respiratory and nervous systems (Samuelsson *et al.*, 1987). These enzymes may also play a role in the degradation of subcellular membranes (Schewe *et al.*, 1986).

Soybean lipoxygenase-1 (SBL-1)[1] is often used as the prototype for studying the homologous family of lipoxygenases from tissues of different species. The reaction with linoleic acid (LA), the standard substrate for SBL-1, preferentially produces 13(*S*)-hydroxyperoxy-9(*Z*),11(*E*)-octadecadienoic acid (LOOH):



(1)

The precise chemical mechanism of lipoxygenase is not yet known and has been surrounded by some controversy [cf. Stubbe (1989) and Wiseman (1989)]. Recently, progress has been made in understanding the structure and function of soybean lipoxygenase-1. The enzyme has been crystallized and its structure determined (Boyington *et al.*, 1993; Minor *et al.*, 1993). On the basis of the crystal structure and other spectroscopic techniques (Scarrow *et al.*, 1994), the ferrous iron in the resting form of the enzyme is described as a

† Supported by a grant from the U.S. Department of Education (to M.H.G.) and the National Institutes of Health (GM25765-19 to J.P.K.).

* To whom correspondence should be addressed.

‡ Current address: Department of Cell Biology, Harvard Medical School, Boston, MA 02115.

⊗ Abstract published in *Advance ACS Abstracts,* September 1, 1996.

[1] Abbreviations: SBL-1, soybean lipoxygenase-1; LA, linoleic acid; D-LA, [$^2H_{31}$]linoleic acid; k_{cat}, first-order rate constant at saturating substrate concentrations; k_{cat}/K_m, second-order rate constant at low substrate concentrations, where a subscript denotes which of the two substrates (linoleic acid and O_2) is extrapolated to very low concentrations; $^D(k_{cat}/K_m)$, k_H/k_D isotope effect on the second-order rate constant, k_{cat}/K_m; $^D(k_{cat})$, k_H/k_D isotope effect on the first-order rate constant, k_{cat}; NDGA, nordihydroguaiaretic acid; dpm, radioactive decompositions per minute; HPLC, high-pressure liquid chromatography.

S0006-2960(96)00985-3 CCC: $12.00

distorted octahedron with four or five protein side chains as ligands: three histidines, the carboxyl terminal of the protein, and possibly an asparagine side chain. Most spectroscopic data suggest that the iron in the active ferric form of the enzyme is octahedral, with six ligands (Dunham *et al.*, 1990; Nelson, 1988b; Van der Heijdt *et al.*, 1992; Zang *et al.*, 1991) and the sixth ligand being ascribed to hydroxide ion (Scarrow *et al.*, 1994). Other than the iron, no other obvious candidate as an enzymatic cofactor has been revealed from the current crystal structures. Two channels from the surface to the active site are apparent. It is postulated that the existence of the two cavities allows separate access of substrate and dioxygen to the active site iron (Boyington *et al.*, 1993). The reduction potential of the active site ferric iron is known to be high (close to 0.6 V, Nelson, 1988a), making lipoxygenase a significantly stronger oxidizing agent than other non-heme iron-containing enzymes. It has not been clear whether O_2 is coordinated to the iron as part of the steady state reaction mechanism, although X-ray absorption measurements performed under nonturnover conditions suggest that O_2 does not bind in a detectable manner to a specific site on the lipoxygenase molecule (Van der Heijdt *et al.*, 1995).

Regarding the catalytic mechanism, the one most widely accepted proposes that hydrogen is abstracted from the C-11 position of substrate to yield a delocalized carbon-based radical, which then reacts with dioxygen, producing a peroxyl radical. EPR has detected a peroxyl-based radical (Nelson *et al.*, 1990, 1994), supporting the idea that C−H bond cleavage produces an organic radical which then reacts with oxygen. A homolytic mechanism, involving a delocalized radical as an intermediate, is also supported by the secondary hydrogen isotope effects that are larger than unity (Glickman *et al.*, 1994b). Kinetic studies have shown that at 25 °C the reaction is partially diffusion-controlled and that the C−H bond cleavage step is also partially rate-determining; this step is characterized by an enormous k_H/k_D kinetic intrinsic isotope effect (Glickman & Klinman, 1994). The precise stage at which oxygen enters the reaction under steady state conditions has not yet been determined. The work presented herein attempts to answer the following questions. (1) Can an enzyme form be detected that is capable of binding dioxygen? (2) Is there an obligatory order to the reaction binding sequence with regard to LA and dioxygen? And, finally, (3) does O_2 participate in any manner in the steady state activation of the C−H bond?

MATERIALS AND METHODS

Materials. Soybean lipoxygenase-1 (SBL-1) was purified from dry soybeans as published (Axelrod *et al.*, 1981). Soybeans were a gift of Pioneer Hi-Bred International (Iowa). Purity was >95% by SDS−PAGE, and the maximal activity was about 180 units/mg (units defined as micromoles of product produced per minute). After purification, lyophilized enzyme was stored at −70 °C. Stock solutions of small amounts of enzyme dissolved in borate buffer (pH 9) served to initiate reaction.

Sodium linoleate was purchased from Sigma Chemical Co., and sodium [$^2H_{31}$]linoleate (isotopic purity, 98%) was from Cambridge Isotope Labs. Substrates were further purified and their concentrations determined as described earlier (Glickman & Klinman, 1994). Stock solutions of either substrate were about 400 μM substrate in 0.1 M borate buffer (pH 9) and were stored at −70 °C up to 1 month.

Unless stated otherwise, all enzyme, substrate, and reaction solutions were in 0.1 M borate buffer (pH 9). All other chemicals were of the highest purity available from Sigma.

Reaction Rates at Varying O_2 Concentrations. Direct comparisons of reaction rates of soybean lipoxygenase-1 with linoleic acid (LA) and [$^2H_{31}$]linoleic acid (D-LA) were determined by measuring oxygen consumption on a Clark oxygen monitor. Reactions were carried out as a function of fatty acid substrate concentrations in 1 mL solutions which were constantly stirred and equilibrated under air at 25 °C. Enzyme was held at 0 °C until use. Reaction was initiated by addition of a 1−5 μL solution of 0.02−0.15 mg/mL SBL (depending on reaction conditions) via a gastight Hamilton syringe to the reaction chamber. The air-equilibrated solution contains 258 μM O_2 at 25 °C; experiments were repeated at a number of different concentrations of oxygen dissolved in reaction solution by passing mixtures of N_2 and O_2 over stirred solutions in the reaction chamber for 20 min. The new oxygen concentration in the solution was calibrated against the value of O_2 dissolved in air-saturated solution at 25 °C (258 μM). The rate of oxygen consumption after initiation of reaction with SBL was then determined as a decrease in dissolved O_2 in the reaction solution. Rates of reaction were measured at O_2 concentrations which varied from 258 to 5 μM. At each O_2 concentration, the substrate concentration was varied between 1.5 and 50 μM LA or D-LA.

Initial rates for each substrate were fitted to the Michaelis−Menten equation using the KaleidaGraph program on a Macintosh computer. Errors for initial rate parameters were from these Michaelis−Menten fits. Up to the first 15% of the reaction was monitored. At substrate concentrations above 35 μM, a brief lag phase was noticed; initial rates were, therefore, measured for the linear portion of reaction after the lag phase. The reaction with D-LA did not show a lag phase but rather an initial burst since 2−3% of the substrate was contaminated with unlabeled linoleic acid; due to the large isotope effect, protonated linoleic acid substrate is preferentially exhausted in the first 3% of the reaction, after which linear kinetics are observed. Thus, for deuterated substrate, the region showing linear kinetics between 3 and 15% conversion of substrate to product peroxide was used to determine initial rates. The kinetic isotope effect was calculated from the ratio of the kinetic parameters determined for each substrate; errors were propagated into ratios from the individual parameters.

In a previous study of lipoxygenase (Glickman & Klinman, 1995), noncompetitive primary deuterium isotope effects were measured by spectrophotometric monitoring of the rate of product formation from H-LA or D-LA at 234 nm. An interesting feature of the experiments reported herein is that the rate of lipoxygenase as determined by oxygen consumption at low substrate concentrations is slower than that determined spectrophotometrically. The difference is due to a K_m for linoleic acid almost 3-fold larger using the Clark oxygen monitor. The maximal rate at saturating concentrations of linoleic acid is similar with both analytical techniques. Very similar (though not identical) increases of K_m were measured by oxygen consumption for linoleic acid and deuterated linoleic acid (Glickman, 1994). It follows that the magnitudes of the isotope effects determined by either method are similar but not identical.

It is not clear what the source of discrepancy is between the values of the observed second-order rate constant measured by the two techniques. One of the differences between the analytical techniques is that the reaction solution is constantly stirred with a rotating magnetic bar in the reaction chamber of the oxygen monitor but not in the spectroscopic cuvette. The second-order rate constant expression includes the steps for substrate binding and release, whereas at saturating substrate, the maximal velocity reflects only steps after formation of the enzyme−substrate complex. The simplest cause for a decrease in the second-order rate constant without a similar decrease in maximal velocity would be a decrease in the association rate of substrate with enzyme; it is possible that the stirring has an effect on the affinity of the enzyme for substrate. It is also possible that specific premicellar aggregates of the fatty acid are the preferred substrate for lipoxygenase. The fatty acid substrate can be present in a number of forms in solution which greatly affect the kinetic parameters of lipoxygenase; the sheer force from rapid stirring could disrupt these complexes, thereby decreasing the effective concentration of substrate available for reaction. We note that *all* of the results presented herein were determined on a Clark oxygen electrode.

Oxygen Binding Experiments. Experiments were performed as published (Tian & Klinman, 1993). In brief, the partial pressure of total O_2 in solution, with and without SBL, is analyzed by separating oxygen from other gases and converting it to CO_2. An isolated reaction chamber of about 100 mL, sufficient for two 50 mL aliquots of reaction solution, is connected to a vacuum line. An air-equilibrated reaction solution, in this case 100 mL of 0.1 M borate buffer (pH 9) at room temperature, is then sealed in the reaction chamber and an aliquot transferred to the vacuum line. O_2 is extracted by degassing under vacuum and sparging with helium for a total of 60 min. A series of four liquid nitrogen traps separates O_2 from water vapor, argon, and CO_2. O_2 is then converted to CO_2 in a furnace and trapped by liquid nitrogen to separate it from the remaining N_2. The partial pressure of CO_2 is determined and correlated to that of oxygen in the initial reaction solution, which is 258 μM at 25 °C. A concentrated enzyme solution equilibrated with air is then injected via syringe through a serum cap into the reaction chamber and the oxygen concentration measured on another aliquot. The final concentration of SBL in the reaction solution was between 50 and 300 μM. The pressure of oxygen in aliquots of the solution with enzyme is thus measured and compared to that of the solution without enzyme. The oxygen concentration in the buffered solution is subtracted from the oxygen content of enzyme-containing solutions, representing oxygen in solution plus oxygen bound to enzyme, to determine the number of moles of dioxygen bound per mole of enzyme in solution. These determinations were performed on solutions containing different forms of lipoxygenase: native (ferrous) enzyme, oxidized (ferric) enzyme, enzyme that was first oxidized and then reduced back to ferrous, and enzyme in the presence of a substrate analog. SBL was oxidized to the ferric form by an excess of product peroxide [13(*S*)-hydroxyperoxy-9(*Z*),11(*E*)-octadecadienoic acid] produced enzymatically from LA. Lipoxygenase was reduced to the ferrous form by nordihydroguaiaretic acid (NDGA) (Kemal, 1987). After reaction, enzyme was desalted by a column (G-50) technique and reconcentrated. Vacenic acid (Sigma), in excess of enzyme concentration and at least 1 mM in reaction solution, was used as a substrate analog.

Tritium Labeling Experiments. Linoleic acid was incubated in tritiated water (TOH) in the presence of lipoxygenase. The reaction with lipoxygenase was stopped before completion, and residual linoleic acid was analyzed for tritium incorporation. Linoleic acid was also incubated in TOH without enzyme for an identical period of time to control for nonspecific incorporation of tritium. Two reaction chambers were linked to an argon line and to a vacuum line. A portion of linoleic acid, which had been purified, dissolved in borate buffer (pH 9), and lyophilized to dryness, was weighed out and dissolved in 0.5 mL of 1 Ci/mL TOH to a final concentration of 1.5 mM linoleic acid in each reaction chamber. A slow stream of argon decreased the concentration of oxygen dissolved in solution, but care was taken not to make it fully anaerobic so as to maintain turnover conditions for lipoxygenase. Enzyme solution, 50 μL of 0.1 mg/mL SBL-1 (180 units/mg), was injected into one of the chambers via syringe to initiate reaction. Both reaction mixtures were incubated for 35 min and the reactions quenched by a mixture of acetic acid and CH_3CN. Samples were then lyophilized and redissolved in H_2O for eight consecutive times until the overall radioactivity decreased to a few hundred thousand dpm. An aliquot from each reaction mixture was subjected to HPLC, in order to separate substrate from product [see Glickman *et al.* (1994) for a previously published procedure].

RESULTS

Magnitude of Steady State Deuterium Isotope Effects as a Function of Linoleic Acid and Dioxygen Concentrations. A full kinetic study of lipoxygenase was performed to determine the order of binding of the fatty acid substrate and dioxygen under steady state conditions. The kinetic parameters of lipoxygenase were deduced by measuring initial rates as a function of unlabeled (LA) or deuterated linoleic acid (D-LA) at a given concentration of O_2, as plotted in Figure 1. These data were replotted to obtain initial rates as a function of O_2 at different concentrations of unlabeled or deuterated linoleic acid (Figure 2). The data in Figure 1a indicate that, when LA is the substrate, both the maximal velocity at saturating substrate, k_{cat}(app), and the second-order rate at low concentrations of LA, $k_{cat}/K_{m(LA)}$(app), decrease as the concentration of the second substrate, O_2, is lowered. In contrast, when D-LA is substituted for LA (Figure 1b), the kinetic parameters are only slightly affected by dioxygen concentrations within the attainable experimental range (258−5 μM O_2).

This characteristic is even clearer in Figure 2; k_{cat}(app) and $k_{cat}/K_{m(O_2)}$(app) both decrease as the LA concentration decreases (Figure 2a). However, when D-LA is the substrate, the reaction is close to saturated with O_2 under all experimental conditions (Figure 2b). Since the observed enzymatic rate is nearly independent of oxygen concentration when D-LA is the second substrate (Figure 2b), the K_m for O_2 is below the lowest oxygen concentration attainable by our experimental procedure (5 μM). The upper limit for the value of $K_m(O_2)$ is thus estimated as 2.5 μM, and K_m may be much smaller.

Extrapolated values for k_{cat}(app) and k_{cat}/K_m(app) for each substrate, obtained from the data in Figures 1 and 2, were

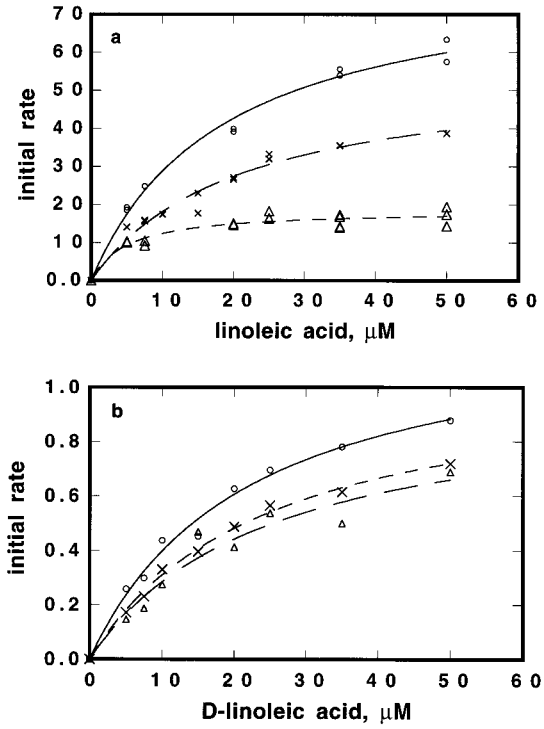


FIGURE 1: Initial rates of lipoxygenase as a function of linoleic acid concentration (a) or deuterated LA concentration (b). Rates of oxygen consumption were determined as described in Materials and Methods and are defined as micromoles of dioxygen consumed per minute per milligram of lipoxygenase. Data are fit to the Michaelis−Menten equation. Examples are shown for 258 μM O_2 (○), 77 μM O_2 (×), and 13 μM O_2 (△).

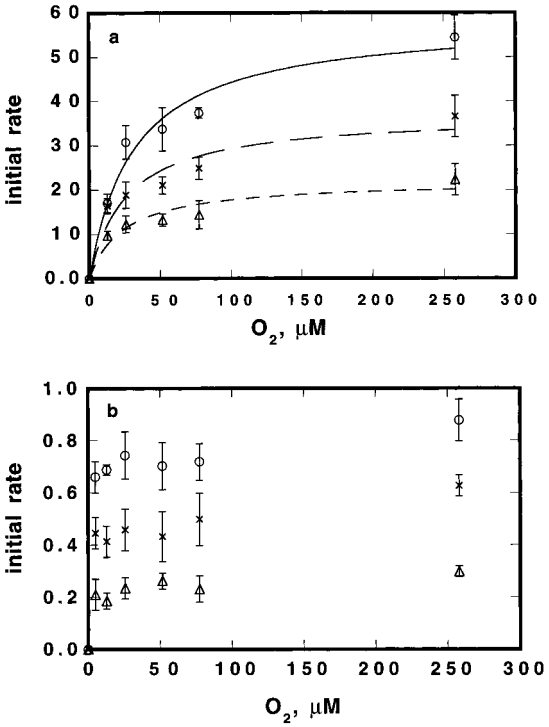


FIGURE 2: Initial rates of lipoxygenase as a function of O_2 with linoleic acid as substrate (a) or deuterated linoleic acid as substrate (b). Rates of oxygen consumption were determined as described in Materials and Methods and are defined as micromoles of dioxygen consumed per minute per milligram of lipoxygenase. Data are fit to the Michaelis−Menten equation. Examples in panel a are shown at 50 μM LA (○), 20 μM LA (×), and 5 μM LA (△).

plotted as a function of the other substrate concentration in Figures 3 and 4, respectively. These secondary kinetic plots

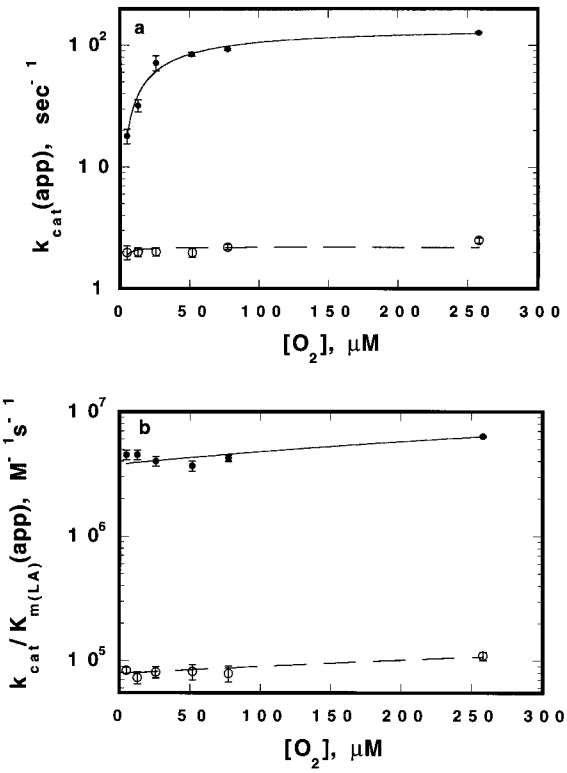


FIGURE 3: Limiting-rate constants. (a) Rates at saturating concentrations of H-LA (●) and D-LA (○) as a function of O_2 concentration. The extrapolated values at infinite linoleic acid concentrations, obtained by fitting the rates of lipoxygenase as a function of H-LA (from Figure 1a) and D-LA (from Figure 1b) at a given O_2 concentration to the Michaelis−Menten equation, are replotted against O_2 concentration for H-LA (●) and D-LA (○) concentrations. Due to the difference of greater than 1 order of magnitude between the rates with D-LA and H-LA, the *y*-axis is displayed as logarithmic. Extracted values representing k_{cat} and $k_{cat}/K_{m(O_2)}$ at high linoleic acid concentrations are in Table 1. Note that only a lower limit could be obtained for $k_{cat}/K_{m(O_2)}$ with D-LA. (b) Rates at low concentrations of H-LA (●) and D-LA (○) as a function of O_2 concentration. Extrapolated values at low linoleic acid concentrations, obtained by fitting results in Figure 1a,b, are plotted against O_2 concentration. The values of $k_{cat}/K_{m(LA)}$ at high O_2 concentrations are summarized in Table 1.

were used to extract the limiting values of k_{cat} and k_{cat}/K_m for each substrate. The ratio of the kinetic parameters obtained from the secondary plots yielded the isotope effects displayed in Table 1. In order to illustrate the pattern in the behavior of isotope effects between concentration extremes, isotopic effects have also been calculated in the intermediate concentration range, and these are presented in Figure 5. From the data in Figures 3a and 4a, we find that the isotope effect on the maximal velocity, $^D(k_{cat})$ is 64 ± 5 and 59 ± 5, respectively. These values are close to the intrinsic isotope effect previously determined for SBL-1 (Glickman & Klinman, 1995). A similar result is observed for the isotope effect on the second-order rate constant at low concentrations of linoleic acid and saturating O_2; $^D[k_{cat}/K_{m(LA)}] = 58 \pm 5$ (Figure 3b) or 59 ± 9 (Figure 4a). In contrast to these enormous isotope effects, the value of the deuterium isotope effect at low concentrations of dioxygen and saturating linoleic acid, $^D[k_{cat}/K_{m(O_2)}]$, is small (Figures 4b and 5a) and represents an upper limit (since, as discussed above, the K_m for oxygen with D-LA is lower than we can measure experimentally, being estimated as ≤2.5 μM). The true value of $^D[k_{cat}/K_{m(O_2)}]$ is, therefore, below 4 and may be as small as unity (Table 1).

Table 1: Kinetic Parameters for SBL-1

parameter	figure	rate constant[a] H-LA	rate constant[a] D-LA	isotope effect[b]
k_{cat} (s^{-1})	3a	140 (±10)	2.2 (±0.1)	64 ± 5
	4a	150 (±8)	2.6 (±0.2)	59 ± 5
$k_{cat}/K_{m(LA)}$ (M^{-1} s^{-1})	3b	6.3 (±0.1) × 10^6	1.1 (±0.1) × 10^5	58 ± 5
	4a	5.4 (±0.5) × 10^6	0.92 (±0.12) × 10^5	50 ± 9
$k_{cat}/K_{m(O_2)}$ (M^{-1} s^{-1})	3a	4.2 (±0.9) × 10^6	>1 × 10^6 [c]	<4
	4b	3.7 (±0.2) × 10^6	~0.96 (±0.6) × 10^6 [d]	~3.8[d]

[a] Extracted by nonlinear fitting of the data in Figures 3 and 4 to the Michaelis−Menten equation. Numbers in parentheses are errors derived from the fit. [b] Isotope effects and propagated errors, obtained by dividing constants for H-LA by those for D-LA. [c] Estimated from k_{cat} for D-LA, divided by a K_m of ≤2.5 μM. [d] Since the rate of lipoxygenase could not be determined at or below K_m for O_2 with D-LA (Figures 1 and 2), the isotope effect is only estimated.

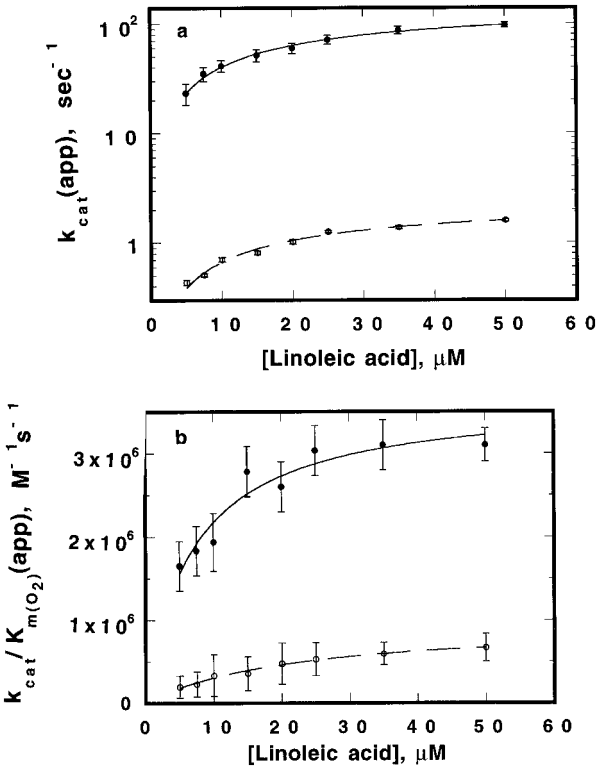


FIGURE 4: Limiting-rate constants. (a) Rates at saturating O_2 as a function of H-LA concentration (●) and D-LA concentration (○). The extrapolated values at infinite O_2 concentrations, obtained by fitting the rates of lipoxygenase as a function of O_2 concentration at given H-LA concentrations (from Figure 2a) and D-LA concentrations (from Figure 2b) to the Michaelis−Menten equation, are replotted against H-LA (●) and D-LA (○) concentrations. Due to the difference of greater than 1 order of magnitude between the rates with D-LA and H-LA, the *y*-axis is displayed as logarithmic. Extracted value representing k_{cat} and $k_{cat}/K_{m(LA)}$ at saturating O_2 concentrations are given in Table 1. (b) Rates at low concentrations of O_2 as a function of H-LA (●) and D-LA (○) concentration. The extrapolated values of low O_2 concentrations, obtained by fitting results in Figure 2a,b, are replotted against H-LA concentration (●) and D-LA concentration (○). The values of $k_{cat}/K_{m(O_2)}$ at high linoleic acid concentrations are in Table 1.

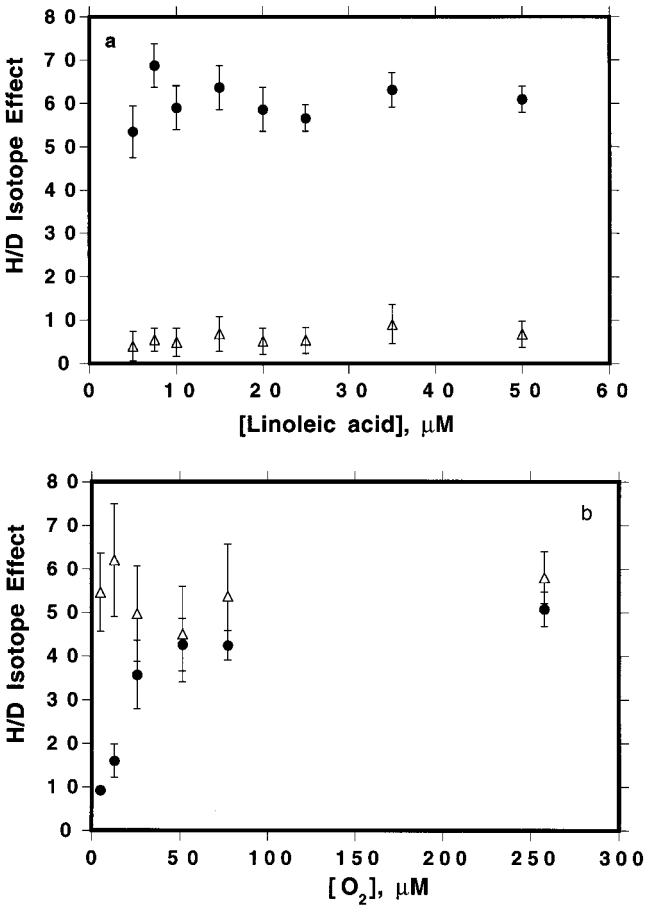


FIGURE 5: Kinetic isotope effects for the lipoxygenase reaction as a function of substrate concentration. (a) The observed experimental values for the isotope effects at high concentrations of dioxygen, $^D(k_{cat})$(app) (●), and at low concentrations of dioxygen, $^D[k_{cat}/K_{m(O_2)}]$(app) (△), as a function of linoleic acid concentration (Figure 4a,b). Within the experimental concentrations, the observed isotope effects decrease significantly with decreasing O_2 concentration but do not appear to be sensitive to linoleic acid concentration. It is important to note that the plotted values of $^D[k_{cat}/K_{m(O_2)}]$(app) represent an upper limit. As evident from Figures 1 and 2, it has only been possible to estimate a lower limit for $k_{cat}/K_{m(O_2)}$ using D-LA. (b) The observed values for the isotope effects at high concentrations of linoleic acid, $^D(k_{cat})$(app) (●), and at low concentrations of linoleic acid, $^D[k_{cat}/K_{m(LA)}]$(app) (△), are plotted as a function of O_2 concentration (Figure 3a,b). The limiting behavior of the isotope effects at very low O_2 is not known, due to the inability to attain $K_{m(O_2)}$ for D-LA (see above and text).

Attempted Detection of Oxygen Binding by Lipoxygenase. To distinguish among possible kinetic mechanisms, the extent of O_2 binding by lipoxygenase was analyzed using a vacuum line/oxygen trap method. Lipoxygenase is known to exist in two forms, which differ with regard to the valence state of the iron. The resting enzyme as isolated from the cell is generally ferrous, whereas the active enzyme is considered to be in the ferric state (deGroot *et al.*, 1975a; Schilstra *et al.*, 1994). As expected for the ferric form of enzyme, we were unable to detect any bound molecular oxygen above a buffer control. We, therefore, attempted to detect oxygen binding to reduced forms of enzyme. Neither the native ferrous form of lipoxygenase nor a form which had been oxidized by product hydroperoxide and reduced by nordihydroguaiaretic acid indicated any binding of oxygen. We

Table 2: Oxygen Binding Properties of SBL-1[a]

enzyme form	iron state[b]	micromoles of enzyme in the sample	background O_2 in buffer[c] (μmol, $\pm$0.3)	total O_2[d] (μmol, $\pm$0.3)	O_2 from protein[j] (μmol, $\pm$0.6)
native	Fe^{2+}	2.0	13.2	13.2	
		5.5	13.2	12.9	
reduced[e]	Fe^{2+}	7.0	13.2	13.2	
		3.0	12.9	12.9	
"yellow" oxidized[f]	Fe^{3+}	15	16.8	16.8	
		15	12.9	12.9	
		15	12.9	12.9	
reduced, after oxidation[g]	Fe^{2+}	4.1	12	12	
		4.1	12	12	
reduced plus vacenic acid[h]	Fe^{2+}	6.6	11.4	11.4	
oxidized plus vacenic acid[h]	Fe^{3+}	6.3	13.2	12.6	
controls[i]					
hemoglobin	Fe^{2+}	6.0	12	18	6
hemoglobin plus reduced SBL-1	Fe^{2+}	6.0	12.0	17.7	5.7
	Fe^{2+}	1.5			

[a] The partial pressure of O_2 dissolved in buffer with or without enzyme was determined after extraction and conversion to CO_2 (see Materials and Methods). [b] The presence of ferric iron was confirmed by measuring absorbance at 330 nm (deGroot *et al.*, 1975; Slappendel *et al.*, 1983).[c] The partial pressure of O_2 dissolved in buffer in the absence of enzyme was determined in each case and converted to micromoles. [d] Total dioxygen in solution of protein dissolved in buffer. [e] Native enzyme reduced by $^1/_2$ × moles of NDGA (0.5:1.0 molar ratio) to ferrous form (Van der Zee *et al.*, 1989; Kemal *et al.*, 1987). [f] Native enzyme oxidized by an excess of lipid hydroperoxide to ferric form. [g] Yellow (Fe^{3+}) SBL-1 reduced by $^1/_2$ × moles of NDGA (0.5:1.0 molar ratio) to ferrous form. [h] SBL-1 in the presence of 1 mM vacenic acid as substrate analog. [i] Detection of dioxygen bound to reduced hemoglobin was performed as published (Tian & Klinman, 1993) in the absence or presence of reduced SBL-1. O_2 bound to hemoglobin in the presence of oxidized SBL-1 could not be determined. [j] From the limits of detection, we estimate that the K_d for O_2 dissociation from SBL-1 is $\geq$3 mM, relative to a concentration of dissolved O_2 in buffer of *ca.* 0.26 mM.

also considered the possibility that oxygen binding was dependent on an enzymatic conformational change triggered by substrate binding. Using saturating levels of vacenic acid (a competitive inhibitor and substrate analog of linoleic acid), we also failed to produce any detectable O_2 binding. As summarized in Table 2, we were unable to find an experimental condition which led to detectable binding of dioxygen to lipoxygenase.

Hydrogen Exchange from the Enzyme−Substrate Complex. In order to assess the reversibility of the C−H bond cleavage under slow turnover conditions (i.e., in the presence of trace dioxygen), lipoxygenase reactions were conducted in highly labeled tritiated water (1 Ci/mL). The reaction with lipoxygenase was stopped before completion, and residual linoleic acid was analyzed for tritium incorporation. From the size of substrate and product peaks after separation by HPLC, it could be concluded that 5−10% of the substrate had been converted to product during the course of the reaction. Analysis of these peaks showed background levels of radioactivity (data not shown). From the specific activity of the water (2×10^7 dpm/μmol), we expected to be able to detect a 0.01% exchange (*ca.* 2×10^3 dpm in unreacted LA). Clearly, if any back-exchange from solvent to substrate occurred in these reactions, it was below this level.

What amount of exchange could we have expected? In order to observe exchange, several conditions are necessary: first, that solvent-derived hydrogen is present in the active site prior to substrate binding; second, that the activated enzyme−substrate intermediate (presumably a carbon-based radical) has access to this solvent-derived hydrogen; and third, that this intermediate is subject to a partitioning between return to substrate or reaction with dioxygen and conversion to product perioxide. The first condition is supported by the sizable solvent isotope effects previously reported in the SBL-1 reaction (Glickman & Klinman, 1995) and suggests that the substrate radical would have access to solvent:

$$\mathrm{ESH} \xleftarrow{k_r} \mathrm{ES}^{\bullet} \xrightarrow{k_f(O_2)} \mathrm{EPOOH} \qquad (2)$$

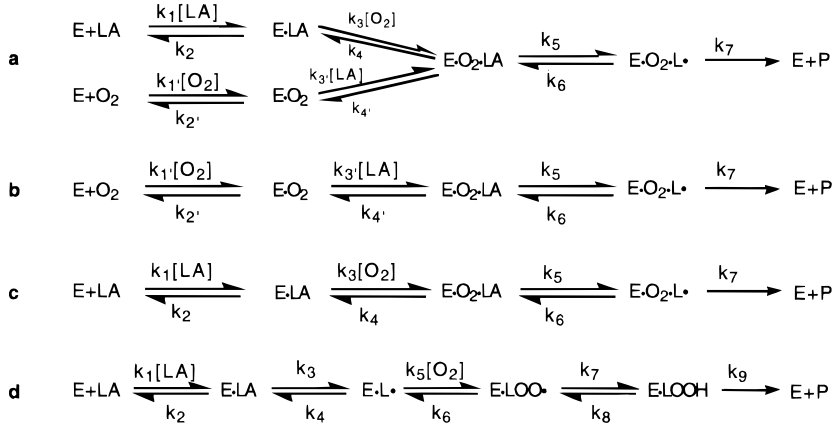
When the oxygen concentration was lowered to trace levels, the return to substrate was expected to be strongly favored. Although these reactions could have been conducted anaerobically, we reasoned that maintenance of low-turnover conditions both would ensure that the results be relevant to steady state conditions and would provide an internal control (% product formation) for quantitation of any measurable exchange. For the observation of *ca.* 10% product formation in 35 min, we estimate that $k_f(O_2) \simeq 0.7\ s^{-1}$ (corresponding to *ca.* 0.15 μM dissolved O_2). Our lack of detectable tritium incorporation indicates k_r is $\leq 7 \times 10^{-5}\ s^{-1}$ for tritium exchange. Although the tritium isotope effect has not yet been measured in this reaction, we estimate that H/T is in the range of $(H/D)^{1.422}$ or 560. Thus, k_r for protium transfer is estimated to be $\leq 0.04\ s^{-1}$.

DISCUSSION

A main task of these studies has been to differentiate between a steady state mechanism that involves a ternary complex (enzyme−linoleic acid−dioxygen) as a prerequisite for catalysis and a mechanism where the chemical step of hydrogen abstraction occurs prior to oxygen binding. In the case of a ternary complex, we can further distinguish between a truly random mechanism where either substrate can bind to free enzyme (Scheme 1a), an ordered binding of dioxygen followed by binding of LA (Scheme 1b), or an ordered biding of LA prior to O_2 (Scheme 1c) (for an example and derivations of isotope effect expressions for these types of reactions, see Ahn and Klinman (1981) and Klinman *et al.* (1980)]. A distinct possibility, whereby O_2 binds after C−H bond cleavage, is shown in Scheme 1d and is described in Table 3.

The simplest case is that of ordered binding. In one case of an ordered binding sequence, LA bind first as a require-

Scheme 1: Possible Mechanisms for the Lipoxygenase Reaction[a]



[a] Part a represents a random-binding mechanism where either substrate can bind to free enzyme. Part b is an ordered-binding mechanism where only dioxygen can bind to free enzyme. Part c describes ordered binding of linoleic acid as a prerequisite for oxygen binding. A mechanism that involves hydrogen abstraction from substrate prior to oxygen binding to enzyme is shown in part d. To simplify these derivations, the step in $k_{cat}/K_{m(LA)}$ that is sensitive to D_2O (Glickman & Klinman, 1995) has been included in the step representing C−H bond cleavage.

Table 3: Isotope Effect Expressions[b] for Reactions Described in Scheme 1a−d[a]

		random binding[d]	ordered binding[d]		O_2 binds after C−H cleavage[c]
KIE	limit	Scheme 1a[e]	Scheme 1b (O_2 first)[e]	Scheme 1c (LA first)[e]	Scheme 1d
$^{D}(k_{cat})$	high LA high O_2	$\dfrac{^{D}k_5+\frac{k_5}{k_7}+{}^{D}K_{eq5}\frac{k_6}{k_7}}{1+\frac{k_5}{k_7}+\frac{k_6}{k_7}}$	$\dfrac{^{D}k_5+\frac{k_5}{k_7}+{}^{D}K_{eq5}\frac{k_6}{k_7}}{1+\frac{k_5}{k_7}+\frac{k_6}{k_7}}$	$\dfrac{^{D}k_5+\frac{k_5}{k_7}+{}^{D}K_{eq5}\frac{k_6}{k_7}}{1+\frac{k_5}{k_7}+\frac{k_6}{k_7}}$	$\dfrac{^{D}k_3+\frac{k_3}{k_9}+\frac{k_3}{k_9}\frac{k_8}{k_7}+\frac{k_3}{k_7}}{1+\frac{k_3}{k_9}+\frac{k_3}{k_9}\frac{k_8}{k_7}+\frac{k_3}{k_7}}$
$^{D}[k_{cat}/K_{m(LA)}]$	high O_2	$\dfrac{^{D}k_5+\frac{k_5}{k_{4'}}+{}^{D}K_{eq5}\frac{k_6}{k_7}}{1+\frac{k_5}{k_{4'}}+\frac{k_6}{k_7}}$	$\dfrac{^{D}k_5+\frac{k_5}{k_{4'}}+{}^{D}K_{eq5}\frac{k_6}{k_7}}{1+\frac{k_5}{k_{4'}}+\frac{k_6}{k_7}}$	1	$\dfrac{^{D}k_3+\frac{k_3}{k_2}}{1+\frac{k_3}{k_2}}$
$^{D}[k_{cat}/K_{m(O_2)}]$	high LA	$\dfrac{^{D}k_5+\frac{k_5}{k_4}+{}^{D}K_{eq5}\frac{k_6}{k_7}}{1+\frac{k_5}{k_4}+\frac{k_6}{k_7}}$	1	$\dfrac{^{D}k_5+\frac{k_5}{k_4}+{}^{D}K_{eq5}\frac{k_6}{k_7}}{1+\frac{k_5}{k_4}+\frac{k_6}{k_7}}$	$\dfrac{^{D}K_{eq3}+\frac{k_3}{k_4}}{1+\frac{k_3}{k_4}}$
$^{D}[k_{cat}/K_{m(O_2)}]^{f}$ $^{D}[k_{cat}/K_{m(LA)}]$	low LA low O_2	$\dfrac{^{D}k_5+\frac{k_5}{k_{4'}+k_4}+{}^{D}K_{eq5}\frac{k_6}{k_7}}{1+\frac{k_5}{k_{4'}+k_4}+\frac{k_6}{k_7}}$	$\dfrac{^{D}k_5+\frac{k_5}{k_{4'}}+{}^{D}K_{eq5}\frac{k_6}{k_7}}{1+\frac{k_5}{k_{4'}}+\frac{k_6}{k_7}}$	$\dfrac{^{D}k_5+\frac{k_5}{k_4}+{}^{D}K_{eq5}\frac{k_6}{k_7}}{1+\frac{k_5}{k_4}+\frac{k_6}{k_7}}$	$^{D}K_{eq3}$

[a] Expressions were derived for the limiting cases at extremes of high and low concentrations of each substrate. [b] Compare to experimental results in Table 1. [c] $^{D}K_{eq3}$ represents the isotope effect on the equilibrium constant k_3/k_4 in Scheme 1d. [d] For simplicity, the steps after the C−H bond cleavage were made irreversible in Scheme 1a−c. [e] In order to correctly compare all the mechanisms shown in Scheme 1a−d, the C−H bond cleavage was made reversible. Simpler expressons for the isotope effect can be obtained—lacking the equilibrium isotope effect term, $^{D}K_{eq5}$, in both the numerator and the denominator—when the chemical step is treated as being irreversible. However, the conclusions are not affected (Glickman & Klinman, 1996). [f] The expression for the isotope effect at low concentrations of both substrates is identical both for $^{D}[k_{cat}/K_{m(O_2)}]$ at a low [LA] and for $^{D}[k_{cat}/K_{m(LA)}]$ at a low $[O_2]$; the later was not determined experimentally.

ment for O_2 binding to enzyme (Scheme 1c); the prediction is that the isotope effect $^{D}[k_{cat}/K_{m(LA)}]$ will be unity under conditions where O_2 is saturating the enzyme. The second-order rate constant $^{D}[k_{cat}/K_{m(LA)}]$ includes steps up to the first irreversible step; at high concentrations of dioxygen, step 1 is basically irreversible, and the isotopically sensitive step (step 5) is not expressed in the isotope effect term for $^{D}[k_{cat}/K_{m(LA)}]$ (Table 1). Since $^{D}[k_{cat}/K_{m(LA)}] \approx 60$ at high O_2 concentrations (Figure 3b and 4a), this scenario can definitely be ruled out as a mechanism for lipoxygenase. Regarding an ordered binding sequence of dioxygen prior to LA (Scheme 1b), the prediction is that $^{D}[k_{cat}/K_{m(LA)}]$ will be independent of the oxygen concentration while $^{D}[k_{cat}/K_{m(O_2)}]$ will go to unity at saturating linoleic acid (Table 1). Within the attainable experimental range of O_2 concentrations, the first statement is valid (Figure 5b). Unfortunately, due to the low K_m value for O_2 with deuterated LA, we could not accurately determine the behavior of $^{D}[k_{cat}/K_{m(O_2)}]$ as a function of LA concentration. Another characteristic of the ordered-binding mechanism in Scheme 1b is that $^{D}[k_{cat}/K_{m(O_2)}]$ is expected to increase as the concentration of LA is decreased until it equals the value of $^{D}[k_{cat}/K_{m(LA)}]$ (cf. Table 3). From the upper limit we have obtained for $^{D}[k_{cat}/K_{m(O_2)}]$ (Figure 5a), there is no evidence that this parameter increases at low linoleic acid concentrations to anywhere approximating the value of 60 measured for $^{D}[k_{cat}/K_{m(LA)}]$. Thus, while

we cannot exclude such a mechanism from the initial rate data, we think it highly unlikely.

If we consider the general case of a random-binding mechanism (Scheme 1a), the isotope effect on k_{cat}/K_m should alternate between two finite values for each of the two substrates. Considering, for example, a mechanism such as that described in Scheme 1a, the isotope effect at low LA concentrations or low O_2 concentrations can be expressed as follows:

$$^{D}\left[\frac{k_{cat}}{K_{m(LA)}}\right]O_2 \rightarrow \infty = \frac{^{D}k_5 + \frac{k_5}{k_{4'}} + {}^{D}K_{eq5}\frac{k_6}{k_7}}{1 + \frac{k_5}{k_{4'}} + \frac{k_6}{k_7}} \quad (3)$$

$$^{D}\left[\frac{k_{cat}}{K_{m(O_2)}}\right]LA \rightarrow \infty = \frac{^{D}k_5 + \frac{k_5}{k_4} + {}^{D}K_{eq5}\frac{k_6}{k_7}}{1 + \frac{k_5}{k_4} + \frac{k_6}{k_7}} \quad (4)$$

While an accurate experimental value for eq 3 is presented in this work, only an upper limit value for eq 4 could be estimated (Figures 3a and 4b); it is evident, however, from the presented results that $^{D}[k_{cat}/K_{m(LA)}] \gg {}^{D}[k_{cat}/K_{m(O_2)}]$. For this result to be consistent with a random-binding mechanism, binding of O_2 to the ternary complex must be much tighter than that of LA. In other words, the rate of linoleic acid release from the ternary complex, $k_{4'}$, must be much faster than the rate of dioxygen release, k_4. This increased "stickiness" of oxygen will show up as a very large commitment, k_5/k_4, decreasing the value of $^{D}[k_{cat}/K_{m(O_2)}]$ compared to $^{D}[k_{cat}/K_{m(LA)}]$. In a practical sense, the kinetic pathway is effectively pushed to ordered binding of O_2 prior to LA (Scheme 1b), due to the enormous difference in magnitude between the isotope effects of $^{D}[k_{cat}/K_{m(O_2)}]$ and $^{D}[k_{cat}/K_{m(LA)}]$. In this extreme case, the mechanism cannot be truly random binding but reduces instead to an ordered mechanism in which O_2 binding precedes linoleic acid. As discussed above, such an ordered mechanism for lipoxygenase cannot be rigorously excluded but is considered unlikely.

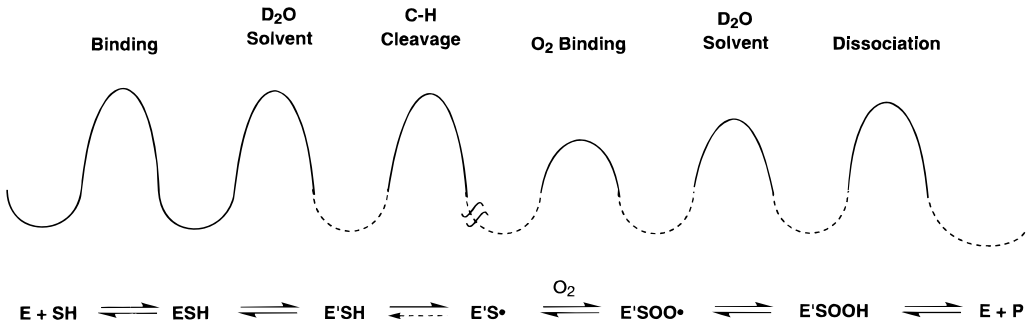
Another distinct mechanism that can explain the observed results is a reaction in which the isotopically sensitive chemical step occurs prior to oxygen binding (Scheme 1d). In this scenario, oxygen binding occurs after the isotopically sensitive step, and a large kinetic isotope effect for C−H bond cleavage is not expected to be expressed in $^{D}[k_{cat}/K_{m(O_2)}]$. In the event that C−H bond cleavage is reversible, an equilibrium isotope effect representing k_3 and k_4 is anticipated for $^{D}[k_{cat}/K_{m(O_2)}]$. In general, primary deuterium equilibrium isotope effects are small compared to kinetic isotope effects and are usually close to unity (Klinman, 1978). The kinetic parameters (Table 3) demonstrate how the isotope effects are influenced by substrate concentrations. This rather unusual enzymatic mechanism is virtually identical to a similar mechanism derived by Cook and co-authors [eqs 19−25 in Cook *et al.* (1993)]. The derivations presented in Table 3 predict that $^{D}[k_{cat}/K_{m(O_2)}]$ will be relatively insensitive to LA concentration and, importantly, be close to or equal to unity for all conditions of linoleic acid concentration. The especially striking characteristic of this type of mechanism is that the isotope effect at low concentrations of dioxygen reflects the equilibrium isotope effect regardless of the concentration of linoleic acid. At the other extreme, isotope effects at high concentrations of dioxygen reflect the kinetic isotope effect. This behavior is fully consistent with our observation that $^{D}[k_{cat}/K_{m(LA)}] \gg {}^{D}[k_{cat}/K_{m(O_2)}]$ under a full range of oxygen and linoleic acid concentrations. We note that the abnormally large intrinsic kinetic isotope effect in the lipoxygenase reaction makes the disparity between $^{D}[k_{cat}/K_{m(LA)}]$ and $^{D}[k_{cat}/K_{m(O_2)}]$ particularly easy to discern.

It is evident that the mechanism in Scheme 1d can explain all the trends in isotope effects observed in the data presented. At first glance, the magnitude of $^{D}[k_{cat}/K_{m(O_2)}] \leq 4$ appears anomolously large. However, as already discussed, the observed value of this isotope effect is an upper limit due to an oxygen K_m value with D-LA which lies outside the experimentally accessible range for O_2 concentrations. Both $K_{m(O_2)}$ with D-LA as substrate and $^{D}[k_{cat}/K_{m(O_2)}]$ are expected to be overestimated. To summarize these results, kinetic studies suggest that molecular oxygen enters the lipoxygenase-catalyzed reaction only after the substrate C−H bond is cleaved. The mechanistic possibility in which dioxygen binds to free enzyme before linoleic acid has not been unambiguously excluded however.

Oxygen Binding by Lipoxygenase. The kinetic studies above indicate two very distinct possibilities: either dioxygen binds tightly to free enzyme (Scheme 1b), or it does not bind to enzyme in the absence of an activated enzyme−substrate complex (Scheme 1d). To distinguish between them, we analyzed for released oxygen using a vacuum line/oxygen trap (cf. Materials and Methods and Table 2). Lipoxygenase in its commercial or "native" form is ferrous, whereas active enzyme is considered to be in the ferric state (deGroot *et al.*, 1975a; Schilstra *et al.*, 1994). As displayed in Table 2, free ferric enzyme does not bind molecular oxygen. This result does not come as a surprise since the iron-containing reversible oxygen carrier proteins (hemoglobin, myoglobin, and hemerythrin) bind oxygen in their ferrous states. Reducing lipoxygenase to its ferrous state (Table 2) does not lead to dioxygen binding, even though other non-heme iron-containing proteins (including other dioxygenases) have the capability to bind molecular oxygen in their ferrous state (Feig & Lippard, 1994; Que, 1980). It is possible that the enzyme is reduced to a form that can then bind oxygen only during the catalytic cycle. Lipoxygenase was thus reduced after it had been first oxidized; this form does not bind O_2 either. Another possibility was that oxygen binding was dependent on an enzymatic conformational change upon substrate binding. However, the presence of vacenic acid, a substrate analog that behaves as a competitive inhibitor, does not produce any oxygen binding with different enzyme forms. Our conclusion from these results is that lipoxygenase does not bind dioxygen in the absence of activated substrate. In conjunction with the models predicted by the kinetic isotope effects above, it would seem that the only plausible mechanism for the lipoxygenase reaction is one in which the C−H bond cleavage takes place prior to any kinetically productive interaction of O_2 with enzyme.

Accessibility of Substrate Intermediate to Solvent. Our working hypothesis is that the hydrogen at position 11 is transferred to an enzymatic group, possibly a hydroxide ion coordinated to iron. After trapping of the substrate-derived radical by dioxygen, the resulting peroxide radical is

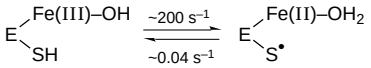
Scheme 2: Free Energy Profile of Lipoxygenase at 25 °C[a]



[a] Summary of reaction steps resolved by this and previous work. With the standard state at the K_m concentration, the energy levels of E and ESH are equivalent. Dotted lines represent energies of intermediates or transition states that are uncertain. Three kinetic barriers leading to the substrate-derived radical have similar heights: substrate binding, an unidentified step sensitive to D_2O solvent, and substrate C−H bond cleavage (Glickman & Klinman, 1995). Molecular oxygen enters the reaction site only after hydrogen abstraction has occurred and under saturating conditions is proposed to react rapidly with activated substrate. The intermediate thus produced is reduced and reprotonated in a step that may be sensitive to D_2O but that is not a rate-determining step at 25 °C (Glickman & Klinman, 1995). Finally, product peroxide is released in a step that is likely to limit the O_2 portion of the reaction and to contribute to net turnover (Glickman, M. H., *et al.*, in preparation). The oxygen portion of the reaction is shown as reversible, whereas the substrate portion of the reaction is best described as an irreversible process.

subsequently reduced and protonated. The identity of the enzymatic group is unknown, and it is unclear whether the hydrogen from position 11 is retained to be inserted at the peroxide position. The tritium exchange experiment attempted to address whether reversibility of C−H bond cleavage could be observed under turnover conditions.

As described in Results, we could not detect any exchange of substrate hydrogen atoms with a tritium label from solvent during the course of the reaction. Given the likelihood of tritium in the active site as Fe(III)−OH(T), the most likely explanation for this observation is an unfavorable partitioning of the linoleyl radical back toward substrate vs trapping with dioxygen, eq 2. From our level of detection for tritium, we therefore estimate that K_{eq} for the interconversion of



is *ca.* 5×10^3.

This finding has implications for the formalism of the mechanism in Scheme 1d. In the event that the substrate-derived radical does not revert readily to starting material, the lipoxygenase mechanism reduces to a ping-pong mechanism. Under these circumstances, $^D[k_{cat}/K_{m(LA)}]$ is predicted to be large at all O_2 concentrations, while the reversible mechanism shown in Scheme 1d predicts that $^D[k_{cat}/K_{m(LA)}]$ will go toward $^DK_{eq3}$ in the limit of very low dioxygen concentration. Once again, our inability to achieve K_m levels of O_2 with D-LA precludes unambiguous conclusions. However, there is no evidence from Figure 5b of a downward trend in $^D[k_{cat}/K_{m(LA)}]$ at low O_2, in support of a largely irreversible C−H bond cleavage process. As predicted for a ping-pong mechanism, $^D[k_{cat}/K_{m(LA)}]$ is close to being independent of O_2 concentration (Figure 3b); by contrast, $^D[k_{cat}/K_{m(O_2)}]$ shows about a 3-fold increase with increasing linoleic acid (Figure 4b). The latter effect may be due to a second (nonsubstrate) site for interaction of linoleic acid with enzyme, as already implicated from the change in $^T[k_{cat}/K_{m(LA)}]$ as a function of linoleic acid concentration (Glickman *et al.*, 1994). Additional kinetic studies suggest the capacity of SBL-1 to bind more than a single molecule of long chain fatty acid (Jones *et al.*, 1992; Wang *et al.*, 1993; Lagocki *et al.*, 1976; Garsen *et al.*, 1972; Butovich *et al.*, 1991). For the future, a more productive tritium incorporation experiment may involve incubation of enzyme under strictly anaerobic conditions to suppress trapping of the substrate radical intermediate by O_2. Given the fact that the isotope effect is much smaller for deuterium than for tritium, examination of exchange in D_2O should also be undertaken.

The lack of tritium label in residual substrate under the conditions of the current experiment supports the conclusion that reaction branching involving loss of substrate radical from enzyme and its conversion back to SH is not likely to be a major contributor to the overall reaction or to be a factor in the very large observed isotope effects (Glickman & Klinman, 1995). If a substrate-derived radical had been lost from enzyme under *ca.* 0.1 μM dissolved O_2, this intermediate may have been expected to react with solvent to some extent, incorporating a tritium label. A second strong argument against reaction branching comes from the data in Figure 5b, where it can be seen that $^D[k_{cat}/K_{m(LA)}]$ remains large as the O_2 concentration is elevated (appearing independent of dioxygen concentration). If branching were occurring to a significant extent at low O_2 levels and was the cause of inflated isotope effects, elevation of the dioxygen concentration would have been expected to reduce the magnitude of $^D[k_{cat}/K_{m(LA)}]$. This is clearly not occurring in the lipoxygenase reaction.

Mechanism of Lipoxygenase. In Scheme 2, we propose a mechanism for lipoxygenase based on the results presented in this paper together with previously published work. Lipoxygenase is unique in that catalysis is achieved via substrate activation instead of the more familiar oxygen activation. Molecular oxygen is proposed to neither bind nor react with lipoxygenase but rather to trap activated enzyme−substrate complex. Linoleic acid binding is partially rate-limiting at pH 9 and 25 °C and is followed by two partially rate-limiting isotopically sensitive steps. The first step is slowed by solvent deuteration, whereas the second step is highly sensitive to substrate deuteration (Glickman & Klinman, 1995). The latter step produces a linoleyl-based radical which upon reaction with O_2 generates a peroxyl radical. The peroxyl radical is subsequently reduced to peroxide anion and protonated to yield bound linoleate hydroperoxide (proposed to be the source of the 585 nm charge-transfer band seen in purple lipoxygenase, Nelson *et*

al., 1995). Release of product to solvent completes the catalytic cycle.

The nature of rate-limiting steps after production of the linoleyl radical can be inferred from the magnitude of deuterium and ^{18}O isotope effects. Solvent deuterium isotope effects on k_{cat} are negligible at 25 °C, but the substrate deuterium isotope effect on k_{cat} is reduced from its maximum value (Glickman & Klinman, 1995). The simplest mechanism attributes partial rate limitation of k_{cat} to the product release step. Such a mechanism is fully compatible with the magnitude of the $^{16}O/^{18}O$ isotope effect of 1.012 for lipoxygenase (Glickman *et al.*, in preparation; Glickman, 1994); similar values of ^{18}O isotope effect have been observed and calculated for the reversible formation of a hydroperoxide species from dioxygen (Tian & Klinman, 1994).

The importance of radical intermediates in lipoxygenase is supported by a number of published results; under anaerobic conditions, lipoxygenase forms a carbon-centered linoleic free radical detected by spin trapping (deGroot *et al.*, 1973). The radical was proposed to react with oxygen to give a peroxyl radical and ultimately fatty acid hydroperoxide as final product (deGroot *et al.*, 1975b). Direct observation of the peroxyl radical was achieved by EPR (Chamulitrat & Mason, 1989). The secondary k_H/k_D isotope effects are normal in magnitude and behavior, consistent with formation of a delocalized pentadienyl radical on the secondary positions (carbons 9, 10, 12, or 13, Glickman *et al.*, 1994). Radical traps can compete with dioxygen for the substrate-based radical intermediate. For instance, chain-breaking antioxidants can inhibit SBL-1 by trapping enzyme-bound radical intermediates produced after C−H bond cleavage and before O_2 addition (Lomnitski *et al.*, 1993; Maccarrone *et al.*, 1995). The oxygen portion of the reaction is likely to be reversible as both linoleyl and peroxyl radicals are detected by EPR (Nelson *et al.*, 1990, 1994), and the enzyme catalyzes the stereospecific exchange of molecular oxygen with the OOH group of the product peroxide (Matthew & Chan, 1983; Nelson *et al.*, 1990). There is no direct evidence as yet that the C−H bond cleavage itself is reversible.

The steady state data presented in this paper, together with pre-steady state kinetic data performed under anaerobic conditions (Egmond *et al.*, 1977; Jonsson *et al.*, 1996), and the fact that neither the crystallographic (Boyington *et al.*, 1993), magnetic susceptibility (Petersson *et al.*, 1985), nor EXAFS studies (Van der Heijdt *et al.*, 1995) detect bound oxygen in the absence of substrate fully support the view that chemistry can be performed on LA in the absence of O_2. We note that our results produce the first kinetic proof that O_2 is neither bound nor activated prior to C−H abstraction during catalysis under normal turnover conditions. We propose that molecular oxygen may not even bind to the active site of lipoxygenase but rather undergoes a rapid, possibly diffusion-controlled reaction with the substrate complex. This last conclusion is based on the fact that the reaction of enzyme with linoleic acid is close to diffusion-controlled, and we expect the rate of binding of dioxygen to be faster than that for the large (^{18}C-containing) linoleic acid. A similar conclusion was reached spectroscopically under nonturnover conditions; X-ray absorption edge and fine structure studies show no evidence that O_2 binds in the vicinity of the iron center upon binding of fatty acid substrate (Van der Heijdt *et al.*, 1995). Interestingly, this mechanism is much closer to that of the autoxidation of lipids (Porter, 1986; Porter & Wujek, 1984) than to model systems for a lipoxygenase type reaction that involves oxygen activation by iron (Rao *et al.*, 1994). The weight of these conclusions focuses attention on the ferric iron center as the locus of substrate oxidation and the origin of the large isotope effects.

ACKNOWLEDGMENT

Dr. Rob Scarrow, Haverford College, is thanked for his critical reading of the manuscript. We also thank Dr. Jeff Wiseman, Glaxo, Inc., for his original suggestion of studying kinetic order in the lipoxygenase reaction.

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BI960985Q