Nature of Rate-Limiting Steps in the Soybean Lipoxygenase-1 Reaction†

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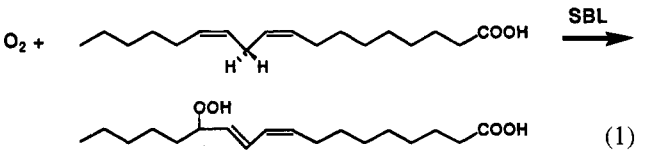
ABSTRACT: A series of kinetic isotope effect experiments were performed with the goal of understanding the nature of rate-limiting steps in the soybean lipoxygenase-1 (SBL-1) reaction. SBL-1 was reacted with linoleic acid (LA) and deuterated linoleic acid (D-LA) under a variety of experimental conditions involving changes in temperature, pH, viscosity, and replacement of H_2O with D_2O. The extrapolated intrinsic primary H/D isotope effect can be estimated to be possibly as large as 80. This value is probably the largest isotope effect published for an enzymatic reaction, and much larger than that predicted from semiclassical models. Due to this large primary isotope effect, the C−D bond cleavage fully limits the rate of reaction under all conditions tested. In the case of protonated linoleic acid, a number of steps are partially rate-limiting at room temperature; three distinct mechanistic steps which include substrate binding, an H_2O/D_2O sensitive step, and C−H bond cleavage have been characterized. Use of glucose as a solvent viscosogen demonstrates that substrate binding is approximately 48% rate-limiting for LA at 20 °C. SBL-1 is one of the few enzymes that fit the definition of a "perfect enzyme", in the sense that further optimization of any single step at room temperature will not significantly increase the overall rate. At lower temperatures, the step sensitive to solvent deuteration begins to dominate the reaction, whereas at higher temperatures, the hydrogen abstraction step is rate-limiting. The pH dependence of k_{cat}/K_m for SBL-1 can be explained as arising from two pK_a's, one controlling substrate binding and the other substrate release. Below pH 7.8, the rate of substrate release increases, thus decreasing the commitment to catalysis and unmasking the large intrinsic isotope effect on the subsequent hydrogen abstraction. An abnormally high pK_a, in the range of 7−8, has been determined for LA in the concentration range employed in these studies. We propose that the negatively charged form of LA, predominating above pH 7.8, is the preferred substrate with larger commitments to catalysis.

Lipoxygenase, a member of a class of enzymes that catalyze the oxidation of unsaturated fatty acids, is found in cyanobacteria, fungi, algae, plants, and mammalian cells [for reviews, see Gardner (1991), Schewe *et al.* (1986), Siedow (1991), and Veldink and Vleigenthart (1984)]. Each species, or even a single tissue, can contain more than one isozyme; high homology is found among these isozymes, suggesting evolutionary relationships (Sigal, 1991). Soybeans contain three or four isozymes of lipoxygenase differing in substrate specificity, positional selectivity, and optimal pH (Hamberg & Samuelsson, 1980; Van Os *et al.*, 1979a,b). Soybean lipoxygenase-1 (SBL-1)[1] is often used as the prototype for its class since soybeans are a cheap and easy source of large quantities of enzyme, which is not surprising considering the high content by weight of fatty acids in soybean seeds. The enzyme is a monomer of 100 kDa and contains a single non-heme iron. Lipoxygenase utilizes two substrates, molecular oxygen and a fatty acid with at least one pair of double bonds flanking a methylene group, to produce a corresponding hydroperoxide. The reaction with linoleic acid (LA), the standard substrate for SBL-1, preferentially produces 13(*S*)-hydroperoxy-9(*Z*),11(*E*)-octadecadienoic acid (LOOH) (eq 1):



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Even though the precise reaction mechanism of lipoxygenase is not known, strong evidence for a radical mechanism has been shown by EPR (Chamulitrat & Mason, 1989; Nelson *et al.*, 1990). It is generally accepted that hydrogen abstraction from C-11 gives rise to a delocalized radical which is then trapped by dioxygen to yield a peroxyl radical of linoleic acid as a catalytic intermediate. SBL-1 has recently been crystallized and its structure determined (Boyington *et al.*, 1993; Wladek *et al.*, 1993). Two enzymatic cavities, possibly one for each substrate, lead to the active site. Within the crystal, the active site iron is in the ferrous form as a distorted octahedron, liganded to three histidines, the C terminal carboxylate (isoleucine), and possibly an asparagine. A large number of lines of inves-

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[1] Abbreviations: SBL-1, soybean lipoxygenase-1; LA, linoleic acid; D-LA, [$^2H_{31}$]linoleic acid; LOOH, 13-hydroperoxy-9,11-octadecadienoic acid; k_{cat}, enzymatic first order rate constant at saturating concentrations of substrate; k_{cat}/K_m, enzymatic second order rate constant at low concentrations of substrate; KIE, kinetic isotope effect; $^D(k_{cat})$, kinetic k_H/k_D isotope effect on the first order rate constant, k_{cat}; $^D(k_{cat}/K_m)$, kinetic k_H/k_D isotope effect on the second order rate constant, k_{cat}/K_m; η/η^0, relative viscosity compared to H_2O at 20 °C; $k_{cat}/K_m(H)$, value of k_{cat}/K_m with LA as substrate; $k_{cat}/K_m(D)$, value of k_{cat}/K_m with D-LA as substrate.

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tigation indicate that the active form of lipoxygenase contains ferric iron in an octahedral geometry with six ligands (Dunham *et al.*, 1990; Nelson, 1988; Van der Heijdt *et al.*, 1992; Zang *et al.*, 1991). Other than the iron, no obvious additional candidate for the enzymatic cofactor was revealed from the current crystal structures.

Kinetic isotope effects (KIE's) are an important tool for elucidating enzyme mechanism. An especially surprising and unique characteristic of SBL-1 is the abnormally large k_H/k_D isotope effects detected independently by two different laboratories (Glickman *et al.*, 1994; Hwang & Grissom, 1994). The magnitude of the observed isotope effect reported for the lipoxygenase reaction is much larger than expected on a single step when accounting for the differences in zero-point energies of the two isotopes in a thermally activated process (Melander & Saunders, 1987). Magnetic spin effects have been ruled out as a source of the large isotope effect (Hwang & Grissom, 1994). A large intrinsic isotope effect could be the consequence of quantum mechanical tunneling of the hydrogen atom (Bell, 1980). An alternate possibility is that the abnormally large observed isotope effect does not arise from a single step in the enzymatic mechanism but is the product of multiplicative terms in a reaction that has more than one isotopically sensitive step and involves reaction branching (Thibblin, 1988; Thibblin & Ahlberg, 1989). As demonstrated in this paper, the observed isotope effect changes significantly with variation of reaction conditions, and any interpretation of kinetic results will need to explain both the magnitude of the isotope effect and its variation with reaction conditions. Thus, there are two mechanistic possibilities: (i) the intrinsic isotope effect on the C−H bond cleavage step is large and rate limitation by other steps decreases the observed KIE under specific conditions, or (ii) the unusually large isotope effect seen under certain conditions is due to multiplicative isotope effects such as arise by reaction branching. We present data and arguments in favor of the former explanation for the variation of isotope effects in the SBL-1 reaction.

In order to make optimal use of kinetic isotope effects, it is necessary to know the intrinsic effect on the isotopically sensitive step. As demonstrated herein, the combination of kinetic studies as a function of pH, temperature, and viscosity allows for an estimation of the magnitude of the intrinsic isotope effect and of the different rate-limiting steps in SBL-1. The general treatment of viscosity effects in enzyme reactions has been implemented and used to evaluate the "stickiness" of substrate (Brouwer & Kirsch, 1982). In other enzymatic systems, viscosity measurements have been used to determine kinetic commitments contributing to a ^{13}C isotope effect (Grissom & Cleland, 1988) and to estimate the intrinsic isotope effect in a competitive experiment (Tipton, 1993). In the present work, the variation of solution viscosity has been used to estimate an extrapolated value for the intrinsic isotope effect from absolute rates of reaction in a noncompetitive procedure. These results, in conjunction with temperature and pH dependencies of the k_H/k_D isotope effect, prove to be a powerful tool for dissecting the different steps in lipoxygenase catalysis and their relative rate limitation.

One fact that can complicate a kinetic analysis of the mechanism of lipoxygenase is that the substrate is not a simple soluble molecule but a long chain fatty acid that aggregates into a number of forms in solution. In this paper, we examine some of the implications this characteristic has on the enzyme kinetics.

EXPERIMENTAL PROCEDURES

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. Lyophilized enzyme was stored at −70 °C. Purity was >95% by SDS PAGE, and the activity was about 180 units/mg.

Sodium linoleate (LA) was purchased from Sigma Chemical Co. and sodium D_{31}-linoleate (D-LA) from Cambridge Isotope Labs. Substrates were further purified by Beckman 332 HPLC on a C_{18} column. Elution buffer A was 50/25/25/0.05% by volume $H_2O/CH_3CN/CH_3OH$/trifluoroacetic acid, and buffer B was 10/65/25/0.05% by volume of $H_2O/CH_3CN/CH_3OH$/trifluoroacetic acid. HPLC solvents were all "Baker analyzed" HPLC grade. The elution gradient started with 100% solvent A (0% solvent B at time 0) and was 0, 100, 100, and 0% solvent B at 5, 15, 40, and 45 min, respectively. Substrate was detected by on-line UV−vis detection at 210 nm. The retention time was about 24 min. Substrate fractions were collected, evaporated to dryness on a speed-vac, and redissolved in 0.1 M borate buffer, pH 9. Stock solutions were about 400 μM substrate and stored at −70 °C up to a month with no noticeable change in concentration. The concentration of substrate was determined by enzymatically converting 1 mL of diluted stock LA to LOOH. The product absorbs at 234 nm with an extinction coefficient of 25 000 M^{-1} cm^{-1}.

Unless stated otherwise, all enzyme, substrate, viscogen, and reaction solutions were in 0.1 M borate buffer, pH 9. Glucose was purchased from Fisher and D_2O (99.9%) from Cambridge Isotope Labs. All other chemicals were of the highest purity available from Sigma.

Methods

Reaction Rates. Initial rates of reaction were measured on a Cary 118 spectrophotometer by following product production at 234 nm. Up to the first 15% of reaction was monitored, which under most conditions did not take more than a few minutes. At the higher substrate concentrations, a brief lag phase was noticed such that initial rates were measured for the linear portion of reaction following the lag phase. The reaction with D-LA did not show a lag phase but rather an initial burst corresponding to 2−3% of substrate and attributed to contamination with unlabeled linoleic acid. Due to the large isotope effect, H-LA is preferentially exhausted in the first 3% reaction, after which time linear kinetics are observed. For each series of experiments, a stock solution of enzyme of about 0.1 mg/mL in 0.1 M borate buffer, pH 9, was prepared. The enzyme solution was further diluted just before reaction as necessary. Reaction mixtures, excluding enzyme, were brought to the temperature of reaction. One to five microliters of an enzyme solution (0.01−0.1 mg/mL, depending on reaction rates at the specific condition) kept at 0 °C was used to initiate reaction by injection via Hamilton syringe to the reaction mixture. Following initiation of reaction with enzyme, mixing was achieved with a Teflon mixer; the cuvette was not taken out of its holder so as to maintain temperature. No effect of

final enzyme concentration on kinetic parameters was observed. For each experimental condition, linoleic acid (or deuterated linoleic acid) concentration was varied between 2.5 and 50 μM. k_{cat} and k_{cat}/K_m parameters for each substrate and at each condition were obtained by nonlinear fit to the Michaelis–Menten equation using the program KaleidaGraph on a Macintosh computer.

Temperature Dependence. Initial rates of reaction were measured at 0.1 M borate, pH 9. The temperature range studied was between 0 and 50 °C. Temperature control of reaction was achieved by the use of water-jacketed cuvettes connected to a circulating Neslab RT210 water bath. As a control, enzyme stability was measured for the temperature range investigated. No loss of activity was noticed for enzyme incubated up to 30 min at all temperatures other than 50 °C. At 50 °C, some loss of enzyme activity was apparent after 5 min. Since initial rate measurements are taken within a few minutes, this loss of activity does not appear to pose a problem. At each temperature, four or five separate determinations of k_{cat} and k_{cat}/K_m were made using either protio- or deuterio-substrate. Slight changes in pH with temperature were not expected to pose a problem as pH 9 is the optimum pH for lipoxygenase and the reaction rate is insensitive to pH in the range pH 8–10 (see below).

Viscosity Studies. Reactions were carried out at different relative viscosities ($\eta_{rel} = \eta/\eta^0$, η^0 is the viscosity of H_2O at 20 °C). Buffer and substrate solutions of 0, 8, 14, 21.5, 26, and 30% by weight glucose, in 0.1 M CHES buffer, pH 9, were prepared corresponding to relative viscosities of 1, 1.25, 1.5, 2, 2.5, and 3, respectively at 20 °C (*CRC Handbook of Chemistry*). Enzymatic measurements at 20 °C were the same as noted above. Sucrose and ethylene glycol were also tried as viscogenic agents but were found to inhibit the SBL-1 reaction. Viscosity studies could not be performed in borate buffer since borate reacts with glucose with a concomitant loss of buffering capacity. The activity of lipoxygenase in 0.1 M CHES or TRIS buffers (pH 9) was found to be within 5% of that determined in borate. Viscosity measurements were repeated at pH 7.18, substituting 0.2 M TRIS buffer for CHES. Viscosity measurements were also performed for the reaction of lipoxygenase and LA at 5 and 37 °C. As the viscosity of liquids is a function of temperature, the relative viscosity of reaction solutions of 0.1 M CHES buffer, pH 9, and 0, 8, 14, 21.5, 26, or 30% by weight glucose were determined by measuring the time it takes for these solutions to flow through a viscosometer kept at 5 and 37 °C. The relative viscosities of these solutions (as compared to distilled water at 20 °C) were found to be 1.85, 2.16, 2.80, 3.60, 4.24, and 5.08 at 5 °C and 0.786, 0.925, 1.17, 1.40, 1.57, and 1.91 at 37 °C, respectively. These values are the average of three to five determinations for each solution at each temperature.

Solvent Isotope Effects. Kinetic measurements were performed in 0.1 M borate in D_2O, pH 9 (pH meter reading 8.6), at temperatures of 25 and 5 °C. Kinetic parameters for protio- and deuterio-LA were compared in those in H_2O-buffered solutions.

pH Studies. Kinetic measurements were carried out at different pH's, using 0.1 M TRIS-HCl in the pH range of 6.5–8.8 and 0.1 M borate buffer in the pH range of 8.6–10.5. The ionic strength was adjusted in all cases to 0.2 M by addition of NaCl. The activity of lipoxygenase was found to vary with changes in ionic strength of solution, with the rate being relatively insensitive to ionic strength around 0.2 M (results not shown). Phosphate buffer, 0.1 M, was tried for the pH range 6.5–7.5 but was found to severely inhibit the reaction [phosphate might be a competitive inhibitor as the phospholipid of linoleic acid is a known substrate of lipoxygenase (Brash *et al.*, 1987)]. Kinetics at pH 8.6–8.8 were measured in solutions of both buffering compounds. No discernible change in kinetic parameters was noticed. Stock solutions of LA and D-LA were prepared at each pH and diluted with the same buffer solution to get the range of concentrations necessary for the kinetic studies. The values of k_{cat}, k_{cat}/K_m, and the observed isotope effect determined in the pH range studied were then fitted as a function of pH with different numbers of parameters using the graphics program KaleidaGraph on a Macintosh computer.

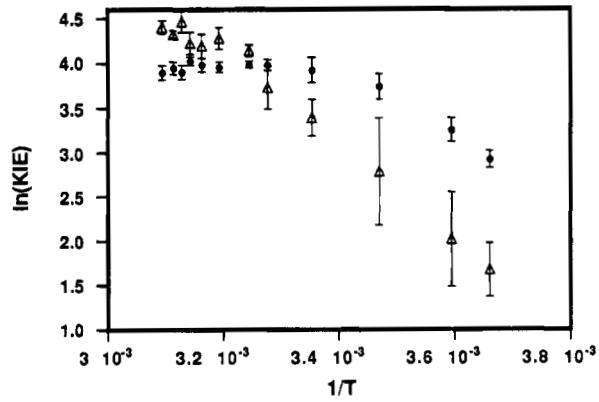

FIGURE 1: Temperature dependence of the observed H/D isotope effects; natural log of $^D(k_{cat}/K_m)$ (triangles) and of $^D(k_{cat})$ (circles) in the temperature range 0–50 °C, pH 9.

Titration of LA. A stock solution of 564 μM sodium linoleate was prepared by dissolving the appropriate amount in doubly distilled water and determining concentration enzymatically as stated above. Solutions were neutralized by dropwise addition of dilute HCl via burette. For instance, 0.2 L of sodium LA (564 μM) was titrated against 0.046 M HCl; larger volumes of LA solution or more dilute HCl were used for more dilute LA titrations. pH of solution after each addition of HCl was measured by pH meter, and the readings were plotted against milliliters of added HCl. The equivalence point was determined from a calculated derivative plot, and pK_a was considered as the half-equivalent. pK_a determinations at different concentrations were achieved by simultaneously diluting the LA and HCl solutions and repeating the titration.

RESULTS

Temperature Dependence of Measured Isotope Effects. Large primary k_H/k_D isotope effects with an unusual temperature dependence have been published for SBL-1 (Glickman *et al.*, 1994). We expand here the temperature range measured. Arrhenius plots of the kinetic isotope effect parameters, $^D(k_{cat})$ and $^D(k_{cat}/K_m)$, are shown in Figure 1. The primary H/D isotope effect on k_{cat} increases from about 18 at 0 °C to a maximum of about 60 and then decreases with further increase in temperature as expected for a single microscopic step (Bell, 1980). The increase in $^D(k_{cat}/K_m)$ is more pronounced than for $^D(k_{cat})$ up to 32 °C, at which point a break in behavior occurs, leading to only a small dependence of isotope effect in temperature. The observed changes in isotope effects with temperature are due primarily to a pronounced nonlinearity in Arrhenius plots for both k_{cat}

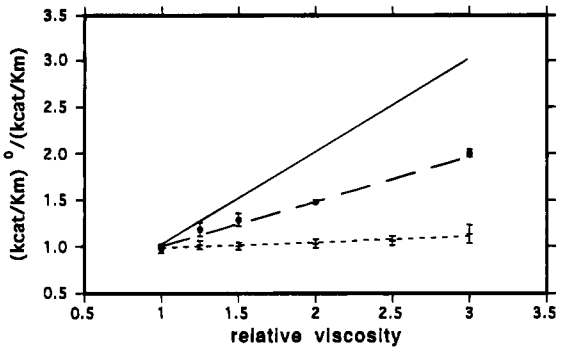

FIGURE 2: Effect of relative viscosity (η/η^0) on normalized values of reciprocal k_{cat}/K_m at 20 °C, pH 9. The slope of the line is 0.48 ± 0.03 for H-LA (circles) and 0.05 ± 0.02 for D-LA (crosses). The solid line is theoretical behavior (slope = 1) for a fully diffusion-controlled bimolecular reaction.

and k_{cat}/K_m using H-LA as substrate. Due to the extremely large k_H/k_D isotope effect observed in this reaction, the expectation is that the isotopically sensitive step will be close to fully rate-limiting under most conditions with D-LA; this view is supported by the observation that the plot of $\ln(k_{cat})$ vs $1/T$ for D-LA is close to linear over the experimental temperature range of 0−50 °C. The following experiments will evaluate the kinetic isotope effect under a variety of conditions in an effort to address whether the magnitude of the observed H/D isotope effect is a reflection of an abnormally large intrinsic isotope effect coupled to the presence of varying degrees of kinetic complexity.

Viscosity Studies. Lipoxygenase is a fast enzyme with k_{cat}/K_m of about 3×10^7 M^{-1} s^{-1} at 20 °C (Figure 4). Viscosity studies can test the assumption that substrate binding is partially rate-limiting. In addition, we show in Section A of the Appendix that an expansion of the Brouwer and Kirsch treatment of viscosity data (Brouwer & Kirsch, 1982) can provide either the intrinsic isotope effect on k_{cat}/K_m or a lower limit to this value for a kinetically complicated mechanism. As seen in Figure 2, the rate of k_{cat}/K_m for a viscosity sensitive reaction, as compared to k_{cat}/K_m at standard relative viscosity ($\eta/\eta^0 = 1$, in H_2O at 20 °C), decreases linearly as the relative viscosity increases. As previously discussed, this presentation will produce a straight line with a slope of 1 or 0, depending on whether a reaction is fully viscosity dependent or independent, respectively (Brouwer & Kirsch, 1982). From Figure 2, the reaction rate of lipoxygenase with H-LA at 20 °C is seen to be 48% viscosity dependent. The effect of viscosity on k_{cat}/K_m for H-LA derives from its effect on K_m. Even though there is a slight increase of k_{cat} with an increase in viscosity (≈10%, Figure 3a), this trend is more than offset by the increase in K_m giving rise to the decrease in k_{cat}/K_m with viscosity. A slight increase of k_{cat} with added viscogen has been seen in a number of published viscosity studies [e.g., Blacklow *et al.* (1988) and Hardy and Kirsch (1984)] and could be due to a change in the hydrophobicity of the solvent, an acceleration of the release of product from the enzyme, or a slight alteration of enzyme structure due to the high solute content in solution. This slight effect on k_{cat} of lipoxygenase proves that the viscosity of the medium does not drastically inhibit or alter the chemical part of the mechanism. The same conclusion can be drawn from the fact that the reaction of lipoxygenase with deuterated linoleic acid is essentially independent of the viscosity of the solution (Figures 2 and 3b), indicating that the results with protonated

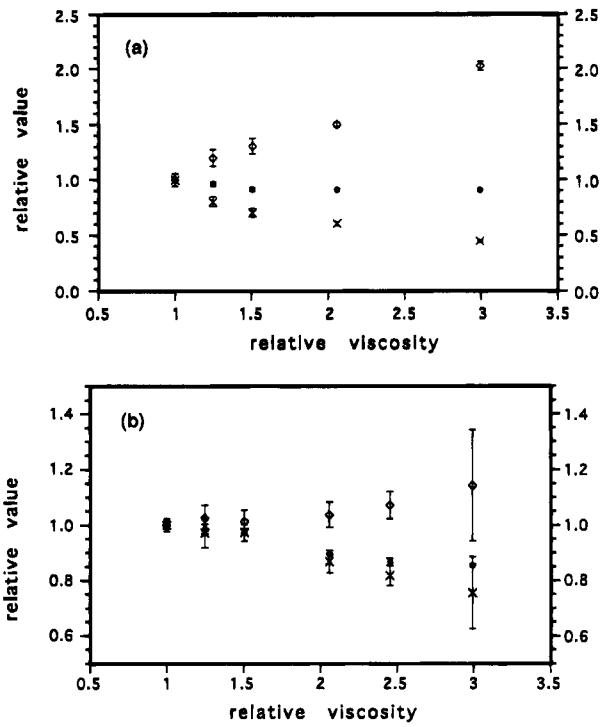

FIGURE 3: Effect of viscosity on the normalized kinetic parameters k_{cat}/K_m (diamonds), k_{cat} (circles), and K_m (crosses) as a function of relative viscosity (η/η^0) at 20 °C, pH 9. (a) For H-LA. (b) For D-LA.

linoleic acid do not arise from an inhibitory effect of glucose. This comparison of H-LA and D-LA allows us to conclude that the effect of viscosity is on binding and/or release steps and not the chemical step.

Deuterated LA, due to its abnormally large isotope effect (Glickman *et al.*, 1994), can serve two functions: as a slow substrate control (see above) and a probe of the intrinsic isotope effect. In the Appendix, we derive a treatment that can use viscosity studies to estimate a lower limit for the intrinsic isotope effect in fast enzyme reactions. Briefly, the enzymatic bimolecular rate follows hyperbolic behavior when analyzed as a function of the reciprocal of the relative viscosity (eq 2):

$$\frac{k_{cat}}{K_m} = \frac{R\eta^0/\eta}{S + \eta^0/\eta} \tag{2}$$

where R is the theoretical extrapolation of k_{cat}/K_m to very low viscosities and relates the binding constant to the forward rates and S, though a complicated expression unique to each system, is a measure of the sensitivity of the reaction rate to changes in medium viscosity. For example, an S of unity means that the reaction is 50% diffusion-controlled in H_2O at 20 °C while values much less than 1 indicate that the enzyme is independent of viscosity. For the simplest enzymatic reaction,

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} E + P \tag{3}$$

$$R = \frac{k^0_1}{k^0_{-1}} k_2 \tag{4}$$

where the superscript on a viscosity sensitive rate constant

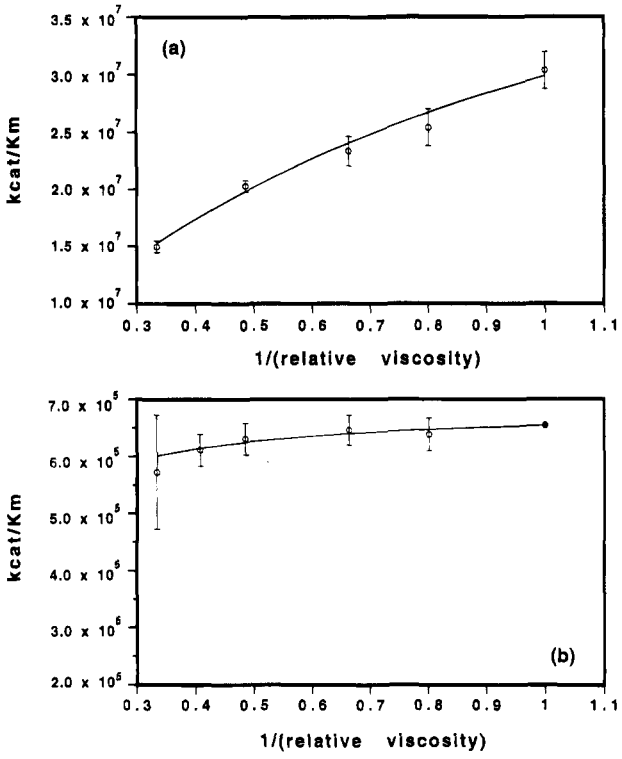

FIGURE 4: k_{cat}/K_m for H-LA (panel a) and D-LA (panel b) as a function of the reciprocal of the relative viscosity. The line is a fit of experimental points to eq 2. (a) $R = 5.72$ (± 0.68) $\times 10^7$ M^{-1} s^{-1}, and $S = 0.92$ (± 0.17). (b) $R = 6.85$ (± 0.48) $\times 10^5$ M^{-1} s^{-1}, and $S = 0.047$ (± 0.007). The units for k_{cat}/K_m are M^{-1} s^{-1}.

denotes the value of this rate constant in H_2O at 20 °C. From plots of k_{cat}/K_m vs η^0/η (Figure 4a,b), we find that the reaction of lipoxygenase with LA is close to 50% diffusion-controlled ($S = 0.92 \pm 0.17$) at room temperature, whereas the reaction with deuterated LA is virtually independent of viscosity ($S = 0.05 \pm 0.02$). The virtue of this presentation lies in the fact that it yields an expression for the intrinsic isotope effect. From the expression of R above, it is seen that for a simple enzymatic mechanism the relationship between the R values for two isotopically labeled substrates describes the intrinsic isotope effect on k_2 ($^Dk_{int}$):

$$\frac{R_H}{R_D} = {}^Dk_{int} \qquad (5)$$

This parameter could be more complicated for reactions other than the simplest case described in eq 3. This value of R_H/R_D in the lipoxygenase reaction (84 ± 12) is found to be identical within experimental error to the large $^D(k_{cat}/K_m)$ determined in the limit of high temperature (Figure 1).

Solvent Isotope Effects. The kinetic parameters for both H-LA and D-LA were analyzed in H_2O and D_2O at two temperatures. At 25 °C, substituting D_2O for H_2O has almost no effect on the kinetic parameters for D-LA or on k_{cat} for H-LA, whereas a rather large isotope effect on k_{cat}/K_m is observed for protio-linoleic acid. From Table 1, it can be seen that the solvent isotope effect on $k_{cat}/K_m(H)$ is 2.5 (±0.5) at 25 °C. At 5 °C, a temperature where we know from the results described as a function of temperature above that the isotopically sensitive step is less rate-limiting, the solvent isotope effect on $k_{cat}/K_m(H)$ increases to 3.7 ± 0.9 and is significant on $k_{cat}(H)$ (2.2 ± 0.2). A slight solvent isotope effect is even noticed on $k_{cat}/K_m(D)$ (1.5 ± 0.2). It is

Table 1: Summary of Solvent Isotope Effects as a Function of Substrate Deuteration and Temperature[a]

	H-LA			D-LA			KIE[b]	
	k_{cat}	K_m	k_{cat}/K_m (10^7)	k_{cat}	K_m	k_{cat}/K_m (10^7)	k_{cat}	k_{cat}/K_m
25 °C								
H_2O	280	11	2.6	6.0	7.0	8.5	48	30
	(8)	(2)	(0.3)	(0.3)	(0.4)	(1.1)	(4)	(6)
D_2O	240	23	1.1	6.5	7.8	8.3	37	13
	(16)	(2)	(0.2)	(0.3)	(0.5)	(1.1)	(7)	(3.0)
SIE[c]	1.2	0.48	2.5	0.92	0.77	1.2		
	(0.1)	(0.09)	(0.5)	(0.09)	(0.07)	(0.3)		
5 °C								
H_2O	89	16	5.4	3.5	4.0	8.6	26	6.3
	(7)	(3)	(0.1)	(0.1)	(0.5)	(1.0)	(3)	(2)
D_2O	40	27	0.15	3.1	5.7	5.9	13	2.5
	(1)	(2)	(0.02)	(0.2)	(0.3)	(0.5)	(1)	(0.5)
SIE[c]	2.2	0.61	3.7	1.1	0.70	1.5		
	(0.2)	(0.12)	(0.9)	(0.1)	(0.09)	(0.2)		

[a] Numbers in parentheses represent errors as determined from replicate determinations. Units for k_{cat} are s^{-1}, for K_m are μM, and for k_{cat}/K_m are M^{-1} s^{-1}. [b] Magnitude of isotope effect when comparing rates of H-LA and D-LA. [c] Magnitude of isotope effect when comparing reactions in H_2O and D_2O.

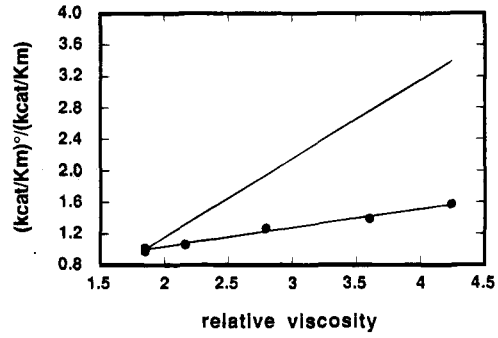

FIGURE 5: Effect of viscosity on reaction with H-LA at 5 °C, pH 9. The slope of the line is 0.24 (±0.04). The solid line is the theoretical behavior for a fully diffusion-controlled reaction.

important to mention that this effect on k_{cat}/K_m is much larger than expected if it were due solely to the change in viscosity of D_2O, since the relative viscosity of heavy water is 1.25. From the previous results (see above), the reaction of lipoxygenase with H-LA at room temperature is 50% viscosity dependent. Therefore, the maximal effect the viscosity of D_2O can have on the rate of the reaction is a factor of 1.125, significantly less than the observed solvent isotope effect. To conclude, a step sensitive to solvent is partially rate-limiting at room temperature and becomes more so as the temperature is lowered, concomitant with a decrease in the observed substrate kinetic isotope effect. At room temperature, the binding of substrate is also partially rate-limiting as seen from the viscosity measurements. The effect of viscosity was, therefore, also studied at several additional temperatures to assess whether the effect of viscosity and D_2O are on the same step or on two separate steps.

Viscosity Measurements at 5 and 37 °C. As the temperature is lowered, the viscosity of the reaction solutions increases, but the same treatment used in Figure 2 for solutions at 20 °C is still effective. In Figure 5, the change in the second order rate constant for the reaction of lipoxygenase with LA at 5 °C is plotted against the change in relative viscosity, producing a slope of 0.24. The reaction

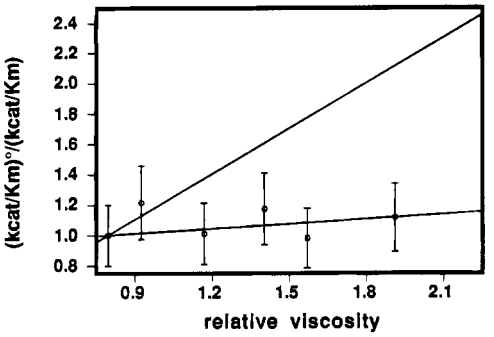

FIGURE 6: Effect of viscosity on reaction with H-LA at 37 °C, pH 9. The solid line is the theoretical behavior for a fully diffusion-controlled reaction.

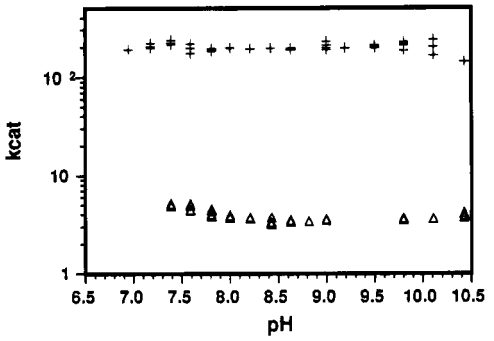

FIGURE 7: pH dependence of k_{cat}: H-LA (crosses) and D-LA (triangles). The units for k_{cat} are s^{-1}.

is concluded to be 24 (±4)% diffusion-limited at 5 °C, compared to 48 (±3)% at 20 °C. In contrast to the solvent isotope effect on k_{cat}/K_m which increases at lower temperatures, both the substrate isotope effect and the viscosity effect on k_{cat}/K_m decrease as the temperature is lowered. Deuteration of the primary position of linoleic acid cancels the sensitivity of the lipoxygenase reaction to viscosity (data not shown), similar to the result at 20 °C. At 37 °C, the second order rate constant (k_{cat}/K_m) of the reaction of lipoxygenase with LA is not sensitive to the viscosity of solution within the experimental error (Figure 6). The reaction of lipoxygenase is most sensitive to viscosity at room temperature, with binding of substrate becoming less rate-limiting as the temperature is either increased or decreased.

pH Studies. Changing the pH of the medium has no effect on k_{cat} of either H-LA or D-LA (Figure 7). However, k_{cat}/K_m for both substrates decreases by over 1 order of magnitude from pH 9 to 7 (Figure 8). Fitting the rates in Figure 8 to an equation with one pK_a gives a mediocre fit since the observed rate decreases faster than that predicted by one pK_a (result not shown). The enzymatic mechanism must be more complicated than just a simple protonation controlling the interaction of substrate with enzyme. Therefore, in trying to fit the results to a possible reaction mechanism, a couple of points were taken into account. First, given the pH independence of k_{cat}, steps involving protonation or deprotonation have to be prior to or concomitant with substrate binding. Second, the fact that the isotope effect $^D(k_{cat}/K_m)$ increases with decreasing pH while the k_{cat}/K_m itself decreases is significant (Figure 9). Since $^D(k_{cat}/K_m)$ is independent of steps prior to substrate binding (e.g., a pH dependent conformational change influencing substrate binding), a pH dependence of substrate release appeared to be a likely candidate for the second pK_a influencing the magnitude of

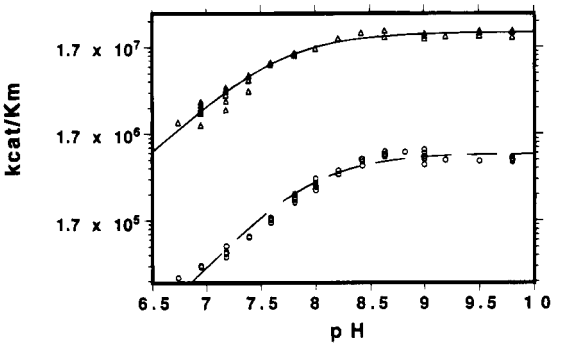

FIGURE 8: pH dependence of k_{cat}/K_m: H-LA (triangles) and D-LA (circles). The line is a fit of experimental points to eq 10, using five parameters: k_1, k_{-1}/k_2, k'_{-1}/k_2, pK_{a1}, and pK_{a2}. pK_{a2} was set at 6.95, the value obtained from Figure 9. The units for k_{cat}/K_m are M^{-1} s^{-1}.

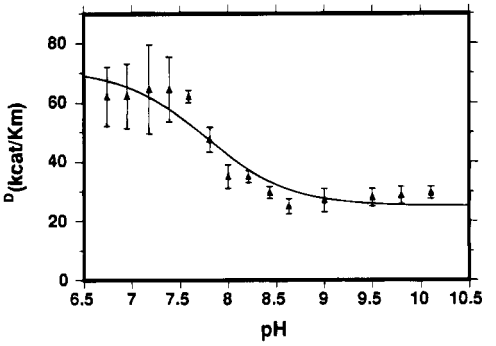

FIGURE 9: pH dependence of $^D(k_{cat}/K_m)$. The line is a fit of experimental points to eq 11, using four parameters: $^Dk_2$, k_{-1}/k_2), k'_{-1}/k_2, and pK_{a2}.

k_{cat}/K_m. Within the experimental error, a single pK_a appeared sufficient to fit the data for $^D(k_{cat}/K_m)$ as a function of pH. As a final point, we note the slight decrease in k_{cat}/K_m at pH above 10.2, which was not pursued in the present treatment.

The simplest enzymatic reaction fulfilling the above requirements is that shown in eq 3 above. The second order rate constant and isotope effect, respectively, are given by

$$\frac{k_{cat}}{K_m} = \frac{k_1 k_2}{k_{-1} + k_2} \tag{6}$$

$$^D(k_{cat}/K_m) = \frac{^Dk_2 + \frac{k_2}{k_{-1}}}{1 + \frac{k_2}{k_{-1}}} \tag{7}$$

where k_1 is "substrate on", k_{-1} is "substrate off", and k_2 includes steps up to the first irreversible step, including the isotopically sensitive step. $^Dk_2$ is the intrinsic isotope effect on this step, and k_2/k_{-1} is termed the commitment to catalysis (Northrop, 1981). The two terms that could have a pH dependence are k_1 and k_{-1}. The pH dependence of k_1 can be described by the presence of a protonated form of enzyme, or substrate, controlled by pK_{a1}:

$$k^{obs}{}_1 = \frac{k_1}{1 + \frac{[H]}{K_1}} \tag{8}$$

The expression for the isotope effect is simpler than for the rate expression in that it includes only one pH sensitive step,

k_{-1}. The observed isotope effect in Figure 9 increases at lower pH, indicating that the commitment, k_2/k_{-1}, must decrease as the pH is lowered. This is possible when the rate of substrate release, k_{-1}, increases as pH is decreased. This condition is fulfilled if the enzyme−substrate complex ([E·S]) releases substrate at two discrete rates, k_{-1} when deprotonated and k'_{-1} when protonated. Accounting for the relative fractions of the two forms of the enzyme−substrate complex controlled by pK_{a2}, the overall rate constant of substrate release is

$$k^{obs}{}_{-1} = \frac{k_{-1} + k'_{-1}\dfrac{[H]}{K_2}}{1 + \dfrac{[H]}{K_2}} \quad (9)$$

Placing the terms for $k^{obs}{}_1$ and $k^{obs}{}_{-1}$ (from eqs 8 and 9) into eqs 6 and 7 yields

$$\frac{k_{cat}}{K_m} = \frac{k_2 k_1 / \left(1 + \dfrac{[H]}{K_1}\right)}{\dfrac{k_{-1} + k'_{-1}\dfrac{[H]}{K_2}}{1 + \dfrac{[H]}{K_2}} + k_2} = \frac{k_1 / \left(1 + \dfrac{[H]}{K_1}\right)}{\dfrac{k_{-1}}{k_2} + \dfrac{k'_{-1}}{k_2}\dfrac{[H]}{K_2}} + 1 \quad (10)$$

$$^{D}(k_{cat}/K_m) = \frac{{}^{D}k_2 + \dfrac{k_2}{k_{-1} + k'_{-1}\dfrac{[H]}{K_2}}}{\dfrac{1 + \dfrac{[H]}{K_2}}{1 + \dfrac{k_2}{k_{-1} + k'_{-1}\dfrac{[H]}{K_2}}}} = \frac{{}^{D}k_2 + \dfrac{1 + \dfrac{[H]}{K_2}}{\dfrac{k_{-1}}{k_2} + \dfrac{k'_{-1}}{k_2}\dfrac{[H]}{K_2}}}{1 + \dfrac{[H]}{K_2}} \quad (11)$$

The outcome of fitting the experimental results to eqs 10 and 11 is seen as the solid lines in Figures 8 and 9; the fitted parameters are presented in Table 2. Release of substrate is controlled by a group with a pK_a of 6.95 (±0.07); above this pH, the group is deprotonated and the enzyme−substrate complex is increasingly committed to catalysis, resulting in a smaller value of the observed isotope effect. The binding of substrate is controlled by a group with a pK_a of 7.43 for H-LA and 7.72 for D-LA. This "outward" shift of observed pK_a's is typical for the faster of two isotopically labeled substrates, as the faster substrate looks "stickier" than the slower one; this also means that the isotopically labeled step is not fully rate-limiting (Cleland, 1977). Under these conditions, the value of pK_{a2} = 7.72 for D-LA is probably closer to the actual pK_a of the group controlling substrate binding. The observed $^{D}(k_{cat}/K_m)$ increases from about 30 above pH 8.5 to around 70 below pH 7.5 (Figure 9). The extrapolated value for the intrinsic isotope effect as seen in Table 2 is $^{D}k_2$ = 80 (±9), indicating that the isotopically sensitive step is rate-limiting at the lower pH range. This

Table 2: Values for Parameters Obtained by Fitting pH Dependencies of $^{D}(k_{cat}/K_m)$, k_{cat}/K_m(H), and k_{cat}/K_m(D) at 25 °C

	source		
parameter	$^{D}(k_{cat}/K_m)^a$	$k_{cat}/K_m(H)^b$	$k_{cat}/K_m(D)^b$
$^{D}k_2$	80 (9)		
k_1 (M^{-1} s^{-1})		$3.5 \cdot 10^7$	$3.5 \cdot 10^7$
k_{-1}/k_2	0.46 (0.08)[f]	0.41	35[d]
k'_{-1}/k_2	8.6 (4.2)	2.1	210[e]
pK_{a2}	6.95 (0.07)	6.95[c]	6.95[c]
pK_{a1}		7.43	7.72

[a] Results of fitting data in Figure 9 to eq 11. [b] Results of fitting data in Figure 8 to eq 10. [c] Obtained from fitting $^{D}(k_{cat}/K_m)$ and consequently fixed in eq 10. [d] The value of $k_{-1}/k_{2(D)}$ divided by the value of $^{D}k_2$ = 80 gives $k_{-1}/k_{2(H)}$ = 0.43. [e] The value of $k'_{-1}/k_{2(D)}$ divided by the value of $^{D}k_2$ = 80 gives $k'_{-1}/k_{2(H)}$ = 2.6. [f] Error bars in parentheses are the output of a nonlinear fit of experimental data points to eq 11. Errors are not given for fit to eq 10 (second and third columns) due to the greater number of parameters.

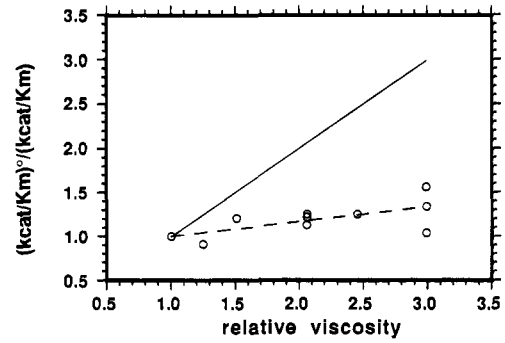

FIGURE 10: Effect of viscosity on reaction with H-LA at 20 °C, pH 7.18. The slope of the line is 0.16 (±0.03). The solid line is the theoretical behavior for a fully diffusion-controlled reaction.

value is similar to the maximal value of $^{D}(k_{cat}/K_m)$ seen from the temperature and viscosity studies above. It is important to note that all three fits in Table 2, $^{D}(k_{cat}/K_m)$, k_{cat}/K_m(H), and k_{cat}/K_m(D), are internally consistent. Both H-LA and D-LA produce identical values of k_1 (3.5×10^7 M^{-1} s^{-1}) and similar values for pK_{a1}. The magnitude of the intrinsic isotope effect $^{D}k_2$ is similar when extracted as a parameter from $^{D}(k_{cat}/K_m)$ either by dividing k_{-1}/k_2 for D-LA by k_{-1}/k_2 for H-LA or by dividing k'_{-1}/k_2 for D-LA by k'_{-1}/k_2 for H-LA. The value of $k_{-1}/k_{2(H)}$ is similar, 0.46 and 0.41, when extracted from $^{D}(k_{cat}/K_m)$ and k_{cat}/K_m(H), respectively.

Viscosity Dependence at Low pH. At low pH, the isotope effect on k_{cat}/K_m is at the upper limit seen for this system. Assuming that the isotopically sensitive step is close to fully rate-limiting at low pH, steps involved in binding of substrate would be less rate-limiting. Viscosity studies at low pH would check this assumption. The effect of increasing the relative viscosity of the medium at pH 7.18 is seen in Figure 10. The bimolecular reaction rate of lipoxygenase with linoleic acid is less viscosity dependent at low pH than at pH 9, about 16 vs 48%, respectively. The rate with D-LA is totally independent of viscosity (data not shown).

Titration of LA. Sodium linoleate in solution was titrated against dilute HCl to determine the pK_a of the substrate of lipoxygenase. The pK_a of linoleic acid is 8.0 (±0.1) for concentrations of LA above 100 μM (Figure 11a). This unusually high pK_a for a carboxylic acid is obtained also for back-titration of linoleic acid to linoleate (Figure 11b). A similar pK_a has been previously determined (Bild *et al.*, 1977); however, the former conditions, 0.1 M linoleic acid

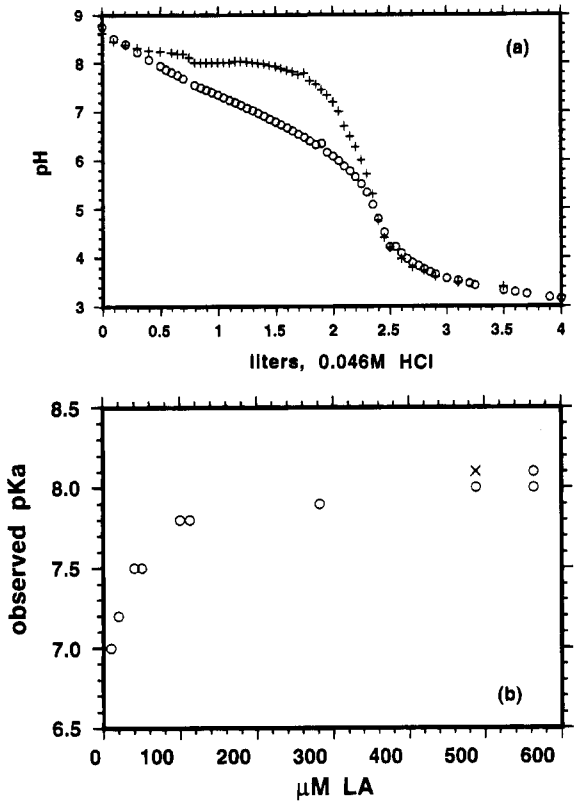

FIGURE 11: pK_a values for linoleic acid. (a) Example of titration curve of 0.2 L of 564 μM sodium linoleate (crosses) with 0.046 M HCl and of 564 μM sodium linoleate in 0.5% TritonX100 (circles) with HCl. Observed pK_a's are 8 and 7.1, respectively. (b) Observed pK_a values for LA at different concentrations. Values obtained are for titration of sodium linoleate with HCl (circles) and for titration of linoleic acid with NaOH (crosses).

dissolved in detergent or 95% ethanol, are far from the catalytic conditions of lipoxygenase. Consequently, it was essential to measure linoleic acid's pK_a at lower concentration and in the absence of chemicals such as ethanol or detergent that are known to interfere with the solubility of fats. From Figure 11a, it can also be seen that linoleic acid is a surprisingly good buffer at and around pH 8 when present at concentrations above 100 μM. At concentrations below 60−100 μM LA, the observed pK_a decreases, and at 10 μM, it is 7.0 (Figure 11b), with linoleic acid losing its buffering capacity and revealing a more typical acid/base titration. However, at such low concentrations of a weak acid, the end point is not pronounced and the observed pK_a inaccurate. It is generally accepted that it is impractical to analyze titration curves of very dilute weak acids or bases (Harris, 1987). Addition of chemicals that interfere with the formation of the soluble forms of fatty acids, such as acid−soaps or micelles, affect the observed pK_a of LA. The detergent TritonX100 lowers the observed pK_a to 7.1 (Figure 11a). NaCl (0.2 M) has a similar effect at high concentration of LA (564 μM) but no effect at 10 and 20 μM (data not shown). This set of experiments shows that LA has associative soluble forms in solution, with unusually high pK_a's ranging from 7 to 8.

DISCUSSION

In this paper, we present extensive kinetic measurements for the reaction of soybean lipoxygenase-1 with linoleic acid. The reaction rate and k_H/k_D isotope effect change dramatically with a number of experimental conditions which include temperature, viscosity, replacing H_2O with D_2O, and pH. The upper limit of the observed isotope effect is the largest reported for an enzymatic system and is greater by approximately one order of magnitude than the isotope effect calculated from vibrational zero-point energies for the two isotopes (Melander & Saunders, 1987). In order to analyze fully the kinetic results, it is essential to estimate the intrinsic isotope effect and to understand the nature of the rate-limiting steps. In particular, does the abnormally large isotope effect originate from a single microscopic step? An observed isotope effect for a catalytic cycle with two isotopically sensitive steps cannot exceed the larger of the two. However, if the two isotopically sensitive steps are separated by an isotopically insensitive branch, then the overall isotope effect expression can include multiplicative terms of the intrinsic isotope effects on the different microscopic steps. The isotopically insensitive branch can yield products (Thibblin & Ahlberg, 1989) or return the reaction to reactants (see section B of the Appendix). In the former case, a branch must yield a product different from that being analyzed since an isotope insensitive branching step leading to the main product can only lower the observed isotope effect (Thibblin & Ahlberg, 1989). In a previous report of abnormally large isotope effects with lipoxygenase from this laboratory, competitive isotope effects were measured by following product formation subsequent to its separation from substrate by HPLC; as described (Glickman *et al.*, 1994), no evidence was obtained for a second product eluting with a retention time different from the expected hydroperoxyl product. Although it is known that lipoxygenase can release substrate-derived intermediates into solution, forming alternate products under anaerobic conditions (De Groot *et al.*, 1973; Garssen *et al.*, 1972), the degree to which alternate pathways occur under initial rate conditions and high-oxygen tension is expected to be quite low [cf. Schlistra *et al.* (1993)]. In section B of the Appendix, equations have been derived which show mechanisms under which multiplicative isotope effect terms can arise from a branching mechanism in which substrate-derived intermediates return to substrate by a pathway different from the main catalytic path. These equations indicate that a very large ratio of side branch to main pathway must be maintained for multiplicative isotope effects to be expressed. Importantly, it is expected that the extent of reaction branching will be sensitive to oxygen concentration, with the degree of branching decreasing as the oxygen tension is increased. In fact, this prediction is opposite to the observed trend in isotope effects with varying oxygen concentrations (M. H. Glickman and J. P. Klinman, unpublished results). For this reason, together with numerous properties of the lipoxygenase reaction summarized in section B of the Appendix, we conclude that reaction branching is highly unlikely to be the cause of the large and variable isotope effects reported in the present study. Such a conclusion is further supported by recent stopped flow experiments completed under anaerobic conditions (Sun and Klinman, unpublished results). These single turnover studies, in which the reduction of active site Fe(III) by substrate has been directly monitored, indicate isotope effects very similar to those reported in the present study under steady state conditions.

We propose that all of the kinetic data presented in this work are consistent with a large intrinsic isotope effect which

varies with reaction condition due to partial rate limitation by steps other than C−H cleavage. We proceed to discuss the specific reaction steps, by analyzing the results obtained under conditions of changing temperature, solvent viscosity, pH, and replacing H_2O with D_2O. We have previously published large primary k_H/k_D isotope effects with an unusual temperature dependence for SBL-1 (Glickman *et al.*, 1994). In the present work, the range of temperature studied has been extended. As shown in Figure 1, a clear break in $^D(k_{cat})$ occurs around 32 °C. Below this temperature, isotope effects decrease with decreasing temperature, whereas above 32 °C, $^D(k_{cat})$ decreases with increasing temperature as expected from theory for a single microscopic step (Bell, 1980). A break is also seen in $^D(k_{cat}/K_m)$, although this is less extreme than for $^D(k_{cat})$. The simplest and most straightforward interpretation of curved temperature dependencies of isotope effects is a change in rate-limiting steps, with the data in Figure 1 implying that the enthalpy of activation for a step other that C−H cleavage is larger than that for hydrogen abstraction. Increasing the temperature is, thus, expected to accelerate preferentially a step(s) different from the isotopically sensitive ones, leading to an isotope effect which approaches the intrinsic value. By comparing isotope effects at 25 °C to the maximal values observed at the high temperature limit, we conclude that the C−H bond cleavage is less than or equal to 50% rate-limiting for k_{cat}/K_m at room temperature; in the case of $^D(k_{cat})$, its slightly larger value at room temperature implies that hydrogen transfer is somewhat more rate-limiting than for k_{cat}/K_m.

If chemistry is not fully rate-limiting, what other steps may be limiting catalysis? SBL-1 is a fast enzyme with a second order rate constant of about 3×10^7 M^{-1} s^{-1} at room temperature (cf. Figure 4a). This rate is near the upper limit of 10^8-10^{10} M^{-1} s^{-1} for diffusion-controlled reactions imposed by the viscosity of aqueous solutions at room temperature (Solc & Stockmayer, 1973); rates of enzymatic encounter with substrate are expected to have lower limits due to the reduced reaction cross section arising from constraints by the enzyme's active site, substrate−enzyme configuration, or solvent reorganization (Solc & Stockmayer, 1973). Thus, lipoxygenase was considered an excellent system in which to examine the effect of added viscosogen on reaction rate. The main conclusion from the analysis presented in this paper is that the reaction of SBL-1 is close to 50% diffusion-controlled at room temperature and pH 9 (Figure 2). Consistent with the trends observed in isotope effects as a function of temperature, this means that the abnormally large KIE of about 30 seen on k_{cat}/K_m under these conditions is at least 50% less than the intrinsic value. Using a method outlined in section A of the Appendix, the lower limit of the intrinsic isotope effect extrapolated from viscosity measurements is 83 (±11). As a result of this huge isotope effect, the reaction of SBL-1 with D-LA is much slower, yielding a second order rate constant in the range of 6×10^5 M^{-1} s^{-1}. This reaction rate is far enough below the diffusion limit for an aqueous reaction to be viscosity independent as has been observed experimentally (Figure 2). Since H- and D-LA are identical substrates, the observed difference in their rates should be due solely to differences in their chemical step. D-LA can, therefore, be used as a slow substrate control, an essential contingent of viscosity studies. The absence of viscosity effects on k_{cat}/K_m(D), or on k_{cat} for either substrate, demonstrates that the viscosity effect on k_{cat}/K_m(H) arises from substrate binding to enzyme. That the step sensitive to viscosity is distinct from that sensitive to isotopic substitution is not surprising, as viscosity sensitive steps different from substrate binding or release are uncommon [an interesting case of a viscosity sensitive catalytic step has been published by Sampson and Knowles, (1992)].

The assumption that the C−H bond cleavage becomes more rate-limiting as the temperature is raised above 32 °C is corroborated by the concomitant decrease in reaction sensitivity to the viscosity of solution. While the reaction is 48% diffusion-controlled at 20 °C (Figure 2), it is virtually insensitive to viscosity at 37 °C (Figure 6). Interestingly, at 5 °C, substrate binding/release is also less rate-limiting (24%, Figure 5) than at room temperature. On the one hand, the k_{cat}/K_m rate is significantly slower at 5 °C, rendering the reaction less likely to be diffusion-controlled. On the other hand, the observed isotope effect $^D(k_{cat}/K_m)$ also decreases (Figure 1), indicating that the C−H bond cleavage is less rate-limiting at lower temperatures. This result indicates that yet another distinct step is involved in the reaction mechanism.

A significant solvent isotope effect (SIE) is observed on k_{cat}/K_m and a smaller SIE on k_{cat} at 25 °C (Table 1); values of both increase at 5 °C. Since a larger SIE is observed on k_{cat}/K_m(H) than on k_{cat}(H), other steps (including C−H abstraction) are more rate-limiting for k_{cat} than k_{cat}/K_m; a similar conclusion was reached from the temperature dependence of the kinetic isotope effect (Figure 1). The magnitude of the observed SIE is rather large and could arise from a solvent-dependent hydrogen transfer from multiple H bond rearrangements involving an enzymatic conformational change upon substrate binding or displacement of water by substrate in the active site. Evidence for bound water in the active site has been published (Nelson, 1988). Since k_{cat}/K_m includes the bimolecular encounter, up to the first irreversible step, and k_{cat} includes steps from the enzyme substrate complex, the SIE seen on k_{cat} and k_{cat}/K_m might be from the same step. It is also possible that the SIE's on k_{cat} and k_{cat}/K_m are due to separate steps; i.e., it is possible that a similar water displacement or other sort of H bond rearrangement upon substrate binding is repeated upon product release. The solvent isotope effect at 25 °C is obliterated when deuterated linoleic acid (D-LA) is used as substrate, demonstrating that the C−H bond cleavage and steps sensitive to solvent deuteration are separate.

The combination of rate and kinetic isotope effect changes with temperature, viscosity, and solvent clearly characterizes three distinct steps: binding of substrate, a solvent-dependent step, and C−H bond cleavage. All three are partially expressed at room temperature; yet, none is fully rate-limiting. At higher temperatures, hydrogen abstraction increasingly limits the reaction, while at lower temperature, the solvent sensitive step dominates. Focusing on the behavior above 32 °C, where C−H cleavage becomes rate-limiting, could be useful in understanding the detailed mechanism of this process. A schematic presentation of the relative rate limitation of the three steps in the lipoxygenase reaction as a function of temperature is displayed in Figure 12. At room temperature, lipoxygenase is close to the diffusion-controlled rate for an enzymatic reaction, with no

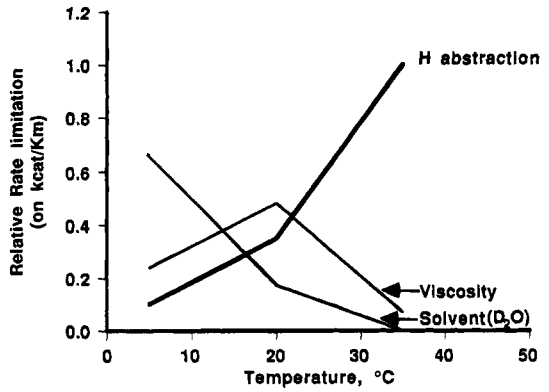

FIGURE 12: Relative rate limitation by different steps on k_{cat}/K_m. Schematic presentation of the relative importance of C−H bond cleavage (thick line), effect of viscosity (substrate binding), and a step sensitive to replacement of H_2O by D_2O, as a function of temperature. At room temperature, soybean lipoxygenase is "a perfect enzyme", close to diffusional controlled, with no one step being fully rate-limiting. At lower temperatures, a solvent-dependent step is dominant, whereas at higher temperatures, C−H bond cleavage limits the reaction rate.

single step being completely rate-limiting. This type of behavior has been referred to by Knowles as "a perfect enzyme" since there should be no further evolutionary pressure to perfect the efficiency of single steps beyond that dictated by the diffusional encounter of enzyme and substrate (Albery & Knowles, 1976; Burbaum *et al.*, 1989). Under this definition, soybean lipoxygenase-1 appears optimized for catalysis at room temperature. Given the fact that mammalian lipoxygenase functions at 37 °C, it would be interesting to see if the behavior of this enzyme approximates that for a plant lipoxygenase at 25 °C.

Varying the pH of reaction solution shows that k_{cat} for both LA and D-LA is invariant with pH (Figure 7). The value of k_{cat}/K_m, however, decreases over one order of magnitude for both substrates as pH is decreased below pH 8 (Figure 8). This is due to a comparable increase in K_m as pH is decreased. It may be worthwhile to add that, although k_{cat} does not change much over the pH range, the enzyme becomes functionally inactive at low pH due to the large increase in K_m. A high K_m does not necessarily result in an inactive enzyme if a physiological concentration of substrate above its K_m value can be achieved. In the case of SBL-1, this is not possible as the solubility of LA decreases with pH, as does the concentration above which the fatty acid forms micelles (Verhagen *et al.*, 1978). It is noted that the micellear form of LA is not a substrate for SBL-1 (Galpin & Allen, 1977). Due to the long extrapolations for k_{cat} at low pH, these values are less reliable than values obtained at pH 7.5 and above. The pH dependence of k_{cat}/K_m and of $^D(k_{cat}/K_m)$, together with the pH independence of k_{cat}, can be explained by invoking two pK_a's: pK_a = 7.72 controlling binding of substrate and pK_a = 6.95 governing substrate release. This mechanism is described by eqs 6−11, with the fitted results summarized in Table 2. The overall rate of substrate release (k^{obs}_{-1} described in eq 9) increases when the group is protonated, or in other words, the substrate becomes "less sticky" in the protonated form [using the nomenclature of Cleland (1977)]. Consequently, the values of both k_{cat}/K_m and the commitment k_2/k_{-1} are reduced, and the value of the observed $^D(k_{cat}/K_m)$ approaches the intrinsic effect. An 18-fold decrease in the value of k_2/k_{-1} over the pH range is obtained from the fit of the isotope effect (Table 2, first column). The intrinsic isotope effect thus fitted is 80 (±9) which is identical to that extrapolated from viscosity studies of 83 (±11). It should be noted that these values have been estimated by fitting the experimental results for k_{cat}/K_m to the simplified model given in eq 3. Since the pattern of observed solvent isotope effects for lipoxygenase implicates a more complex kinetic scheme, the value obtained from viscosity effects may be an underestimate of the intrinsic isotope effect (cf. section A of the Appendix). A value of $(k_1/k_{-1})k_2$ can be obtained from both fits; $R = 5.7 \times 10^7$ M^{-1} s^{-1} (Figure 4) and 8.5×10^7 M^{-1} s^{-1} (second column, Table 2). The two values are within the same order of magnitude. The discrepancy could be due to the difference in reaction conditions: 20 vs 25 °C and 0.2 M CHES, pH 9, vs 0.1 M borate, pH 9, for the viscosity studies and the pH experiments, respectively. In addition, as mentioned in section A of the Appendix, the extrapolated value of $R = (k_1/k_{-1})k_2$ obtained from viscosity measurements will include an additional internal commitment(s) if present, which could lower the estimated value of R from viscosity effects. Thus, parameters extracted from both treatments are consistent. The overall treatment is supported by the observation that the reaction is only 16% diffusion-limited at pH 7.18 (Figure 10) compared to 48% at pH 9 (Figure 2). As anticipated from the increase in isotope effect with decreasing pH, the decreased sensitivity to solvent viscosity at lower pH supports an increasing rate limitation by the C−H bond cleavage step.

In attempting to ascribe mechanistic significance to pK_{a1} and pK_{a2}, it would be of great value to know whether these arise from ionization of substrate or enzyme. A group with an apparent pK_a of about 7.7 is involved with substrate binding. There are three possibilities. First, the ionized form of the carboxyl group on the substrate is the preferred form of substrate. Second, an ionizable side chain on the enzyme is in direct interaction with substrate. Third, ionization of a remote side chain generates an enzyme form which is responsible for substrate binding. The second possibility is unlikely since better stabilization of a negatively charged carboxylic acid at high pH would require that an enzymatic side chain become more positive after deprotonation. Can the ionizable function group be on the substrate? Carboxylic acids are considered to have pK_a's in the range of 4.9 (March, 1968). However, direct titration of linoleic acid has shown that, under certain conditions, LA has a pK_a of 8 (Figure 11a,b). This result can be attributed to the presence of multimeric forms of fatty acids in solution. Although fatty acids exist as either free fatty acids or micelles at very low concentrations and concentrations above the critical micelle concentration (CMC), respectively, intermediate concentrations of fatty acid may lead to a substantial fraction of the lipid in dimeric ions or higher (premicellar) aggregates. Formation of acid−soaps, for example, occurs over a wide concentration range, and the subsequent ionization of acids within these complexes can occur at pH values well above 7 (Eagland & Franks, 1965). The observed pK values of such solutions increase with chain length of the lipid (Lucassen, 1966). Linoleic acid, an 18-carbon chain, is no exception to this trend, and a pK_a of 7.9 has been published (Bild *et al.*, 1977), albeit for conditions that are far from those used in lipoxygenase assays. Acid−soap formation is an associative process and as such should be sensitive to reaction conditions. As seen in this study, addition of

detergent lowers the observed pK_a of LA (Figure 11a) as does a decrease in the concentration of LA itself (Figure 11b). From the data in Figure 11b, the apparent pK_a for linoleic acid is between 7 and 8 when measured in the concentration range of LA used for kinetic studies (2.5−50 μM), with the caveat that the accuracy of pK_a's for LA decreases with increasingly dilute solutions (cf. Experimental Procedures).

We, therefore, propose that the pK_a controlling substrate binding arises from the ionization of substrate and that the ionized form of LA is the preferred substrate. This view is supported by the observation that (1) SBL-1 has a greater affinity for charged substrates and a lower one for neutral ones, regardless of the pH of solution, and that (2) linoleyl sulfate, negatively charged at both pH 7 and 9, is a better substrate for SBL-1 than linoleate at both pH's (Bild *et al.*, 1977). It was further noted by Bild *et al.* that SBL-1 has the same activity with linoleyl sulfate at pH 6.8 as it does with LA at pH 9, whereas the neutral methyl linoleate is a poor substrate even at pH 9. The value of k_{cat}/K_m for linoleate sulfate changed little with pH, and a 10-fold increase in K_m was noted for neutral substrates compared to their negatively charged analogs. We notice a similar increase in K_m for linoleic acid when the pH is decreased from pH 9 to 7. It is well-known that the reaction of SBL-1 is highly specific for the single product 13*S*-HPOD at pH 9 but produces a mixture of 13*S*-HPOD and 9*S*-HPOD at pH 6 [cf. Gardner (1989)]. As Gardner discusses, oxidation of LA to either 9*S*- or 13*S*-HPOD is spatially identical if the substrate is arranged head to tail in opposite orientations. Such an orientation will be more probable when LA is protonated.

The second observed pK_a in the SBL-1 reaction, pK_{a2}, occurs within the enzyme−substrate complex, arising from either bound substrate or enzyme. Since the unusually high pK_a of linoleic acid arises from cooperative forms in solution, one would expect that a downward shift of this pK_a would be noticed upon binding to enzyme. While the pK_a of the enzyme substrate of 6.95 is lower than that of substrate in solution, it is still higher than that expected for a single molecule of substrate, especially if in the vicinity of a positively charged enzyme side chain. Thus, the more likely assignment for this pK_a is to a functional group on the protein. However, until we have a structure for the complex of SBL-1 with its fatty acid substrate, the possibility remains open that LA can bind to protein in a multimeric form. Such an effect might offer an explanation for the earlier observation that the magnitude of deuterium isotope effects measured with LA and SBL-1 were found to be a function of the LA concentration (Glickman *et al.*, 1994). A structure for the E·S complex of SBL-1 should also shed light on the role of the side chain(s) ligating the carboxylate of substrate. Possible candidates for such a role include Lys483 (Boyington *et al.*, 1993) and His499 or Arg707 (M. Amzel, personal communication). Modifying one arginine residue of SBL-1 decreases the interaction with linoleic acid in a manner consistent with our observations (Matsui *et al.*, 1995).

We note that the optimum pH of SBL-2 and SBL-3 is around 7 (Arens *et al.*, 1973; Christopher *et al.*, 1970), a pH range where we show that linoleic acid is protonated. It is, therefore, possible that SBL-2 and SBL-3 react with neutral substrates whereas the natural substrates of SBL-1 are negatively charged at physiological pH. For instance, it has been shown *in vitro* that SBL-1 is an acceptable catalyst of charged unsaturated phospholipids (Brash *et al.*, 1987; Eskola, 1983; Kondo, 1993). SBL-1 might, therefore, play a role in the degradation process of the phospholipid bilayer.

To summarize our observations, SBL-1 has an extremely large intrinsic k_H/k_D isotope effect on the C−H cleavage step. Reaction branching, involving two distinct isotopically sensitive steps as a source of the large observed isotope effects, does not appear to be an explanation for these results. All the kinetic data presented are consistent with a hydrogen abstraction step with an intrinsic k_H/k_D in the range of 80, with this step not fully rate-limiting at 25 °C. Commitments which are sensitive to external conditions, such as temperature, pH, viscosity, solvent (this study), or substrate concentration (Glickman *et al.*, 1994), determine the expression of the observed kinetic isotope effect. The reaction rate at 25 °C and standard assay conditions is partially limited by at least three distinct steps: binding of substrate; a step sensitive to solvent, possibly a conformational change; and C−H bond cleavage. The encounter of enzyme with substrate is close to 50% rate-limiting at room temperature. After substrate binding, a step involving H bond rearrangement, possibly displacement of H_2O from the active site, or a conformational change is observed. These characteristics make soybean lipoxygenase-1 one of the few enzymes that fit the definition for a perfect enzyme (Albery & Knowles, 1976; Burbaum *et al.*, 1989).

One unanswered question from this work is the physical origin of the large intrinsic isotope effect. Although similar isotope effects have been observed for perdeuterated LA and 11,11-dideuterated LA (Glickman *et al.*, 1994), we have not yet examined the oxidation of substrate containing a single deuterium at C-11. Thus, it is conceivable that SBL-1 has a highly unusual mode for C−H activation involving both the *R*- and *S*-hydrogens at C-11. This seems unlikely, given the small secondary isotope effect (k_H/k_T = 1.19) reported with human lipoxygenase (Brash *et al.*, 1986). However, we cannot rule out the possibility that the properties of SBL-1 are markedly different from those for enzymes from different sources. For the future, experiments will be focused on addressing whether the origin of the large intrinsic isotope effect could be due to quantum mechanical tunneling.

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APPENDIX

A: Kinetic Treatment of Viscosity Measurements and a Method for Approximating Intrinsic Isotope Effects. Kinetic isotope effects have been used extensively in the study of

enzyme mechanisms. The kinetic complexity of enzymatic reactions imposes a significant barrier to a full analysis of isotope effects. Isotope effects on single steps are often not fully expressed in the overall rate equation, resulting in smaller observed isotope effects. Accurate measurement of intrinsic isotope effects is essential for the interpretation of steady state isotope effect data. From a comparison of the observed isotope effect to the intrinsic, one can obtain information on both the kinetic and chemical mechanism.

There are a few existing methods for extracting intrinsic isotope effects: (1) varying reaction conditions, in order to increase the observed isotope effect by decreasing commitments, thus rendering the isotopically sensitive step more rate-limiting [cf. Klinman (1972)]; (2) simultaneous measurement of more than one isotope effect [cf. Hermes *et al.* (1982)]; and (3) comparison of deuterium and tritium isotope effects [cf. Northrop (1975)], a useful modification of which is described by Miller and Klinman (1983). Viscosity measurements have been used in competitive isotope effect experiments to extract internal commitments (Grissom & Cleland, 1988) or the intrinsic isotope effect (Tipton, 1993). We show here how viscosity studies can also be used in direct rate measurements of two isotopically labeled substrates to separate external commitments from internal ones. In the absence of internal commitments, viscosity studies can be added to this list of methods for isolating intrinsic isotope effects. Kinetically, this is similar to the first option above, since it requires changing reaction conditions. It does not, however, require the isotopically sensitive step to be fully rate-limiting, leading instead to the intrinsic isotope effect upon extrapolation.

We present here a slight modification of the kinetic manipulations previously used to analyze the diffusion component of enzyme-catalyzed reactions (Brouwer & Kirsch, 1982; Loo & Erman, 1977; Nakatani & Dunford, 1979). This treatment is general and based solely on two assumptions: (1) Each and every microscopic bimolecular association or dissociation is diffusion-controlled. (2) The rate of a microscopic diffusion-controlled step is inversely proportional to the viscosity of the medium as dictated by the Stokes–Einstein equation (Caldin, 1964). In a simple form, a microscopic association or dissociation rate constant can be denoted as

$$k = (\text{constant})_T \frac{1}{\eta} \tag{A1}$$

where η is the absolute viscosity of solution and T is temperature. Therefore, the relationship between the rate constants for a single diffusion-controlled step, k_1, at viscosities η and η^0, is

$$k_1\eta = k^0{}_1\eta^0 \quad \text{or} \quad k_1 = k^0{}_1\frac{\eta^0}{\eta} \quad \text{or} \quad k_1 = k^0{}_1\frac{1}{\eta^{rel}} \tag{A2}$$

where the relative viscosity $\eta^{rel} = \eta/\eta^0$ is the viscosity of solution compared to aqueous solution at 20 °C.

We now look at some simple enzymatic mechanisms. In the following treatments, only k_{cat}/K_m is derived as k_{cat} does not include substrate binding steps and, therefore, has limited value in viscosity studies. The simplest mechanism to describe an enzymatic reaction is

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} E + P \tag{A3}$$

$$\frac{k_{cat}}{K_m} = \frac{k_1k_2}{k_2 + k_{-1}} \tag{A4}$$

As mentioned above, $k_1\eta = k^0{}_1\eta^0$, and $k_{-1}\eta = k^0{}_{-1}\eta^0$; therefore, k_{cat}/K_m can be described in terms of relative viscosity:

$$\frac{k_{cat}}{K_m} = \frac{k^0{}_1k_2\dfrac{\eta^0}{\eta}}{k_2 + k^0{}_{-1}\dfrac{\eta^0}{\eta}} \quad \text{or} \quad \frac{k_{cat}}{K_m} = \frac{k^0{}_1k_2\dfrac{1}{\eta^{rel}}}{k_2 + k^0{}_{-1}\dfrac{1}{\eta^{rel}}} \tag{A5}$$

In the treatment by Brouwer and Kirsch (1982), eq A5 was rearranged according to Nakatani and Dunford (1979) to give

$$\frac{k_{cat}}{K_m} = \frac{k^0{}_1\dfrac{\eta^0}{\eta}}{1 + \dfrac{k^0{}_{-1}}{k_2}\dfrac{\eta^0}{\eta}} \tag{A6}$$

or by taking the reciprocal

$$\frac{1}{k_{cat}/K_m} = \frac{1}{k^0{}_1}\frac{\eta}{\eta^0} + \frac{k^0{}_{-1}}{k^0{}_1k_2} \tag{A7}$$

Plotting $1/(k_{cat}/K_m)$ vs relative viscosity will yield the rate constant for association (Brouwer & Kirsch, 1982) from the slope of the line. It is also possible to plot a slightly different rearrangement of eq A5 to yield a simple and general presentation which is valid for the mechanism in eq A3 and for most standard enzymatic reactions:

$$\frac{k_{cat}}{K_m} = \frac{\dfrac{k^0{}_1k_2}{k^0{}_{-1}}\dfrac{\eta^0}{\eta}}{\dfrac{k_2}{k^0{}_{-1}} + \dfrac{\eta^0}{\eta}} \tag{A8}$$

or in a general formate

$$\frac{k_{cat}}{K_m} = \frac{R_m\dfrac{\eta^0}{\eta}}{S_D + \dfrac{\eta^0}{\eta}} = \frac{R_m\dfrac{1}{\eta^{rel}}}{S_D + \dfrac{1}{\eta^{rel}}} \tag{A9}$$

In this presentation, k_{cat}/K_m shows hyperbolic behavior with respect to $1/\eta^{rel}$. This presentation is mathematically identical to the Michaelis–Menten equation of enzyme kinetics but with a variable of $1/\eta^{rel}$ instead of substrate concentration. This formulation facilitates the comparison of different enzymatic systems, as it seems to be a general and simple presentation of k_{cat}/K_m as a measure of two constants. (1) R_m is the maximal rate attainable at infinitely low viscosities. R_m is a measure of how the intrinsic forward step(s) relates to the binding constants. The strength of this presentation is that R_m is (as shown below) an identical expression for most simple enzymes. Barring any internal commitments, $R_m = (k^0{}_1/k^0{}_{-1})k_2$. (2) S_D is a measure of how sensitive $k_{cat}/$

K_m is to viscosity. For more complex mechanisms, S_D can be rather complicated (analogous to K_m in the Michaelis–Menten equation).

The two limiting cases for k_{cat}/K_m are, therefore,

at very low viscosities:

$$\frac{k_{cat}}{K_m} = \frac{k^0{}_1 k_2}{k^0{}_{-1}} \qquad \left(\frac{\eta^0}{\eta} = \frac{1}{\eta^{rel}} \rightarrow \infty\right) \quad \text{(A10)}$$

at very high viscosities:

$$\frac{k_{cat}}{K_m} = k^0{}_1 \frac{\eta^0}{\eta} \qquad \left(\frac{\eta^0}{\eta} = \frac{1}{\eta^{rel}} \rightarrow 0\right) \quad \text{(A11)}$$

Given the limited range of $1/\eta^{rel}$ which can be accessed experimentally, a physically meaningful extrapolation to $1/\eta^{rel} \rightarrow \infty$ will depend on the properties of the enzyme system (i.e., the magnitude of S_D).

For simple enzyme kinetics, k_{cat}/K_m at infinitely high viscosities is dependent solely on the association constant, k_1, since under these conditions binding is fully rate-limiting.

$$\frac{k_{cat}}{K_m} = k^0{}_1 \frac{\eta^0}{\eta} = k_1$$

As the above equations are general, why is it that some enzymes are viscosity sensitive and others are not? The difference lies in the S_D constant, which determines what is "high viscosity" for each enzyme system. Enzymes that are considered diffusion-controlled in an aqueous buffer ($\eta^0/\eta = 1$) are those with an S_D factor much greater than 1, making k_{cat}/K_m linear in viscosity. These are enzymes whose substrates are sticky such that the catalytic step (k_2) is faster than substrate release. Similar to the parameters V_{max} and K_m, R_m tends to be a simpler parameter than S_D. R_m is a product of intrinsic rate constants, usually including the equilibrium binding steps and the first forward steps following binding. As such, R_m can be used to extract the isotope effect on k_2. For instance, in a hydrogen transfer reaction, deuteration of the primary hydrogen on the substrate would have little or no effect on k_1 or k_{-1}. By performing viscosity studies on the enzymatic reaction with protonated and deuterated substrates, and determining R_m(H) and R_m(D), it is possible to estimate a value for the magnitude of the isotope effect on k_2 (eq A12 below). We note that deuteration of the primary position on the substrate could lower the rate of reaction, even to the extent that the reaction would become viscosity insensitive. This should pose no hindrance to the treatment proposed for estimating the isotope effect on k_2 as the parameter compared is the maximal extrapolated rate (R_m). The rate of the slower, labeled substrate would simply be closer to the value of R_m.

$$\frac{R_{m(H)}}{R_{m(D)}} = \frac{k_{2(H)}}{k_{2(D)}} = {}^D k_2 \quad \text{(A12)}$$

In the preceding treatment, a number of more elaborate enzyme systems are considered, showing that the above treatment is a general one, and does not lose its effectiveness in complex enzyme reactions.

Example 1: The simplest "realistic" enzyme mechanism includes substrate binding, product release, and a catalytic step.

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} EP \xrightarrow{k_3} E + P \quad \text{(A13)}$$

In this case, k_1, k_{-1}, and k_3 are viscosity dependent, and k_2 and k_{-2} are isotopically sensitive.

$$\frac{k_{cat}}{K_m} = \frac{k_1 k_2 k_3}{k_{-1}k_{-2} + k_{-1}k_3 + k_2 k_3} = \frac{k^0{}_1 \frac{\eta^0}{\eta} k_2 k^0{}_3 \frac{\eta^0}{\eta}}{k_{-2}k^0{}_{-1}\frac{\eta^0}{\eta} + k^0{}_{-1}k^0{}_3 \frac{\eta^0}{\eta}\frac{\eta^0}{\eta} + k_2 k^0{}_3 \frac{\eta^0}{\eta}} = \frac{k^0{}_1 k_2 k^0{}_3 \frac{\eta^0}{\eta}}{k_{-2}k^0{}_{-1} + k_2 k^0{}_3 + k^0{}_{-1}k^0{}_3 \frac{\eta^0}{\eta}} = \frac{(k_2 k^0{}_1/k^0{}_{-1})\frac{\eta^0}{\eta}}{\frac{k_{-2}k^0{}_{-1} + k_2 k^0{}_3}{k^0{}_{-1}k^0{}_3} + \frac{\eta^0}{\eta}} \quad \text{(A14)}$$

Once again, $R_m = k^0{}_1 k_2 / k^0{}_{-1}$, as in the case shown previously. S_D is more complicated in this instance; however, the important information for the determination of the isotope effect on k_2 is contained in R_m:

$$\frac{R_{m(H)}}{R_{m(D)}} = \frac{k_{2(H)}}{k_{2(D)}} = {}^D k_2$$

We note that, if we had used a double reciprocal plot, as suggested in eq A7 above, the slope would now be $(1/k^0{}_1)(1 + k^0{}_{-1}/K_{(eq)2}k^0{}_3)$. For a reaction that is close to diffusion-limited ($k^0{}_{-1}/k^0{}_3 < 1$), plotting $1/(k_{cat}/K_m)$ against the relative viscosity would yield the rate constant for association as the slope [cf. Brouwer and Kirsch (1982)].

Example 2: An enzymatic reaction with two substrates.

$$E + A \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} EA \underset{k_{-2}}{\overset{k_2[B]}{\rightleftharpoons}} EAB \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} EP \xrightarrow{k_4} E + P \quad \text{(A15)}$$

Without going into detail, $(k_{cat}/K_m)_A$ as a function of relative viscosity is

$$\frac{k_{cat}}{K_m} = \frac{\frac{k^0{}_1 k^0{}_2[B]}{k^0{}_{-1}k^0{}_{-2}} k_3 \frac{\eta^0}{\eta}}{\frac{k^0{}_{-1}k^0{}_{-2}k_{-3} + k^0{}_{-1}k_3 k^0{}_4 + k^0{}_2[B]k_3 k^0{}_4}{k^0{}_{-1}k^0{}_{-2}k^0{}_4} + \frac{\eta^0}{\eta}} \quad \text{(A16)}$$

Once again, R_m is a simple product of a forward intrinsic step and the equilibrium binding steps. It is just as easy to isolate the intrinsic isotope effect:

$$\frac{R_{m(H)}}{R_{m(D)}} = \frac{k_{3(H)}}{k_{3(D)}} = {}^D k_3$$

(on the condition that the concentration of the second substrate, [B], is not too high).

Example 3: As a final example, the mechanism considered the most probable for lipoxygenase [substrate binding, C–H bond cleavage (formation of a radical), oxygen trapping, and

release of product] is derived:

$$E + LA \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ELA \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} E^*LA \underset{k_{-3}}{\overset{k_3[O_2]}{\rightleftharpoons}} E^*LA{\cdot}O_2 \xrightarrow{k_4} E + P \quad \text{(A17)}$$

$$\frac{k_{cat}}{K_m} = \frac{\frac{k^0_1}{k^0_{-1}}k_2\frac{\eta^0}{\eta}}{\frac{k^0_{-1}k^0_{-3}k_{-2} + k^0_3[O_2]k_2k^0_4 + k^0_{-1}k_{-2}k^0_4}{k^0_{-1}k^0_3[O_2]k^0_4} + \frac{\eta^0}{\eta}} \quad \text{(A18)}$$

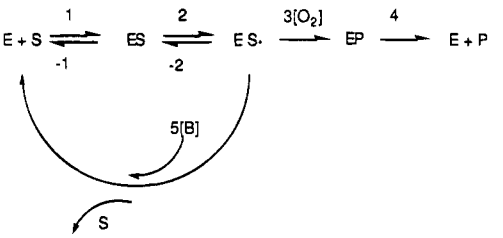
For more complicated mechanisms, which include partially rate-limiting nonviscosity sensitive and nonisotopically sensitive conformational steps, the respective equations become more complex, although the general format stays intact. Such reactions will contain an internal commitment, and R_m will include a factor for the internal commitment. In these instances, $R_m(H)/R_m(D)$ gives a lower limit for the intrinsic isotope effect. We note, however, that since this approach will be restricted to fast enzymes (i.e., those with at least partially rate-limiting diffusion steps), the contribution of rate-limiting conformational changes to the net rate expression may not be very significant.

B: Reaction Branching as a Possible Explanation for Large Observed Isotope Effects in the Lipoxygenase Reaction. In this section of the Appendix, we discuss reaction branching as a possible source of multiplicative terms in the isotope effect expression for lipoxygenase. Large observed isotope effects, where the isotope effect on any single microscopic step is smaller than the overall kinetic isotope effect, can arise if the reaction includes (1) at least two distinct isotopically sensitive steps and (2) an isotopically insensitive step that lies between the isotopically sensitive step and which leads either to a product that is different from the main product being monitored or back to reactants or a previous intermediate [cf. Thibblin (1988) and Thibblin and Ahlberg (1989)]. Returning to a previous species in an isotopically insensitive manner (even though the reaction of the species is isotopically sensitive) would not violate laws of microscopic reversibility if the branch were dependent on an external property (such as the solvent pool as a source of protons). If reaction branching is dependent on external parameters, it may lead to an isotope effect that is dependent on reaction conditions. In this way, the behavior of reaction branching may look similar to the often seen behavior of enzyme reactions in which the magnitude of the observed isotope effect changes due to a change in rate-determining steps. This makes branching a relevant concern when trying to understand the nature of the lipoxygenase reaction.

Carbon-based radicals derived from linoleic acid have been detected, using spin traps in solution, during the reaction of lipoxygenase in the absence of dioxygen (de Groot *et al.*, 1973; Garssen *et al.*, 1972). Under normal (aerobic) turnover conditions, abortion of the lipoxygenase reaction does not seem to occur to a significant extent; it has been estimated that a fatty acid-based radical dissociates from the enzyme once in every 200 catalytic turnovers (Schilstra *et al.*, 1993). It is not clear, however, whether these released radical intermediates react to products or return to reactants. The most straightforward case, that of branching giving rise to two different products, is probably not applicable for lipoxygenase, as analysis of reaction products by HPLC does not detect any additional products in significant quantities (Glickman *et al.*, 1994). In addition, a branch in the mechanism that gives rise to two products which are indistinguishable experimentally (for example, by retention time on HPLC or UV absorbance) can only lower the observed isotope effect, as the rate of product production and, therefore, the expression of the kinetic isotope effect will have contributions from both the isotopically sensitive and insensitive pathways.

We focus, therefore, on a mechanism for lipoxygenase that includes an isotopically insensitive branch that can regenerate free enzyme and substrate. The observed isotope effect can change if the branch is dependent on external conditions, another molecule or even temperature, such that the ratio between the subsequent isotopically sensitive reaction step and the isotopically insensitive branch is dependent on this parameter. Considering what we know about lipoxygenase, we consider a possible reaction mechanism involving branching from a substrate-derived radical intermediate, ES• (Scheme 1):

Scheme 1



As shown, steps 2, −2, and 3 are isotopically sensitive. The isotopically insensitive branch, 5, is dependent on an external factor, [B], which controls the extent of branching and could be due to temperature, pH, detergent, another molecule of substrate, or another reactant that increases the rate of regeneration of free enzyme.

Using the King Altman method, we derived the overall rate expression for this mechanism:

$$(k_{cat})^{app}_{O_2} = \frac{k_2k_3[O_2]k_4}{k_2k_4 + k_4k_5[B] + k_{-2}k_4 + k_3[O_2]k_4 + k_2k_3[O_2]} \quad \text{(B1)}$$

The isotope effect on k_{cat} is given in eq B2:

$$^Dk_{cat} = \left[{}^Dk_2\,{}^Dk_3 + \frac{1}{k_5[B]}({}^Dk_3k_2 + {}^Dk_3{}^DK_{eq2}k_{-2} + {}^Dk_2k_3[O_2] + k_2k_3[O_2]/k_4)\right] \Big/ \left[1 + \frac{1}{k_5[B]}(k_2 + k_{-2} + k_3[O_2] + k_2k_3[O_2]/k_4)\right] \quad \text{(B2)}$$

We can denote a "commitment" factor, C:

$$C = \frac{1}{k_5[B]}(k_2 + k_{-2} + k_3[O_2] + k_2k_3[O_2]/k_4) \quad \text{(B3)}$$

The isotope effect expression includes a term that is a

multiple of two intrinsic isotope effects. Under conditions where this is the dominate term in the expression, the observed isotope effect would appear abnormally large relative to that expected for a single microscopic step. Only when the commitment is small, however, $C \ll 1$, will the multiplicative term, ${}^{D}k_2\,{}^{D}k_3$, be expressed. Assuming saturation with oxygen (as $K_m(O_2)$ for lipoxygenase is 40 μM, while the concentration of molecular oxygen in air-saturated solutions at 25 °C is 258 μM (Glickman and Klinman, unpublished results), this condition will be fulfilled when

$$k_5[B] \gg \left(1 + \frac{k_2}{k_4}\right)k_3[O_2] \quad \text{(B4)}$$

This condition necessitates a large extent of branching; the branch rate, $k_5[B]$, must be significantly larger than the forward rate, $k_3[O_2]$, for the observed kinetic isotope effect to represent a multiplicative term.

Similar conclusions are extracted from the expression of k_{cat}/K_m:

$$k_{cat}/K_m = \frac{k_1k_2k_3[S][O_2]}{k_2k_5[B] + k_{-1}k_5[B] + k_{-1}k_3[O_2] + k_2k_3[O_2] + k_{-1}k_{-2}} \quad \text{(B5)}$$

$${}^{D}(k_{cat}/K_M) = \left[{}^{D}k_2\,{}^{D}k_3 + {}^{D}k_3\frac{k_2}{k_{-1}} + \frac{1}{k_5[B]}\left({}^{D}k_2k_3[O_2] + \frac{k_2}{k_{-1}}k_3[O_2] + {}^{D}k_3\,{}^{D}K_{eq2}k_{-2}\right)\right] / \left[1 + \frac{k_2}{k_{-1}} + \frac{1}{k_5[B]}\left(k_3[O_2] + \frac{k_2}{k_{-1}}k_3[O_2] + k_{-2}\right)\right] \quad \text{(B6)}$$

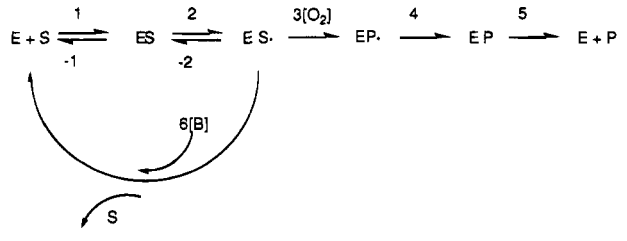
For the multiplicative term to be expressed at saturating O_2, the following condition must be fulfilled:

$$C = \frac{k_2}{k_{-1}} + \frac{1}{k_5[B]}\left(k_3[O_2] + \frac{k_2}{k_{-1}}k_3[O_2]\right) = \frac{k_2}{k_{-1}} + \frac{k_3[O_2]}{k_5[B]}\left(1 + \frac{k_2}{k_{-1}}\right) \ll 1 \quad \text{(B7)}$$

This last condition will not be fulfilled if $k_2 > k_{-1}$. Even when $k_2 < k_{-1}$, $k_5[B]$ still has to be much greater than $k_3[O_2]$ for the commitment to be small, thereby unmasking the multiplicative term, ${}^{D}k_2\,{}^{D}k_3$.

In this last example, the oxygen binding step was assumed to be isotopically sensitive for simplicity. Chemically, a slightly more reasonable case would be to separate the step involving interaction with oxygen into two steps: oxygen trapping of the substrate-derived radical followed by reprotonation of the product-based radical (Scheme 2):

Scheme 2



According to Scheme 2, steps 2, −2, and 4 are isotopically sensitive. Step 3 can be assumed to be fast under the experimental conditions of saturating oxygen. Interestingly, neither k_{cat} nor k_{cat}/K_m shows multiplicative isotope effect terms (eqs B8 and B9):

$${}^{D}k_{cat} = ({}^{D}K_{eq2}k_{-2}k_4k_5 + k_2k_4k_5 + {}^{D}k_2k_3[O_2]k_4k_5 + {}^{D}k_4k_2k_3[O_2]k_5 + k_2k_3[O_2]k_4 + {}^{D}k_2k_4k_5k_6[B])/(k_{-2}k_4k_5 + k_2k_4k_5 + k_3[O_2]k_4k_5 + k_2k_3[O_2]k_5 + k_2k_3[O_2]k_4 + k_4k_5k_6[B]) \quad \text{(B8)}$$

$${}^{D}(k_{cat}/K_m) = ({}^{D}k_2k_{-1}k_6[B] + k_2k_6[B] + {}^{D}K_{eq2}k_{-1}k_{-2} + {}^{D}k_2k_{-1}k_3[O_2] + k_2k_3[O_2])/(k_{-1}k_6[B] + k_2k_6[B] + k_{-1}k_{-2} + k_{-1}k_3[O_2] + k_2k_3[O_2]) \quad \text{(B9)}$$

In a similar manner, one can derive expressions for other mechanisms. Results analogous to those of Scheme 1 above can be obtained for reactions branching from other points in the mechanism and even for cases that branch to a previous intermediate (the ES complex, for instance, rather than back to free enzyme). In all cases relevant to lipoxygenase, where multiple isotope effect terms exist, they will only be expressed when the ratio of the isotopically insensitive return pathway is very significant compared to the second isotopically sensitive forward step to products.

To conclude, the examples shown here raise a number of caveats regarding the relevance of a branching mechanism as the source of the abnormally large isotope effects in the lipoxygenase reactions.

(1) What is the second isotopically sensitive step? A prerequisite of any branching mechanism giving rise to large observed isotope effects is two isotopically sensitive steps. The C−H(D) bond cleavage on C-11 of linoleic acid is one isotopically sensitive step. The reprotonation of the peroxyl radical of linoleic acid could be the second isotopically sensitive step; however, this requires retention of the labeled hydrogen from substrate and reinsertion at the C-13 position. As the dioxygen is inserted on C-13 in an antarafacial manner to the methylene hydrogen cleaved on C-11 (Hamburg, 1971; Nikolaev *et al.*, 1990), this does not seem to be a likely explanation.

(2) Branching from a common enzymatic intermediate to two products can be ruled out. If these products are indistinguishable under the conditions of analysis, branching can only decrease the intrinsic isotope effect. In the case of two distinguishable products, one should be able to detect significant amounts of the second reaction product. Previous studies, using HPLC to separate reactants from products, has failed to detect a second product (Glickman & Klinman, 1994).

(3) What is the chemistry involved in a branch leading back to substrate, such as presented in this Appendix? Release of a carbon-based radical appears unlikely since this species would be expected to react preferentially with molecular dioxygen in solution to give peroxide, rather than to protonate and regenerate substrate. It is possible that another reactant or molecule of substrate could enter the active site and reprotonate the activated linoleic acid.

(4) Deriving the kinetic equations for the most plausible branching mechanism for lipoxygenase (Scheme 2) fails to indicate any multiplicative terms in the observed isotope

effect. It is possible to derive less germane mechanisms that do include multiplicative isotope effect terms (e.g., Scheme 1). However, in these cases, a strict ratio between two pathways must be maintained to express the multiplicative term in the overall kinetic isotope effect. For the mechanism of Scheme 1, this requires that the radical derived from substrate reprotonate more rapidly than it is trapped by oxygen, an unlikely possibility. Further direct evidence against this possibility comes from the observation that the size of the deuterium isotope effects increases as the oxygen tension is increased (Glickman and Klinman, unpublished results). According to the mechanism in Scheme 1, increased oxygen concentrations would be expected to decrease the magnitude of the observed isotope effect, in direct conflict with the observed results.

Branching does seem to occur, in a reaction condition dependent manner, with lipoxygenase. Furthermore, branching can give rise to observed kinetic isotope effects larger than those expected on single microscopic steps. This type of mechanism, however, does not seem to be the explanation for the abnormally large kinetic isotope effects seen under a wide range of conditions in the lipoxygenase reaction.

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