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Comparative Study of [17]O and [18]O Isotope Effects As a Probe for Dioxygen Activation: Application to the Soybean Lipoxygenase Reaction[#]

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Abstract: We present the first comparative study of [17]O and [18]O isotope effects in a dioxygen-dependent enzymatic reaction. The binding of dioxygen to soybean lipoxygenase is expected to be near the diffusive limit, introducing the possibility of a magnetic isotope effect with [17]O reacting faster than [16]O. However, the experimental data indicate a purely mass effect among the ^{16}O, [17]O, and [18]O isotopes. A reaction mechanism for soybean lipoxygenase is proposed in which dioxygen undergoes reversible reaction with a substrate derived radical, followed by a slow release of the bound lipid hydroperoxide.

Introduction

Lipoxygenase is a non-heme iron protein catalyzing the oxidation of unsaturated fatty acids to hydroperoxides. The reaction can be formulated as two half reactions, involving first, hydrogen atom abstraction from substrate to yield a substrate based radical and second, the trapping of this radical by dioxygen to yield a lipid hydroperoxyl radical species that is then reduced and protonated to the product hydroperoxide (cf. ref 1 and references therein). Although hydrogen isotope effects have proven extremely informative with regard to the first half reaction,[1,2,3] the nature of the oxygen activation step has been less well understood. In recent years, oxygen-18 isotope effects have begun to emerge as a tool for the exploration of dioxygen binding/activation in a number of protein systems.[4,5] Using soybean lipoxygenase (SBL-1), we present the first comparative study of oxygen-17 and oxygen-18 effects as a probe of the mechanism of dioxygen interaction within an enzymatic reaction.

Hydrogen abstraction from linoleic acid by SBL-1 has been shown to display a k_H/k_D isotope effect of 20 to 80 at pH 9 and 25 °C[6,7] with the magnitude of this effect undergoing further increase upon elevation of the temperature and decrease in the pH.[2] From the dependence of the substrate isotope effect on solvent viscosogen and D$_2$O, the half reaction with fatty acid

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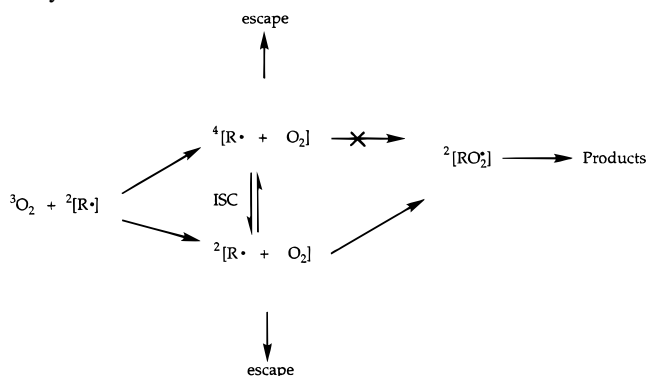
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Scheme 1. Reaction of Atmospheric Oxygen with the Enzyme-Bound Radical[a]

[a] Atmospheric oxygen (triplet) interacts with the enzyme-bound radical (doublet; the [ ] symbolizes the enzyme active site) forming a geminate radical pair that can be a doublet or a quartet at a ratio of 1:2, respectively. For a case in which the rate of intersystem crossing (ISC) between spin states is similar to competing reactions, a magnetic isotope effect can be apparent (see body of text for details).

has been concluded to be 50% diffusion controlled at 25 °C, fully rate limited by the C–H bond cleavage step at 31 °C and above, and partially rate limited by a D_2O dependent step at 5 °C.[2] At 25 °C, the magnitude of the substrate isotope effect on k_{cat}/K_m for linoleic acid (reflecting the binding and activation of substrate) is similar to that on k_{cat} (reflecting the hydrogen atom abstraction from substrate, its interaction with dioxygen and product desorption). The data imply that both C–H activation and a second, possibly oxygen dependent step contribute to the rate limitation of k_{cat}. Steps involving oxygen include (i) the chemical trapping of the substrate derived radical by dioxygen, (ii) conversion of a lipid hydroperoxyl radical intermediate to the hydroperoxide, and (iii) the desorption of product hydroperoxide from enzyme.

Recent evidence indicates that under steady state conditions the C–H bond cleavage from linoleic acid occurs prior to the formation of a catalytically productive interaction of enzyme with molecular oxygen.[1] Given the evidence for partial rate limitation of k_{cat}/K_m for linoleic acid by its binding at 25 °C ($k_{cat}/K_m = 10^7 – 10^8$ M^{-1} s^{-1}, ref 2), it was expected that the reaction of the substrate derived radical with dioxygen would approach the diffusion limit. A number of studies have shown that the reaction of molecular oxygen with organic radicals can exhibit magnetic isotope effects.[8–10] In the reaction of atmospheric dioxygen with organic radicals, the electronic configuration of ground state molecular oxygen is a triplet whereas the organic radical is a doublet. The reaction intermediate is a geminate radical pair (R•, O_2) with three unpaired electrons and, thus, can exist as a quartet or a doublet in a statistical ratio of 2:1 (Scheme 1). Since the product peroxyl radical is a doublet, only the less populated doublet configuration of the geminate pair is reactive. Intersystem crossing from the quartet to the reactive doublet, through hyperfine interactions with the magnetic nucleus [17]O, can increase the rate of product formation. The initially formed geminate radical pair will maintain its initial spin information for about 10^{-9} s, the usual time scale for a diffusional controlled process. Although the observed k_{cat}/K_m for reaction of dioxygen with SBL-1 is *ca.* 10^7 M^{-1} s^{-1},[1] this

may reflect a relatively small cross section of enzyme that yields productive collisional encounters (i.e., the statistically corrected rate constant could approach $10^8 – 10^9$ M^{-1} s^{-1}). In this instance, nuclear magnetic spin effects for quartet to doublet conversion became a possibility, predicting faster reaction for the magnetic nucleus [17]O than for either of the nonmagnetic nuclei [16]O or [18]O (*cf.* refs 11–13 for overviews of magnetic isotope effects).

Previous measurements of oxygen isotope effects in enzymatic systems have focused on the mass differences between [16]O and [18]O as the origin of the isotope effect. With the recognition that the reaction of O_2 with SBL-1 may be close to the diffusion limit, a possible role for magnetic isotope effects is now considered.

Materials and Methods

Oxygen Isotope Effect Determination. The reaction was performed by analyzing the isotopic constitution and quantity of O_2 left in solution at different time points after initiation of reaction. A reaction solution of 70 mL, containing *ca.* 1.3 mM linoleic acid in 0.1 M borate buffer at pH 9, and 25 °C, was saturated with O_2 by repeated freezing and evacuating under vacuum, and bubbling with natural abundance [17]O and [18]O pure oxygen. Reaction was initiated by addition of concentrated SBL-1 via syringe through a serum cup in the sealed reaction flask. Concentration and isotopic constitution of residual O_2 was measured at time points after initiation by two methods—direct oxygen analysis, and analysis after conversion to CO_2.

Oxygen Analysis after Conversion to CO_2. The reaction procedure is similar to that previously described.[4] At different time points, aliquots of SBL-1 reaction solution were quenched and then degassed. The unreacted O_2 was separated from contaminating CO_2 and H_2O and converted to CO_2 in a furnace and trapped in a sealed glass tube. In this manner, ^{16}O/^{18}O isotope effects were measured as a competitive experiment by using natural abundance isotopes of oxygen, leading to isotope effects on k_{cat}/K_m for oxygen. After the isolation of O_2 and its conversion to CO_2 in a furnace, CO_2 pressure was measured to determine reaction progression [*f* in eq 1]. The ratio of ^{18}O/^{16}O in CO_2 was determined by mass-spectral analysis (Krueger Enterprises, MA). The analysis of the experimental data is similar to that described previously.[4] Isotope effects at each time point were calculated from

$$^{18}k = \frac{1}{1 + \dfrac{\ln(R/R_0)}{\ln(1-f)}} \tag{1}$$

where ^{18}k is the ^{16}O/^{18}O isotope effect, R is the ratio of the masses [18]O and [16]O in the sample (R_0 is the same ratio at time zero, before addition of enzyme), and f is the fractional conversion of substrate to product. This reaction procedure does not allow for measurement of [17]O/^{16}O isotope effects due to the presence of *ca.* 1% [13]C in the CO_2 product.

Direct Analysis of Oxygen from Reaction Solution. Prior to initiation of reaction, a small amount of N_2 was introduced to serve as a marker; since nitrogen is not consumed throughout the reaction, the ratio of O_2 to N_2 yields the percent conversion of oxygen to product [for use in eq 1]. The reaction was then sealed in the reaction chamber, followed by the transfer of a 15-mL aliquot to the vacuum line. This aliquot was sparged with He to degas and O_2 separated from CO_2 and H_2O with use of liquid N_2 traps. O_2 was trapped onto previously cleaned molecular sieve beads in a small sample tube connected to the vacuum line. A small volume of concentrated enzyme solution was then injected via syringe through a serum cap, and the oxygen from three more aliquots was trapped in a similar fashion. Oxygen samples were further cleaned and analyzed as previously described.[14–16] Each sample was analyzed for O_2 and N_2 content and the ratio of gases used

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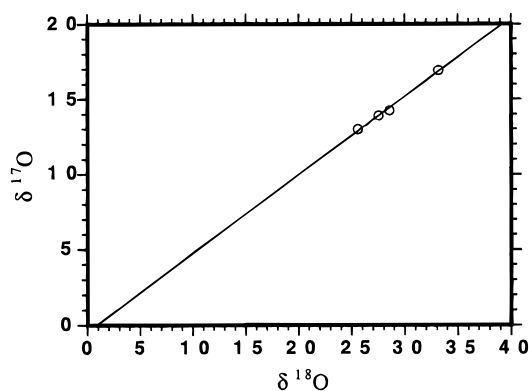(16) Thiemens, M. H.; Jackson, T. *Geophys. Res. Lett.* **1988**, *15*, 639–642.

Figure 1. Three-isotope plot for the reaction of lipoxygenase with molecular oxygen at 25 °C. Enrichment factors for ^{17}O and ^{18}O in residual oxygen as a function of product formation are plotted against each other. The slope is 0.52 ± 0.03. Initial conditions are 1 mM natural abundance O_2 and about 1.3 mM linoleic acid. Relative isotopic ratios of starting oxygen are $\delta^{17}O = 13.00 \pm 0.06$ and $\delta^{18}O = 25.57 \pm 0.04$. The errors associated with $\delta^{17}O$ and $\delta^{18}O$ are determined by replicate analysis.

to determine the extent of reaction. The isotopic composition of oxygen was determined by mass spectral analysis. Typical errors associated with these mass spectrometric analyses are 0.04−0.06‰ for $^{17}\delta$ and $^{18}\delta$.

$^{16}O/^{18}O$ Isotope Effect as a Function of O_2 Concentration in Solution. The reaction proceeded as described above. Isotope effects were determined by comparing every two time points throughout the reaction, using the ratio of isotopes, R, in the first of the two as the ratio at time zero, R_0. The oxygen concentration is considered the mean between the two points as described,[17] by determining the partial pressure of O_2 (after converting to CO_2) and knowing the initial concentration to be 1 mM O_2. In Figure 2a the isotope effect is calculated by comparing the isotopic ratio at each time point to that at time zero, whereas in Figure 2b every two time points are compared to each other. This treatment produces more data points than experimental time points (since it compares isotopic ratios between every two time points and not just in comparison to initial time), thereby creating values that are not fully independent from each other. In calculating the average isotope effect we have only used the points in Figure 2a. Figure 2b is shown to emphasize the fact that the $^{16}O/^{18}O$ isotope effect is independent of oxygen concentration as the reaction progresses to lower oxygen concentration (initial conditions of saturating linoleic acid and O_2, without removal of product peroxide from the reaction solution).

Results and Discussion

The results of measuring both oxygen-17 and oxygen-18 isotope effects in the SBL-1 reaction are shown in Figure 1. Published data for oxygen-18 effects in other O_2 dependent enzymatic reactions have involved conversion of residual dioxygen to carbon dioxide prior to analysis by isotope ratio mass spectrometry.[4,5,17,18] The present data were obtained by[19,20] direct analysis of unreacted dioxygen, to circumvent the overlap of carbon-13 in carbon dioxide with the oxygen-17 label. In our study of the relationship between ^{17}O and ^{18}O isotope effects, the notation of delta values has been used:

$$\delta^{18}O(\%) = 1000 \, (^{18}R/^{18}R_{std} - 1) \qquad (2)$$

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where $^{18}R = ^{18}O/^{16}O$, for comparison to a standard ($^{18}R_{std}$, the ratio for standard mean ocean water or SMOW). In the case of ^{17}O, $^{17}R = ^{17}O/^{16}O$ and $^{17}R_{std}$ is also from SMOW. As illustrated in Figure 1, enrichment of both ^{17}O (y axis) and ^{18}O (x axis) occurred with increasing conversion of substrate to product (starting from isotopic ratios of $\delta^{17}O = 13.00 \pm 0.06$ and $\delta^{18}O = 25.57 \pm 0.04$). This demonstrates a discrimination between light and heavy isotope effects in the segment of the SBL-1 reaction that involves interaction of the substrate derived radical with dioxygen (described by the kinetic parameter k_{cat}/K_m for dioxygen, which includes steps from oxygen binding up to its first irreversible step).

The slope and sign of the line in Figure 1 allow direct analysis for mass dependent vs magnetic spin dependent processes. In the former case, the difference in reaction rate of two isotopes stems primarily from the difference in zero point vibrational energy between isotopes in the initial and final states.[19] The vibrational frequency is dependent on the reduced mass such that:

$$\nu_1/\nu_2 = (\mu_2/\mu_1)^{1/2} \qquad (3)$$

where ν is the vibrational frequency for the light (1) and heavy (2) isotopes and μ is the reduced mass. For the purposes of this study, we are interested in a comparison of isotope effects for pairs of isotopes:[19]

$$r = \frac{\ln(k_1/k_3)}{\ln(k_1/k_2)} = [1 - (\mu_1/\mu_3)^{1/2}]/[1 - (\mu_1/\mu_2)^{1/2}] \qquad (4)$$

where k is the rate constant (or fractionation factor) for isotopes 1, 2, or 3. The significance of r is similar to the exponent in the familiar Swain−Schaad relationship used in a comparison of reaction rates for protium, deuterium and tritium.[20] In the case of reactions at oxygen ($m_1 = 16$, $m_2 = 17$, $m_3 = 18$), the simplified model presented in eq 3 predicts that r should be 1.92 for a mass dependent isotope effect.[21] By contrast, a magnetic isotopic fractionation would cause the nucleus with a non-zero spin (e.g., ^{17}O) to react faster than the nonmagnetic isotopes (^{16}O and ^{18}O). In this instance, the magnitude of k_1/k_2 would be inverse when $1 = ^{16}O$ and $2 = ^{17}O$, leading to a negative sign for r. For the data in Figure 1 we have used a different formalism from eq 4, relating instead the isotopic enrichment for one isotope (^{17}O) to that for the second isotope (^{18}O).[22] Idealized plots of the enrichment factors $\delta^{17}O$ vs $\delta^{18}O$ indicate a positive slope of $1/r = 0.52$ for the case of a purely mass dependent isotope effect and a negative slope for a purely magnetic dependent isotope effect (not shown). A reaction that contains contributions from both mass and magnetic origins would be expected to have a slope somewhere between the two extreme cases. The positive slope of the experimental data in Figure 1 indicates a significant mass dependence for the lipoxygenase reaction rate. Further, the magnitude of the experimentally observed slope in Figure 1 (0.52 ± 0.03) is identical with that predicted for a purely mass dependent isotope effect. Despite the expectation of a close to diffusion controlled reaction of dioxygen with lipoxygenase at room temperature, the origin of the oxygen isotope effect in this reaction arises purely from mass effects.

The data in Figure 1, when combined with fractional conversion data [cf. eq 1], lead to values for the individual isotope effects of $k_{16}/k_{18} = 1.0115 \,(\pm 0.0013)$ and $k_{16}/k_{17} = $

(21) A similar value for r results from more sophisticated models of heavy atom isotope effects at room temperature (Huskey, W. P. In *Enzyme Mechanism from Isotope Effects*, Cook, P. F., Ed.; CRC Press: Boca Raton, FL, 1991).
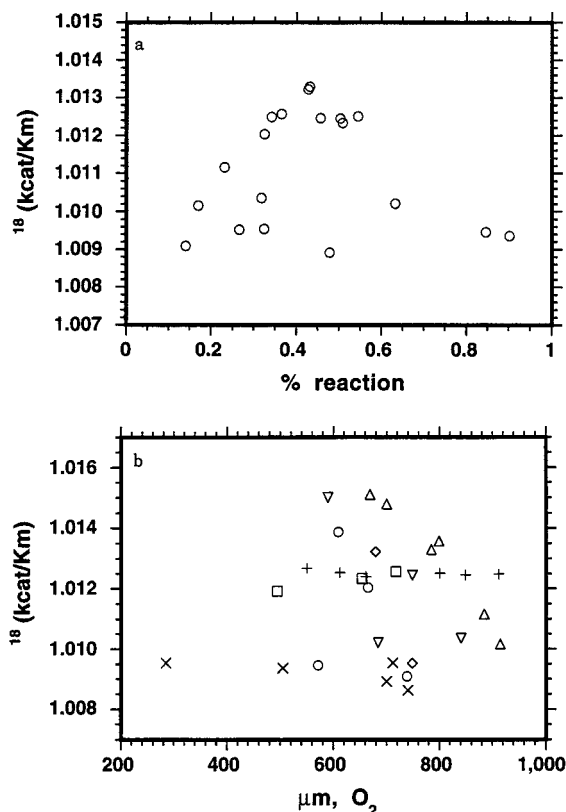
Figure 2. (a) $^{18}(k_{cat}/K_m)$ as a function of percent reaction. The $^{16}O/^{18}O$ at each time point was determined as described in refs 4 and 5. Six independent experiments are overlaid on the graph represented by the different symbols. Averaging all points produced an isotope effect of $^{18}(k_{cat}/K_m) = 1.0111\ (\pm 1.0016)$. (b) $^{18}(k_{cat}/K_m)$ as a function of mean oxygen concentration. Mean oxygen concentration was determined by comparing the O_2 concentration in every two time points throughout the reaction. Independent experiments are represented by the different symbols.

1.00549 (± 0.00092). These can be compared to k_{16}/k_{18} isotope effects determined by another procedure in which unreacted dioxygen is converted to carbon dioxide prior to analysis by isotope ratio mass spectrometry.[4] The magnitude of the isotope effect as measured in this fashion, $k_{16}/k_{18} = 1.0110\ (\pm 0.0016)$, is independent of either fractional conversion of oxygen to product (Figure 2a) or mean oxygen concentration (Figure 2b, initial conditions of 1 mM O_2, 1.3 mM linoleic acid, 25 °C, and pH 9).

The absence of a magnetic isotope effect, together with the magnitude of k_{16}/k_{18}, yields considerable insight into the chemical nature of the reaction of dioxygen with lipoxygenase. Two assumptions underlie our subsequent analysis of the observed oxygen isotope effects: first, that steps other than an initial diffusional encounter of O_2 with enzyme limit k_{cat}/K_m for oxygen and second, that addition of O_2 to the enzyme-bound substrate radical is reversible. The first assumption is supported by the absence of a magnetic oxygen isotope effect and the latter assumption derives from experiments of Nelson *et al.* indicating formation of $E-S^\bullet$ following anaerobic incubation of soybean lipoxygenase with product hydroperoxide.[23]

The most straightforward mechanism for SBL-1 involves rapid trapping of molecular oxygen by the substrate-bound radical, followed by reduction of the hydroperoxyl intermediate and loss of product hydroperoxide from the enzyme. By using the experimentally measured and calculated equilibrium ^{18}O isotope effects for formation of reduced oxygen species,[4] it is possible to predict the following values for the three component steps of SBL-1.
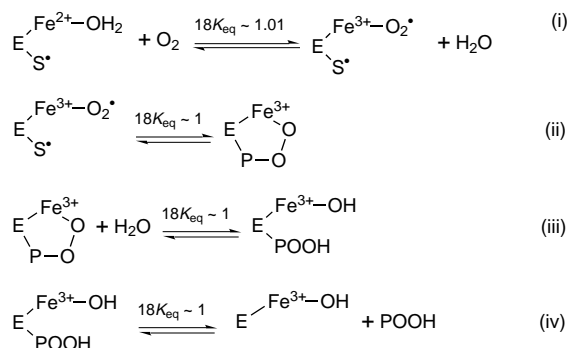
Mechanism I
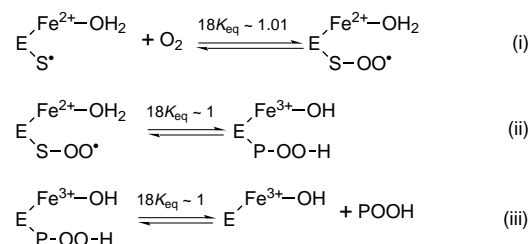
We note that the above mechanism involves the addition of dioxygen to the carbon (position 13) of the linoleic acid substrate, whereas previous data on ^{18}O isotope effects have focused on either the transfer of protons or the complex of metal ions to oxygen concomitant with its reduction. However, experimentally measured isotope effects, in which oxygen interacts with the iron sites of the reversible oxygen carriers hemoglobin, myoglobin, and hemerythrin, are remarkably close to the values calculated by using force fields for protonated intermediates of reduced oxygen.[4] Thus, we expect that changes in force constants at oxygen will parallel changes in bond order, being relatively insensitive to the nature of the atom attached to the reduced oxygen center.

In the case of mechanism I, the observed $^{18}(k_{cat}/K_m)$ arises from a reversible interaction of O_2 with the substrate radical, step (i), followed by two isotopically insensitive steps, steps (ii) and (iii). Clearly either step (ii) or step (iii) could be rate determining. The earlier observation of a small solvent isotope effect on k_{cat} for protonated substrate may implicate step (ii) as the rate determining step for the k_{cat}/K_m of oxygen. However, given the fact that substrate binding is 50% rate limiting for the reaction of linoleic acid with enzyme under the same conditions of pH and temperature as the present experiments, we consider it likely that step (iii) represents the kinetically limiting barrier. As shown in eq (ii) above, the conversion of the hydroperoxyl radical intermediate to hydroperoxide involves a transfer of a proton and electron from the $Fe^{2+}-OH_2$ to regenerate $Fe^{3+}-OH$, the resting, active form of enzyme. This step is mechanistically analogous to the first half reaction of SBL-1, in which a one proton, one electron reduction of $Fe^{3+}-OH$ to $Fe^{2+}-OH_2$ is postulated to be the driving force in substrate oxidation.

As an alternative to mechanism I, it is formally possible to write mechanism II, which involves an initial, reversible interaction of O_2 with the active site iron to form ferric superoxide as the first intermediate, followed by the formation of a metal bound hydroperoxide that then undergoes protonation and dissociation from enzyme.

Mechanism II

The predictions regarding ^{18}O isotope effects are similar to those from mechanism I, where the magnitude of the observed isotope

effect arises from an initial reaction with oxygen to form superoxide, which is followed by one of three possible rate determining steps: radical recombination of the substrate radical with superoxide, step (ii), proton transfer to the product hydroperoxide, step (iii), or dissociation of product from enzyme, step (iv). The reaction shown in step (ii) is unlikely to be rate determining. Analogous to mechanism I, the small, observed solvent isotope effect may implicate step (iii), whereas the partially rate determining binding of substrate under the conditions of these experiments would point toward step (iv) as the step controlling k_{cat}/K_m for oxygen.

In deciding between mechanism I and II, the chemical reactivity of E–S• vs E–Fe^{2+}–OH_2 toward dioxygen is expected to be the major determinant. Even if Fe^{2+} constitutes a formal site for dioxygen binding, the likelihood of a further one-electron transfer from iron to oxygen to form a ferrous superoxide complex appears unlikely, given the high reductive half potential estimated for the active site iron of SBL-1 [*ca.* 0.6 V[24]]. Further comparison of the bond dissociation energy for free linoleic acid [*ca.* 80 kcal/mol[25]] to the reductive half potential for the active site iron [*ca.* 0.6 V (14 kcal/mol in the direction of Fe(II) oxidation)[24]] implicates the substrate radical as the far more likely site of initial reactivity with molecular oxygen (mechanism I). The apparent reversibility of the chemical step in the half reaction involving O_2,[23,26] which contrasts with the step involving hydrogen abstraction from linoleic acid,[1] indicates preferential stabilization of the substrate radical complex in relation to subsequent oxygenated intermediates. Cleavage of P–OOH may also be assisted by the relatively low concentration (0.25 mM) of dissolved oxygen.

The results described herein suggest a number of future studies with SBL-1. In light of the strong evidence for radical intermediates in the various stages of the SBL-1 reaction, it is perhaps surprising that no evidence was found previously for a dependence of k_{cat} on external magnetic field.[7] This may be the result of the complexity of the k_{cat} parameter for SBL-1 at room temperature, which reflects *both* the formation of the substrate radical and its subsequent reaction with molecular oxygen. Reexamination of the effect of external magnetic field under conditions where a single step has been isolated kinetically could yield interesting results. In attempting to differentiate between steps (ii) and (iii) in mechanism I as the rate limiting step, it would be of interest to examine the effects of solvent viscosgens on the k_{cat}/K_m for oxygen. Similarly, a detailed investigation of pH and solvent D_2O effects on k_{cat}/K_m for oxygen may allow further mechanistic distinctions to be made regarding the steps leading from the enzyme-bound substrate radical to product hydroperoxide.

In conclusion, we have demonstrated a relationship between ^{17}O and ^{18}O isotope effects in the SBL-1 reaction that implicates a purely mass dependent process for oxygen binding and reaction. Despite an initial oxygen reactivity that is likely to be close to the diffusion limit, magnetic spin effects are not a factor for SBL-1. We note that the mass spectrometric technique used for this study is sufficiently sensitive to determine $^{16}O/^{17}O$ isotope effects using natural abundance ^{17}O, thereby introducing a new probe for study of the mechanism of the interaction of dioxygen with the range of known oxygen utilizing enzymes. Additionally, this technique validates indirect oxygen determinations via the conversion of dioxygen to carbon dioxide and the analysis of isotope ratios in CO_2, as used in earlier studies of enzyme reactions.[4,5] Future detection of magnetic isotope effects in dioxygen dependent biological reactions appears most probable for systems in which reaction between enzyme and oxygen is both near the diffusive limit and operationally irreversible. This latter property would obviate possible rate limitation by step(s) downstream from the initial oxygen activation step, which can obscure a magnetic isotope effect.

(22) The delta formalism is common in atmospheric chemistry and geochemistry where the time of initiation of reaction and, hence, fractional conversion of reaction are unknown. This is a particularly useful way to represent multiple heavy atom isotope effects, since delta values (eq 2 in text) bypass the use of logarithmic values for numbers close to unity.

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