Unusually Large Deuterium Isotope Effect in Soybean Lipoxygenase Is Not Caused by a Magnetic Isotope Effect

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Herein, we report that soybean lipoxygenase (EC 1.13.11.12) exhibits a large deuterium kinetic isotope effect on the enzyme-catalyzed oxygenation of the 1,4-diene moiety in [11,11-$^2H_2$]-linoleate.[1,2] At 24.2 °C, we observe $^{D}V_{max} = 36 \pm 3$ and $^{D}(V_{max}/K_m) = 28 \pm 2$. This is one of the largest kinetic isotope effects known for an enzymatic reaction,[3] and it is difficult to explain simply by a difference in ^{1}H and ^{2}H zero-point energies. In this report, we have eliminated the difference in nuclear magnetic moment between ^{1}H and ^{2}H as a cause of the large isotope effect.

A previous report estimated $^{D}V_{max} = 8.7 \pm 0.4$ and $^{D}(V_{max}/K_m) = 7.6$ at 25 °C and pH 9.0, as determined by comparing the kinetic parameters with unlabeled linoleate and [11,11-$^2H_2$]-linoleate of 95% dideuterium isotopic purity.[4] We have repeated this measurement with [11,11-$^2H_2$]linoleate of 99.4% dideuterium isotopic purity[5] and found the kinetic isotope effect to be considerably larger. In a direct comparison of the kinetic parameters determined by spectrophotometrically following (13*S*)-hydroperoxy-9(*Z*),11(*E*)-octadecadienoic acid product at 234 nm,[6] $^{D}V_{max} = 36 \pm 3$ and $^{D}(V_{max}/K_m) = 28 \pm 2$.

To test for the presence of a potent inhibitor in the deuterated linoleate, assays with unlabeled linoleate were doped with increasing amounts of [11,11-$^2H_2$]linoleate. No significant decrease in rate was observed until the amount of deuterated linoleate added was greater than 10%.

In order to quantify the kinetic isotope effect obtained by deuterated and unlabeled substrate competing in the same assay, the isotopic composition of residual substrate was determined by mass spectrometry.[7] Under internal competition conditions, $^{D}(V_{max}/K_m) = 28 \pm 9$ at 24.2 °C.[8] The large standard error is due to the inherent unsuitability of determining large kinetic isotope effects by following residual substrate.[9] The only interpretation possible from this measurement is that $^{D}(V_{max}/K_m)$ is indeed large and greater than 19.

There are at least four general explanations for a deuterium kinetic isotope effect that exceeds the maximum value of about 7–9 for mass kinetic isotope effects: (1) a "magnetic" kinetic isotope effect on C–^{1}H and C–^{2}H bond homolysis and radical-pair recombination due to a difference in nuclear magnetic moment between ^{1}H and ^{2}H;[10,11] (2) quantum mechanical tunneling of ^{1}H relative to ^{2}H;[12] (3) branching to an alternate reaction pathway;[13] and (4) multiplicative isotope effects on synchronous (coupled) bond scission at multiple isotopically labeled sites.

To address the first possibility of a magnetic isotope effect, we have determined the magnetic field dependence of the lipoxygenase reaction. A magnetic isotope effect can only be a factor in radical-pair or biradical reactions.[10] The nuclear magnetic moment of the relevant hydrogen isotopes are for ^{1}H, $\mu_N = 2.79$, and for ^{2}H, $\mu_N = 0.86$ (in units of the nuclear magneton, 5.05×10^{-27} JT^{-1}). In the early moments following C–H bond homolysis, intersystem crossing (ISC) due to hyperfine interactions in the C· ·H radical pair will populate the three triplet spin states ($T_{0,\pm1}$) equally in the absence of a magnetic field. As the applied magnetic field is increased, the $T_{\pm1}$ levels are no longer degenerate with the singlet state (S_0), and ISC is decreased. The increased singlet population will enhance nonproductive radical-pair recombination. The greater nuclear magnetic moment of ^{1}H in the homolysis of a C–^{1}H bond will lead to *less* nonproductive radical-pair recombination (relative to homolysis of a C–^{2}H bond) and result in a greater isotopic discrimination against ^{2}H. A further increase in the overall observed kinetic isotope effect may result: any magnetic spin-induced isotope effect will be multiplicative with a mass isotope effect such that the product of a mass isotope effect of ≈9 and a magnetic spin enhancement of ≈4 could lead to an observed kinetic isotope effect in excess of 30.

Plausible mechanisms with radical intermediates have been proposed for lipoxygenase.[14,15] Furthermore, magnetic field

(1) A preliminary report of this work was presented at the 203rd National Meeting of the American Chemical Society, San Francisco, CA, March, 1992.

(2) For an independent report of the large deuterium isotope effect, see: Glickman, M.; Wiseman, J. S.; Klinman, J. P., *J. Am. Chem. Soc.*, preceding paper in this issue.

(3) For another report of a large isotope effect in an enzymatic reaction, see: Reinsch, J.; Katz, A.; Wean, J.; Aprahamian, J.; McFarland, J. T. *J. Biol. Chem.* **1980**, *255*, 9093–9097.

(4) Egmond, M. R.; Veldink, G. A.; Vliegenthart, J. F. G.; Goldingh, J. *Biochem. Biophys. Res. Commun.* **1973**, *54*, 1178–1184.

(5) [11,11-$^2H_2$]Linoleic acid was synthesized by the procedure of Tucker et al: Tucker, W. P.; Tove, S. B.; Kepler, C. R. *J. Labelled Compd.* **1971**, *7*, 11–15. Isotopic enrichment was 99.4% dideuterated as determined by GC–MS at 70 eV.

(6) Soybean lipoxygenase was obtained from Sigma Chemical Co. as the $(NH_4)_2SO_4$ precipitate and desalted by dialysis and gel permeation chromatography on Sephadex G-10 in 0.1 M NaOAc, pH 5.6, and 10% glucose. SDS–polyacrylamide gel electrophoresis showed the enzyme to be greater than 80% pure, with a minor contaminating band of higher electrophoretic mobility. Deuterated and unlabeled linoleic acid was purified by C-18 HPLC (CH_3CN, 60% in H_2O initially, followed by a linear gradient to 100% CH_3CN), collected anaerobically, and stored at −20 °C in the dark. Assay conditions were 24.2 °C, 100 mM Ches (2-[*N*-cyclohexylamino] ethanesulfonic acid) or borate (as indicated), pH 9.00, and 1-cm-pathlength cuvette.

(7) Unlabeled and [11,11-$^2H_2$]linoleate were mixed to give a 80:20 mixture. The isotopic mixture was incubated with enzyme in 100 mM borate, pH 9.00, at a final concentration of 48.1 μM total linoleate and quenched at 32% reaction by the addition of 2 N HCl. Linoleic acid was extracted 3 times with ethyl ether, and the solvent was removed by rotary evaporation. The resulting isotopic mixture of linoleic acid was purified by HPLC as described in ref 6 above. Isotopic analysis was performed by GC–MS at 70 eV at a probe temperature of 70 °C. The intensity at 282 amu ($m + 2$ relative to the *unlabeled* linoleic acid at $m = 280$ amu) was taken as a measure of dideuterium incorporation. The contribution to the $m + 1$ and $m + 2$ (and $m + 3$ and $m + 4$ for dideuterated linoleic acid) peaks from naturally-occurring ^{13}C was accounted for by measuring the intensities of these peaks in the unlabeled and dideuterated linoleic acid prior to mixing. As a test of ^{1}H/^{2}H isotopic ratio quantification by mass spectrometry, samples containing known amounts of unlabeled and dideuterated linoleic acid were combined and the isotopic ratio was determined by mass spectrometry. A linear relationship of the known isotopic composition to the mass-spectrometrically measured isotopic composition was observed in the range from 0 to 60% dideuterated linoleic acid.

(8) Isotopic data and the method of calculating the kinetic isotope effect from residual substrate are given in supplementary material.

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(15) For evidence of radical intermediates, see: (a) Nelson, M. J.; Cowling, R. A. *J. Am. Chem. Soc.* **1990**, *112*, 2820–2821. (b) Funk, M. O.; Carroll, R. T.; Thompson, J. F.; Sands, R. H.; Dunham, W. R. *J. Am. Chem. Soc.* **1990**, *112*, 5375–5376. (c) Van der Zee, J.; Eling, T. E.; Mason, R. P. *Biochemistry*, **1989**, *28*, 8363–8367. (d) Nelson, M. J.; Seitz, S. P.; Cowling, R. A. *Biochemistry* **1990**, *29*, 6897–6903. (e) Cucurou, C.; Battioni, J. P.; Thang, D. C.; Nam, N. H.; Mansuy, D. *Biochemistry* **1991**, *30*, 8964–8970.

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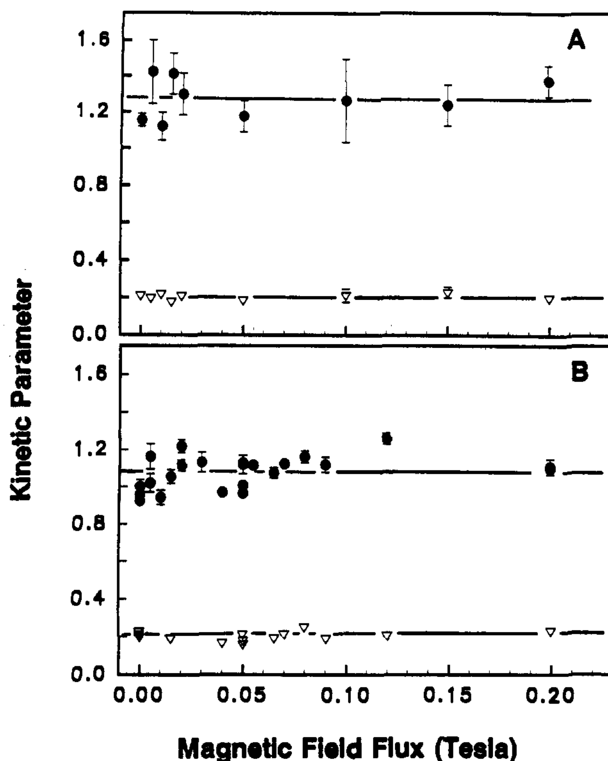

Figure 1. Magnetic field dependence of lipoxygenase kinetic parameters with linoleate as substrate. Assay conditions: 100 mM Ches, pH 9.00, 24.2 °C. Each point represents the indicated kinetic parameter obtained from fitting the substrate saturation isotherm to $d[P]/dt = V_{max}[S]/K_m + [S]$ by nonlinear methods: ● = V_{max} in μM min^{-1}; ∇ = V_{max}/K_m in min^{-1}. The standard error for data points without plotted error bars is smaller than the symbol. (A) Unlabeled linoleate: [lipoxygenase] = 0.09 nM in the assay, and each data point is the average of at least two separate experiments. (B) Deuterated linoleate: [lipoxygenase] = 2.7 nM in the assay.

effects on radical-based enzymatic reactions (including lipoxygenase) have been proposed but not demonstrated experimentally.[16] Recently, our research group has shown the first magnetic field effect on an enzymatic reaction that contains a radical pair (adenosylcobalamin-dependent ethanolamine ammonia lyase).[17] To investigate whether the large isotope effect in lipoxygenase is the result of a magnetic isotope effect on a kinetically significant radical pair in the enzymatic reaction, we have determined V_{max}, V_{max}/K_m, $^DV_{max}$, and $^D(V_{max}/K_m)$ each as a function of magnetic flux density.

Figure 1 shows the absence of any magnetic field dependence of the kinetic parameters V_{max} and V_{max}/K_m as determined with unlabeled and deuterated linoleate.[18] Daily variations in enzymatic activity were corrected by normalizing all rates in a standard assay without an applied magnetic field. Figure 2 shows the absence of any magnetic field dependence to the isotope effects $^DV_{max}$ and $^D(V_{max}/K_m)$. The data in Figure 2 were obtained by completing kinetic isotherms with unlabeled and deuterated linoleate as quickly as possible at the indicated magnetic flux density. Since the data in Figure 2 are ratios, no normalization to a standard rate was required. In an experiment that produced data similar to those shown in Figure 1A, no magnetic field dependence was observed to the lipoxygenase oxygenation of arachidonic acid either (data obtained at pH 10.00 are presented in the supplementary material).

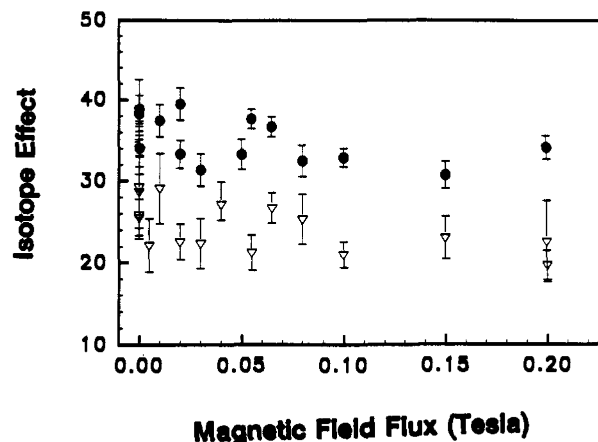

Figure 2. Magnetic field dependence of the deuterium kinetic isotope effects: ● = $^DV_{max}$; ∇ = $^D(V_{max}/K_m)$.

Since the size of the deuterium isotope effect does not change with applied magnetic field in the range 0–0.20 T, the unusually large kinetic isotope effect cannot be due to differences in the nuclear magnetic moment of ^1H and ^2H. We have also demonstrated conclusively that the kinetic parameters of lipoxygenase are not influenced by a magnetic field below 0.2 T.[16a] However, lipoxygenase may still catalyze the oxygenation of dienes by a radical mechanism. If the radical-pair lifetime is outside of the optimal window of about 10^{-10}–10^{-6} s for ISC or if electron spin relaxation by the proximal non-heme iron is dominate, then no magnetic field dependence of the kinetic parameters will be observed. If either of these conditions exists, the unusually large isotope effect still could not be ascribed to the difference in nuclear magnetic moments between ^1H and ^2H.

In summary, the unusually large deuterium kinetic isotope effect of $^DV_{max} = 36 \pm 3$ and $^D(V_{max}/K_m) = 28 \pm 2$ is unassailable. Our observations and the independent report of Glickman, Wiseman, and Klinman[2] show the large isotope effect to be independent of enzyme, substrate source, laboratory, or assay method. We have shown that magnetic spin interactions appear to be unimportant in producing the large isotope effect. A mechanism to account adequately for this large effect awaits further investigation.

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Supplementary Material Available: Isotopic data and calculations for determining the deuterium isotope effect by mass spectrometry; V_{max} and V_{max}/K_m vs magnetic field with arachidonate as substrate (2 pages). This material is contained in many libraries on microfiche, immediately follows this article in the microfilm version of the journal, and can be ordered from the ACS; see any current masthead page for ordering information.

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(18) Kinetic measurements of enzymatic activity were performed as described in ref 6. In order to follow spectrophotometrically the rate of product formation as a function of magnetic field, a Beckman DU monochromator with solid-state electronics was retrofitted with an electromagnet. The cell holder was a thermostated brass block containing a Hall probe to measure the magnetic field flux. The magnetic field was homogeneous to ±2% within the area of the cuvette, and the long-term stability was better than 0.1%. The photomultiplier tube was shielded from the applied magnetic field with μ metal and low-carbon, high-iron steel.