Extremely Large Isotope Effects in the Soybean Lipoxygenase–Linoleic Acid Reaction§

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Kinetic isotope effects have been used extensively in the study of enzyme mechanisms. Considering only zero-point energy differences, a fully expressed primary kinetic (k_H/k_D) isotope effect is expected to have an upper limit of 7–10 at 25 °C.[1] For enzymatic reactions, there are only a few published results slightly exceeding this limit.[2] These results are attributed mainly to tunneling. We report here a very large and unique (k_H/k_D) isotope effect for the oxidation of linoleic acid by soybean lipoxygenase.

Soybean lipoxygenase (SBL) catalyzes the stereospecific oxidation of 9,12-(*Z*,*Z*)-octadecadienoic acid (LA) to 13-hydroperoxy-9,11-(*Z*,*E*)-octadecadienoic acid.[3] Even though the precise mechanism of SBL is not yet known, it is widely accepted that a hydrogen abstraction from C-11 gives rise to a pentadienyl radical, which is then trapped by dioxygen.[4]

Competitive primary (k_H/k_D) isotope effects were obtained by measuring the discrimination between protonated and deuterated substrates, reacting with SBL.[5] Two remarkable results are shown (Figure 1A). The magnitude of $^D(V/K)$ is dependent on the initial substrate concentration and reaches an upper limit greater than 60.[9] Since competitive experiments give V/K isotope effects,[10] which are expected to be concentration independent, these data indicate an additional effect of substrate which alters the mechanism. One possible interpretation would be a "large" intrinsic isotope effect and a substrate-dependent commitment, possibly due to multiple forms of substrate arising from premicellar aggregates or a substrate-dependent isomerization of enzyme. We also cannot rule out a real change in mechanism in which the chemistry of the hydrogen abstraction has been altered. Interestingly, $^D(V/K)$ does not change as substrate is converted to product (Figure 1B), hinting that the critical concentration is substrate plus product. It has been observed that both substrate and product have a complex effect on the kinetics of SBL.[11]

Noncompetitive deuterium isotope effects were measured with a different set of isotopically labeled substrates.[12] The values obtained for $^D(V/K)$ (low substrate conditions) at 0 °C are similar for competitive and noncompetitive experiments (9 ± 1, 6 ± 2) and for published results obtained at 0 °C (8.0)[13a] and 25 °C (8.2 ± 1.4).[13b] However, at 35 °C, the noncompetitive isotope effect increases to the anomalous values of $^D(V/K)$ = 57 ± 5, and $^D(V)$

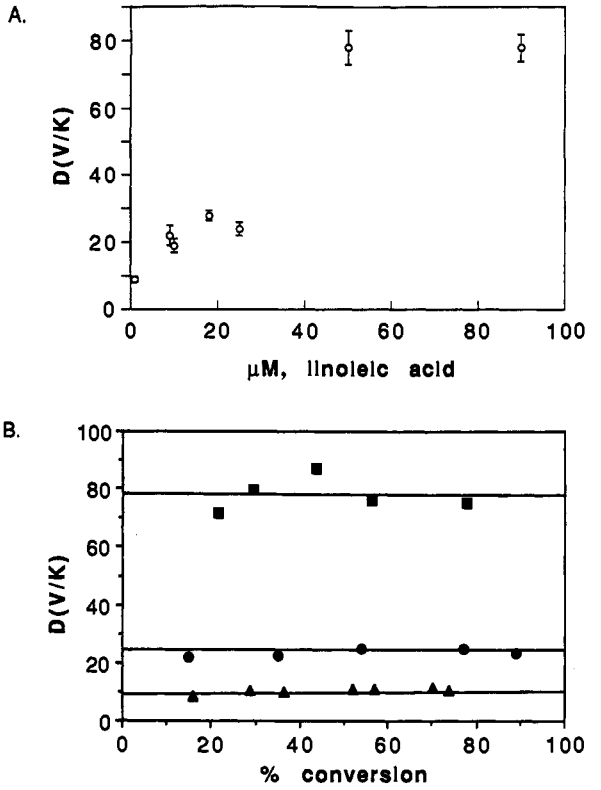

Figure 1. (A) Competitive (k_H/k_D) isotope effect as a function of total initial substrate concentration. Data were collected at pH 9, 0 °C, air saturation of O_2. A total of 3–6 experiments were performed at 1, 9, 18, 50, and 90 μM LA. Two determinations were done at 10 and 25 μM LA. (B) Independence of the competitive (k_H/k_D) isotope effect on percent conversion of substrate to product at three levels of initial linoleic acid concentration: ▲, 1 μM LA; ●, 25 μM; ■, 90 μM. Each line represents different time points taken from a single experiment.

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§ Abbreviations: LA, linoleic acid; SBL, soybean lipoxygenase; *V*, maximal enzyme velocity at saturating substrate; *V*/*K*, enzyme velocity as substrate concentration nears zero; KIE, kinetic isotope effect; $^D(V)$, deuterium isotope effect on V; $^D(V/K)$, deuterium isotope effect on *V*/*K*.

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(5) The procedure is similar to that described in ref 8. Reactions were all in borate buffer (0.1 M, pH = 9) and equilibrated on ice. Trace amounts of [11,11-$^2H_2$,9,10,12,13-^{3}H]LA (synthesized[6]) and [1-^{14}C]LA (NEN, 99%) were mixed with LA (Sigma) to different total concentrations. After addition of SBL (purified[7]), the reaction was quenched at different time points. Aliquots were separated on a Beckman 332 HPLC on a C_{18} column. Two major compounds, corresponding to substrate and product, were detected using on-line spectrophotometric and radiochemical detectors. After correction for background, over 95% of C-14 and tritium were accounted for in substrate and product peaks. There was a small and consistent difference between the tritiated substrate (at t = 0) and tritiated product (at t = ∞), representing a small, nonenzymatically active contaminant in the starting material. This was subtracted from all time points. Pooling of the radiolabeled peaks was made sufficiently large to circumvent any differential fractionation of C-14 and tritium on the column. Radioactivity in product and remaining substrate was measured on an LKB 1029 liquid scintillation counter. The isotope effect was calculated from the expression $^D(V/K) = \ln(1-f)/\ln((1-f)R/R_0)$.[14a] Secondary isotope effects were measured in an identical fashion, using [9,10,12,13-^{3}H]LA and [1-^{14}C]LA. The former was synthesized using the same synthetic protocol as that for [11,11-$^2H_2$,9,10,12,13-^{3}H]LA,[6] thereby serving as a control for the synthetic procedure.

(6) Synthesized according to: Tucker, W. P.; Tove, S. B.; Kepler, C. R. *J. Labelled Compd.* **1970**, *7*, 11–15. Compound purity was determined as 97% and isomeric purity as 95%.

(7) Purified from soybeans (gift of Pioneer Hi-bred International) according to: Axelrod, B.; Cheesbrough, T. M.; Laakso, S. *Methods Enzymol.* **1981**, *71*, 441–451.

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(9) Since dideuterated substrates were used, $^D(V/K)$ reflects both primary and α-secondary effects. However, the latter effects are expected to be quite small, *ca.* 20%.[10]

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(12) Reaction rates were determined for different concentrations of LA, [$^2H_{31}$]LA (Cambridge Isotope Labs, 98%), and [11,11-$^2H_2$]LA (gift from Ron P. Potman, Unilever Research Labs, Vlaardingen Nederland; original isotopic purity of 95%) by monitoring the change in absorbance at 234 nm on a Cary 118 spectrophotometer after addition of SBL. Temperature control was achieved with a water-jacketed cuvette holder. Isotope effects were calculated from the values of V and V/K obtained for each substrate. Prior to use, all three substrates were repurified in our lab by HPLC on C_{18} columns.

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Table 1. Comparison of Competitive and Noncompetitive Isotope Effects in the SBL Reaction[a]

substrate pair	reaction conditions	primary KIE	secondary KIE[b]
[1-^{14}C]LA vs [9,10,12,13-^{3}H,11,11-$^{2}H_2$]LA,	competitive, 0 °C (low substrate)	$^{D}(V/K) = 9 \pm 1$	
[1-^{14}C]LA vs [9,10,12,13-^{3}H]LA,	competitive, 0 °C		$^{T}(V/K) = 1.16 \pm 0.04$
LA vs [$^{2}H_{31}$]LA,	noncompetitive, 0 °C	$^{D}(V/K) = 6 \pm 2$ $^{D}(V) = 17 \pm 2$	
LA vs [$^{2}H_{31}$]LA,	noncompetitive, 35 °C	$^{D}(V/K) = 65 \pm 9$ $^{D}(V) = 57 \pm 5$	
LA vs [$^{2}H_{31}$]LA,	noncompetitive, 25 °C	$^{D}(V/K) = 30.5 \pm 6.5$ $^{D}(V) = 47.6 \pm 1.9$	
LA vs [11,11-$^{2}H_2$]LA,	noncompetitive, 25 °C	$^{D}(V/K) = 27.4 \pm 3.8$ $^{D}(V) = 43.4 \pm 1.9$	
[11,11-$^{2}H_2$]LA vs [$^{2}H_{31}$]LA	noncompetitive, 25 °C		$^{D}(V/K) = 1.13 \pm 0.36$ $^{D}(V) = 1.10 \pm 0.03$

[a] Experimental procedures are explained in refs 5 and 12. [b] Secondary isotope effects are β or γ (C-9,10,12,13), not α (C-11).

= 65 ± 9 (Table 1). This is especially surprising since isotope effects usually decrease with an increase in temperature.[14b]

Secondary isotope effects were measured in both competitive and noncompetitive fashions (Table 1). The competitive result, $^{T}(V/K)_{sec} = 1.16 \pm 0.04$), is independent of substrate concentration or reaction progress, normal in sign and magnitude, and identical to the published value.[8] Normal secondary (k_H/k_D) isotope effects are also observed noncompetitively using alternately labeled substrates. As expected, $^{T}(V/K)_{sec} > {}^{D}(V/K)_{sec}$. These secondary results prove that the large primary isotope effects observed are not a result of additive effects due to the multiple isotopic labels in both types of experiments. In addition, a secondary isotope effect greater than unity at a vinylic carbon is consistent with the formation of an allylic radical.[15a,b]

In respect to the noncompetitive experiments, several controls were performed. Incubation of SBL with [$^{2}H_{31}$]LA prior to use in experiments with LA showed no decrease in activity. The rate of reaction for a 50:50 mixture of protio- and perdeuterio-labeled LA was exactly as calculated for protio-LA at half this total concentration, ruling out any inhibition due to a contaminant in [$^{2}H_{31}$]LA. Additionally, rate was observed to be linear in enzyme concentration. Overall, the observed isotope effects are independent of substrate source, synthetic procedure, or assay method.

Isotope effects on the order of magnitude of 60 seem to be the largest published primary (k_H/k_D) isotope effects in an enzymatic system. This result is as large as or larger than reported k_H/k_D effects in model systems at room temperature.[16] Because of the complexity of enzymatic reactions, isotope effects on single steps are often not fully expressed, resulting in smaller observed isotope effects than in the corresponding model systems. Large primary deuterium isotope effects in this same system, SBL and LA, have been observed independently by Grissom and Hwang.[17] The behavior of the isotope effect seems to indicate a uniquely large intrinsic isotope effect on the C–H cleavage step and a commitment sensitive to external factors such as substrate concentration and temperature. This implies that the C–H cleavage is not fully rate limiting at all conditions monitored. In future studies, we will investigate other parameters that affect the expression of the intrinsic isotope effect in this system. Reaction branching as a cause of the large isotope effect[18] can be ruled out since only two peaks corresponding to substrate and product are seen on the HPLC traces. One possible explanation for such a large isotope effect is hydrogen tunneling. This possibility will be pursued by a number of means including detailed analysis of the relationship between deuterium/tritium and protium/tritium isotope effects and their temperature dependencies. In addition, there are other circumstances that could lead to unusual isotope effects; very large isotope effects have been reported for formation of an iron–hydrogen bond[19] and also in the radical abstraction of hydrogen by a peroxide radical.[20] A linoleate-derived radical has also been implicated in an unusually large primary (k_H/k_D) isotope effect in the oxidation of cholesterol by SBL and LA.[21]

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