Large Competitive Kinetic Isotope Effects in Human 15-Lipoxygenase Catalysis Measured by a Novel HPLC Method

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Human lipoxygenases are an increasingly important area of study because of their implications in a number of inflammatory diseases[1,2] and cancer growth regulation.[3,4] The generally accepted mechanism for the lipoxygenases involves a H-atom abstraction at C-3 of the 1,4-diene in the substrate,[5] with subsequent trapping of the pentadienyl radical by oxygen, forming the hydroperoxide product. [6−8] Hydrogen atom abstraction for soybean lipoxygenase (SLO-1) is the rate-determining step (RDS) above 32 °C and proceeds through a quantum-mechanical tunneling pathway. [9−11] In the current paper, we report a large, temperature independent (above 30 °C) kinetic isotope effect (KIE) ($^{D}[k_{cat}/K_m] = 47 \pm 7$) for human 15-lipoxygenase (15-HLO) indicating that the H-atom abstraction step for 15-HLO also proceeds through a quantum-mechanical tunneling pathway. At low temperature and low substrate concentration, 15-HLO displays a temperature-dependent $^{D}[k_{cat}/K_m]$, indicative of multiple rate-limiting processes under these conditions, despite the fact that its k_{cat} is 45-fold less than that of SLO-1.

The $^{D}[k_{cat}/K_m]$ measurements presented here were possible due to a novel method which determines the competitive primary $^{D}[k_{cat}/K_m]$ isotope effect of 15-HLO by measuring the relative amounts of the protonated and per-deuterated product. Critical to this technique was our discovery of conditions under which RP-HPLC achieves baseline separation of the per-deuterated 13-HPOD from the protonated 13-HPOD (Figure 1).[12,13] A competitive method is essential for 15-HLO because the enzyme displays both an initial lag phase (13-HPOD activation)[14] and auto-inactivation[15] which make KIE measurements by noncompetitive methods extremely difficult.

The results of the temperature dependence of the KIE for both 15-HLO and SLO-1 are shown in Figure 2. The remarkable feature is that the magnitude of $^{D}[k_{cat}/K_m]$ for 15-HLO at 5 μM LA (38 °C) is large (47 ± 7), comparable to that of SLO-1 (48 ± 5), and temperature independent at high temperatures (above 30 °C).[16] These results strongly suggest that C−H cleavage for 15-HLO is fully rate limiting above 30 °C and proceeds through a quantum-mechanical tunneling pathway. The $^{D}[k_{cat}/K_m]$ of 15-HLO at 5 μM LA is temperature dependent between 5 and 30 °C, indicative of partial rate limitation by steps other than C−H cleavage. Comparable temperature dependence results,

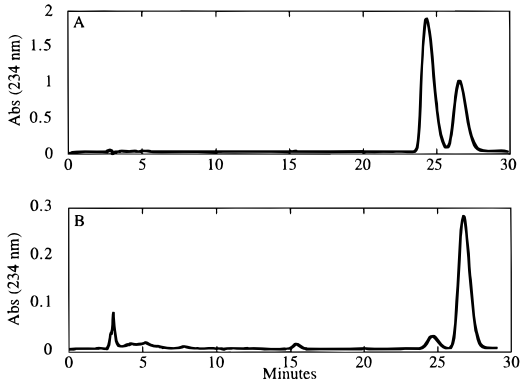

Figure 1. RP−HPLC traces. (A) A completion reaction which determines the 13-HPOD ratio (1.9:1, per-deutero to protio) and (B) a 5 mM, 20 °C, 15-HLO reaction with a $^{D}(k_{cat}/K_m) \approx 22$, quenched at less than 5% completion. The small peaks around 3 and 15 min are artifacts of the sample workup.

Scheme 1

$$E+LA \underset{k_{-1}}{\overset{k_1[LA]}{\rightleftharpoons}} E\bullet LA \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} E\bullet L^{\bullet} \underset{k_{-3}}{\overset{k_3[O_2]}{\rightleftharpoons}} E\bullet LOO^{\bullet} \underset{k_{-4}}{\overset{k_4}{\rightleftharpoons}} E\bullet LOOH \xrightarrow{k_5} E+P$$

using noncompetitive kinetics, were demonstrated previously for SLO-1,[10] which indicate that SLO-1 and 15-HLO may have a common mechanism even though their enzymatic rates are dramatically different (15-HLO, $k_{cat} = 6.2\ s^{-1}$, SLO-1, $k_{cat} = 280\ s^{-1}$).[10,17]

The kinetic mechanism proposed for SLO-1 can be minimally described by Scheme 1.[18] According to Scheme 1, $^{D}[k_{cat}/K_m]$ would be described by eq 1,

$$^{D}[k_{cat}/K_m] = (k_{cat}/K_m)^{H}/(k_{cat}/K_m)^{D} = (k_2^{H}/k_2^{D} + k_2^{H}/k_{-1}^{H})/(1 + k_2^{H}/k_{-1}^{H}) \quad (1)$$

where substrate release (k_{-1}) and C−H bond cleavage (k_2) are

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(1) Samuelsson, B.; Dahlen, S.-E.; Lindgren, J. A.; Rouzer, C. A.; Serhan, C. N. *Science* **1987**, *237*, 1171−1176.
(2) Sigal, E. *Am. J. Physiol.* **1991**, *260*, L13-L28.
(3) Rioux, N.; Castonguay, A. *Carcinogenesis* **1998**, *19*, 1393−1400.
(4) Nie, D.; Hillman, G. G.; Geddes, T.; Tang, K. Q.; Pierson, C.; Grignon, D. J.; Honn, K. V. *Cancer Res.* **1998**, *58*, 4047−4051.
(5) The natural substrate for 15-HLO is arachidonic acid and linoleic acid for SLO-1; however, both enzymes react with either substrate.
(6) Abbreviations: SLO-1, soybean lipoxygenase-1; 15-HLO, human 15-lipoxygenase; LA, linoleic acid, 9,12-(*Z*,*Z*)-octadecadienoic acid; OA, oleic acid; FA, fatty acid; 13-HPOD, 13-hydroperoxy-9,11-(*Z*,*E*)-octadecadienoic acid; RP-HPLC, reverse phase-HPLC; $^{D}[k_{cat}/K_m]$, $(k_{cat}/K_m)^{H}/(k_{cat}/K_m)^{D}$; RDS, rate-determining step; ES-MS, electrospray-mass spectroscopy.
(7) DeGroot, J. J. M. C.; Veldink, G. A.; Vliegenhart, J. F. G.; Boldingh, J.; Wever, R.; Van Gelder, B. F. *Biochim. Biophys. Acta.* **1975**, *377*, 71−79.
(8) Gardner, H. W. *Biochim. Biophys. Acta* **1989**, *1001*, 274−281.
(9) Glickman, M. H.; Wiseman, J. S.; Klinman, J. P. *J. Am. Chem. Soc.* **1994**, *116*, 793−794.
(10) Glickman, M. H.; Klinman, J. P. *Biochemistry* **1995**, *34*, 14077−14092.
(11) Hwang, C.-C.; Grissom, C. B. *J. Am. Chem. Soc.* **1994**, *116*, 795−796.

(12) The method involves reacting a protio/perdeutero LA mixture, of known molar ratio, with lipoxygenase in an appropriate buffer. The per-deuterated substrate (D_{32}-LA, 98% deuterated, Cambridge Isotope Labs) is enzymatically treated with SLO-1 to remove the trace protio substrate (H_1-D_{31}-LA). Both protio and depleted per-deutero substrates are RP-HPLC purified and stored in ethanol at −80 °C. The reaction is monitored at 234 nm with a P-E Lambda 4 and stopped with an acid quench at less than 5% total LA consumption. It is important to note that diode array spectrophotometers degrade 13-HPOD and are unsuitable for the experiment. The acidified reaction mixture is extracted with methylene chloride containing 5% (w/v) trimethyl phosphite which quantitatively reduces the labile 13-HPOD to an alcohol. (The reduction of the 13-HPOD is only necessary when comparing the RP-HPLC data with the ES-MS data.) The methylene chloride layer is evaporated to dryness under vacuum, reconstituted in 50 μL of methanol, injected onto a C18 column (Higgins Analytical, 5 mm, 250 × 4.6 mm), and eluted at 1 mL/min (isochratic mobil phase, 74.9% methanol:25% H_2O:0.1% acetic acid). The molar protio/per-deutero 13-HPOD ratios are equated to the corresponding peak area ratios and the competitive KIE, $^{D}[k_{cat}/K_m]$,[13] is then calculated as $([P - H]/[P - D])([S_o - D]/[S_o - H])$. This RP-HPLC-based method for determining molar protio/per-deutero 13-HPOD ratios gave results identical (within experimental error) to those obtained by ES-MS. We see no appreciable side products at any wavelength for 15-HLO or SLO-1, indicating no reaction branching as previously shown for SLO-1.[9,11] Both 15-HLO and SLO-1 were purified as previously described.[21,24]
(13) Melander, L.; Saunders: W. H. *Reaction Rates of Isotopic Molecules*; R. E. Krieger Publishing: Florida, 1987.
(14) Egmond, M. E.; Fasella, P. M.; Veldink, G. A.; Vliegenhart, J. F. G.; Boldingh, J. *Eur. J. Biochem.* **1977**, *76*, 469−479.
(15) Gan, Q. F.; Witkop, G. L.; Sloane, D. L.; Straub, K. M.; Sigal, E. *Biochemistry* **1995**, *34*, 7069−7079.
(16) The 15-HLO reactions were performed at pH 7 because the enzyme inactivates rapidly at elevated temperatures and pH.
(17) Gan, Q.-F.; Browner, M. F.; Sloane, D. L.; Sigal, E. *J. Biol. Chem.* **1996**, *271*, 25412−25418.
(18) Glickman, M. H.; Klinman, J. P. *Biochemistry* **1996**, *35*, 12882−12892.

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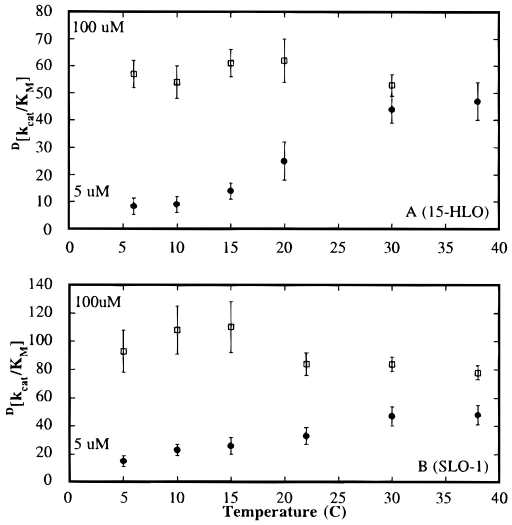

Figure 2. Variable temperature KIE experiments with (A) 15-HLO, (solid circles) 5 μM LA in 100 mM phosphate buffer (pH 7) and (open squares) 100 μM LA in 100 mM borate buffer (pH 9.2). Borate (pH 9.2) is required because LA is not soluble in phosphate buffer (pH 7) above ~50 μM. (B) SLO-1, (solid circles) 5 μM LA and (open squares) 100 μM LA, both in 100 μM borate buffer (pH 9.2).

the primary determinants for $^{D}[k_{cat}/K_m]$ (This assumes $k_2 = k_{cat}$ and the multiple steps indicated at low temperature are included in k_2). The $^{D}[k_{cat}/K_m]$ increases to a maximum of k_2^H/k_2^D when commitment (k_2^H/k_{-1}^H) is small and decreases, approaching 1, when commitment is large. This assumes that the intrinsic k_2^H/k_2^D remains unchanged. The reaction mechanism for 15-HLO is presumed to proceed through a comparable pathway; however, k_{cat} for 15-HLO is ~45-fold less than that of SLO-1. The abstraction rate is most likely reduced in 15-HLO because of a change in the redox potential of the iron. If H-atom abstraction is thought of as a two-step process, a loss of an electron and a proton, then the driving force can be related to the redox potential ($\Delta E°$) of the Fe^{3+}/Fe^{2+} couple and the pK_a of the $Fe^{2+}-OH_2$.[19] Recent MCD and EPR spectroscopic results on 15-HLO and a soybean coordination mutant, N694H SLO-1, suggest that the ligation change from Asn_{694} (SLO-1) to a stronger ligand, His_{544} (15-HLO), reduces the redox potential of the iron and subsequently lowers the rate of the abstraction.[20,21] Given the above kinetic scheme, a decrease in k_2 by 45-fold, as seen for 15-HLO, would establish it as the sole RDS. Nevertheless, this is not the case since the temperature dependence indicates that 15-HLO has multiple rate-limiting steps at low temperature and low substrate concentration. This is consistent with H-atom abstraction for 15-HLO actually consisting of several discrete steps, all of whose rates may be coupled to the reduction potential of the iron and thus lowered accordingly. Spectroscopic and kinetic studies are currently in progress to identify the nature of the multiple steps involved in the H-atom abstraction at low temperature and low substrate concentration.

Another striking characteristic of both 15-HLO and SLO-1 is that the temperature dependencies of their $^{D}[k_{cat}/K_m]$'s are highly influenced by substrate concentration, as seen by the increase of $^{D}[k_{cat}/K_m]$ at low temperatures and high LA concentration (100 μM) (Figure 2). At 100 μM LA, the $^{D}[k_{cat}/K_m]$ for 15-HLO remains at ~57, across the temperature range (5−30 °C) (Figure 2A), while the $^{D}[k_{cat}/K_m]$ for SLO-1 is above 80 from 5° to 40 °C (Figure 2B). These are the first published results demonstrating that substrate concentration affects the temperature dependence of the $^{D}[k_{cat}/K_m]$ for both SLO-1 and 15-HLO and indicates that C−H cleavage becomes rate limiting at high substrate concentration, regardless of temperature. A similar increase in $^{D}[k_{cat}/K_m]$ at low temperature is effected by the addition of oleic acid (OA), a competitive inhibitor,[22] for both SLO-1 and 15-HLO (Table 1).

Table 1. Variable Fatty Acid Concentration KIE Experiments of 15-HLO (100 mM Tris Buffer, 109 mM NaCl, pH 7.8, 5 °C) and SLO-1 (100 mM Borate Buffer, pH 9.2, 5 °C)[a]

	total FA (μM)			
	5	13	19	40
KIE-SLO-1				
LA (only)	19(6)	38(7)	46(1)	n/d[b]
LA(5 μM)+OA		28(1)	34(2)	43(7)
	total FA (μM)			
	5	10	15	30
KIE−15-HLO				
LA (only)	15(2)	22(2)	33(3)	n/d
LA(5 μM)+OA		30(4)	45(4)	65(2)

[a] All of the measurements with OA have 5 μM LA in order to measure the 13-HPOD ratios. [b] n/d: not determined.

This is significant with regard to eq 1 because, as a simple competitive inhibitor, OA would not affect k_2; however, we observe an increase in $^{D}[k_{cat}/K_m]$ with increased OA concentration, which suggests either a change in the H-atom tunneling (i.e., the intrinsic k_2^H/k_2^D) or the substrate release (k_{-1}).

Previously, Klinman and co-workers observed substrate concentration dependence for SLO-1 at a single temperature (0 °C) and proposed it was due to either premicellular substrate aggregates or a substrate-dependent protein conformational change.[9] To investigate these two hypotheses further, the reaction with both enzymes was carried out at high substrate concentration in D_2O buffer at varying temperatures. A solvent isotope effect (SIE, $^{D}[k_{cat}/K_m]^{H2O}/^{D}[k_{cat}/K_m]^{D2O}$) of 2 was observed for SLO-1 at 5 °C consistent with a H-bond dependent conformational change being partially rate limiting (Table 2). 15-HLO also demonstrates a SIE

Table 2. Variable Temperature KIE Experiments of 15-HLO and SLO-1 with 75 μM LA in 100 mM Borate Buffer (pH 9.2, pD 8.8)

	5 °C	20 °C	30 °C	38 °C
KIE-SLO-1				
H_2O	97 (11)	90 (4)	84 (11)	74 (9)
D_2O	55 (10)	70 (3)	76 (5)	69 (4)
KIE−15-HLO				
H_2O	42 (5)	n/d[a]	42 (4)	n/d
D_2O	31 (4)	n/d	42 (4)	n/d

[a] n/d: not determined.

at low temperature, albeit of a lower magnitude. The LA/OA concentration dependence and the SIE are consistent with a LA/OA concentration-dependent conformational change which may suggest the presence of an allosteric site for both SLO-1 and 15-HLO, as previously proposed for SLO-1.[23] A solvent isotope effect (SIE) was also seen for SLO-1 using noncompetitive methods ($^{D}[k_{cat}/K_m]^{H2O}/^{D}[k_{cat}/K_m]^{D2O} \approx 2.5$, 5 °C),[10] but it is unclear if it is due to the same enzymatic process as seen in this paper. Mutants of both the 15-HLO and SLO-1 are currently being prepared to investigate the factors regulating these effects further.

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(19) Gardner, K. A.; Mayer, J. M. *Science* **1995**, *269*, 1849−1851.
(20) Zhang, Y.; Gan, Q. F.; Pavel, E. G.; Sigal, E.; Solomon, E. I. *J. Am. Chem. Soc.* **1995**, *117*, 7422−7427.
(21) Holman, T. R.; Zhou, J.; Solomon, E. I. *J. Am. Chem. Soc.* **1998**, *120*, 12564−12572.
(22) Van der heijdt, L. M.; Schilstra, M. J.; Feiters, M. C.; Nolting, H. F.; Hermes, C.; Veldink, G. A.; Vliegenthart, J. F. G. *Eur. J. Biochem.* **1995**, *231*, 186−191.
(23) Wang, Z. X.; Killilea, S. D.; Srivastava, D. K. *Biochemistry* **1993**, *32*, 1500−1509.
(24) Sloane, D. L.; Leung, R.; Barnett, J.; Craik, C. S.; Sigal, E. *Protein Eng.* **1995**, *8*, 275−282.