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Kinetic isotope effects in the oxidation of arachidonic acid by soybean lipoxygenase-1

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ABSTRACT

The reaction of soybean lipoxygenase-1 with linoleic acid has been extensively studied and displays very large kinetic isotope effects. In this work, substrate and solvent kinetic isotope effects as well as the viscosity dependence of the oxidation of arachidonic acid were investigated. The hydrogen atom abstraction step was rate-determining at all temperatures, but was partially masked by a viscosity-dependent step at ambient temperatures. The observed KIEs on k_{cat} were large (~100 at 25 °C).

Lipoxygenases are non-heme iron-dependent proteins that catalyze the oxidation of polyunsaturated fatty acids to hydroperoxides.[1] These enzymes occur in plants and animals, and have also been detected in certain bacteria.[2,3] Lipoxygenase products are further elaborated by downstream enzymes to give a variety of physiological regulators and pain mediators. In plants, lipoxygenases convert fatty acids into jasmonates and aldehydes, which are involved in signaling, germination and senescence.[4] Soybean lipoxygenase-1 (sLO-1) exhibits high structural similarity with mammalian lipoxygenases despite only sharing 25% sequence identity.[5] sLO-1 acts on polyunsaturated fatty acids in which a 1,4-diene unit is located six carbons away from the methyl terminus (ω-6 fatty acids). Its natural substrate is linoleic acid (LA), a C18 bisunsaturated fatty acid, which it converts into 13-hydroperoxyoctadienoic acid (13-HPODE). sLO-1 can also catalyze the oxidation of arachidonic acid (AA), a C20 tetraunsaturated fatty acid found in animals, to 15-hydroperoxyeicosatetraenoic acid (15-HPETE).

Lipoxygenases abstract a hydrogen atom from the substrate to form a radical, which then reacts with molecular oxygen.[6,7] In resting lipoxygenase, the iron is in the ferrous form and the enzyme is inactive.[8] Before catalysis can occur, the iron must be converted to the active ferric hydroxide form by autooxidized compounds. A subsequent proton-coupled electron transfer from the substrate to the ferric hydroxide forms an intermediate radical and a ferrous species (Fig. 1).[9,10] After stereoselective reaction of the substrate radical with molecular oxygen, the peroxyl radical oxidizes the iron back to the active ferric state and the peroxide product is released from the enzyme.

Lipoxygenases have drawn considerable attention due to the large kinetic isotope effects (KIEs) exhibited in reactions with LA.[11–14] The observed values of 50–100 for k_{cat} are much larger than the semi-classical limit. Quantum chemical tunneling in the rate-determining hydrogen atom transfer step, mediated by protein dynamics, has been proposed to account for these large values.[15,16] Thus far, KIE studies have focused on LA due to the availability of its deuterium-labeled analogs. Previously, our laboratory reported the synthesis of site-specifically deuterium-labeled AA analogs.[17–19] With these compounds in hand, the reaction of sLO-1 with AA was investigated, revealing a large isotope effect of 97 for k_{cat} and a more modest effect of 8.0 for k_{cat}/K_m, compared to 64 and 25 for the corresponding parameters for the sLO-1-catalyzed oxidation of LA under similar conditions. In addition, an unusual isotope effect on substrate inhibition by AA was discovered.[20] In this contribution, the temperature dependence of these isotope effects is presented as well as studies to assess whether the hydrogen atom abstraction step is fully rate-limiting with AA as substrate. Furthermore, the substrate inhibition by AA is shown to be consistent with a high affinity of this fatty acid for the ferrous form of the enzyme.

Previously, the unusual observation was made that substrate inhibition was alleviated by isotopic substitution at the site of hydrogen atom abstraction in the reaction of sLO-1 and AA.[20] Based on a series of experimental observations including a more than 10-fold decrease in the K_m for oxygen with deuterium-labeled substrate,[20] a model was proposed to account for the unusual observation. For protiated substrate, the reaction of the substrate radical with O_2 (k_{ox}) was proposed to compete with dissociation of the radical intermediate from the enzyme (k_d) (Fig. 2). The inactive ferrous enzyme generated by substrate radical dissociation could then be sequestered by AA, giving rise to the observed substrate inhibition (Fig. 2). Three different explanations were suggested for the finding that substrate inhibition is not observed with LA despite KIEs of similar size. HPODE, the oxidation product with LA, could be more effective than HPETE, the oxidation product of AA, at reoxidizing the ferrous enzyme back to the active form. Alternatively, AA might have a higher binding affinity for the

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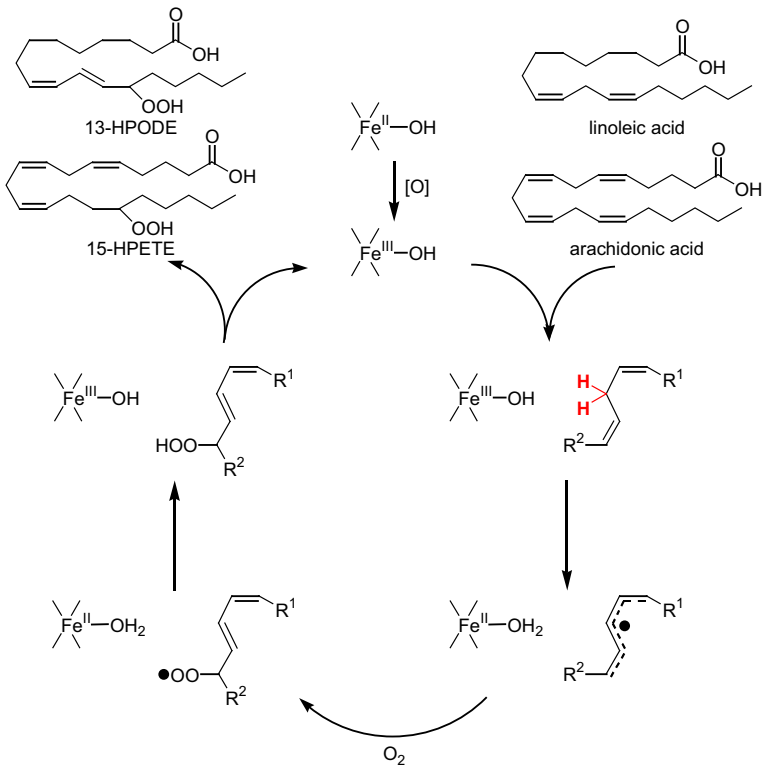

Figure 1. Oxidation of linoleic acid and arachidonic acid to 13-HPODE and 15-HPETE, respectively, catalyzed by soybean lipoxygenase-1.

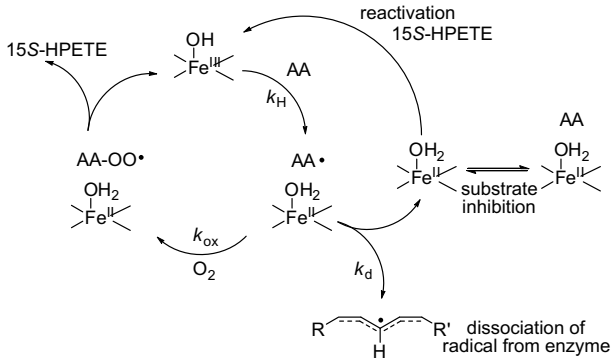

Figure 2. Proposed model for substrate inhibition of the sLO-1 reaction with AA. The relatively slow capture of the substrate radical with O_2 $k_{cat}/K_{m,O_2}$ results in the dissociation of a subset of radicals from the ferrous enzyme. AA can bind this inactive enzyme. The oxygen capture of radical when using deuterium-labeled substrate is more efficient and no substrate inhibition is observed.

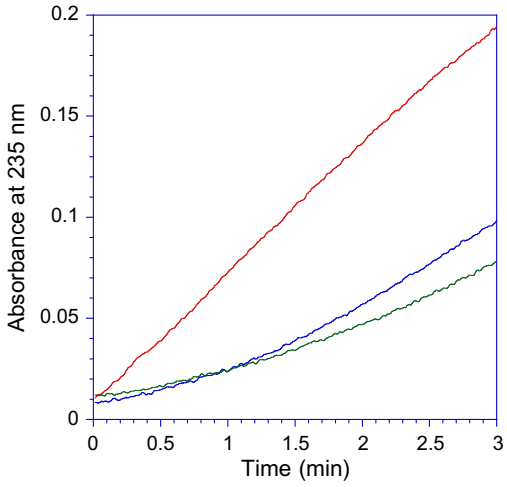

Figure 3. Formation of oxidized product in the reaction of sLO-1 with LA (red), AA (green), and a combination of the two substrates (blue). In all cases, the reaction was initiated by addition of enzyme. The assay was performed in oxygen-saturated sodium borate buffer (100 mM, pH 10.0) without the addition of activator. The substrate and enzyme concentrations were 60 and 0.75 nM, respectively.

reduced enzyme than LA. Finally, k_d might be larger when AA is the substrate.[20] A subsequent independent study showed that the two hydroperoxides (HPODE and HPETE) are in fact equally efficient at oxidizing ferrous sLO-1.[21] In this work, we set out to investigate whether AA has a higher affinity for the ferrous enzyme than LA. Since direct binding studies are complicated by the requirement of rigorous anaerobic conditions as well as by an allosteric binding site on sLO-1,[22–25] an indirect measurement was used to compare the relative affinities of LA and AA for the ferrous enzyme. Lag phases are typically observed in sLO-1 assays as the inactive ferrous form is oxidized to the active ferric form. We noticed that the lag phase with HPLC-purified AA was significantly longer than that observed with purified LA (Fig. 3), suggesting a higher affinity of AA for the ferrous enzyme. The longer lag phase with AA is consistent with a previous report,[26] but the difference between the two substrates was more pronounced in this work (see Supporting information). To rule out that the LA sample contained an oxidized impurity that resulted in more efficient oxidation of the ferrous form of sLO-1, AA was first incubated with the enzyme before the addition of LA. The rate of product formation again indicated that the presence of AA significantly increased the observed lag phase. The long lag times were also observed when LA and AA were presented at the same time (Fig. 3). Hence, these observations support a higher affinity of AA than LA for the ferrous form of sLO-1. The current work does not rule out, however, a lower affinity of the arachidonyl radical for the reduced enzyme compared to the linoleyl radical (i.e. larger k_D for the former).

In the presence of 13-HPODE, the lag phase is effectively suppressed, and therefore the temperature dependence of the KIEs in the reaction of sLO-1 with AA and 13,13-d_2-AA was determined in the presence of this activator. The use of 13,13-d_2-AA results in both primary and secondary KIEs, but the secondary KIE is expected to be very small compared to the large primary KIEs. Furthermore, the use of dideuterated substrate prevents any erosion in the stereoselectivity of hydrogen atom abstraction as a result of the large KIE; such a reduced stereoselectivity was previously reported for stereospecifically singly deuterium-labeled LA.[27] With 13,13-d_2-AA, $^{D}k_{cat}$ decreased over the temperature range studied, from 150 at 5 °C to 82 at 35 °C (Fig. 4). On the other hand, $^{D}k_{cat}/K_m$ was much smaller and exhibited very little temperature dependence, with the values at all temperatures close to within experimental error. The K_m values for 13,13-d_2-AA are very small (<2 μM) and have relatively large uncertainty due to the limit of detection using non-competitive methods (see Supporting information). As a result the $^{D}k_{cat}/K_m$ presented here is an upper limit and could be even smaller. Competitive methods may provide more accurate values.

Arrhenius plots using the values for k_{cat} provide an apparent activation energy of 1.4 ± 0.4 kcal/mol for the protiated AA and 4.1 ± 0.6 kcal/mol for the dideuterated analog (Fig. 5). For comparison, the corresponding energies for the reaction with LA were 1.8 ± 0.4 kcal/mol for protiated substrate and 2.2 ± 0.3 kcal/mol for dideuterated substrate.[15] These low energies suggest tunneling is occurring for both isotopes in the reaction of AA, similar to previous conclusions for the reaction of LA. Due to the large errors in the pre-exponential terms, no conclusions can be drawn from the value of A_H/A_D.

The temperature dependence of solvent isotope effects (SIEs) was investigated next to probe if any steps involving solvent or solvent exchangeable positions on the enzyme were kinetically significant. However, the SIE on k_{cat} remained close to unity over the temperature range investigated (Fig. S1). The SIE on k_{cat}/K_m was also temperature-independent and close to unity.

In a final set of experiments, the dependence of the reaction rate on viscosity was determined. The k_{cat}/K_m values for the oxidation of AA by sLO-1 (3.1×10^7 M s^{-1} at 25 °C) approach diffusion-controlled rates. If the encounter of substrate and enzyme is (partially) rate-limiting, the rates should decrease in a predictable manner with increasing viscosity.[28] The viscosity of the reaction buffer was varied by the addition of glucose as described previously for similar studies for the oxidation of LA by sLO-1.[13] For a completely diffusion-controlled reaction, the dependence of the rate on viscosity has a slope of 1.[28] At 20 °C, the rate of oxidation of AA by sLO-1 was 56 ± 5% diffusion-controlled (slope of 0.56, Fig. 6). The corresponding values at 5 and 37 °C were 18 ± 4% and 28 ± 5% respectively (Figures S3 and S4). This pronounced viscosity effect at ambient temperature, which dissipates at higher and lower temperatures, mirrors the behavior observed in the reaction of sLO-1 with LA.[13]

The kinetic behavior in the reaction of sLO-1 with AA can be compared to that with LA. For both substrates, a very large substrate KIE is observed on kcat that greatly exceeds the semi-classical limit. Furthermore, for both substrates the activation energies are very small for both protiated and deuterated substrates. Therefore, the enzymes appear to promote similar tunneling contributions for these fatty acids. These findings are in contrast to human 15-lipoxygenase-1 that displays very different substrate KIEs on kcat with LA (~40)[29] compared to AA (~10).[30] Similarly,

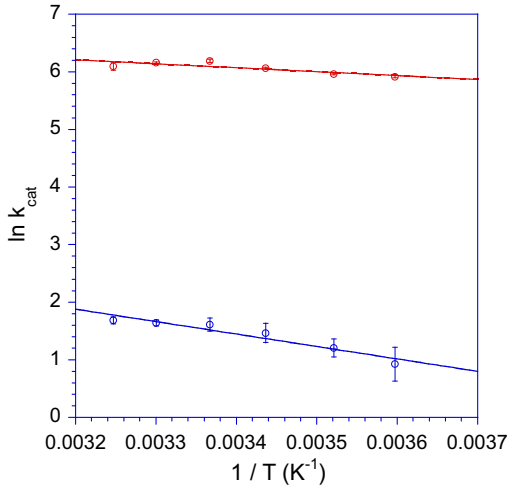

Figure 5. Arrhenius plot for the reaction of sLO-1 with AA (red line) and 13,13-d_2-AA (blue line) between 5 and 35 °C. The assay was performed in oxygen-saturated sodium borate buffer (100 mM, pH 10.0) in the presence of 13-HPODE (16 μM).

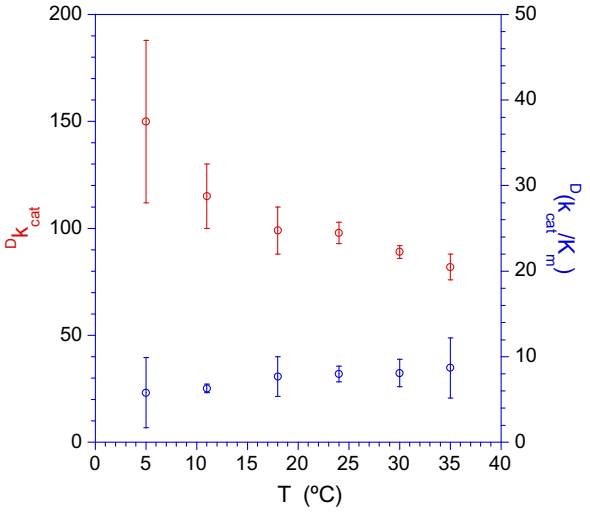

Figure 4. Temperature dependence of the KIE of the reaction of sLO-1 with AA and 13-d_2-AA. $^{D}k_{cat}$ is shown in red while $^{D}(k_{cat}/K_m)$ is shown in blue. The assays were performed in oxygen-saturated sodium borate buffer (100 mM, pH 10.0) in the presence of 13-HPODE (16 μM) to activate the enzyme.

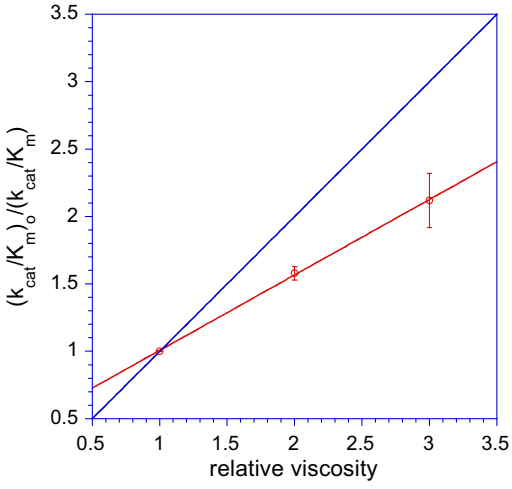

Figure 6. The effect of viscosity on the reaction of sLO-1 and AA at 20 °C (red line). The blue line indicates the behavior of a fully diffusion-controlled reaction. The assays were performed in CHES buffer (100 mM, pH 10.0) in the presence of 13-HPODE (16 μM) to activate the enzyme.

the KIEs on kcat/Km are also quite different for the reaction of sLO-1 with AA and LA. For LA, D(kcat/Km) is large (25) at 20 °C with the rate being limited to a large extent (48% at pH 9) by diffusion.[13] The reaction with AA is also limited largely by diffusion (56%) but a much more noticeable masking of the KIE is observed (~8). A solvent isotope sensitive step is not responsible for this more pronounced decrease in the KIE on kcat/Km. We tentatively conclude that a slower off-rate of AA results in increased commitment to catalysis which results in smaller observed KIEs. The higher affinity of AA for the ferrous form of sLO-1 and the lower Km for catalysis (15 μM for AA versus 39 μM for LA) are consistent with a higher affinity of AA for the enzyme.

In summary, the reaction of sLO-1 with AA displays many similarities in its kinetic behavior compared to that with LA. In both cases, the hydrogen abstraction step displays high KIEs on k_{cat} due to tunneling contributions. The only difference in the reactions is the lack of solvent-dependent rate-limiting steps at lower temperatures with AA as substrate, a smaller KIE on k_{cat}/K_m and a higher affinity of the enzyme for AA.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2008.08.108.

References and notes

1. Brash, A. R. *J. Biol. Chem.* **1999**, *274*, 23679.
2. Kuehn, H.; Thiele, B. J. *FEBS Lett.* **1999**, *449*, 7.
3. Porta, H.; Rocha-Sosa, M. *Microbiology* **2001**, *147*, 3199.
4. Gardner, H. W. *Biochim. Biophys. Acta* **1991**, *1084*, 221.
5. Knapp, M. J.; Klinman, J. P. *Biochemistry* **2003**, *42*, 11466.
6. McGinley, C. M.; van der Donk, W. A. *Chem. Commun.* **2003**, *23*, 2843.
7. Nagel, Z. D.; Klinman, J. P. *Chem. Rev.* **2006**, *106*, 3095.
8. Schilstra, M. J.; Veldink, G. A.; Vliegenthart, J. F. G. *Biochemistry* **1994**, *33*, 3974.
9. Lehnert, N.; Solomon, E. I. *J. Biol. Inoorg. Chem.* **2003**, *8*, 294.
10. Nelson, M. J.; Chase, D. B.; Seitz, S. P. *Biochemistry* **1995**, *34*, 6159.
11. Glickman, M. H.; Wiseman, J. S.; Klinman, J. P. *J. Am. Chem. Soc.* **1994**, *116*, 793.
12. Hwang, C.-C.; Grissom, C. B. *J. Am. Chem. Soc.* **1994**, *116*, 795.
13. Glickman, M. H.; Klinman, J. P. *Biochemistry* **1995**, *34*, 14077.
14. Lewis, E. R.; Johansen, E.; Holman, T. R. *J. Am. Chem. Soc.* **1999**, *121*, 1395.
15. Jonsson, T.; Glickman, M. H.; Sun, S.; Klinman, J. P. *J. Am. Chem. Soc.* **1996**, *118*, 10319.
16. Knapp, M. J.; Klinman, J. P. *Eur. J. Biochem.* **2002**, *269*, 3113.
17. Peng, S.; Okeley, N. M.; Tsai, A.-L.; Wu, G.; Kulmacz, R. J.; van der Donk, W. A. *J. Am. Chem. Soc.* **2001**, *123*, 3609.
18. Peng, S.; McGinley, C. M.; van der Donk, W. A. *Org. Lett.* **2004**, *6*, 349.
19. Peng, S.; Okeley, N. M.; Tsai, A.-L.; Wu, G.; Kulmacz, R. J.; van der Donk, W. A. *J. Am. Chem. Soc.* **2002**, *124*, 10785.
20. Peng, S.; van der Donk, W. A. *J. Am. Chem. Soc.* **2003**, *125*, 8988.
21. Ruddat, V. C.; Mogul, R.; Chorny, I.; Chen, C.; Perrin, N.; Whitman, S.; Kenyon, V.; Jacobson, M. P.; Bernasconi, C. F.; Holman, T. R. *Biochemistry* **2004**, *43*, 13063.
22. Wang, Z. X.; Killilea, S. D.; Srivastava, D. K. *Biochemistry* **1993**, *32*, 1500.
23. Mogul, R.; Johansen, E.; Holman, T. R. *Biochemistry* **2000**, *39*, 4801.
24. Mogul, R.; Holman, T. R. *Biochemistry* **2001**, *40*, 4391.
25. Ruddat, V. C.; Whitman, S.; Holman, T. R.; Bernasconi, C. F. *Biochemistry* **2003**, *42*, 4172.
26. Smith, W. L.; Lands, W. E. M. *J. Biol. Chem.* **1972**, *247*, 1038.
27. Rickert, K. W.; Klinman, J. P. *Biochemistry* **1999**, *38*, 12218.
28. Brouwer, A. C.; Kirsch, J. F. *Biochemistry* **1982**, *21*, 1302.
29. Segraves, E. N.; Holman, T. R. *Biochemistry* **2003**, *42*, 5236.
30. Jacquot, C.; Wecksler, A. T.; McGinley, C. M.; Segraves, E. N.; Holman, T. R.; van der Donk, W. A. *Biochemistry* **2008**, *47*, 7295.