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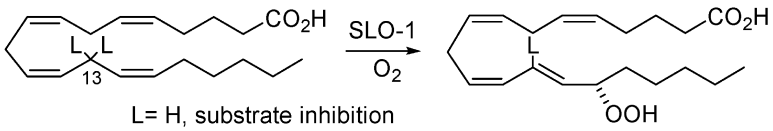

L= H, substrate inhibition
L = D, no substrate inhibition

15*S*-HPETE

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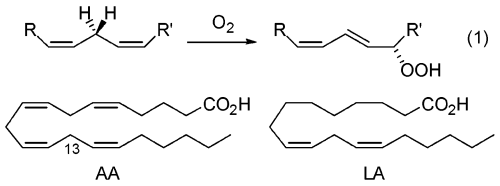
An Unusual Isotope Effect on Substrate Inhibition in the Oxidation of Arachidonic Acid by Lipoxygenase

Sheng Peng and Wilfred A. van der Donk*

Department of Chemistry, University of Illinois at Urbana-Champaign, 600 South Mathews Avenue, Urbana, Illinois 61801

Received May 6, 2003; E-mail: vddonk@uiuc.edu

Soybean lipoxygenase-1 (SLO) catalyzes the oxidation of lipids containing 1,4-*cis*,*cis*-diene units (eq 1). The enzyme has received much attention for its unusually large kinetic isotope effects (KIEs) in the oxidation of linoleic acid (LA),[1a−d,h,j] which have been attributed to environmentally coupled quantum mechanical tunneling[1e] governed by protein dynamics.[1f] This model has been evaluated recently with several site-directed mutants.[1g] In this report, we present a complementary approach to assess the importance of substrate structure through the use of isotopically labeled arachidonic acid (AA). These studies uncovered an unprecedented isotope effect on substrate inhibition.



Fatty acids with 18 carbons such as LA are substrates for plant LOs, whereas fatty acids with 20 carbons such as AA are substrates for human LOs. In contrast to the extensive studies with LA, no reports have focused on KIEs with AA due to the lack of available substrate deuterated at the position of hydrogen atom abstraction (13*S*). We recently reported the synthesis of such compounds for our studies on the cyclooxygenase reaction[2,3] and used these materials here for the investigation of SLO. Kinetic analysis of the reaction of the recombinant enzyme expressed in *E. coli* with unlabeled AA showed strong substrate inhibition, whereas the use of LA did not show inhibition at concentrations up to 90 μM (Figure 1). Unexpectedly, when 13,13-d_2-AA was used as substrate, no substrate inhibition was observed. Several experiments were conducted to rule out interference by impurities in the substrate preparations.[4] Substrate samples purchased from various suppliers and HPLC-purified prior to use all displayed substrate inhibition, as did synthetic AA labeled at C11.[3] The strongest indication that substrate inhibition is a function of a chemical event at C13 was obtained with 13*R*-d_1-AA and 13*S*-d_1-AA. The latter substrate did not display substrate inhibition, whereas its enantiomer showed strong inhibition (Supporting Information). To the best of our knowledge, this is the first example of apparent complete alleviation of substrate inhibition due to isotope substitution.[5]

The unusual suppression of substrate inhibition prompted further investigation of its origin. Because inhibition is observed with unlabeled but not labeled substrate, micelle or premicelle formation can be ruled out, consistent with the reported critical micellar concentration of 180 μM at pH 10.[6] The most widely accepted mechanism of SLO involves activation of the inactive ferrous resting state of the enzyme by peroxide impurities present in the fatty acid substrate.[8−10] The ferric enzyme then abstracts a hydrogen atom[11]

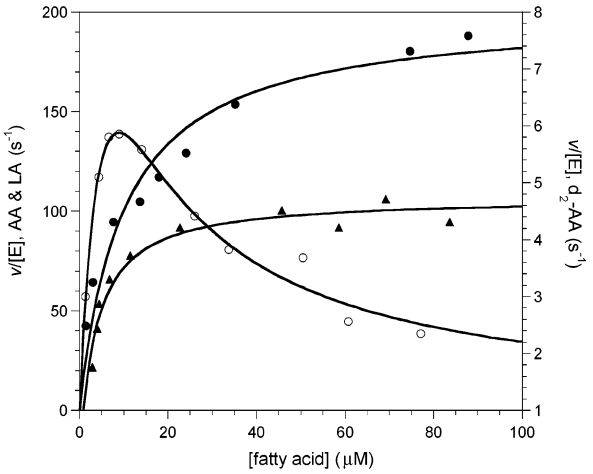
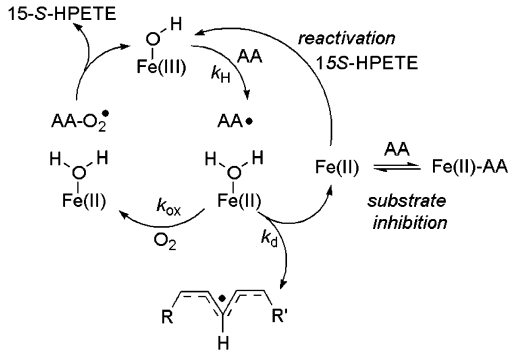


Figure 1. Concentration dependence of the initial rates of the oxidation of LA (●), AA (○), and 13,13-d_2-AA (▲, right *Y*-axis) at 25 °C, 0.1 M borate buffer, pH 10, $[O_2] = 225$ μM. Solid lines were obtained by fitting to the Michaelis−Menten equation (LA, 13,13-d_2-AA) or by using the generic equation for substrate inhibition (AA): $v = k_{cat}[E][AA]/[K_a + [AA] + ([AA]^2/K_i)]$.

Scheme 1



from a bisallylic position to generate a delocalized radical that reacts with molecular oxygen to produce a peroxyl radical (Scheme 1).[12] This radical reoxidizes the ferrous enzyme to afford the product and regenerate the ferric enzyme. With LA as substrate, the organic radical has been proposed to dissociate from the enzyme at low oxygen concentrations (k_d).[9,10,13] Substrate binding to the resulting ferrous protein then causes substrate inhibition.[14,15] We probed in a variety of ways whether the inhibition observed with arachidonic acid is consistent with such a model. Addition of external peroxide activators should compete with arachidonic acid to bind to the ferrous enzyme, and indeed addition of 13*S*-HPOD, the product of SLO oxidation of LA, at increasing concentrations eliminated substrate inhibition (Figure 2).[7] With 16 μM HPOD, the k_{cat} was 431 ± 19 s^{-1} and the $K_{m,AA}$ was 11.3 ± 1.6 μM, close to the values

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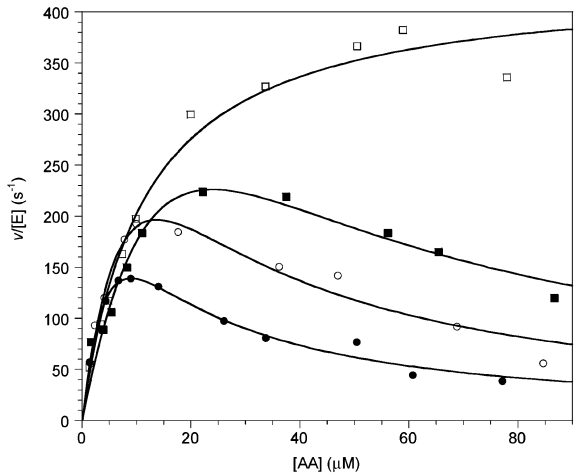

Figure 2. Initial rates of the oxidation of AA by SLO without 13-HPOD (●), and with 0.32 μM 13-HPOD (○), 1.6 μM 13-HPOD (■), and 16 μM 13-HPOD (□), pH = 10, 25 °C.

for oxidation of LA (413 ± 34 s^{-1} and 39 ± 8.7 μM, respectively). 15*S*-HPETE was much less effective in suppressing substrate inhibition, suggesting the C20 fatty acid peroxide is less efficient in reoxidizing the enzyme.[16] Whereas these experiments support the view that formation of the ferrous enzyme results in substrate inhibition with AA, it does not explain why this behavior is not observed with 13,13-d_2-AA. The difference between the labeled and unlabeled substrate is not expected to change the rate of radical dissociation (k_d) significantly. Hence, the only manner in which partitioning between a productive turnover (k_{ox}) and radical dissociation (k_d) can be affected is if the rate of reaction with oxygen is changed by the isotopic substitution. The K_{m,O_2} with LA is very low (8 μM),[14] and the rate of reaction of oxygen with the enzyme-bound radical approaches the diffusion-controlled limit.[1d] We postulated that this step might be less efficient with AA. Previous studies on LA have shown that a large KIE on C−H bond cleavage results in a large isotope effect on K_{m,O_2}, which was used to investigate the order of binding of the substrates.[1d] A similar effect with AA could lead to a situation in which the available oxygen in air-saturated buffer ($[O_2] \approx 256$ μM) fully saturates the E−S· form of the enzyme with deuterium labeled substrate but not with unlabeled AA. Using an oxygen electrode, we determined the K_{m,O_2} to be 99 μM for unlabeled AA and <10 μM for 13-d_2-AA under conditions where substrate inhibition is absent (16 μM HPOD, 60 μM AA), consistent with the proposed origin of the isotope effect on substrate inhibition. The model also predicts that at higher oxygen concentration (i.e., oxygen saturated buffer) the inhibition for unlabeled AA should be relieved as was observed (data not shown).[17] Hence, the data support the mechanism in Scheme 1, and the difference between labeled and unlabeled AA is due to a much lower K_{m,O_2} for the former.

The KIEs for the SLO-catalyzed oxidation of AA at 25 °C were determined under conditions when substrate inhibition was not present (16 μM HPOD, 1.1 mM O_2). Values of 97 ± 5 for k_{cat} and 8.01 ± 0.89 for k_{cat}/K_m were determined, as compared to 64 ± 5 and 58 ± 5 for the corresponding reaction with [U-^{2}H]-LA.[1d] Hence, the extremely large KIEs on k_{cat} are not restricted to the reaction of SLO with LA. The lower isotope effect on $k_{cat}/K_{m,AA}$ probably reflects the rate being limited in part by a diffusion-controlled encounter of SLO and AA ($k_{cat}/K_{m,AA} = 3.9 \times 10^7$ M^{-1} s^{-1}). Further studies to interrogate the details of the hydrogen atom abstraction step are underway. Our studies presented here suggest that the striking difference observed in the behavior with LA and AA is caused by (1) an increased K_{m,O_2} with AA, (2) a large KIE on K_{m,O_2}, and (3) a diminished efficiency of the product 15*S*-HPETE to reoxidize the ferrous form to the active ferric form. We cannot rule out that the enzyme may also have a decreased affinity for the intermediate organic radical derived from AA as compared to the linoleyl radical, which would also favor partitioning to the ferrous enzyme resulting in substrate inhibition (Scheme 1).

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Supporting Information Available: Figures of the concentration dependence of the initial rates of oxidation (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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