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Kinetic investigation of human 5-lipoxygenase with arachidonic acid

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ABSTRACT

Human 5-lipoxygenase (5-LOX) is responsible for the formation of leukotriene (LT)A_4, a pivotal intermediate in the biosynthesis of the leukotrienes, a family of proinflammatory lipid mediators. 5-LOX has thus gained attention as a potential drug target. However, details of the kinetic mechanism of 5-LOX are still obscure. In this Letter, we investigated the kinetic isotope effect (KIE) of 5-LOX with its physiological substrate, arachidonic acid (AA). The observed KIE is 20 ± 4 on k_{cat} and 17 ± 2 on k_{cat}/K_M at 25 °C indicating a non-classical reaction mechanism. The observed rates show slight temperature dependence at ambient temperatures ranging from 4 to 35 °C. Also, we observed low Arrhenius prefactor ratio (A_H/A_D = 0.21) and a small change in activation energy ($E_a(D) - E_a(H)$ = 3.6 J/mol) which suggests that 5-LOX catalysis involves tunneling as a mechanism of H-transfer. The measured KIE for 5-LOX involves a change in regioselectivity in response to deuteration at position C7, resulting in H-abstraction form C10 and formation of 8-HETE. The viscosity experiments influence the $^{H}k_{cat}$, but not $^{D}k_{cat}$. However the overall k_{cat}/K_M is not affected for labeled or unlabeled AA, suggesting that either the product release or conformational rearrangement might be involved in dictating kinetics of 5-LOX at saturating conditions. Investigation of available crystal structures suggests the role of active site residues (F421, Q363 and L368) in regulating the donor–acceptor distances, thus affecting H-transfer as well as regiospecificity. In summary, our study shows that that the H-abstraction is the rate limiting step for 5-LOX and that the observed KIE of 5-LOX is masked by a change in regioselectivity.

Lipoxygenases are non-heme iron-containing enzymes, present in bacteria, plants as well as mammals catalyzing the stereospecific and regiospecific peroxidation of natural polyunsaturated fatty acids to hydroperoxy derivatives.[1,2] The products of lipoxygenase reactions are involved in a variety of biological functions in almost all phyla such as signaling, germination and senescence.[3,4] Human 5-LOX is implicated in a variety of diseases ranging from asthma to cancer, thus gaining importance as a potential therapeutic target[5–7] and emphasizing the need for further investigation of its catalytic mechanism.

5-LOX catalyzes a 2-step reaction where arachidonic acid (AA) is oxygenated, resulting in formation of 5(*S*)-hydroperoxy-6(*E*),8(*Z*),11(*Z*),14(*Z*)-eicosatetraenoic acid (5-HPETE), with subsequent formation of leukotriene A_4 (LTA_4). During the first step, 5-LOX binds its substrate AA and abstracts the *pro-S* hydrogen, H_7, from position C7. After radical rearrangement, O_2 is inserted at position C5 resulting in the formation of 5(S)-HPETE. In the second step, abstraction of the *pro-R* hydrogen, H_{10}, from position C10 generates the epoxide LTA_4[8] (Fig. 1).

Isotope-labeling studies provides a handle to investigate the mechanism of action of various enzymes. Lipoxygenases, especially soybean LOX, have attracted considerable attention due to the large kinetic isotope effects (KIEs) exhibited in reactions with linoleic acid (LA). However, there is dearth of studies investigating the mechanism of action of lipoxygenases with arachidonic acid as substrate. To investigate the catalytic mechanism of human 5-LOX, we used arachidonic acid (AA) and 7,7-d_2-arachidonic acid (7,7-d_2AA) as substrates. Although the double label of 7,7-d_2-arachidonic acid results in a combination of primary and secondary KIEs, we decided to prepare this compound to avoid any complications from a potential change in stereoselectivity in the hydrogen atom abstraction step, as has been reported in earlier studies.[1] The reaction of AA and 7,7-d_2-AA with 5-LOX was monitored at 235 nm for the formation of HETEs as shown in Figure S1. 13-HPODE was added to oxidize Fe^{2+} to Fe^{3+}, thus reducing the lag phase of the reaction. By fitting the Michaelis–Menten rate

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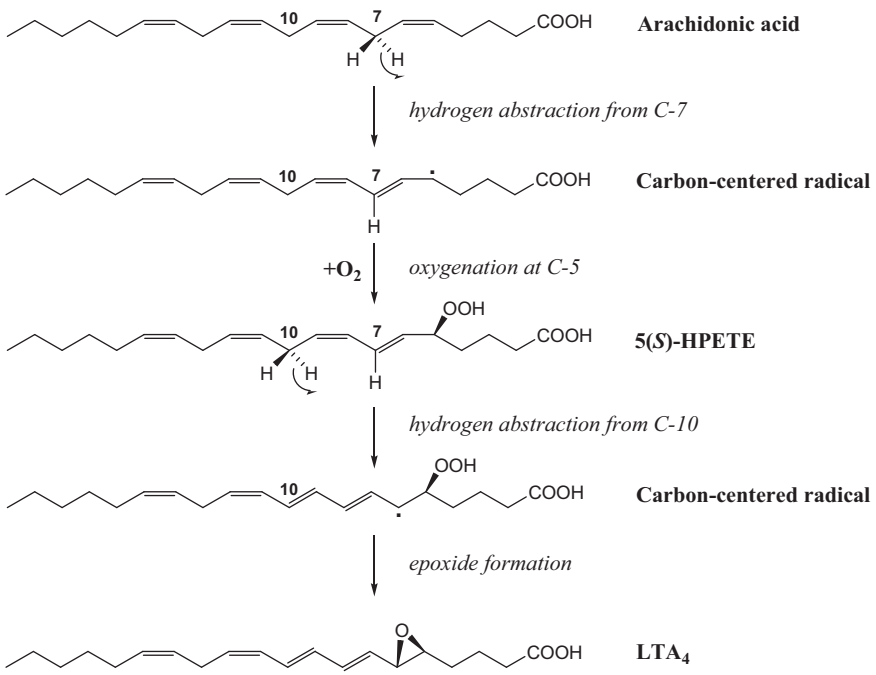

Figure 1. The catalytic reaction of 5-LOX. First, H-abstraction occurs specifically at C7 to form first 5-HPETE. 5-HETE is formed by reduction of 5-HPETE. The reaction further proceeds by a second H-abstraction at C10 resulting in the formation of epoxide LTA_4.

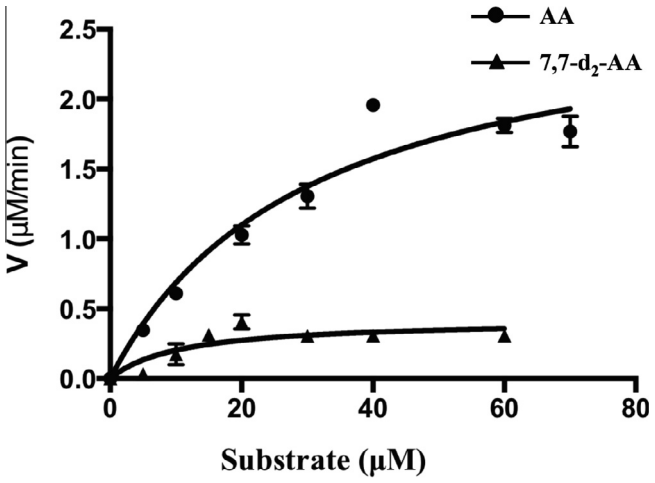

Figure 2. Steady state kinetics of 5-LOX with arachidonic acid (AA) (circles) and deuterated arachidonic acid (7,7-d_2AA) (triangles) to calculate KIE by non-competitive method using UV–Vis Spectrophotometer.

equation to the data, the kinetic parameters shown in Figure 2 and Table 1 were obtained.

The apparent primary kinetic isotope effect on k_{cat} and k_{cat}/K_M at 25 °C was 6.0 and 2.3, respectively when comparing AA and 7,7-d_2-AA kinetics. These values are smaller than the previously reported numbers under similar conditions for the oxidation of perdeuterated LA by lipoxygenases, which include a $^{D}k_{cat}$ of 80 and 48 for soybean LOXs and 15-LOX-1, respectively, but 10 for 15-LOX-1 and AA.[9–11] As mentioned in previous reports, one possible mechanism resulting in lower isotope effects involves a change in regioselectivity in response to deuteration.

Table 1
Kinetic parameters for the reaction(s) of 5-LOX with AA and 7,7-d_2AA using UV–Vis spectrophotometer. Standard deviations from three independent experiments are indicated in parentheses

	V_{max} (μM/min)	K_M (μM)	k_{cat} (s^{-1})	k_{cat}/K_M ($\mu M^{-1}\ s^{-1}$)
AA	2.56 (0.25)	22.3 (6.49)	0.06	0.054
7,7-d_2-AA	0.42 (0.06)	11.14 (5.12)	0.01	0.023

To investigate a possible shift in regioselectivity, the product composition of the reactions of 5-LOX with arachidonic acid and its deuterated analog (5–30 μM), were determined by HPLC after quenching the reactions with methanol. To quantify the analysis, 13-HPODE was added to the kinetic assays as described above and the product distribution was determined. The representative chromatograms for the reactions of 5-LOX with 30 μM of AA and 7,7-d_2-AA, are shown in Figure S3. We observed a significant reduction in 5-HETE formation, along with upsurge in rate of 8-HETE formation (Fig. 3A and B).

The reaction of 5-LOX with unlabeled AA generated products from hydrogen abstraction at C7 (95% 5-HETE) with minor products derived from abstraction at C10 (5% 8-HETE). With 7,7-d_2-AA the selectivity remained skewed towards C7 hydrogen abstraction (59%) compared to C10 hydrogen abstraction products (41% 8-HETE), but the selectivity was significantly reduced compared to unlabeled AA. The data analysis, after product profiling at substrate concentrations (5–40 μM), provided an apparent $^{D}k_{cat}$ and $^{D}k_{cat}/K_M$ of 20 and 17, respectively, markedly higher than those observed earlier in our study. Thus, our results show that 5-LOX exhibits isotope sensitive branching, however the extent to which the product distribution changed was smaller. An analogous shift in regiospecificity induced by deuterium-labeling has been reported for arachidonic acid in 12-LOX and 15-LOX2.[12] Additionally, oxygenation of 8,11,14-eicosatrienoic acid (lacking the Δ5 double bond) takes place by hydrogen abstraction at C10 and oxygen insertion at C8.[13]

The dependence of the steady-state velocity on AA concentration appears to follow Michaelis–Menten kinetics up to 40–50 μM (further increase in AA concentration results in significant substrate inhibition), with $V_{max} = 2.56 \pm 0.25$ μM/min and $K_M = 24 \pm 6$ μM. The data presented in this study show that 5-LOX has turnover rates ($k_{cat} = 0.06\ s^{-1}$) lower than the other known LOXs.[14] Comparing the catalytic rate (k_{cat}) of 5-LOX, when AA or