Steric Control of Oxygenation Regiochemistry in Soybean Lipoxygenase-1

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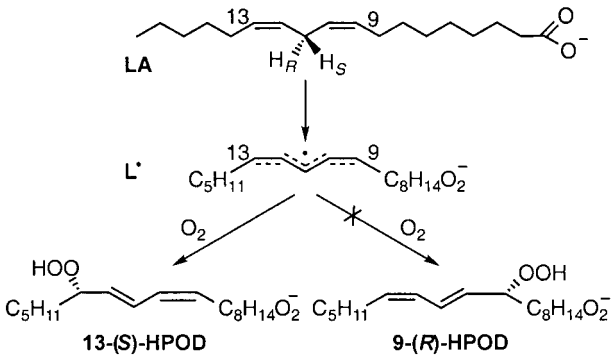
Mammalian lipoxygenases catalyze the formation of important biological secondary messengers and have been implicated in carcinogenesis[1] and inflammation.[2] While the catalytic reaction of all known lipoxygenases leads to the formation of a substrate-derived radical intermediate, most lipoxygenase products are highly regio- and stereopure.[3] Soybean lipoxygenase-1 (SLO), the prototypical model lipoxygenase, catalyzes the oxidation of linoleic acid (LA) by O_2, producing 13-(*S*)-hydroperoxyoctadecadienoic acid (HPOD). The first chemical step in this reaction[4] is abstraction of the pro-*S* hydrogen atom from C-11 by the active-site Fe^{3+}-OH to generate a putatively delocalized carbon based radical ($L^{\bullet}$) and Fe^{2+}-OH_2. This intermediate reacts with O_2 to regenerate Fe^{3+}-OH and the product 13-*(S)*-HPOD (Scheme 1). Reactions in solution between carbon radicals and triplet O_2 typically lead to many isomers and are generally diffusion limited.[5] While SLO reacts with O_2 at a rapid rate ($k_{cat}/K_M(O_2) = 4.4 \times 10^6\ M^{-1}\ s^{-1}$), this reaction remains highly selective for O_2 attack at C-13. This has led to the notion that the active-site Fe^{2+} influences the site of O_2 insertion via a purple $(Fe\text{-}OOL)^{2+}$ intermediate.[18] However, this idea is difficult to reconcile with the fact that H-abstraction by Fe^{3+}-OH is antarafacial to O_2 insertion.[6] Inspection of the crystal structure for SLO[7] reveals a side channel intersecting the substrate pocket near the reactive C-11, proposed[7] to permit O_2 access. The current report details our investigation into the role played by bulky hydrophobic residues in controlling the regiospecificity of SLO. Steady-state kinetics and product distribution data from single-point mutants of SLO show that Leu^{546} and Leu^{754} grant selectivity for 13-(*S*)-HPOD by blocking O_2 access to C-9 of LA, and that O_2 enters the active site via the postulated side channel. These results indicate that SLO controls O_2 access to the intermediate $L^{\bullet}$, and suggest that Fe^{2+} plays a secondary role in oxygenation.

The X-ray crystal structure of SLO revealed the LA pocket, cavity IIa,[7] as a twisted channel, constricted at the Fe^{3+}-OH by the side chains of Leu^{546} and Leu^{754} (Figure 1). A sharp bend near Fe^{3+}-OH is introduced by the side chains of Gln^{495} and Ile^{538}. On the basis of extensive experiments with many lipoxygenases[8−13] models have been published[6,10,11,13] describing substrate binding in which the reactive C-11 lies near the constriction formed by

Scheme 1. Simplified Chemical Mechanism of SLO[a]



[a] WT-SLO produces predominantly 13-(*S*)-HPOD at high pH.

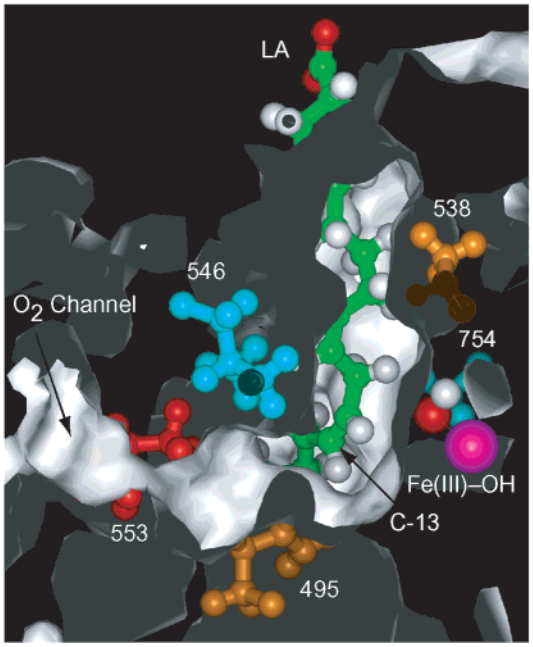


Figure 1. Substrate cavity and O_2 access channel of SLO shown as a white surface. The substrate LA (C, green; H, white; O, red) has been modeled with C-1 toward the top of the figure, and with the C-9−C-13 moiety of substrate between Ile^{538} and Gln^{495} (orange). The postulated O_2 channel, on top of Ile^{553} (red), intersects the substrate cavity close to the C-13 position of LA. Leu^{546} and Leu^{754} (blue) constrict the LA channel, preventing O_2 access to C-9 of LA. Fe^{3+}-OH is presented as CPK spheres (Fe, magenta; O, red; H, white).[16]

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(16) All modeling was performed using InsightII/Discover software (Biosym/MSI, San Diego, CA). The CVFF force field was used, and the pro-*S* hydrogen of LA was constrained to lie within 3.0 Å of the oxygen atom of Fe(III)-OH. Only residues within the substrate cavity (channel IIa[7]) were minimized, with the exception that Fe and its ligands were held fixed. The internal Connolly surface was constructed by use of a 0.8 Å solvent sphere. Using a larger solvent sphere (1.4 Å) resulted in a surface that was interrupted by a few amino acid side chains (Ile^{553}, Leu^{564}) which could easily reorient to form a continuous surface, as noted before.[7]
(17) An O_2 electrode was used to determine initial rates at constant [LA] as a function of $[O_2]$, at pH 9.0, 20 °C. Kinetic parameters were determined by nonlinear least-squares fitting to the Michaelis−Menten equation. Rates were normalized for Fe content of the enzymes (ICP-AES).
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Table 1. Kinetic Constants[a] (pH 9.0, 20 °C) and Product Distributions (pH 10.0, 23 °C) for WT-SLO and Mutants

			% HPOD[c]			
enzyme	k_{cat} (s^{-1})	$k_{cat}/K_M(O_2)$ ($\mu M^{-1}s^{-1}$)	13(*S*)	13(*R*)	9(*S*)	9(*R*)
WT-SLO	210	4.4	95	3	<1	2
Ile^{553}→Ala	209	6.1	97	1	<1	<1
Gln^{495}→Ala	120	2.4	95	2	1	2
Ile^{538}→Ala	110	3.2 (0.2)	93	3	2	3
Leu^{546}→Ala	3.0	0.4 (0.3)	85	2	3	10
Leu^{754}→Ala	0.18	>0.2[b]	62	12	10	16
Ile^{553}→Phe	105	0.72	95 (2)	2	1	2

[a] Corrected for Fe content; see Supporting Information. Standard error is less than 15% of the parameter, unless specified in parentheses. [b] $K_M(O_2)$ was too low to be measured (≤1 μM). [c] Average of two separate experiments. The estimated error ((run 1 − run 2)/2) is less than 1, unless noted in parentheses.

Leu^{546} and Leu^{754}. Such binding predicts that Gln^{495} and Ile^{538} will interact with C-14 and C-8 of L•, respectively, which would disfavor a planar pentadienyl moiety.[7] The side channel bordered by Ile^{553} intersects cavity IIa between Leu^{546} and Gln^{495}, a position that would be occupied by C-13 of bound L•. Additionally, this channel encounters the active site on the face of cavity IIa opposite that of Fe^{2+}. It seems feasible that O_2 enters through this channel, reacting with substrate before encountering Fe^{2+}. To test this hypothesis, the bulkiness of critical side chains near this intersection was changed by site-directed mutagenesis,[14,15] and the characteristics of the O_2 reaction with L• were compared to those of WT-SLO.

Table 1 contains a summary of kinetic data[17] for these mutants and WT-SLO. The overall rate of catalysis (k_{cat}) is limited by H• abstraction, whereas the rate of O_2 chemistry ($k_{cat}/K_M(O_2)$) reflects only those steps from O_2 encounter to product release. The mutants exhibited three types of behavior, depending on how the rates of overall catalysis and of O_2 chemistry were affected by the mutation. Ile^{553}→Ala has little effect on the rates of overall catalysis (k_{cat}) and oxygenation chemistry ($k_{cat}/K_M(O_2)$), while Gln^{495}→Ala and Ile^{538}→Ala reduce these parameters by *ca.* 2-fold. Leu^{546}→Ala and Leu^{754}→Ala greatly reduce overall catalysis (k_{cat} decreases by *ca.* 100−1000-fold), while having a lesser effect on oxygenation chemistry. Finally, Ile^{553}→Phe is interesting in that it greatly reduces the rate of oxygenation chemistry relative to WT-SLO and Ile^{553}→Ala, while leaving k_{cat} relatively unchanged. This suggests that Ile^{553}→Phe impedes O_2 access to the carbon radical intermediate (L•), and supports the view that O_2 enters the active site through the channel that passes residue 553.

The radical intermediate L• has generally been depicted as a pentadienyl radical, in which radical character is spread over C-9−C-13, generating greater stability than for an allyl radical. This would make C-9 and C-13 equally reactive to O_2 attack, barring accessibility differences. However, the side chains of Ile^{538} and Gln^{495} provide sufficient bulk to disfavor the planar pentadienyl intermediate,[7] raising the possibility that L• is an ene−allyl radical. If the unpaired electron density of L• were localized near C-13, in the form of a Δ^9-[11,12,13] allyl radical, then O_2 would be expected to selectively react at C-13. However, an EPR investigation into the identity of L• showed predominantly a Δ^{12}-[9,10,11] ene−allyl radical,[18] suggesting that radical character is localized near C-9. The Gln^{495}→Ala and Ile^{538}→Ala mutations should each reduce the amount of strain that might favor a localized ene−allyl radical. However, these mutations had only modest effects on the rate of oxygenation, suggesting that such an ene−allyl radical is not formed during turnover, or else that there is rapid interconversion between the Δ^{12}-[9,10,11] and Δ^9-[11,12,13] radicals. Additionally, these mutants produce the same product distribution as WT-SLO (vide infra).

Table 1 lists the stereo- and regiochemical product distributions for each enzyme, as determined by chiral HPLC analysis.[19,20] It is immediately apparent that the dominant product for WT-SLO is 13-(*S*)-HPOD, with trace amounts of 13-(*R*), 9-(*S*), and 9-(*R*) isomers. This product distribution is observed for all other mutants, except for Leu^{546}→Ala and Leu^{754}→Ala. Both of these mutants replace a bulky Leu side chain with that of Ala at the constriction within the substrate cavity. It is noteworthy that Leu^{546}→Ala yields 13-(*S*)- and 9-(*R*)-HPOD, the stereoisomers formed by addition of O_2 on the same face of L•; inverse (head-to-tail) substrate binding would yield the 9-(*S*) isomer.[6] Leu^{754}→Ala yields an increase not only in the 9-(*R*) isomer, but also a similar increase in the 13-(*R*) and 9-(*S*) isomers, with ca. 3% of the all-trans-HPOD isomers formed as well (supplementary material). The reasons for the formation of these other isomers are unclear, but Leu^{754} does sit roughly adjacent to the Fe^{3+}-OH, and removal of this bulky side chain could easily permit the LA substrate to adopt alternative binding modes. These data indicate that the constriction in cavity IIa formed by Leu^{546} and Leu^{754} sterically prevents O_2 access to the C-9 position within the putative pentadienyl radical, thereby imparting regioselectivity to this reaction. This obviates any requirement for the $(Fe\text{-}OOL)^{2+}$ intermediate during turnover, suggesting that purple lipoxygenase is not catalytically relevant.

These results present a departure from typical views of substrate-activating oxygenases, in that O_2 has seldom been thought to require a discrete and separate access channel. The present analysis shows how simple sterics can control the reaction of an enzyme-bound radical with O_2, and it provides a structural rationale for product distributions within the lipoxygenase family. Other substrate-activating oxygenases,[21,22] such as the cofactor-generating activity of TOPA-containing amine oxidase,[23] may also sterically direct O_2 attack to a specific site of the substrate, thereby controlling product identity.

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Supporting Information Available: Chromatograms showing peak assignments and a table listing Fe content of each enzyme (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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