Control of the Position of Oxygen Delivery in Soybean Lipoxygenase-1 by Amino Acid Side Chains within a Gas Migration Channel*

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Lara Collazo* and Judith P. Klinman†

From the Department of Molecular and Cell Biology, Department of Chemistry, and the California Institute for Quantitative Biosciences (QB3), University of California, Berkeley, California 94720

Understanding gas migration pathways is critical to unraveling structure-function relationships in enzymes that process gaseous substrates such as O₂, H₂, N₂, and NH₃. This work investigates the role of a defined pathway for O₂ in regulating the peroxidation of linoleic acid by soybean lipoxygenase 1. Computational and mutagenesis studies provide strong support for a dominant delivery channel that shuttles molecular oxygen to a specific region of the active site, thereby ensuring the regio- and stereospecificity of product. Analysis of reaction kinetics and product distribution in channel mutants also reveals a plasticity to the gas migration pathway. The findings show that a single site mutation (I553W) limits oxygen accessibility to the active site, greatly increasing the fraction of substrate that reacts with oxygen free in solution. They also show how a neighboring site mutation (L496W) can result in a redirection of oxygen toward an alternate position of the substrate, changing the regio- and stereospecificity of peroxidation. The present data indicate that modest changes in a protein scaffold may modulate the access of small gaseous molecules to enzyme-bound substrates.

Structure-function studies of enzymes have moved beyond an exclusive focus on active site protein side chains, with the growing recognition that distal residues can have a significant impact on catalytic bond cleavage events (1, 2). Topological features within the protein matrix, such as cavities and channels, can also play key roles in the flux of biomolecules. For proteins with gaseous substrates, e.g. O₂, H₂, N₂, and NH₃, specific channels have been proposed either to transport reactive intermediates between active sites within a multifunctional enzyme (3) or to guide these small molecules from the solvent to the active site. The likely importance of the latter is relevant to a wide range of enzymes that includes oxidases, monooxygenases, nitrogenses, and hydrogenases (4–17). The involvement of functionally evolved gas channels represents a significant departure from earlier held views in which random, transient fluctuations within a protein were proposed to facilitate the delivery of gaseous substrates to their site of binding and/or reactivity.

Studies of lipoxygenases have aided in this shift in perception, given their requirement to capture O₂ via highly regio- and stereospecific pathways. As illustrated in Fig. 1 for the reaction catalyzed by soybean lipoxygenase-1 (SLO-1), the delocalized nature of the free radical intermediate generated from the preferred substrate linoleic acid (LA) predicts that four unique products will be produced in equal amounts in solution. By contrast, native SLO-1 produces the 13S-hydroperoxide product (13S-HPOD) in greater than 90% yield for reaction of WT enzyme under optimal conditions. The precise mechanism by which SLO-1, as well as other lipoxygenases, maintain the regio- and stereospecificity of product peroxidation has engendered a variety of proposals that include: (i) an alteration in substrate binding (from head to tail first) as a means of reversing the enantiomeric specificity of O₂ addition to a fixed carbon center; (ii) the interchange of a single active site residue between Gly and Ala to alter the position (but not the face) of O₂ attack; and (iii) a directed movement of O₂ through the protein matrix toward a spatially defined position of substrate that is capable of simultaneously controlling both the position and face of O₂ attack on a substrate-derived free radical intermediate (4, 16, 18).

Although all three of the above factors may play a role in the evolution of lipoxygenases that catalyze the formation of stereo- and regiochemically distinct hydroperoxides, there remains the generic question of how each enzyme manages a transit of O₂ from bulk solvent toward the reactive carbon of buried substrate. The x-ray structure of SLO-1 is quite informative in this regard, implicating a putative gas channel that appears “crimped” in its middle at the residue Ile553 (18). Earlier support for the involvement of this channel came from the generation of an I553F mutant enzyme that showed a reduction in rate for the O₂-dependent portion of the SLO-1 reaction accompanied by only a modest impact on the initial C–H abstraction step and the position/stereochemistry of substrate peroxidation (3). The reaction mechanism invoked for I553F involves protein breathing modes capable of relieving a mutation-induced “steric impasse” toward O₂ (19). In the present

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† Present address: Genentech, Inc., One DNA Way, South San Francisco, CA 94080.

To whom correspondence should be addressed. Tel.: 510-642-2668; Fax: 510-643-4500; E-mail: klinman@berkeley.edu.

The abbreviations used are: SLO-1, soybean lipoxygenase-1; LA, linoleic acid; HPDOD, hydroperoxyoctadecadienoic acid; ILS, implicit ligand sampling; HODE, hydroxyoctadecadienoic acid.
The ILS tool was applied to the molecular dynamics simulation of SLO to assess regions favorable for oxygen migration within the enzyme. The ILS technique allows for the generation of a three-dimensional map of the Gibbs free energy cost, or \( \Delta G_s(O_2) \), associated with transferring an oxygen molecule from a vacuum to a particular position inside the protein (28). \( \Delta G_s(O_2) \) was evaluated for every 1 \( \text{Å}^3 \) volume element throughout the SLO system. Within each of these elements, oxygen was placed in 20 different rotational orientations at each of 27 different positions on a \( 3 \times 3 \times 3 \) grid. Five thousand protein conformations were sampled from the 10-ns trajectory. A three-dimensional free energy map was generated describing the distribution of oxygen throughout SLO. Regions of the enzyme likely to be occupied by oxygen were identified by drawing isoenergery surfaces—that is, plotting all points in a protein for which the \( \Delta G_s(O_2) \) is below a certain value. The results were visualized in VMD, the molecular visualization program in which the ILS method is realized (21).

Site-directed Mutagenesis and Protein Expression and Purification—Seven SLO mutants were generated through site-directed mutagenesis according to the QuickChange II protocol (Agilent Technologies). HPLC-purified primers were purchased from Eurofins MWG Operon, and sequencing was performed by UC Berkeley Sequencing Facility. The V564F lipoxygenase mutant was expressed and purified using the SLO pT7-7 plasmid in *Escherichia coli*, as described previously (29, 31). WT SLO and all other mutants were expressed and purified as His tag fusion proteins using the SLO pET-30Xa/LIC plasmid, acquired from Professor Betty Gaffeney at Florida State University. These enzymes were expressed and purified as described previously, with several modifications (31). His-tagged SLO was eluted with wash buffer containing 250 mM imidazole and dialyzed overnight (4 °C) into 20 mM Bis-Tris (pH 6.6) buffer. The dialyzed eluate from nickel-nitrilotriacetic acid chromatography was concentrated and injected onto an FPLC system previously, with several modifications (31). His-tagged SLO was eluted with wash buffer containing 250 mM imidazole and dialyzed overnight (4 °C) into 20 mM Bis-Tris (pH 6.6) buffer. The dialyzed eluate from nickel-nitrilotriacetic acid chromatography was concentrated and injected onto an FPLC system with a 6-ml UNO S6 column (Bio-Rad) for separation by cation exchange chromatography. A salt gradient for protein elution was run from 0.0 to 0.5 M NaCl in 20 mM Bis-Tris (pH 6.6) buffer. SLO activity was assayed in fractions by monitoring the formation of HPOD at 234 nm upon the addition of 5 M linoleic acid in 0.1 M borate (pH 9.0). Fractions containing SLO activity were combined, dialyzed into 0.1 M borate (pH 9.0), concentrated, and stored at −80 °C.

Enzyme Kinetics—Oxygen electrode and UV kinetic assays were performed for WT SLO and each mutant lipoxygenase as described previously (19, 29, 31). Oxygen concentration was varied from 30 to 1365 \( \mu \text{M} \). LA concentration was varied from 2 to 80 \( \mu \text{M} \). All kinetic assays were performed at 20 °C and in 0.1 M borate (pH 9.0). Reported estimates of \( k_{\text{cat}} \) are derived from oxygen electrode studies rather than UV kinetic data, allowing extrapolation rather than correction for saturating \( O_2 \).

Enzyme Regio- and Stereospecificity—The reactions were incubated at 20 °C in 0.1 M borate (pH 9.0) and 100 \( \mu \text{M} \) LA for 5 h after initiation with enzyme. The reactions at elevated and depressed \( O_2 \) concentrations were performed using a Clark-type electrode from Yellow Springs Inc. and a YSI-5300 biological oxygen monitor. The reaction mixtures were quenched at...
Oxygen Channel in SLO-1

FIGURE 2. A and B, computational analysis of O2 transport in SLO using CAVER. The surface of SLO is depicted in gray, and the catalytic iron, a hallmark of the active site, is represented as a magenta sphere. A, eight channels (green) were detected extending from the buried active site in SLO to the exterior of the protein. B, channel A (green) was identified as the most favorable route from the active site to the solvent according to a throughput metric computed by CAVER that favors short, wide channels. Residues within 1.5 Å of the bottleneck of channel A are depicted in orange and are listed here, sorted from closest to the iron center: Ile$^{553}$, Ile$^{547}$, Leu$^{496}$, and Val$^{564}$. C and D, computational analysis of O2 transport in SLO using ILS. SLO is represented by a gray surface, and the catalytic iron atom is depicted as a magenta sphere. C, the −0.5 kT isoenergy surface (cyan) highlights multiple, preferred pockets for oxygen to inhabit throughout the SLO protein matrix. D, the −2.5 kT isoenergy surface (cyan) imposes a more stringent requirement than the −0.5 kT surface, including only the most favorable regions for oxygen migration in the protein matrix. Note the pocket to the left of the catalytic iron, which is located closest to the active site.

pH 4 (glacial acetic acid) and extracted into dichloromethane. Reaction products were taken to dryness under a stream of N2 and stored at −80 °C until HPLC analysis. Reaction products were injected onto a reverse phase HPLC system with a Luna C18 5-μm column (0.46 × 25 cm; Phenomenex). An isocratic solvent system of 78% methanol and 21.9% water with 0.1% acetic acid was employed to elute HPOD. A flow rate of 0.9 ml/min was used. HPOD was collected, taken to dryness under N2, and reduced via incubation for >1 h with a solution of 1.9 mM triphenylphosphine in methanol. The hydroxyoctadecadienoic acid (HODE) generated was taken to dryness under N2 and stored at −80 °C until chiral phase HPLC analysis.

HODE was injected onto a chiral phase HPLC system with a Chiralcel OD-3 column (0.46 × 25 cm; Chiral Technologies, Inc.). An isocratic solvent system of 1.55% isopropanol, 0.3% acetic acid, and 98.2% hexanes was employed to separate the 13S, 13R, 9S, and 9R isomers. A flow rate of 2 ml/min was used. Assignment of the four HODE isomeric peaks was accomplished using standards of 13S-, 13R-, 9S-, and 9R-HODE acquired from Cayman Chemical.

Results

Computational Prediction of the Preferred Route(s) for Oxygen Delivery to the SLO-1 Active Site—Two parallel computational techniques, CAVER and implicit ligand sampling, were employed to probe gas migration within SLO-1. The CAVER 3.0 software tool was used to identify pathways linking the SLO active site to the solvent. Assigning a bottleneck radius of 0.9 Å for passage of O2 through the protein, eight routes were detected (Fig. 2A) with the length, average radius, and minimum radius, summarized in Table 1. Pathways were also ranked from most favorable (A) to least favorable (H) by CAVER, based on a throughput parameter that takes into account channel length as well as width. Channel A (Fig. 2B) was identified by CAVER as the most favorable route for oxygen to travel from the solvent to the active site. This channel exhibits the highest throughput (0.4) and largest bottleneck radius (1.1 Å), as well as the second largest mean radius and second shortest total length. Four residues were identified within 1.5 Å of the bottleneck of channel A: Ile$^{553}$, Ile$^{547}$, Leu$^{496}$, and Val$^{564}$ (Fig. 2B). In short, our analysis indicates that although multiple pathways link the buried SLO active site and solvent, channel A is identified as the preferred route for oxygen delivery to the site of catalysis.

In parallel with the geometric algorithm employed by CAVER, ILS simulations were performed to detect energetically favorable regions for oxygen to occupy within SLO. This technique infers gas migration pathways by generating a three-dimensional map of the Gibbs free energy cost associated with placing an oxygen molecule anywhere in the protein. Lower free energy values are associated with a greater probability of finding oxygen. Regions of SLO likely to be occupied by oxygen were mapped by drawing free energy isosurfaces at −0.5 kT (Fig. 2C) and −2.5 kT (Fig. 2D). Together, these energy maps indicate that although pockets favorable for the gas exist
throughout SLO, dioxygen tends to concentrate near the active site. Interestingly, the largest pocket outlined by the −2.5 kT isosurface directly overlaps with the top-ranked pathway identified by CAVER, channel A (Fig. 3). This pocket, which is adjacent to the active site, is also located in close proximity to the four bottleneck residues of channel A. Together, energetic and geometric insights from ILS and CAVER, respectively, point to a single pathway for oxygen delivery and implicate four residues in modulating oxygen access to the active site.

**Introducing Larger Amino Acid Side Chains into Channel A Disrupts Oxygen Trafficking, as Evidenced by Altered Kinetic Parameters for O2**—To assess the functional role of channel A, steric bulk was introduced at bottleneck residues 553, 547, 496, and 564 via site-directed mutagenesis. The impact of mutations on oxygen delivery was assessed by determining $k_{cat}/K_m(O_2)$, $K_m(LA)$, and the respective $k_{cat}/K_m$ parameters (Table 2). Focusing on the insertion of Trp at the four positions, the change in $k_{cat}/K_m(LA)$ is negligible for positions 547 and 564 in relation to WT. By contrast, Trp at positions 553 and 496 leads to quite significant changes in $k_{cat}/K_m(LA)$, with values that are only $10^{-3}$ to $10^{-2}$ of WT. In the case of $k_{cat}/K_m(O_2)$, all of the selected mutants indicate rate reductions, ranging from 2-fold ($V_564W$) to $10^4$-fold ($I_553W$). This behavior is mirrored in $K_m$ values of 132–744 μM O2, compared with 38 μM for WT SLO. A key issue is the degree to which an increase in side chain size decreases $k_{cat}/K_m(O_2)$ to a greater extent than $k_{cat}/K_m(LA)$. This feature is evaluated in the final column of Table 2, highlighting the largest impact of Trp occurring at positions 553 and 496 on $O_2$. In support of a straightforward analysis of the observed $K_m(O_2)$ and $k_{cat}/K_m(O_2)$ parameters, we note the absence of an elevation of the $K_m$ of channel A for any of the variants interrogated here. All previous studies of mutants of SLO-1 (cf. Refs. 29, 30, and 32) support an unchanged rate determining step that is controlled by C–H cleavage, implying that $K_m = K_d$ for both WT and mutant variants. The consequences of Trp at positions 553 and 496 become even more apparent following analysis of the product distribution among the four possible HPODs (cf. Fig. 1).

**Diminishing O2 Availability at the Active Site of WT SLO-1 Disrupts the Regio- and Stereospecificity of Product**—In addition to kinetic parameters, the regio- and stereospecificity of the SLO-catalyzed reaction provides a measure of oxygen availability at the active site. Significant loss of regiospecificity, for example, has been documented in the reaction of LA with SLO under low oxygen conditions, where the ratio of 13 HPOD to 9 HPOD approaches 1:1 (33). Diminished oxygen availability is thought to result in increased dissociation of the linoleyl radical intermediate into solution, where it can react indiscriminately with oxygen. Our investigation of the SLO-catalyzed reaction at low oxygen confirms previous findings with respect to regio- and stereospecificity and furthermore discerns the proportion of 13S-, 13R-, 9R-, and 9S-HPOD produced at low $O_2$ (Fig. 4). At ambient oxygen, 13R-, 9S-, and 9R-HPOD are present in small amounts and account in total for <10% of HPOD produced. At 28 μM $O_2$, by contrast, a substantial amount of 13R-, 9S-, and 9R-HPOD is generated. The alternate isomers are produced in approximately equivalent amounts, each accounting for ~21% of total HPOD produced. These results support the hypothesis that reduced oxygen availability leads to nonspecific oxygen insertion during the peroxidation of LA by SLO and serve as controls for the impact of site-specific mutagenesis and altered oxygen tension on product distribution.

**Introducing Steric Restriction into Channel A Disrupts the Regio- and Stereospecificity of Product HPODs**—The impact of mutation on SLO-1 regio- and stereospecificity provides one important probe of the disruption of oxygen trafficking. This is seen to be most dramatic for three SLO mutants, I553W, L496F, and I496W (Table 3). In accordance with the kinetic data (Table 2), analysis of product distribution implicates important roles for positions 553 and 496 in directing O2 from the solvent to the correct position and face within the substrate-derived pentadienyl radical.

Replacement of Ile553, which is fully conserved in all soybean lipoxygenases, by Trp and reaction with LA produces 45% 13S-HPOD, whereas the WT enzyme generates 93% 13S-HPOD. A substantial amount of 13R-, 9S-, and 9R-HPOD is thus produced with I553W. These isomers account for >50% of HPOD and, importantly, are seen to be generated in roughly equivalent amounts. The regio- and stereospecificity of the I553W mutant resembles that of the WT reaction at low $O_2$, consistent with restricted oxygen availability and the resulting increased dissociation of the linoleyl radical to solution. This explanation is supported by the significantly elevated $K_m(O_2)$ determined for this mutant (744 μM $O_2$). We note that introducing phenylala-

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**TABLE 1**

Characterization of eight channels detected and ranked by CAVER

<table>
<thead>
<tr>
<th>Channel</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>E</th>
<th>F</th>
<th>G</th>
<th>H</th>
</tr>
</thead>
<tbody>
<tr>
<td>Length (Å)</td>
<td>21</td>
<td>20</td>
<td>25</td>
<td>41</td>
<td>50</td>
<td>44</td>
<td>109</td>
<td>133</td>
</tr>
<tr>
<td>Mean radius (Å)</td>
<td>1.6</td>
<td>1.4</td>
<td>1.5</td>
<td>1.8</td>
<td>1.5</td>
<td>1.4</td>
<td>1.3</td>
<td>1.3</td>
</tr>
<tr>
<td>Bottleneck radius (Å)</td>
<td>1.1</td>
<td>0.9</td>
<td>1</td>
<td>0.9</td>
<td>0.9</td>
<td>1</td>
<td>0.9</td>
<td>0.9</td>
</tr>
<tr>
<td>Throughput</td>
<td>0.4</td>
<td>0.3</td>
<td>0.5</td>
<td>0.2</td>
<td>0.1</td>
<td>0.1</td>
<td>0.002</td>
<td>0.0003</td>
</tr>
</tbody>
</table>

**FIGURE 3.** SLO depicted with the −2.5 kT isosurface computed by ILS, as well as channel A and its bottleneck residues identified by CAVER. The largest pocket outlined by the −2.5 kT isosurface (cyan) directly overlaps with channel A (green), the top-ranked channel detected by CAVER. This region is also in close proximity to the bottleneck residues identified by CAVER (orange), highlighting the accordance of these two techniques. Overlap of the −2.5 kT isosurface with other channels detected by CAVER (B–H) is comparatively small, consistent with channel A representing the biologically relevant pathway.
Oxygen Channel in SLO-1

**TABLE 2**

Kinetic characterization of SLO mutants

The experiments were conducted at 20 °C, pH 9. \( k_{\text{cat}} \) is at saturating concentrations of both LA and O₂, whereas the \( k_{\text{cat}}/K_m \) indicated is at saturation with regard to the alternate substrate.

<table>
<thead>
<tr>
<th>SLO</th>
<th>( k_{\text{cat}} )</th>
<th>( K_m ) (LA)</th>
<th>( K_m ) (O₂)</th>
<th>( k_{\text{cat}}/K_m ) (LA)</th>
<th>( k_{\text{cat}}/K_m ) (O₂)</th>
<th>Impact of mutation on ( k_{\text{cat}}/K_m )</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>211 ± 9 (230 ± 15)</td>
<td>20 ± 2 (18 ± 3)</td>
<td>38 ± 5 (31 ± 3)</td>
<td>11 ± 1 s⁻¹ µM⁻¹</td>
<td>6 ± 1 s⁻¹ µM⁻¹</td>
<td></td>
</tr>
<tr>
<td>I553F</td>
<td>102 ± 8</td>
<td>19 ± 4</td>
<td>142 ± 54</td>
<td>5 ± 1</td>
<td>0.7 ± 0.3</td>
<td>3.8</td>
</tr>
<tr>
<td>I553W</td>
<td>0.40 ± 0.04</td>
<td>25 ± 5</td>
<td>744 ± 142</td>
<td>0.016 ± 0.004</td>
<td>0.0005 ± 0.0001</td>
<td>18</td>
</tr>
<tr>
<td>I547F</td>
<td>66 ± 5</td>
<td>5.0 ± 0.3</td>
<td>44 ± 11</td>
<td>22 ± 3</td>
<td>1.5 ± 0.4</td>
<td>8</td>
</tr>
<tr>
<td>I47W</td>
<td>44 ± 2</td>
<td>5 ± 1</td>
<td>132 ± 26</td>
<td>9 ± 1</td>
<td>0.9 ± 0.1</td>
<td>5.5</td>
</tr>
<tr>
<td>L496F</td>
<td>44 ± 3</td>
<td>5 ± 1</td>
<td>132 ± 26</td>
<td>9 ± 2</td>
<td>0.3 ± 0.1</td>
<td>16</td>
</tr>
<tr>
<td>L496W</td>
<td>1.6 ± 0.1</td>
<td>11.0 ± 0.8</td>
<td>171 ± 33</td>
<td>0.15 ± 0.01</td>
<td>0.009 ± 0.002</td>
<td>9.1</td>
</tr>
<tr>
<td>V564F</td>
<td>60 ± 1</td>
<td>6.0 ± 0.4</td>
<td>46 ± 5</td>
<td>10 ± 1</td>
<td>1.3 ± 0.1</td>
<td>4.2</td>
</tr>
<tr>
<td>V564W</td>
<td>99 ± 5</td>
<td>8.0 ± 0.7</td>
<td>32 ± 6</td>
<td>12 ± 1</td>
<td>3 ± 1</td>
<td>2.2</td>
</tr>
</tbody>
</table>

The data for I553F are from Ref. 19.

TABLE 3

Regio- and stereospecificity of product formation with mutant forms of SLO-1

The experiments were performed under the conditions of pH 9, 20 °C, and ambient O₂ concentration (284 µM). The errors range from 6 to 18% of the stated values.

<table>
<thead>
<tr>
<th>SLO</th>
<th>13S HPOD</th>
<th>15R HPOD</th>
<th>9S HPOD</th>
<th>9R HPOD</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>%</td>
<td>%</td>
<td>%</td>
<td>%</td>
</tr>
<tr>
<td>I553F</td>
<td>93</td>
<td>3</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>I553W</td>
<td>95</td>
<td>2</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>I547F</td>
<td>45</td>
<td>16</td>
<td>22</td>
<td>17</td>
</tr>
<tr>
<td>I547W</td>
<td>93</td>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>L496F</td>
<td>93</td>
<td>2</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>L496W</td>
<td>63</td>
<td>9</td>
<td>14</td>
<td>14</td>
</tr>
<tr>
<td>V564F</td>
<td>89</td>
<td>2</td>
<td>4</td>
<td>5</td>
</tr>
<tr>
<td>V564W</td>
<td>86</td>
<td>3</td>
<td>5</td>
<td>5</td>
</tr>
</tbody>
</table>


done at position 553 does not disrupt regio- and stereospecificity in the same way as tryptophan. Despite exhibiting an elevated \( K_m(O_2) \), the I553F mutant produces >90% 13S-HPOD. The introduction of phenylalanine at position 553 reduces the rate at which O₂ reaches the substrate derived radical (19) while leaving the regio- and stereospecificity of product formation intact. We propose that the key factor in determining the fidelity of product formation is the ratio of rate constants for trapping of the bound pentadienyl intermediate by O₂ versus its release from enzyme. The insertion of Trp at position 553 has clearly tipped the balance toward a solution-based reaction of substrate with O₂, in a manner resembling the impact of reduced O₂ concentration on the product distribution of WT enzyme.

Regio- and stereospecificity is also dramatically disrupted in the reaction of both Leu⁴⁹⁶ mutants with LA. Reaction with the phenylalanine and tryptophan mutants produces 63 and 31% 13S-HPOD that is accompanied by a substantial amount of 13R-, 9S-, and 9R-HPOD; together, these alternate isomers account for 37 and 69% of HPOD produced in reactions with L496F and L496W (Table 3). It is significant that the 13R, 9S, and 9R isomers are produced in unequal amounts, contrasting with the behaviors of WT SLO at low oxygen and the I553W mutant. This is particularly apparent for the L496W mutant, where the 9S and 9R isomers each account for 29 and 30% of HPOD produced compared with 10% for the 13R isomer. The disparity is more subtle in the case of L496F, where the 9S and 9R isomers account for 14% of HPOD produced compared with 9% for 13R-HPOD. We conclude that the altered regio- and stereospecificity of the Leu⁴⁹⁶ mutant reactions cannot be simply explained by a restricted availability of O₂ at the active site that results in increased loss of the substrate radical to solvent. Rather, the elevated amount of 9R and 9S-HPOD suggests a gain of function for the Leu⁴⁹⁶ mutations, i.e. the opening up of an alternate route for gas migration that allows O₂ to access carbon 9 of the substrate radical. We note that earlier studies (31) have discussed a changeover from the production of 13S- to 13R-HPOD products in the event of a “reverse binding” of substrate, under conditions where the fatty acid carboxylate of substrate becomes protonated and can orient itself toward the protein interior. Whereas this phenomenon may account for behaviors at pH 7, it is an unlikely explanation for the behavior reported herein at pH 9. With the expectation that reverse binding will not be sensitive to increased O₂ levels and to further test out the hypothesis that the behavior of the Ile⁵⁵³ and Leu⁴⁹⁶ variants is due to perturbations in a gas channel, we examined the impacts of O₂ concentration on product distributions with I553W and L496W.

**FIGURE 4. Impact of diminished O₂ on the regio- and stereospecificity of WT-SLO-1.**

**TABLE 3**

Regio- and stereospecificity of product formation with mutant forms of SLO-1

The experiments were performed under the conditions of pH 9, 20 °C, and ambient O₂ concentration (284 µM). The errors range from 6 to 18% of the stated values.

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</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>%</td>
<td>%</td>
<td>%</td>
<td>%</td>
</tr>
<tr>
<td>I553F</td>
<td>93</td>
<td>3</td>
<td>3</td>
<td>2</td>
</tr>
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<td>I553W</td>
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<td>2</td>
</tr>
<tr>
<td>I547F</td>
<td>45</td>
<td>16</td>
<td>22</td>
<td>17</td>
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**Ref. 4.**
increasing [O₂] has an impact that goes beyond simply increasing 
product formed as seen for I553W. This observation indicates that 
carbon 9) in L496W.

Barrier for O₂ reactivity through the normal channel (that leads 
to carbon 13) than for the newly introduced channel (leading to 
carbon 9) in L496W.

generated by the I553W reaction similarly increases from 45% 
at ambient O₂ to 65% at 1365 μM O₂. Increased O₂ can be 
concluded to partially restore the regio- and stereospecificity of 
this mutant reaction, indicating that additional O₂ compensates 
for impaired oxygen delivery in the mutant.

Elevated O₂ is also able to partially restore the regio- and 
stereospecificity of the L496W mutant reaction (Fig. 5), but not 
by uniformly decreasing the amount of 13R, 9S, and 9R isomers 
produced as seen for I553W. This observation indicates that 
increasing [O₂] has an impact that goes beyond simply increas-
ing oxygen availability in relation to radical dissociation, as 
observed for I553W at high O₂. As noted earlier, a decrease in 
radical dissociation would affect the formation of the 13R, 9S, 
and 9R product isomers equally. In contrast, elevated [O₂] in 
L496W influences the partitioning of O₂ between oxygen trans-
port pathways, reducing the reaction at carbon 9 and increasing 
product formed via the pathway leading to a reaction at carbon 
13. This result strongly supports the conclusion of a higher 
barrier for O₂ reactivity through the normal channel (that leads 
to carbon 13) than for the newly introduced channel (leading to 
carbon 9) in L496W.

**Discussion**

Enzymes are complex systems that contain a variety of pock-
ets, clefts, and channels throughout the protein matrix. These 
internal structural features can play an essential role in tuning 
enzyme function by modulating the flux of small molecules 
within the protein. For example, in flavo-monoxygenases and 
oxidases, oxygen delivery channels have been shown to guide 
oxide from the solvent through preorganized protein cavities 
and direct it to the reacting C₄a atom of the flavin cofactor (14, 
34). In 12/15 lipoxygenase, a preferred route for oxygen travel 
has been identified that links the solvent and buried active site 
(6). Similarly, crystallographic and computational studies have 
also implicated oxygen access paths in cyclooxygenase, copper 
amine oxidase, cholesterol oxidase, and cytochrome c oxidase 
(8, 10, 11). The nuanced role of the protein matrix in influenc-
ing catalysis is not restricted to oxygen delivery. In hydroge-
nases, gas channels have been reported to selectively filter mol-
cules such as oxygen and carbon monoxide to prevent inactivation at the active site (12, 17, 35). Recent investigation 
of heme nitric oxide/oxygen binding domains has suggested 
that tunnels not only direct gases to the heme iron but also 
modulate reversible gas binding (16). Importantly, our work is 
the first to directly implicate an oxygen channel in regulating 
regio- and stereospecificity by explicitly showing the produc-
tion of alternate product isomers upon obstruction of the 
channel.

Lipoxygenases provide ideal context in which to examine 
oxogen migration because product positional and stereoche-
my, in addition to reaction kinetics, can report on oxygen tar-
getting to the active site (36). In this work, the functional role of 
oxogen migration pathways was examined in SLO-1, the best 
studied member of the lipoxygenase family. Our computational 
and experimental findings support a role for a single delivery 
channel within SLO-1 that shuttles oxygen to the active site 
and ensures its regio- and stereospecific insertion during catalysis.

To examine oxygen trafficking within SLO-1 from both 
a structural and energetic perspective, two computational 
approaches were employed in parallel. ILS was used to identify 
regions favorable for oxygen travel throughout the protein 
whereas CAVER was employed to visualize pathways leading 
continuously from the active site to the exterior of the protein. 
Although these tools identify multiple pockets and pathways 
throughout the enzyme (Fig. 2), both converge on a single 
channel deemed most competent for oxygen delivery (Fig. 3) 
(20, 28) Interestingly, this channel is consistent with a cavity originally 
identified in the crystal structure of SLO and previously pro-
posed as an O₂ channel (4, 18, 32).

The functional role of this putative oxygen pathway was con-
firmed by introducing steric bulk at its bottleneck residues via 
site-directed mutagenesis. Introducing tryptophan at positions 
553 and 496, in particular, dramatically disrupts oxygen traf-
ficking to the active site, as evidenced by alterations in Michaelis 
constants and $k_{cat}/K_m(O_2)$ (Table 2) and reaction regio- 
and stereospecificity (Table 3) that, importantly, is dependent on 
the exogenous O₂ level (Fig. 5). These observations point to a 
role for these residues as gatekeepers of a channel that shuttles 
oxogen to the active site.

Examining the regio- and stereospecificity of the I553W and 
L496W mutant reactions further reveals that introducing steric 
restriction at these positions disrupts oxygen trafficking in dif-
frent ways. Tryptophan at position 553 diminishes oxygen 
availability at the SLO active site, effectively starving the 
enzyme for oxygen. Accordingly, the I553W reaction exhibits 
impaired regio- and stereospecificity similar to the reaction of 
WT SLO-1 at low oxygen (Table 3 and Fig. 4). This observation, 
coupled with the 20-fold increase in $K_m(O_2)$ for the I553W 
mant, is proposed to reflect limited oxygen availability at the 
active site and increased dissociation of the linoleyl free radical 
intermediate into solution that then undergoes reaction with 
O₂ in a random manner. Consistent with this hypothesis, per-
forming the I553W mutant reaction at high [O₂] partially res-
The changes in impact on oxygen availability at the active site, as evidenced by Figure 6). Accordingly, O₂ availability at the active site is diminished, as evidenced by the large decrease in \( k_{\text{cat}} / K_m(O_2) \), an increased Michaelis constant for oxygen, and an increased proportion of equal levels of 13R-, 9S-, and 9R-HPOD produced in the peroxidation of LA that is partially reversed at elevated O₂ concentration. The L496W mutation, by contrast, appears to unlock an alternate pathway to the active site (Fig. 6C), significantly increasing the proportion of 9S- and 9R-HPOD produced in the peroxidation of LA in an O₂-dependent manner. These new findings, which extend previous evidence for a gas channel in SLO-1 (4), may provide insight into mechanisms for altered positions of oxygen delivery with the larger lipoxygenase family, as well as offer guidelines for the de novo design of enzymes that require gaseous molecules as cosubstrates.

**Author Contributions**—L. C. performed the experiments. J. P. K. and L. C. designed the experiments and wrote the manuscript.

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