4-2020

Understanding How Human Lipoxygenases Bind Molecular Oxygen and Arachidonic Acid Substrate

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by

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Undergraduate honors thesis under the direction of

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Submitted to the LSU Roger Hadfield Ogden Honors College in partial fulfillment of the Upper Division Honors Program.

April 2020

Louisiana State University & Agricultural and Mechanical College Baton Rouge, Louisiana

Abstract

The primary substrate for animal lipoxygenases is arachidonic acid, and the oxygenated products of these fatty acids are classified as eicosanoids. Eicosanoids are potent signaling molecules that play many roles in human homeostasis and certain disease states. The human lipoxygenase of key interest in the Newcomer lab is 5-lipoxygenase (5-LOX), which has been implicated in many human diseases including asthma, atherosclerosis and cancer. This research will lead to a better understanding of how human lipoxygenases bind molecular oxygen, and arachidonic acid substrate. While "closed" lipoxygenase structures have been solved, a lipoxygenase structure bound to both arachidonic acid and molecular oxygen is yet to be resolved. Furthermore, an open conformation of 5-LOX is yet to be revealed. Therefore, we aimed to resolve lipoxygenase structure bound to oxygen and arachidonic acid using a homologue of human 5-LOX, coral 8R-PSWT and xenon as an oxygen mimetic. The strategy to resolve the open structure of 5-LOX lies in crystallizing a 5-LOX mutant which is characterized by relatively high stability, and a high rate of turnover.

List of Abbreviations

5-lipoxygenase (5-LOX)

5-hydroperoxyeicosatetraenoic acid (5-HPETE)

8R-lipoxygenase (8R-LOX)

8R-lipoxygenase pseudo-wildtype (8R-PSWT)

Center for Advanced Microstructures and Devices (CAMD)

Fast Performance Liquid Chromatography (FPLC)

Polyethylene glycol (PEG)

Introduction

The lab of Marcia Newcomer in the Department of Biological Sciences utilizes protein crystallography to reveal the structure and function of enzymes linked to inflammation. Our focus in this structural biology lab is a class of enzymes named lipoxygenases. Lipoxygenases are enzymes that add molecular oxygen to a regio-specific carbon on a lipid (1). The primary substrate for animal lipoxygenases is arachidonic acid, and the oxygenated products of these fatty acids are classified as eicosanoids. Eicosanoids are potent signaling molecules that play many roles in human homeostasis and certain disease states (2). The most notorious eicosanoids are the prostaglandins from the cyclooxygenase pathway, with the production of these prostaglandins being therapeutically inhibited by ibuprofen, aspirin or naproxen (3). The human lipoxygenase of key interest in the Newcomer lab is 5-lipoxygenase (5-LOX), which has been implicated in many human diseases including asthma, atherosclerosis and cancer (4). The coral 8R-lipoxygenase from the Caribbean Sea whip coral *Plexaura homomalla* is a model enzyme for the human 5-lipoxygenases due to its robust expression level, protein stability, and amenability to structural studies. Structural studies that probe mechanistic details of the enzyme mechanisms for two lipoxygenases are described here.

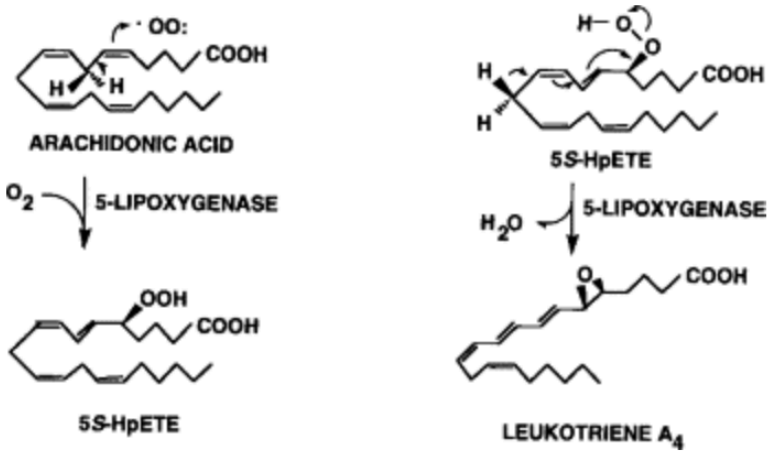


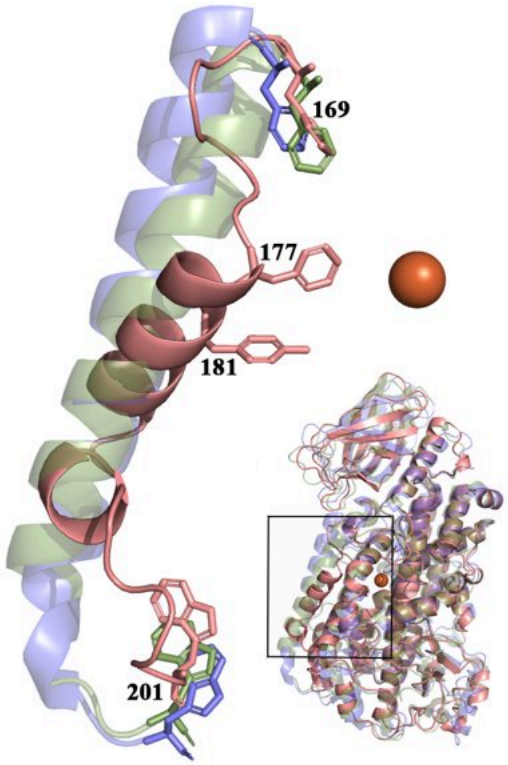
Fig.1 Mechanisms of the reactions catalyzed by the 5-lipoxygenase. HpETE is the abbreviation for hydro-peroxyeicosatetraenoic acid. **Credit:** William L. Smith, Frank A. Fitzpatrick, in New Comprehensive Biochemistry, 1996.

Human 5-LOX is notoriously unstable, losing 50% of its activity within 10 hours (5). Therefore, it is convenient to work with a stable model enzyme such as coral 8R-LOX, which shares about 40% sequence identity with human 5-LOX (6). 8R-LOX, on the other hand, retains >95% of its activity after storage at 277 K for four months (5). Furthermore, 8R-PSWT has been much easier to crystallize than human 5-LOX. A deletion mutant of 8R-LOX in which the corresponding membrane-insertion loop is absent (Δ41–45:GSLOX) was engineered (5). The removal of a membrane-insertion loop required for Ca^{2+}-dependent membrane targeting of 8R-LOX improves the solubility of the protein, while the enzyme activity in a membrane-free assay is unaffected. Removal of the membrane-insertion loop dramatically increased the protein yield from bacterial cultures and the quality of the crystals obtained, as well as increasing the water solubility of the protein. Taken together, this allows the proteins to pack closer together, which increases crystallization and diffraction efficacy, and resulted in a better than 1 Å improvement in the resolution of the diffraction data (5). The deletion mutant displays wild-type activity in a

membrane-free assay, however, Ca^{2+} does not promote membrane binding of the mutant and does not stimulate enzyme activity in a membrane-based assay (7). Thus, we refer to this enzyme as pseudo wild-type in accordance with its native-like structure and catalytic activity in the absence of membranes.

The stability of 8R-lipoxygenase pseudo-wildtype (8R-PSWT) enzyme has been essential in revealing multiple structures of this enzyme, including the structure of the enzyme-substrate complex. 8R-PSWT has been crystallized in an anaerobic chamber with the presence of the substrate, arachidonic acid (8). Preparing protein crystals in an anaerobic chamber allows for the lipoxygenase to bind one of its substrates, arachidonic acid, in the absence of its other substrate, molecular oxygen. If both oxygen and arachidonic acid are present the 8R-lipoxygenase will transform the arachidonic acid to product, rendering trapping the substrate-lipoxygenase complex improbable. Revealing the molecular details of substrate binding of a substrate-protein complex allows a more detailed understanding of how the molecular machine functions. The next step to understanding the complete catalytic cycle of lipoxygenases is discovering where the molecular oxygen binds in the active site of the 8R-lipoxygenase. In the 8R-PSWT project, we aimed to capture a structure with not only enzyme and substrate, but also molecular oxygen. In order to capture the structure of this enzyme we used an oxygen mimetic, xenon. In order to do so we would bombard crystals with xenon in a high-pressure chamber. We proposed crystallizing 8R-PSWT with xenon and arachidonic acid would hold the enzyme in a bound conformation, without a reaction turning over. In addition to O_2 forcing the reaction to turn over, it is not usually observed in protein crystal structures and xenon has successfully identified O_2 binding sites in previous literature (9,10). Xenon was first used to detail where hemoglobin binds oxygen in 1965 (11).

In contrast to the “open” structure of 8R-LOX, previous studies have revealed the “closed” structure of human 5-LOX. In order to understand how this enzyme binds substrate, we aim to resolve an open structure. In order to do so, we mutated glycine 174 and aspartate 176 to alanine residues, creating a G174A/D176A-Stable-5-LOX mutant. The Stable-5-LOX enzyme was created using a similar approach to 8R-PSWT. The enzyme lacks a membrane insertion loop and a pair of proximal cysteines (4). These mutations lead to an increase in melting temperature and half-life, while maintaining intermediate 5*S*-HPETE and the leukotriene A_4 production (4). 5-LOX has an uncharacteristic α2 helix which contributes to one edge of the active site. The 5-LOX α2 helix is 3-4 turns shorter than α2 helix of 8R-PSWT, and is positioned at a slightly different angle, which limits access to the enzyme’s catalytic site (4). Preliminary data supports our hypothesis that the G174A/D176A mutations extend the α2 helix, opening the enzyme and allowing greater access to the catalytic site.



B. **A.**

Fig. 2 The positioning of helix α2 is unique in 5-LOX
(A) A 5-LOX cartoon is rendered in pink, 15-LOX in blue and 8R-LOX in green. Conserved aromatic amino acids (F169,W201) that flank the region are in stick rendering. F177 and Y181 that make up the "cork" that helps define the active site are in stick. The catalytic iron is an orange sphere. (B) A full overlay of the three structures in which it is apparent that, with the exception of α2, the secondary structural elements in the enzymes are conserved. The box indicates the region amplified in (A). **Credit: (4)**

The Glycine amino acid is located just prior to an alpha helix which blocks the active site of the protein. Glycine is a known "helix breaker," and we hypothesize that mutating this amino acid to alanine, which favors a helical structure, un-blocks the active site. In the closed structure of 5-LOX the aspartate residue forms a hydrogen bond with Q413, which keeps the enzyme closed. Mutating this amino acid to an alanine prevents the formation of such bond, which should allow the enzyme to spend more time in an open conformation. Kinetic assays have revealed G174A/D176A has a much higher rate of turnover than wild-type 5-LOX. A proteolysis assay, which allows proteases to "cut" proteins in unstructured regions, is often used as a proxy for evaluating protein stability and flexibility. When the G174A/D176A variant is evaluated in a proteolysis assay with an inhibitor, SCTH33, low levels of proteolysis are observed. This mutant's resistance to proteolysis supports the idea it has little conformational flexibility. Taken with the enzyme's high turnover rate our data points to G174A/D176A as a more stable protein that spends more time in the open conformation as compared to other 5-LOX mutants. This would increase the likelihood we are able to resolve the open structure using protein crystallography. We are screening for G174A/D176A crystals, with a suspected competitive inhibitor, SCTH33, which we hypothesize maintains the enzyme in an open conformation. We will use x-ray diffraction to collect data on the G174A/D176A structure with bound substrate, arachidonic acid.

Methods

Plasmid Preparation

8R-PSWT and D174A/G176A mutants were constructed using the QuikChange II XL site-directed mutagenesis kit (Agilent). All mutations were verified by sequencing. Plasmid DNA was purified using the Wizard system (Promega).

Protein synthesis and Purification

The ROS2 (DE3) E. coli cells were transformed with the 8R-PSWT and G174A/D176A 5-LOX recombinant plasmids. For large scale protein purification, the cells were grown in Terrific Broth (Alpha Bioscience, MD) with 100 µg/ml chloramphenicol, 100 µg/ml carbenicillin. The cultures were shaken at 250 rpm for 4 hours at 37 °C and then the temperature was reduced to 20 °C and the cultures were grown overnight. The cells were harvested and pelleted by centrifugation and frozen at −80 °C for preservation.

Pellets are thawed and weighed to begin the purification process. The cells were suspended in 3ml/gm Bugbuster (Novagen) with 1 µM pepstatin, 1 µM leupeptin, 100 µM PMSF and 2 KU/gm DNAse I (all from Sigma). The mixture was stirred on ice for 30 min and subjected to three rounds of sonification. The homogenized cell solution was lysed by passing the mixture through a French pressure cell. The lysate was centrifuged at 40000 g for 45 min. This centrifugation separates insoluble cell materials from the soluble proteins. The resulting supernatant was loaded onto a HisTrap 5ml cobalt Sepaharose (GE Healthcare) column equilibrated in 50 mM Tris-HCl (pH 8.0), 500 mM NaCl, and 20 mM imidazole on an AKTA FPLC system (GE Healthcare). The column binds 8R-PSWT and G174A/D176A 5-LOX by the 6x-histidine tag. Subsequently, the column was washed with ten column volumes of the above

buffer and then the protein was eluted by applying a linear gradient of 50 mM Tris-HCl (pH 8.0), 500 mM NaCl and 200 mM imidazole. This mostly purified solution is dialyzed overnight to remove excess salt and imidazole from the elution buffer. The next day, dialyzed protein is concentrated to a loading volume of 1 mL, syringe filtered to remove particulate matter, and then loaded onto the size exclusion column in fast protein liquid chromatography instrument. The peak fractions were concentrated in an Amicon Ultra 30K (Millipore) filter to a final volume of 500 µl. Protein was concentrated to a final concentration of 10mg/ml as detected by a NanoDrop spectrometer (Thermo Scientific). The protein was flash frozen in liquid N_2 and stored at −80 °C until usage. The same protocol was followed for the purification of G174A/D176A, but protein was concentrated to a final concentration closer to 5.0mg/ml.

Crystallization Screening and Data Collection

8RPSWT

Previous literature cites sitting drop vapor diffusion in 5-8% PEG-8000, 5% glycerol, 0.2 M $CaCl_2$, 0.1 M imidazole acetate, pH 8.0 as premium crystallization conditions (6). Cryschem plates (24 well) from Hampton research were used for sitting drop vapor diffusion. Preliminary screenings for 8RPSWT crystals were performed in and around these conditions in an aerobic environment. All crystallization solutions were filtered and degassed before they were transferred to the anaerobic chamber. After collecting a series of hits in the above conditions, screening was performed in a Coy anaerobic chamber at the Gulf Coast Protein Crystallography Consortium beamline at the Center for Advanced Microstructures and Devices (CAMD, Louisiana State University). The crystals were mounted using a cryo-protecting solution of 10%

PEG-8000, 25% glycerol, 0.02 M $CaCl_2$, 0.1 M imidazole acetate, pH 8.0 (7), and X-ray diffraction data were collected at CAMD.

G174A/D176A

Crystallization screens were performed using PEG ION2 from Hampton Research, and LMB2 from Molecular Dimensions. Trays were placed on a shake table to ensure wells were mixed to homogeneity. Protein samples were incubated for at least an hour with a final concentration of 0.5mM SCTH33 before they were added to the crystallization drop. All crystallization trials were performed using cryschem plates (24 well) from Hampton research. PEG ION2 and LMB2 screens yielded hits, and additional trays were set up in and around those conditions.

Results

Early screening for 8R-PSWT revealed aerobic hits near and around the conditions cited in previous literature 5-8% PEG-8000, 5% glycerol, 0.2 M $CaCl_2$, 0.1 M imidazole acetate, pH 8.0 (6). I reproduced aerobic crystals successfully in said conditions. In anaerobic conditions, early crystal screens seemed to have the best growth in higher PEG-8000 and $CaCl_2$ conditions. However, later screens revealed reverse in this trend as the best crystals were grown in PEG-8000 conditions as low as 2 or 3%. I tried to vary protein and well drops at all conditions, but I could not make sense of any trends. The reproducibility of the crystals in anaerobic conditions was non-existent. In anaerobic and aerobic conditions, I screened over 1200 crystallization conditions. Although the crystals were not reproducible, we collected both aerobic and anaerobic crystals for diffraction at CAMD, but no diffraction data could be obtained. The crystals did not

diffract due to unknown issues. Potential problems include the quality of the crystals and issues with cryo-protection conditions.

As the 8R-PSWT proved to be more difficult than we had imagined, we shifted our focus to a new 5-LOX mutant enzyme, G174A/D176A. Proteolysis and kinetic assays had shown promising results and supported our hypothesis of G174A/D176A maintaining a more stable open conformation. I began broad matrix crystallization screening for G174A/D176A, beginning with the PEG ION2 and LMB2 screening kits. Crystal hits were found in conditions 17 (4% volume/volume Tacsimate pH 8.0 12% PEG 3350 weight/volume) and 28 (0.2M sodium formate pH 7.0 20% PEG 3350 weight/volume) in the PEG ION2 screen. In the LMB 2 screen, condition 26 (3.5% wieght/volume PEG 6000, 0.1 M Bis-Tris propane pH 7.1, 0.1 M KCl) yielded a hit. Following these hits, screening was performed in and around the aforementioned conditions. We've varied pH's, KCl concentrations, and PEG concentrations. G174A/D176A crystals have been consistently reproducible in a range of 3-7% PEG 3350 or PEG 6000. Although we have seen a high reproducibility, the crystals are much too small to collect data on at this point.

Discussion

Working on the 8R-PSWT portion of this project illuminated to me the meticulous nature of basic science research. Initially, we developed a few crystals aerobically and the project seemed to be moving at a great pace. However, when the work was transferred to the anaerobic chamber, we found vastly fewer crystals in similar conditions. Also, the reproducibility of the crystals suffered. Finally, the crystals we were able to grow did not diffract sufficiently for data collection.

Although our data collection suffered, I developed bacterial transformation, protein expression, and purification skills. In the search for anaerobic crystals, I chased trends in every direction. I was severely frustrated, as crystallization trends seemed to shift from tray to tray. However, this frustration developed me into a more careful and meticulous crystallographer. As I was having a difficult time reproducing crystals and optimizing conditions, I exhausted my protein samples. I was forced to repeat transformation, expression, and purification experiments a countless number of times. As this project proved more difficult than originally imagined, we shifted our attention to the G174A/D176A project, given the promising proteolysis and kinetic results.

This project did not yield excellent results, but it equipped me with the tools to succeed in other crystallization projects, including the G174A/D176A project. Given the transformation and purification methods of the two enzymes are so similar, it took no time to familiarize myself with the protocol. In our early crystallization efforts, we have reproduced crystals with ease. The next step is to improve the size and quality of crystals, which we are attempting to do by improving the purity of our protein sample.

Acknowledgements

I would like to thank Dr. Nathan Gilbert for his guidance, leadership, and mentorship over the past 4 years. I would like to thank Mrs. Susan Laborde for her assistance in expression and purification protocols, especially during the periods of school in which my attention was drawn away from the lab. I would like to thank Eden McMillin Gallegos for her help in the lab, and her support through this process. I would like to thank Dr. Granger Babcock and Dr. Johnna Roose for being members of my thesis committee. Thank you, Dr. Marcia Newcomer, for

allowing me the opportunity to join your lab four years ago and supporting my research and ambitions throughout my college career. Lastly, I would like to thank my family and friends for their unconditional support of my schoolwork and scholarly activities.

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Vita

Austin Primeaux was born June 14th, 1998 in Lafayette, Louisiana. He attended Broadmoor Elementary School, Edgar Allen Martin Middle School, and Lafayette High School, graduating in May 2016. He has since been pursuing a Bachelor of Science in Biochemistry while achieving Sophomore Honors Distinction and striving for College Honors through the Ogden Honors College. He began his research in August of 2016 under the guidance of Dr. Marcia Newcomer, and has thoroughly enjoyed the challenges of research, as well as bonding with the fellow members of the Newcomer Lab.